Amlan K. Patra *Editor*

Dietary Phytochemicals and Microbes

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Preface

Plants produce an enormous array of phytochemicals arising from various biosynthetic pathways. More than 200,000 defined structures of phytochemicals have been recognized[1] and about 20,000 phytochemicals have been identified from edible plant sources such as fruits, vegetables and grains.[2] The phytochemicals are used for various purposes such as pharmaceuticals, agrochemicals, flavours, fragrances, colouring agents, biopesticides and food additives. These plant bioactive compounds are not essential for normal physiological functions, but the importance of certain plant bioactives has been well recognized for health promoting activities such as immuno-modulation, prevention of cancer and cardiovascular diseases, anti-aging and anti-diabetics.[3]

Humans have utilized the bioactive principles of different plants for various beneficial physiological properties including antimicrobial properties for many centuries. The ancient records provide evidence of their use by Chinese, Indian, Egyptian, Greek, Roman and Syrian dates back to about 5,000 years. However, interests of using medicinal plants declined in the twentieth century with the availability of effective synthetic antimicrobial drugs. The growing concerns over bacterial resistance to antibiotics and chemical residues in animal derived foods have led to a resurgence of interests to use phytochemicals as alternatives to antibiotics, other chemotherapeutic agents, and chemical and growth promoting antibiotic feed additives. Consequently, the trends of market sales of natural plant products have been rising tremendously in recent years. Phytochemicals exhibit antibacterial, antiviral and antifungal activities against a wide range of pathogenic and non-pathogenic

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microorganisms.[4] The antimicrobial properties of phytochemicals are being explored to utilize as potential antimicrobial drugs for prevention and cure of microbial diseases including methicillin resistant *Staphylococcus aureus* and multiple resistant strains of bacteria, and also as feed additives in livestock production system. The *in vivo* evaluation of safety and efficacy of emerging plant bioactives is essential for an extended period of time before they can be recommended for use. Identification of particular genes for the target phytochemicals and the genetic engineering of the biosynthetic pathways could overexpress the targeted genes to produce greater concentrations of phytochemicals,[5] and could meet the growing demands of plant bioactive substances.

Recently, the number of publications focusing the investigation of phytochemicals as antimicrobial compounds has been increasing exponentially. For instance, a PubMed search on the 'antimicrobial' or 'antiviral' or 'antibacterial' properties of 'plant' in the title/abstract provides an estimate of 689 articles until 2000, 2,186 articles during 2001–2010 and 400 articles in 2011 (November 25, 2011). Recognizing the beneficial health effects of phytochemicals, a number of books have been published, but there is no book published exclusively on the antimicrobial properties of phytochemicals. Emphasizing the importance of phytochemicals as an antimicrobial agents and the possibility of dietary phytochemicals and microbial interactions in the gastrointestinal tracts, this book provides the current knowledge on the effects of dietary plant secondary metabolites on beneficial and pathogenic microbes so that researchers, professors and students could get comprehensive information in these areas. The recent updates on the antimicrobial and antiviral properties of numerous recently reported phytochemicals and their mechanisms of antimicrobial actions have been provided comprehensively in several chapters. Some of the chapters have critically discussed the beneficial and adverse effects of antimicrobial and stimulatory activities of dietary phytochemicals on the rumen microbial populations, and gut microbial populations of humans and animals. Microbial adaptation and resistance of microbes to phytochemicals have also been highlighted. The synergetic interactions of phytochemicals and antimicrobial drugs have been discussed in some parts of the chapters. On the applied aspects, the use of phytochemicals against drug resistance microbes, to treat microbial diseases, for food preservation, to inhibit methanogenic archaea in the rumen, and to modulate lipid biohydrogenating microbial populations to increase conjugated linoleic acids in animal-derived foods have been presented in different chapters of this book. The well-known researchers in their respective fields have written the chapters of this book, and the information included in this book would be extremely valuable to the researchers, professors, pharmacists and postgraduate students in clinical microbiology, pharmacology and animal sciences including agriculture and pharmaceutical industry.

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Kolkata, India Amlan Kumar Patra

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Chapter 1
An Overview of Antimicrobial Properties of Different Classes of Phytochemicals

Amlan Kumar Patra

Abstract Plants produce a great diversity of phytochemicals, the beneficial properties of which have been used by humans for centuries since the advent of human civilization. With the discovery of effective and potent antimicrobial compounds, these synthetic antimicrobial compounds are widely used to prevent and cure microbial diseases. However, the development of antibiotic resistant strains of bacteria, reduced efficacy and safety of antimicrobials and the search of new antimicrobials against emerging incurable diseases by conventional antimicrobial agents have revived to explore phytochemicals as an alternative to synthetic antimicrobial compounds. Although numerous studies have been conducted *in vitro* and *in vivo* in the recent years on the efficacy of plant phytochemicals as antimicrobial agents, this chapter provides an overview of the antimicrobial properties of some major group of phytochemicals, namely, different phenolic compounds, alkaloids, saponins, iridoids and secoiridoids, polyacetylenes, glucosinolates, terpenoids, sulfinate, limonoids (tetranortepenoids) and anthranoids against pathogenic bacteria, fungi, viruses and commensal bacteria in the intestinal tracts of humans and animals. This chapter also discusses their antimicrobial mechanisms of action, the efficiency of different groups of phytochemicals against multiple-drug resistant bacteria, the effect of active dietary phytometabolites on the beneficial and pathogenic microbes of the gastrointestinal tracts and the outcomes of combination of phytofactors and drugs interactions.

Keywords Phytochemicals • Medicinal plants • Antimicrobial • Antiviral • Antifungal • Mechanism of action

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1.1 Introduction

Plants contain a wide array of phytochemicals, which have traditionally been utilized for centuries in folk medicines or ethnomedicines. The earliest information on the medicinal use of plants comes from China in 5000 BC (Greathead 2003), from India (in Rigveda and Atharvaveda) in 2000 BC (Ramawat et al. 2008), from Mesopotamia in 2600 BC (Newman et al. 2000), and also from Egypt in about 1550 BC (Davidson and Naidu 2000). The natural medicines were widely used until the first half of the twentieth century, when a shift towards synthetic medicines that were more effective, patentable and highly profitable, occurred (Tyler 1999). However, there have been increasing interests towards use of natural chemicals in medicinal purposes in recent years. These ethnomedicines are encouraging for both the public and national health care institutions as alternatives to synthetic drugs due to relatively lower incidences of adverse reactions compared to modern conventional pharmaceuticals along with their reduced cost (Nair et al. 2005).

Recently, the growing occurrences of multi-drug resistant strains of bacteria and the appearance of strains with decreased susceptibility to antibiotics have led to a resurgence of research interests in the discovery of novel antimicrobial agents from natural sources for therapeutic and preventive purposes against microbial diseases, food preservatives and feed additives in the animal industry. The ethnopharmacologists, botanists, microbiologists and natural-product chemists are constantly in search of medicinal efficacy of plants and their phytochemicals, since the reported data so far available on plants are comparatively meager compared to the vast number of plant population. Plants produce a great diversity of compounds. The structures of close to 50,000 compounds have already been elucidated and there are perhaps hundreds of thousands of such compounds in plants (Pichersky and Gang 2000). Only a few of these are part of 'primary' metabolic pathways (those common to all organisms). The rest are secondary metabolites or phytochemicals whose biosynthesis is restricted to selected plant groups (Pichersky and Gang 2000). Phytochemicals can be divided into many major classes depending upon the chemical structures, botanical origins, biosynthesis pathways or biological properties. The most phytochemical classification scheme is based on chemical structures such as phenolics, alkaloids, saponins, terpenoids, limonoids, polyacetylenes and secoiridoids and so on. Numerous studies have been conducted *in vitro* and *in vivo* in the recent years on the efficacy of plant phytochemicals as antimicrobial agents. This paper presents the antimicrobial properties of some major group of phytochemicals against pathogenic bacteria, fungi and virus, and beneficial microbes of the gastrointestinal tracts and their mechanism of action.

1.2 Phenolic Compounds

Phenolic compounds are a group of phytochemicals, which have a phenol structure, i.e. an aromatic benzene ring bearing at least one hydroxyl substituent (Robbins 2003; Vermerris and Nicholson 2006). Phenolic compounds are commonly found

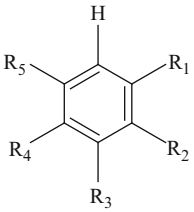
throughout the plant kingdom, where they protect the plants from microbial infections, ultraviolet radiation and chemical stressors. This large and diverse group of phytochemicals is classified into many subclasses depending upon chemical structures and occurrence in plants. The commonly categorized subclasses of phenolic compounds are simple phenolics (resorcinol and phloroglucinol), phenolic acids and aldehydes, coumarins, flavonoids, chalcones, aurones, benzophenones, xanthones, stilbenes, benzoquinones, naphthaquinones, anthraquinones, betacyanins, lignans, and polyphenols (proanthocyanidin, galloyl, hexahydroxydiphenyl ester, hydroxy cinnamic acid, and phloroglucinol derivatives) (Vermerris and Nicholson 2006; Handique and Baruah 2002). The detailed structures and chemistry of these phenolic compounds are presented elsewhere (Vermerris and Nicholson 2006). Foods containing phenolics are becoming an important part of diets due to their potential anti-oxidative properties. Besides, these compounds have also potent anti-microbial properties.

1.2.1 Phenolic Acids and Aldehydes

The phenolic acid and aldehyde group of phenolic compounds is characterized by the presence of a carboxylic acid or aldehyde group substituted on a phenol (Table 1.1; Vermerris and Nicholson 2006). The naturally occurring phenolic acids generally have two characteristic constitutive carbon frameworks: the hydroxycinnamic and hydroxybenzoic structures (Robbins 2003). Majority of cinnamic and benzoic acid derivatives in plants are linked through ester, ether or acetal bonds to structural components, polyphenols, organic acids (quinic, maleic, tartaric and shikimic acid), glucose and terpenes (Robbins 2003). Chlorogenic acid is an ester of quinic acid and caffeic acid. Some aldehyde analogues of phenols (e.g. vanillin) are also grouped with phenolic acids (Robbins 2003). The numbers and positions of the hydroxyl and other groups on the aromatic ring can produce a large number of compounds in this subclass (Robbins 2003; Vermerris and Nicholson 2006). Phenolic acids are present in a wide range of plants including in many common foods such as tea, coffee and berries. Besides, phenolic acids and aldehydes could be formed by the intestinal microbial biotransformation of other phenolic compounds in the intestine, where they may influence intestinal microbiota.

A number of simple phenols and phenolic acids possess antibacterial, antiviral and antifungal activities against a wide range of microbes, but at different concentrations. Gallic acid and *p*-hydroxybenzoic acid reduced the viability of *Camplylobacter jejuni* at concentrations as low as 1 mg/L (Ganan et al. 2009). Synaptic acid, vanillic acid, and caffeic acid were microbicidal at concentrations starting at 10 mg/L. Ferulic acid and cumaric acid were effective at a concentration of 100 mg/L (Ganan et al. 2009). Ozçelik et al. (2011) recently tested some phenolic acids such as gallic acid, caffeic acid, chlorogenic acid, and quinic acid for their *in vitro* antiviral, antibacterial, and antifungal activities. All these phenolic acids were inhibitory to herpes simplex virus type 1 (HSV-1), whereas gallic acid, chlorogenic acid and quinic acid showed potent antiviral effect against parainfluenza virus type 3 at the therapeutic range of 0.8–0.05 mg/L.

Table 1.1 Chemical structures of some phenolic acids found naturally in plants and foods (Robbins 2003; Vermerris and Nicholson 2006; Cueva et al. 2010)



Common name	R_1	R_2	R_3	R_4	R_5
Cinnamic acid	–CH.CH.COOH	H	H	H	H
p-Coumaric acid	–CH.CH.COOH	H	H	–OH	H
Ferulic acid	–CH.CH.COOH	H	$–OCH_3$	–OH	H
Sinapic acid	–CH.CH.COOH	H	$–OCH_3$	–OH	$–OCH_3$
Caffeic acid	–CH.CH.COOH	H	–OH	–OH	H
Benzoic acid	–COOH	H	H	H	H
Salicylic acid	–COOH	–OH	H	H	H
p-Hydroxybenzoic acid	–COOH	H	H	–OH	H
Vanillic acid	–COOH	H	$–OCH_3$	–OH	H
Syringic acid	–COOH	H	$–OCH_3$	H	$–OCH_3$
Protocatechuic acid	–COOH	H	–OH	–OH	H
Gentisic acid	–COOH	–OH	H	H	–OH
Gallic acid	–COOH	–OH	–OH	–OH	–OH
Veratric acid	–COOH	H	$–OCH_3$	$–OCH_3$	H
Syringealdehyde	–CHO	H	$–OCH_3$	–OH	$–OCH_3$
Vanillin	–CHO	H	$–OCH_3$	–OH	H
Phenylacetic acid	$–CH_2$.COOH	H	H	H	H
2-hydroxyphenylacetic acid	$–CH_2$.COOH	–OH	H	H	H
Phenylpropionic acid	$–CH_2.CH_2$.COOH	H	H	H	H
3-hydroxyphenylpropionic acid	$–CH_2.CH_2$.COOH	H	–OH	H	H
2-hydroxyacetophenone	–HCO	–OH	H	H	H

In general, antibacterial activity of phenolic acids is stronger against Gram-positive bacteria than Gram-negative bacteria (Merkl et al. 2010; Cueva et al. 2010). The outer membrane of Gram-negative bacteria provides them with a hydrophobic surface structure that is able to exclude certain hydrophilic molecules, making them inherently resistant to many antimicrobial agents including phenolic acids (Alakomi et al. 2007; Cueva et al. 2010). Gram-positive bacteria are enclosed in a plasma membrane covered by a thick peptidoglycan wall and lack an outer membrane (Alakomi et al. 2007; Cueva et al. 2010). Although, phenolic acids are effective against Gram-negative bacteria, their antimicrobial effect is strain dependent (e.g. different strains of *Escherichia coli*; Cueva et al. 2010).

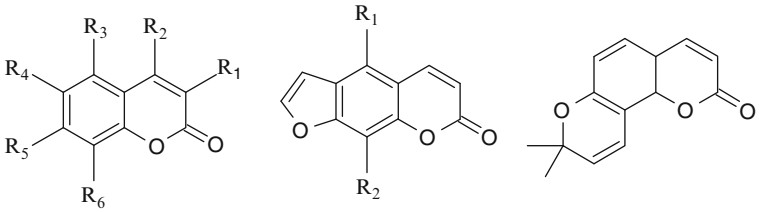
Phenolic compounds are usually poorly absorbed in the small intestine, and thus most of the dietary phenolics accumulate in the colon (Clifford 2004; van Duynhoven et al. 2011). Therefore, higher concentrations of phenolic acids may reach in the intestine than the concentrations in diets. Phenolics may selectively suppress or stimulate

the growth of certain members of intestinal microbiota, which may influence microbial population dynamics in the gastrointestinal tract (Tzounis et al. 2008). Chlorogenic, quinic and gallic acids stimulated growth of *Lactobacillus collinoides* relative to control cultures (no additive) up to concentrations of 1 g/L of tomato broth media. In contrast, growth of *Lactobacillus brevis* was little affected during early incubation, which has been suggested to be due to metabolism of these acids (Stead 1994).

From structure-activity relationship, phenols having different alkyl chain length with hydroxyl groups could be important for antimicrobial actions (Kubo et al. 1995). p-Hydroxybenzoic acid, protocatechuric, gentisic acid, vanillic acid, ferulic acid, caffeic acid and their methyl, ethyl, propyl and butyl esters were investigated for antibacterial action. It has been reported that the antimicrobial effect of phenolic acids derivatives increased with the increasing length of the alkyl chain (Merkl et al. 2010). The presence of hydroxyl groups on the phenol groups and oxidized status of phenol groups also determine the toxicity of microbes. The fluidity of the cell membrane could be disturbed with increasing hydrophobic alkyl chains. The phenolic acids could enter the molecular structure of the membrane with the polar hydroxyl group oriented into the aqueous phase by hydrogen bonding and nonpolar carbon chain aligned into the lipid phase by dispersion forces (Kubo et al. 1995). Thus, when the hydrophilic force exceeds hydrophobic one, the activity tends to disappear. Also, the number and position of substitutions in the benzene ring of the phenolic acids and the saturated side-chain length influenced the bacteriocidal effects of phenolic acids against the different microorganisms, but in different ways against Gram-positive and Gram-negative bacteria (Cueva et al. 2010). For example, Cueva et al. (2010) showed that for benzoic and phenylacetic acids, *E. coli* was inhibited in the following order of potency: non-substituted > 4-hydroxy-3-methoxy- > 3-hydroxy- > 4-hydroxy- > 3,4-dihydroxy-substituted acid. For phenylpropionic acids, the order differed slightly: nonsubstituted > 4-hydroxy- > 3-hydroxy- >3,4-dihydroxysubstituted acid. However, the potency of phenolic acids was in different order for *Lactobacillus* spp. For benzoic acids, the order of potency was: 4-hydroxy- > 3-hydroxy- > non-substituted > 4-hydroxy-3-methoxy- > 3,4-dihydroxy-substituted acids, except for *Lactobacillus coryniformis* CECT 5711 (4-hydroxy-> non-substituted > 3-hydroxy > 4-hydroxy-3-methoxy-substituted acids). For phenylacetic acids, growth inhibition of lactobacilli was on the order of non-substituted > 3-hydroxy- > 4-hydroxy- > 3,4-dihydroxy-substituted acids. For phenylpropionic acids, growth inhibition was as follows: non-substituted > 4-hydroxy- > 3-hydroxy > 3,4-dihydroxy-substituted acids, except for *Lactobacillus fermentum* CECT 5716 (3-hydroxy > non-substituted > 4-hydroxy- and 3,4-dihydroxy-substituted acids) and *Lactobacillus plantarum* LCH17 (non-substituted > 3-hydroxy- > 4-hydroxy-> 3,4-dihydroxy-substituted acids).

1.2.2 Coumarins

Coumarins are naturally found in many families of plants (Apiaceae, Asteraceae, Fabiaceae, Rosaceae, Rubiaceae, Rutaceae and Solanaceae) and microorganisms,



Coumarins with substituents in benzene or pyrone ring | Furocoumarins | Pyranocoumarins

Fig. 1.1 Basic chemical structures of different types of coumarins

and approximately 1,000 coumarins have been isolated from these sources (Weinmann 1997; Smyth et al. 2009). Coumarins can be classified into five groups depending upon the structure, i.e. coumarins with substituents in benzene ring, coumarins with substituents in pyrone ring, furocoumarins, pyranocoumarins, and coumarin dimmers (Fig. 1.1; Smyth et al. 2009).

Coumarins exhibit a broad diversity for antimicrobial activity. O-acetylcolumbianetin, edultin, cniforin A, columbianadin and imperatorin isolated from the fruits of *Cnidium monnieri* (L.) Cuss exerted a little to no appreciable growth-inhibition of Gram-positive and Gram-negative bacteria (Ng et al. 1996). An amino-coumarin – 7-amino-4-methylcoumarin showed broad-spectrum antibacterial and antifungal activities (Liu et al. 2008). Melliou et al. (2005) studied the antibacterial activity of pyranocoumarins using an agar disc diffusion method. Seselin, xanthyletin, 5-hydroxyseselin, and 7-hydroxyalloxanthyletin had no antibacterial effects. Coumarin derivatives such as 5-methoxyseselin and its brominated derivatives, alloxanthoxyletine, the acetylated derivatives, and dipetalolactone were active against all the tested bacteria. A seselin derivative, 3-bromo-4-benzoyloxyseselin showed moderate activity, while three coumarins containing acetoxy groups in pyrano ring were only active against the two Gram-positive bacteria. A new coumarin – cajanuslactone isolated from pigeon pea leaves showed anti-bacterial activity against *Staphylococcus aureus* (ATCC 6538), and the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were 0.031 and 0.125 mg/mL, respectively (Kong et al. 2010). Some seselin derivatives, including derivatives of 5-methoxyseselin, were found to be potent against human immunodeficiency virus (HIV) (Xie et al. 1999).

It has been suggested that the presence of oxygenated substituents in the ether or ester form usually enhances the antibacterial activity, while the presence of free hydroxyl group reduces the activity (Melliou et al. 2005). This fact could be at least partially attributed to the reduced lipophilicity of the hydroxyl derivatives, which hinders the penetration through the bacterial cell wall (Melliou et al. 2005).

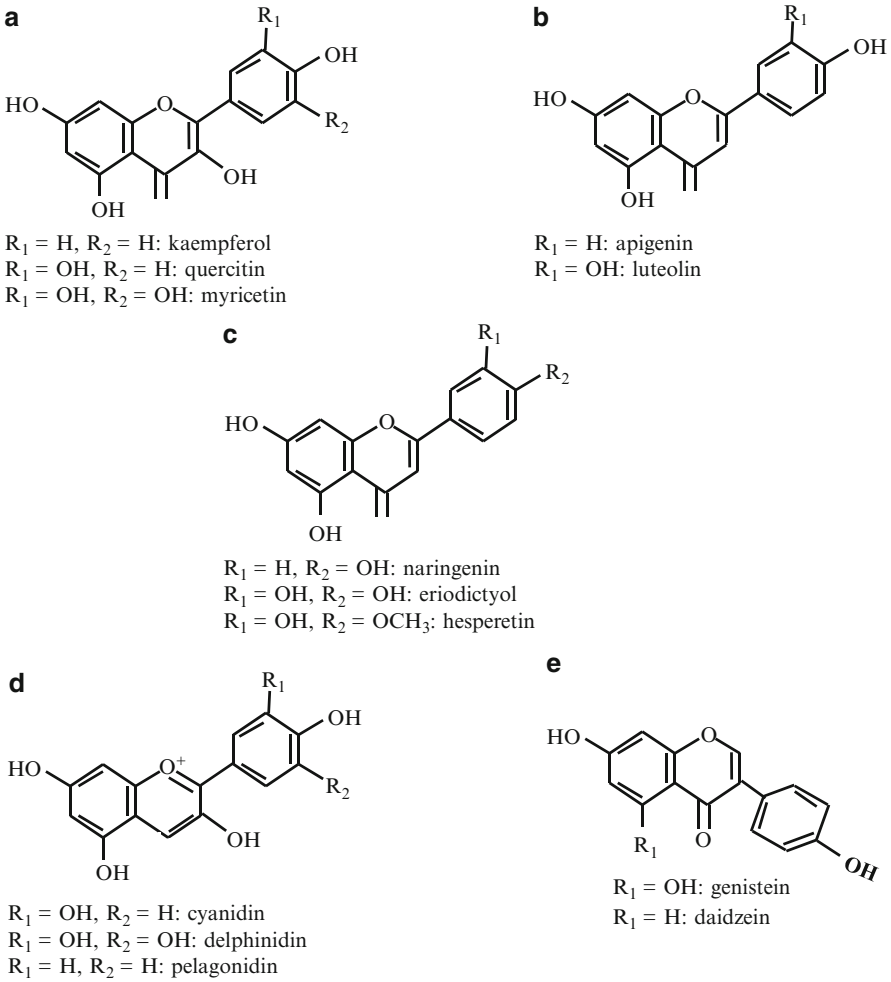


Fig. 1.2 The chemical structures of flavonoids; (**a**) flavonol, (**b**) flavone, (**c**) flavanone, (**d**) anthocyanidins and (**e**) isoflavone

1.2.3 Flavonoids

Flavonoids are one of the largest groups of secondary metabolites that are distributed in various plant species. They have significant antioxidant properties, which are beneficial for health. These polyphenolic compounds are constructed basically with an A and C ring of benzo-1-pyran-4-quinone and a B ring. The main classes of flavonoids (Fig. 1.2) are (1) flavones (basic structures), e.g. luteolin, apigenin, diosmetin, chrysoeriol, tangeretin, sinensetin, gardenin, vitexin and baicalein; (2) flavonols

(having a hydroxyl group at the 3-position), e.g. kaempferol, quercetin, galangin, datiscetin, morin, robinetin, isorhamnetin, tamarixetin, quercetagetin and myricetin; (3) flavanones (2–3 bond saturated), e.g. hesperetin, taxifolin, eriodictyol and naringenin; (4) flavan-3-ol, e.g. catechin and epicatechin; (5) isoflavone, e.g. genistein, daidzein and coumestrol; (6) anthocyanidins: cyanidin, delphinidin, pelargonidin and peonidin (Crozier et al. 2006). The majority of flavonoids commonly remain conjugated with sugars as glycosides.

Numerous flavonoid derivatives showed antiviral activity against a wide range of viruses such as HSV, HIV, coxsackie B virus, coronavirus, cytomegalovirus, poliomyelitis virus, rhinovirus, rotavirus, poliovirus, sindbis virus, and rabies virus (De Bruyne et al. 1999; Evers et al. 2005; Nowakowska 2007). Ozçelik et al. (2011) investigated the effects of quercitin, apigenin, genistein, naringin, silymarin and silibinin against HSV-1 and PI-3 virus. All flavonoids inhibited HSV-1 activity, but only genistein inhibited parainfluenza type-1 (PI-1) activity. Of the three flavonoids (baicalin, rutin and naringin) examined by Ng et al. 1996, baicalin was found to be the most potent in inhibiting the growth of *S. aureus*: 11 of the 16 strains tested were inhibited at 128 mg/L. However, no inhibitory activity of rutin and naringin against *S. aureus* was observed at 128 mg/L. At this concentration, naringin and baicalin inhibited two strains and rutin inhibited one strain of the eight *P. aeruginosa* strains tested. The flavonoids compounds display different mode of antiviral action. For instance, baicalein probably block human cytomegalovirus infection at entry level while the primary mechanism of action for genistein may be to block immediate-early protein functioning off human cytomegalovirus (Evers et al. 2005). Both these flavoinoids did not inhibit the virus replication (Evers et al. 2005).

Puupponen-Pimia et al. (2001) investigated 13 falovonoid compounds (apigenin, (+)-catechin, chlorogenic acid, cyanidin chloride, delphinidin chloride, isoquercitrin, kaempferol, cyanidin-3-glucoside (kuromanin), luteolin, myricetin, pelargonidin chloride, quercetin dehydrate and rutin trihydrate), and 4 phenolic acids (caffeic acid, 3-coumaric acid, ferulic acid, trans-cinnamic acid) on 7 Gram-positive lactic acid bacteria of intestines, Gram-negative *E. coli* CM 871 and *Salmonella*. Myrecetin strongly inhibited the growth of *Lactobacillus* as well as *E. coli*, but did not affect *Salmonella*. Luteolin was weakly inhibitory to Gram-positive lactic acid bacteria but not to Gram-negative bacteria. The anthocyanidins pelargonidin, delphinidin and cyanidin, as well as cyanidin-3-glucoside, only inhibited growth of *E. coli* and had no effect on other bacterial strains (Puupponen-Pimia et al. 2001). However, phenolic acids did not inhibit lactic acid bacteria, but inhibited Gram-negative *E. coli* and *Salmonella* sp.

Hatano et al. (2005) discussed that some prenylated flavonoids such as licoricidin (an isoflavan) effectively suppressed the antibiotic resistance of methicillin-resistant *S. aureus* (MRSA) compared to other flavonoids. The addition of 4 μg/mL of licoricidin shifted the MIC of oxacillin from 128–256 to 8–16 μg/mL, and 8 μg/mL of licoricidin reduced it to less than 0.5 μg/mL. The requirement for dimethylallyl or equivalent substituents suggests the importance of affinity for the bacterial cell membrane.

Phenolic acids show greater antimicrobial potency than their corresponding flavonoids precursors such as the monomers (+)-catechin and (−)-epicatechin (Ganan et al. 2009; Cueva et al. 2010). Therefore, microbial transformations of dietary flavonoid compounds in the intestine could lead to more potent microbial-inhibitory compounds (phenolic acids) and could reach greater concentrations in the intestine. This may selectively influence intestinal bacteria species, and therefore could affect the diversity and metabolic activity of the intestinal microbiota, including the transformation of phenolics in the gut (Cueva et al. 2010).

Epigallocatechin gallate exerted strong antibacterial growth against Gram-positive bacteria than against Gram-negative bacteria (Yoda et al. 2004; Engels et al. 2009). It has been stated that Gram-positive bacteria absorb more epigallocatechin gallate into their peptidoglycan cell wall and aggregate its presence, while Gram-negative bacteria do not aggregate and absorb less epigallocatechin gallate (Ikigai et al. 1993; Engels et al. 2009) because of the repulsive negative charge of lipopolysaccharides on the surfaces of Gram-negative bacteria. The binding of epigallocatechin gallate to peptidoglycan disrupts its function in osmotic protection, cell division, and cell wall biosynthesis (Yoda et al. 2004). Detailed information of antimicrobial activities of flavonoids has been discussed elsewhere in this book (Chap. 2).

1.2.4 Polyphenols

Some phenolic acids (ellagic and gallic acids) or flavonoids (flavan-3-ol, flavan-3-4-diol or flavan-4-ol) in plants are esterified or polymerized into dimeric, oligomeric or polymeric compounds. Most abundantly present polyphenolic compounds in plants are tannins, which are usually of two types: hydrolysable tannins (HT) and condensed tannins (CT). The HT are complex molecules with a polyol as a central core such as glucose, glucitol, quinic acids, quercitol and shikimic acid that is partially or totally esterified with a phenolic group, i.e. gallic acid (3,4,5-trihydroxy benzoic acid; gallotannins) or gallic acid dimmer hexahydroxydiphenic acid (ellagitannins) (Haslam 1989). The CT (proanthocyanidins) are mainly polymers of the flavan-3-ols (epi)catechin and (epi)gallocatechin units, which are linked by C4-C8 and C4-C6 interflavonoid linkages (Ferreira et al. 1999; Hagerman and Butler 1989).

The polyphenols also exert a wide range of antibacterial and antifungal activities. Ellagitannin extracts inhibited a range of pathogenic organisms including *Vibrio cholerae, Shigella dysenteriae* and *Campylobacter* spp. (Silva et al. 1997; Puupponen-Pimia et al. 2002). Puupponen-Pimia et al. (2005) reported that berry extracts exhibit selective inhibitory properties against intestinal bacteria such as *Staphylococcus, Salmonella, Listeria* and *Lactobacillus* strains, and the selective inhibitory actions varied with berry extracts. In general, pathogenic *Staphylococcus* and *Salmonella* were sensitive to various berry extracts and ellagitannins fractions, while pathogenic *Listeria* and beneficial *Lactobacillus* were not inhibited.

Rauha et al. (2000) studied antimicrobial effects of some berry extracts against food spoilage and poisoning bacteria. The widest antibacterial activity was present in berries belonging to the genus *Rubus* (cloudberry and raspberry) that are rich in ellagitannins. Ellagic acid has been reported to exhibit a dose-dependent inhibitory effect (IC50 = 1 mM) on *Helicobacter pylori* isolated from peptic ulcer patients (Chung 1998). Tannins isolated from *Dichrostachys cinerea* roots exerted antimicrobial effects against *S. aureus*, *E. coli*, *Shiegella* spp. and *P. aeruginosa* with MIC of the tannins ranging between 4.0 and 5.5 mg/mL, while the MBC ranging between 4.5 and 6.0 mg/mL (Banso and Adeyemo 2007). Gallotannins extracted from the mango seed kernel inhibited the growth of Gram-positive food spoilage bacteria and decreased the growth of Gram-negative *E. coli*, but did not affect lactic acid bacteria (Engels et al. 2009). The antibacterial properties of cranberry juice with inhibition of *E. coli* adherence to mucosal surfaces by cranberry juice is reported to be associated with the presence of proanthocyanidins (Howell et al. 1998).

Many polyphenols have antiviral activities against different types of viruses (De Bruyne et al. 1999; Cheng et al. 2002). It has been suggested that prodelphinidin B-2 3′-*O*-gallate (a proanthocyanidin gallate isolated from green tea leaf) showed anti-HSV-2 properties with the mechanism of inhibiting the attachment and penetration between cells and viruses possibly through the instability of viral glycoproteins (Cheng et al. 2002). The structure and functional groups of the polyphenol compounds may determine the effectiveness of the antiviral activities (De Bruyne et al. 1999).

The content of small-molecular phenolic compounds have greater influence on the antibacterial activity of extracts than tannins (Nazaruk et al. 2008). Thus, polyphenols could be cleaved by bacterial enzymes to form a number of phenolic acids in the intestine, where they may influence the microbial populations (Bock and Ternes 2010). Engels et al. (2009) recently studied the effects of gallotannins with different galloyl units from mango seed kernel on various Gram-positive and Gram-negative bacteria. Gallotannins showed antibacterial activities with MICs ranging from 0.1 g/L for *S. aureus* to 3.3 g/L for *Pediococcus acidilactici*. They also observed that degree of galloylation did not affect the growth of bacteria. It has been suggested that the antibacterial activities of gallotannins are due to their strong affinity for iron and the inactivation of membrane-bound proteins (Engels et al. 2009). It has also been shown that gallotannins changed the morphology of *Bacillus subtilis*, which has been hypothesized due to inhibition of cell division by binding of gallotannins to the cell wall or inhibition of enzymes involved in cell separation (Engels et al. 2009).

1.2.5 Naphthoquinones

Naphthoquinones are widely distributed in plants, fungi, and some animals. Lapachol, plumbagone, juglone and lawsone are naturally occurring naphthoquinones

of plant origin that have antimicrobial effects against various pathogenic bacteria and fungi. Adeniyi et al. (2000) reported that two dimeric naphthoquinones, diospyrin and isodiospyrin, isolated from the root of *Diospyros piscatoria* (Gurke), a common ingredient in several folk medicines, exhibited a broad spectrum of antibacterial activity against *S. pyogenes* and *S. pneumoniae* (MICs of diospyrin ranged from 1.56 to 50 μg/mL) *Salmonella choleraesuis* serotype *typhi* (*S. typhi*) and *Mycobacterium chelonae* (MICs of diospyrin were between 25 and 100 μg/mL). Isodiospyrin was more active than its racemic isomer diospyrin (MICs against Gram-positive bacteria ranged from 0.78 to 50 μg/mL, while those against *Pseudomonas aeruginosa* and *S. typhi* ranged from 50 to 100 μg/mL). Another naphthoquinones, lapachol and β-lapachone, found in species of *Tabebuia,* had relevant effects against *Candida albicans, Candida tropicalis,* and *Cryptococcus neoformans,* and were more active than the reference standard, ketoconazole. Lapachone showed strong antimicrobial activity than lapachol against the fungi (Guiraud et al. 1994). Methanol extract from the dried inner bark of *Tabebuia impetiginosa* exhibited potent antibacterial activity against *H. pylori* which contained lapachol and anthraquinones (Park et al. 2006).

1.3 Alkaloids

Alkaloids have been defined as N-heterocyclic basic metabolites, although the definition does not clearly separate from other N-containing compounds. Alkaloids have been classified in many ways depending upon biogenic precursors or carbon skeleton characteristics. They have a great structural diversity compared with other classes of phytochemicals. Alkaloids are generally known according to their carbon skeleton structures. Pyridine (e.g. piperine), piperidine, quinoline, indole, pyrrolidine, quinazoline, isoquinoline, glyoxaline, lupinane, tropan, phenanthridine, imidazoline, alkaloidal amines and terpenoid types of alkaloids are commonly found in plants (Hegnauer 1988).

Alkaloid fractions isolated from *Strychnos potatorum* L.f. (Loganiaceae) seeds, which were of indole type, were tested for their antimicrobial properties against some pathogenic Gram-positive, Gram-negative and acid-fast bacteria and fungi. These fractions had shown considerable antimicrobial activity against both bacteria and fungi at the tested concentrations (100 and 200 μg/mL). Further, the growth of *Proteus vulgaris*, *S. aureus, Salmonella typhimurium, Vibrio cholerae, Mycobacterium tuberculosis, Aspergillus niger* and *C. albicans* were significantly inhibited (Mallikharjuna and Seetharam 2009). Similarly, two benzophenanthridine alkaloids, dihydrochelerythrine and dihydrosanguinarinealkaloid constituents of *Bocconia arborea* showed considerable antimicrobial activity against Gram-positive and Gram-negative bacteria and *C. albicans* (Navarro and Delgado 1999).

Sensitivity of DNA and RNA viruses to alkaloids may differ. Ozçelik et al. (2011) investigated various alkaloids namely yohimbine and vincamine (indole-type), scopolamine and atropine (tropane-type), colchicine (tropolone-type), allantoin

(imidazolidine-type), trigonelline (pyridine-type) as well as octopamine, synephrine, and capsaicin (exocyclic amine-type) for their antiviral activities against DNA virus herpes simplex (HSV-1) type 1 and RNA virus parainfluenza type-3 (PI-3). All the alkaloids were effective against HSV-1 at 0.05–1.6 mg/L, but atropin and octopamine showed potent antiviral activities against PI-3 at 0.05–0.8 mg/L (Ozçelik et al. 2011). Antibacterial alkaloids from *Chelidonium majus* Linn, i.e. benzo[c] phenanthridine-type alkaloids, 8-hydroxydihydrosanguinarine, 8-hydroxydihydrochelerythrine were potently active against MRSA strains with MICs/MBCs ranged from 0.49 to 15.63 and 1.95 to 62.50 μg/mL, respectively (Zuo et al. 2008).

1.4 Organosulphur Compounds

There are two rich sources of organosulphur compounds from plants; (1) Alliaceae family containing alliin-alliinase system and (2) Cruciferae (Brassicacae) family e.g. *Brassica juncea*, *Wasabia japonica* (wasabi), *Armoracia rusticana* (horseradish) and *Brassica oleracea* (cauliflower) containing glucosinolate-myrosinase (Mithen 2006). A number of sulphur-containing compounds can be derived from these plants through the action of myrosinase and alliinase enzymes.

1.4.1 Thiosulfinate

The primary sulphur-containing constituents in *Alliums* spp. (e.g. *A. sativum* (garlic), *A. cepa* (onion), *A. porrum* (leek)) and *Brassica* spp. (e.g. cabbage, kale, cauliflower and turnip) are *S*-alk(en)yl-L-cysteine sulphoxides and γ-glutamyl-*S*-alk(en)yl-L-cysteine sulphoxides (Block et al. 1992; Ross and Milner 2007; Fig. 1.3). The content of S-alk(en)yl-L-cysteine sulphoxides in garlic may range from 0.53% to 1.3% of fresh weight with *S*-allyl-L-cysteine sulphoxide (alliin) being the largest contributor. By the action of alliinase enzyme present inside the cells, these compounds are converted into thiosulfinate (a functional group consisting of the linkage R-S(=O)-S-R'), which are then spontaneously and enzymatically converted into a large array of volatile compounds, e.g. diallyl disulphide, diallyl trisulphide, allyl methyl disulphide and dipropyl and disulphide (Mithen 2006).

Antimicrobial activities of garlic and onion against a wide range of Gram-positive and Gram-negative bacteria, virus and fungi are known for many years (Ankri and Mirelman 1999). The antifungal activities of garlic oils appear to be more than the antibacterial activity (Avato et al. 2000). Extracts of garlic exhibit the most potent antibacterial activity, followed by onion, and *Brassica* including cabbage (Kyung and Lee 2001). The principal antimicrobial compounds of *Allium* and *Brassica* are allicin (*S*-allyl-L-propene thiosulfinate) and methyl methanethiosulfinate, respectively (Kyung and Lee 2001). These compounds are derived from *S*-allyl and *S*-methyl derivatives of L-cysteine sulfoxide, respectively. Avato et al. (2000)

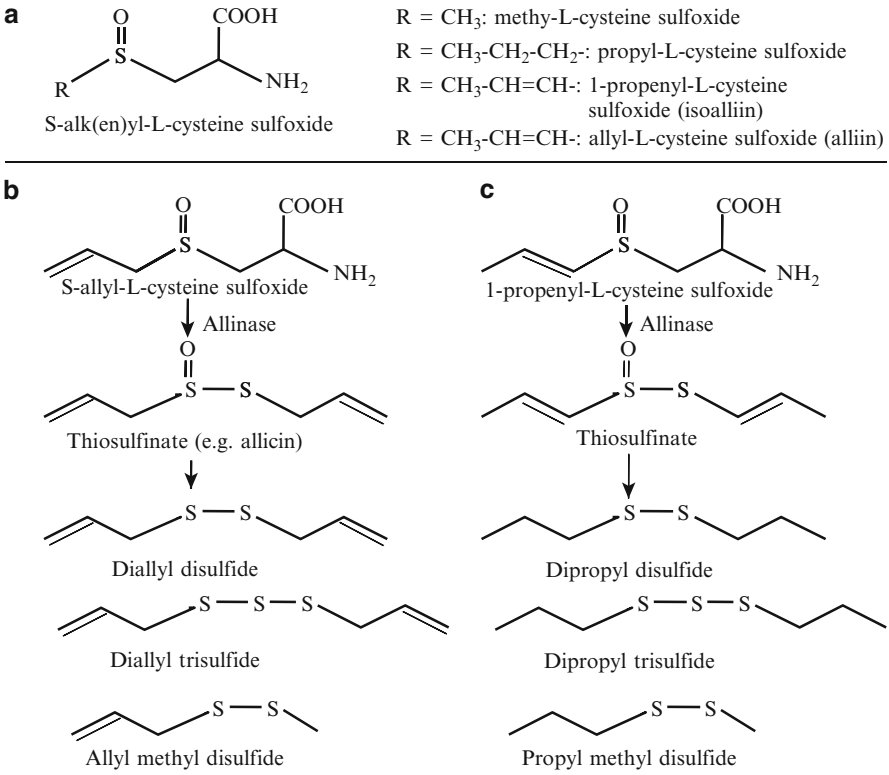


Fig. 1.3 Major thiosulfinate compounds found in Alliaceae family: (**a**) organosulfur compounds in intact plants, (**b**) compounds Produced from allyal cystein sulfoxide (in garlic) and (**c**) 1-propenyl cystein sulfoxide (in onion) by aliinase

tested different mixtures of garlic distilled oils containing diallyl disulfide (DDS) and diallyl trisulfide (DTS), ranging from 1% to 51% and 88% to 38%, respectively, against yeasts (*C. albicans, C. tropicalis* and *B. capitatus*), Gram-positive bacteria (*S. aureus* and *B. subtilis*) and Gram-negative bacteria (*P. aeruginosa* and *E. coli*). Incubation of garlic extracts made up of 1% DDS and 88% DTS did not show growth inhibition against all the tested microorganisms, whereas garlic oils with higher quantities of DDS showed significant inhibitory activity, increasing with the increase of DDS amount, thus implicating the DDS as the active antimicrobial agent (Avato et al. 2000). It has been reported that allicin (MIC, 6 μg/mL; MBC, 6 μg/mL) was more potent than DDS (MIC range, 100–200 μg/mL; MBC range, 100–200 μg/mL), its corresponding sulfide, but of a strength similar to that of diallyl tetrasulfide (MIC range, 3–6 μg/mL; MBC range, 3–6 μg/mL) against *H. pylori* (O'Gara et al. 2000). Kyung and Fleming (1997) investigated the different S-compounds found in cabbages on the growth of 15 bacteria and 4 fungi. *S*-Methyl-L-cysteine sulfoxide, sinigrin, and dimethyl sulfide at 500 ppm did not inhibit the

growth of any of the bacteria and yeasts. Dimethyl disulfide at 500 ppm retarded the growth of some bacteria, but was not bactericidal to any of the test microorganisms. Dimethyl trisulfide, methyl methanethiosulfinate and methyl methanethiosulfonate had MICs of 200 ppm, between 50 and 200 ppm, and between 20 and 100 ppm, respectively for bacteria, and 20 ppm, between 6 and 10 ppm and between 50 and 500 ppm for yeasts, respectively (Kyung and Fleming 1997).

There are numerous reports showing the effectiveness of garlic or allicin as antimicrobial agents in comparison to antibiotics (Fujisawa et al. 2009; Cai et al. 2007). Also, allicin with antibiotics may synergistically augment the antimicrobial actions (Cai et al. 2007; An et al. 2009). Besides, thiosulfinates and their derivatives show promising activity against multidrug resistant bacteria including MRSA (Ankri and Mirelman 1999; Fujisawa et al. 2009). The main mode of action of thiosulfinate derivatives have been proposed to be due to its chemical reaction with the thiol groups of various enzymes (Ankri and Mirelman 1999) and thus antimicrobial properties of allicin may be abolished by cysteine, coenzyme A and glutathione (Fujisawa et al. 2009). Antimicrobial activity of the diallyl sulfides has been reported to increase with the number of sulfur atoms (O'Gara et al. 2000).

1.4.2 Glucosinolates

Glucosinolates are the sulphur-containing metabolites found in large number of edible plants. Over 120 glucosinolates are present in 16 families of dicotyledonous angiosperms, most of which are clustered within the Brassicaceae and Capparaceae (Fahey et al. 2001). Allyl (sinigrin) and 3-butenyl (gluconapin) glucosinolate are found in brown mustard, *p*-hydroxybenzyl glucosinolate in white mustard, allyl and other glucosinolate in horseradish and wasabi, methylthiopropyl in cabbage and 2-hydroxy 3-butenyl glucosinolate in rapeseed (Fig. 1.4; Fahey et al. 2001; Mithen 2006).

The antibacterial and antifungal properties of glucosinolates are known for a long time (Fahey et al. 2001). Intact glucosinolates do not show antimicrobial action, but the hydrolysis products of glucosinolates are active against various microorganisms (Manici et al. 1997; Tierens et al. 2001). Aires et al. (2009a) observed that the *in vitro* growth inhibition and the sensitivities of the individual bacteria are influenced by the structure of glucosinolates and their hydrolysis products. The most effective glucosinolate hydrolysis products were the isothiocyanates; sulforaphane and benzyl isothiocyanate were the strongest inhibitory against the growth of human pathogenic bacteria. Regarding action of glucosinolates products on the type of bacteria, 4-methyl sulfinyl butylisothiocyanate exhibited antibacterial activity against a larger range of bacteria. Indole-3-carbinol had some inhibitory effects against the Gram-positive bacteria, but had no effect against the Gram-negative bacteria. Indole-3-acetonitrile had some inhibitory activity against the Gram-negative bacteria. Glucosinolates, nitriles and amines were ineffective at the doses up to 3 μmol (Aires et al. 2009b). Saavedra et al. (2010) evaluated the *in vitro* antibacterial actions of

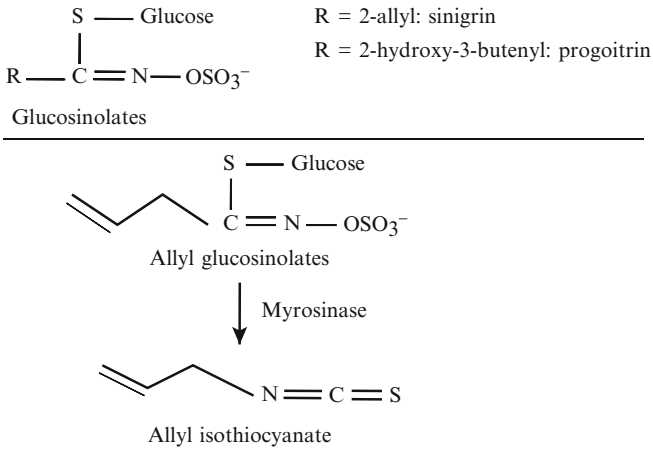


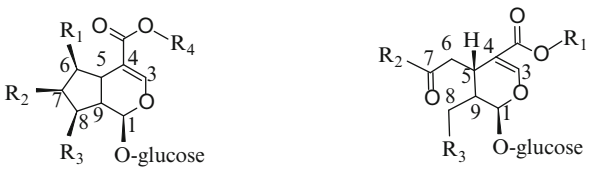
Fig. 1.4 Common glucosinolates found in Brassicaceae family: (**a**) in intact plants and (**b**) enzymatic conversion of allyl glucosinolate to allyl isothiocyanate, a potent antibacterial compound

different classes of common dietary phytochemicals, i.e. simple phenolics – tyrosol, gallic acid, caffeic acid, ferulic acid, and chlorogenic acid; chalcone – phloridzin; flavan-3-ol – (−) epicatechin; secoiridoid – oleuropein glucoside; glucosinolate hydrolysis products – allyl isothiocyanate, benzyl isothiocyanate and 2-phenylethyl isothiocyanate) against four pathogenic microbes. All of the isothiocyanates had significant antimicrobial activities, while the phenolics were much less efficient. No antimicrobial activity was observed with phloridzin. Allyl isothiocyanate from cabbage had an MIC between 50 and 500 ppm for bacteria and between 1 and 4 ppm for yeasts (Kyung and Fleming 1997).

1.5 Iridoids and Secoiridoids

Iridoids is a group of cyclic monoterpenoids having iridane skeleton (cis-2-oxabicyclo-(4.3.0)-nonane), which mostly remain as glycosides (Fig. 1.5; Perez et al. 2005). Secoiridoids derive from iridoids by the elimination of the link 7–8 to yield the basic structure (Perez et al. 2005). This group of phytochemicals is found in a number of folk medicinal plants and many of them possess significant biological and pharmacological activities (Dinda et al. 2009).

A number of iridoids and secoiridoids (nepetalactones from Serbian *Nepeta* species**,** Nestorović et al. 2010; plumericin and isoplumericin from the stem-cut latex of *Himatanthus sucuuba*, Silva et al. 2010; Cantleyoside dimethyl acetal from the aerial parts of *Pterocephalus perennis*; Graikou et al. 2002**)** from different plants (Chinese medicinal plant *Cymbaria mongolica*, Dai et al. 2002; aerial parts of the



Iridoids

Loganin,

R_1=H, R_2=OH, R_3=CH_3, R_4=CH_3

Loganic acid,

R_1=H, R_2=OH, R_3=H, R_4=H

Secoiridoids

Secologanin,

R_1=CH_3, R_2=H, R_3=CH_2

Fig. 1.5 Structures of iridoids and secoiridoids

Argentinean plant *Caiophora coronata,* Khera et al. 2003; aerial parts of *Verbena littoralis* (Verbenaceae), Castro-Gamboa and Castro 2004; roots of *Patrinia rupestris,* Yang et al. 2006b) have been reported to have antibacterial and antifungal properties. Three iridoids, phloyoside1, phlomiol, and pulchelloside 1, isolated from the rhizomes of the Iranian flora *Eremostachys laciniata* (Lamiaceae) had low to moderate levels of antibacterial activity (MIC = 0.05–0.50 mg/mL) against five bacterial strains, *Bacillus cereus*, *Citrobactor freundii*, *Proteus mirabilis*, *P. aeruginosa*, *S. aureus* (Modaressi et al. 2009). Out of these three compounds, pulchelloside 1 showed highest antibacterial activity against *B. cereus*, penicillin- resistant *E. coli*, *P. mirabilis* and *S. aureus* with an MIC value of 0.05 mg/mL.

Nestorović et al. (2010) investigated the nepetalactones content in the methanol extracts of the shoot cultures of three endemic Serbian Nepeta species: *Nepeta rtanjensis*, *N. sibirica* and *N. nervosa*, and evaluated the antimicrobial activity of these extracts against eight bacterial strains *E. coli*, *P. aeruginosa*, *S. typhimurium*, *Listeria monocytogenes*, *Enterobacter cloacae* (human isolate), *B. cereus* (clinical isolate), *Micrococcus flavus* and *S. aureus,* and eight fungal species: *Aspargillus flavus*, *Aspargillus fumigatus*, *Aspargillus niger*, *Fusarium sporotrichoides*, *Fulvia fulvum, Penicillium funiculosum, P. ochrochloron* and *Trichoderma viride*. *Trans, cis*-nepetalactone was present in shoots of *N. rtanjensis*, while *cis,trans*-nepetalactone stereoisomer was present in *N. sibirica*. No nepetalactone was observed in shoots of *N. nervosa*. All these extracts had significant antibacterial and antifungal activities against all the tested species. *N. rtanjensis* extract showed the strongest antibacterial activity with MIC of 50 μg/mL. *N. nervosa* and *N. sibirica* extracts showed antibacterial activities with MIC of 50–100 and 100 μg/mL, respectively. Similarly, *N. rtanjensis, N. nervosa* and *N. sibirica* extracts showed MIC of 25–5, 50–100 and 25–100 μg/mL, respectively. The presence of trans-nepetalactone in *N. rtanjensis* extract was probably responsible for strongest activity against bacteria and fungi, while cis-nepetalactone in *N. sibirica* extract showed higher antibacterial and antifungal activity than that of *N. nervosa* extract.

Iridoids compounds also exhibit potent antiviral action. A number of swerilactones, which are secoiridoids, isolated from endemic Chinese herb *Swertia mileensi* exhibited significant *in vitro* anti-hepatitis B virus activity on the Hep G 2.2.15 cell line with IC_{50} values ranging from 1.53 to 5.34 μM (Geng et al. 2009a, b, 2011). Iridoid aglycone moieties, but not its glycosides, exhibit the antiviral activities. Zhang et al. (2009) studied an anti-hepatitis C virus pseudoparticles (HCVpp) entry essay on both aqueous and methanol extracts of the flowering tops of *Lamium album*. Iridoid glucoside lamalbid isolated from the methanol extract was inactive against HCVpp, whereas its aglycone, and epimers named lamiridosins A and B present as major constituents in the aqueous extract significantly inhibited *in vitro* HCV entry (IC50 value of 2.31 μM). These were nontoxic to the Hep G2 2.2 cells at a concentration of 50 μg/mL. They also demonstrated that the parent iridoid glycosides did not show anti-HCV entry activity, but the aglycones of shanzhiside methyl ester, loganin, loganic acid, verbenalin, eurostoside and picroside II exhibited significant anti-HCV entry and anti-infectivity activities.

1.6 Saponins

Chemically, saponins are a group of high molecular-weight glycosides, in which saccharide chain units (1–8 residues) are linked to a triterpene (triterpene saponins) or steroidal (steroid saponins) aglycone moiety, i.e. sapogenin (Fig. 1.6). They occur in a wide variety of plants with triterpene saponins (in soybean, alfalfa, quillaja, and guar), and are more widely distributed in nature than steroidal (in yucca, tomato, and oats) saponins (Hostettmann and Marston 1995). The steroidal saponins may possess furostanol or spirostanol (e.g. smilagenin and sarsapogenin) moiety. The saccharide chains are commonly attached at the C3 position (monodesmosidic), but some sapogenins contain two saccharide chains (bidesmosidic) attached at the C3 and C17 (via C28) position (Vincken et al. 2007). A large number of saponins could be possible depending upon the modifications of the ring structure of aglycone moieties and number of sugars added to it, and in turn producing different biological properties.

Many plant extracts containing saponins from various plants and purified saponins show antimicrobial activities at different concentrations (Sen et al. 1998; Avato et al. 2006). However, the types of saponins exhibit different spectra of antimicrobial effects. Oleanolic acid isolated from the root bark of *Newbouldia laevis* have broad-spectrum antimicrobial activity against 6 Gram-positive, 12 Gram-negative bacterial species and three *Candida* species (Kuete et al. 2007). β-sitosterol-3-*O*-β-d-glucopyranoside isolated from this plant also showed antibacterial effects on three Gram-positive, six Gram-negative bacterial species and three *Candida* species. A saponin fraction from the stem of *Y. schidigera* exhibited potent growth-inhibitory activity with MIC ranging from 31.3 to 125 μg/mL against certain food-deteriorating yeasts (*C. albicans*), film-forming yeasts (*Debaryomyces hansenii, Pichia nakazawae, Zygosaccharomyces rouxii*), dermatophytic yeasts

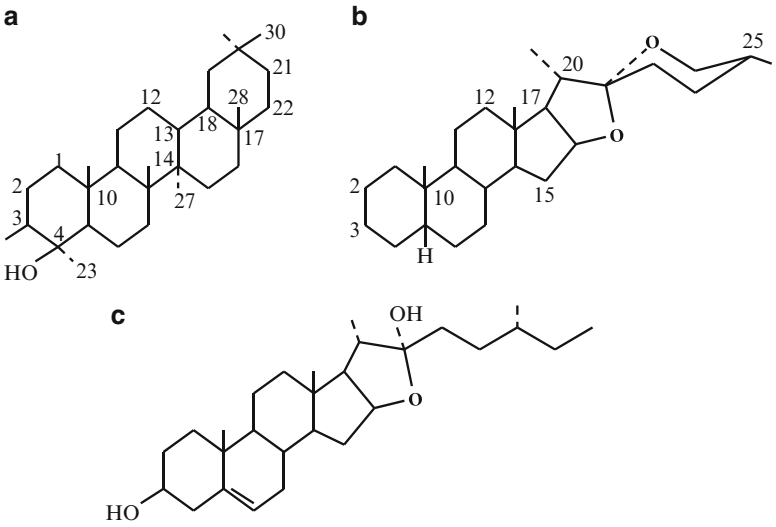


Fig. 1.6 Chemical structures of sapogenins: triterpenoid, for example, oleanane (**a**); steroids, for example, spirostanol (**b**) and furostanol (**c**)

(*Candida famata, Hansenula anomala, Pichia carsonii*), and against brewer's yeast (*Saccharomyces cerevisiae*) (Miyakoshi et al. 2000). Different saponins, i.e. tigogenin from *Tribulus terrestris*, dioscin from the rhizomes of *Smilacina atropurpurea*, minutosides from bulb of *Allium leucanthum* were very active against different fungal strains such as *C. albicans*, *C. glabrata* and *Cryptococcus neoformans* (Zhang et al. 2006a, b; Barile et al. 2007). Saponins appear to have stronger activities against fungi, and act by disrupting the membrane integrity of fungal cells.

Different extraction procedures and storage may affect the antimicrobial action of saponins probably due to chemical transformation of saponins (Guclu-Ustundag and Mazza 2007). Commercially produced quillaja (*Quillaja saponaria*) and yucca (*Yucca schidigera*) saponins showed different antibacterial activities against *E. coli*, suggesting that saponins from various commercial sources differ in their biological activities (Sen et al. 1998). In this study, commercial saponin-rich quillaja and yucca extracts exhibited antibacterial activity against *S. aureus* and *E. coli* at different concentrations. The antimicrobial activity of saponins may also be modified by the pH of media. The tea saponins exhibited greater antimicrobial activities against Gram-positive *S. aureus* (MIC <0.006 vs. >0.2), Gram-negative *E. coli* (MIC 0.003 vs. >0.2) and *C. albicans* (MIC <0.006 vs. >0.2) at low pH 4 than high pH 8.5 (Li et al. 2009).

Some saponins, in general, exhibit stronger antimicrobial activity against Gram-positive bacteria than against Gram-negative bacteria (Avato et al. 2006). Saponins fraction from soapnut pericarps (*Sapindus mukurossi*, Tanaka et al. (1996) and guar (*Cyamopsis tetragonoloba*, Hassan et al. 2010a, b) showed greater antibacterial activity against Gram-positive bacteria than against Gram-negative

bacteria. Conversely, saponins isolated from orchid tree (*Bauhinia variegata* L.) bark exhibited greater antibacterial activity for Gram-negative bacteria than Gram-positive bacteria at concentrations ranging from 2.5 to 10 mg/mL (Morrissey and Osbourn 1999).

The relationships between saponin structures and antimicrobial activity are strongly noted. The structure of sapogenin moiety, chain length and composition of sugars influences the antimicrobial activities. The *Y. schidigera* saponin fraction possessing a trisaccharide chain without any oxygen functionalities at C-2 and/or C-12 of the aglycone exhibited potent anti-yeast activity, while saponins with 2b-OH or 12-keto groups showed very weak or no activity. Low activity was observed for saponins with a disaccharide chain and no activity was observed for the aglycones obtained after acid hydrolysis (Miyakoshi et al. 2000). Yang et al. (2006a) noted that no activity was observed in the hecogenin saponins when its sugar moiety was less than four monosaccharide units. Pentaglycoside was more active than tetraglycoside and shows extended antifungal spectrum against *A. fumigatus*. In the diosgenin saponin series, saponins with only triglycosides are active against *C. albicans* and *C. glabrata*, while the diosgenin saponins with monoglycoside and diglycoside did not show any activity. Again, within the group of tigogenin saponins, their antifungal capacity was slightly influenced by the composition of the sugar moiety. The replacement of a glucosyl unit with a xylosyl unit showed enhanced activity against *A. fumigatus*. Avato et al. (2006) suggested that the sugar moiety is not important for the antimicrobial efficacy from their study since antibacterial activity increased from the saponin extracts to the sapogenin samples.

1.7 Terpenoids/Essential Oils

Terpenoid compounds derive from a basic structure of C5 isoprene units. They are classified according to the number of isoprene unit involved for their synthesis, i.e. monoterpenoid (C10), sesquiterpenoids (C15), diterpenoids (C20), sesterterpenoids (C25) and triterpenoids (C30). They can be acyclic (myrcene and geraniol), monocyclic (cymene and carvacrol), bicyclic (pinene) and tricyclic with different groups (alcohol, phenol, and aldehyde). The most commonly occurring essential oils (EO) are included in two chemical groups (Fig. 1.7): terpenoids (monoterpenoids and sesquiterpenoids) and phenylpropanoids, which are synthesized through mevalonate and shikimic acid metabolic pathways, respectively (Gershenzon and Croteau 1991; Calsamiglia et al. 2007). Among these two classes, terpenoids are the more diversified group of plant bioactives abundantly found in many herbs and spices (Gershenzon and Croteau 1991). Within terpenoids, the most important components of EO of the majority of plants belong to the monoterpenoids and sesquiterpenoids (Gershenzon and Croteau 1991; Calsamiglia et al. 2007). Phenylpropanoids have a side chain of three carbons bound to an aromatic ring of C6 (Calsamiglia et al. 2007). Phenylpropanoids are less abundant compounds of EO compared with terpenoid family, but some plants contain in significant proportions. The EO are a group of

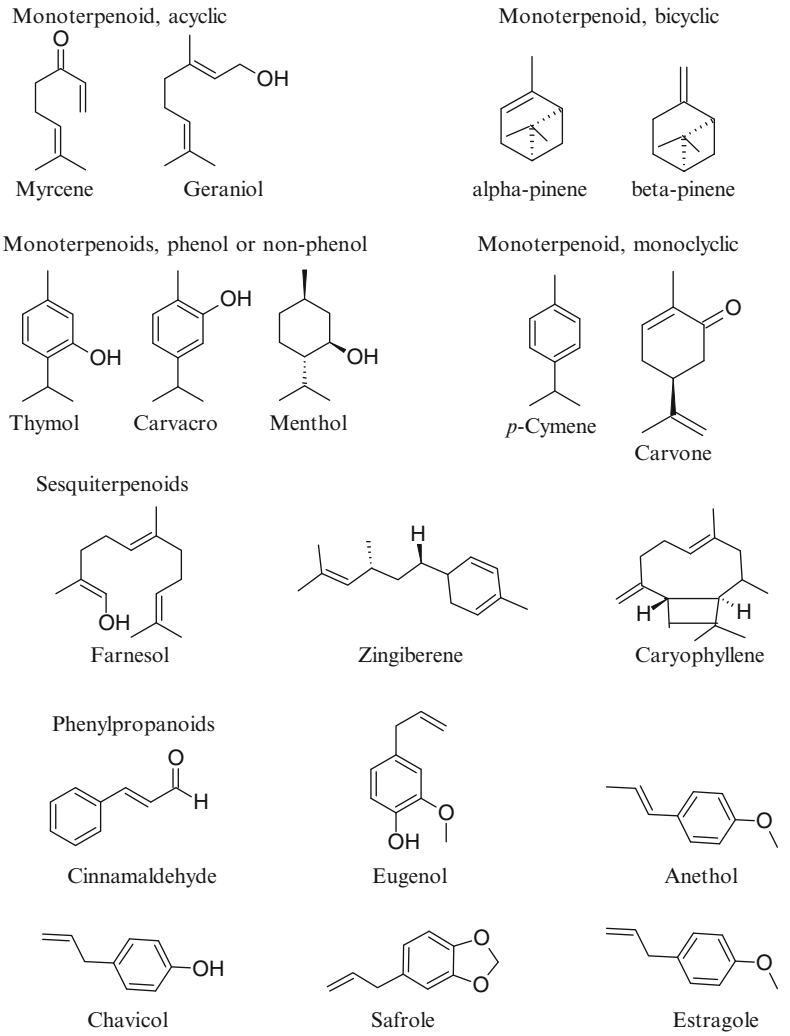


Fig. 1.7 Chemical structures of different components of essential oils

secondary plant metabolites obtained from volatile fractions of plants by steam distillation process (Gershenzon and Croteau 1991). The EO are used traditionally by humans, for many centuries, which provide characteristic flavor and aroma specific to many plants, and are used as antimicrobial agents and preservatives. The EO have diverse chemical composition, nature and biological properties. The EO can be obtained from flowers, petals, leaves, stems, fruits, roots and barks and the concentrations of EO in these parts depends upon the stage of growth, environmental conditions (Hart et al. 2008).

A number of EO are known for their strong anti-microbial activities against many pathogenic and non-pathogenic bacteria and fungi. Curcumin and its derivatives,

the phenylpropanoids, are the principal compounds in rhizome of *Curcuma longa* (turmeric), which exhibit antibacterial properties against different bacteria and fungi. Essential oil fractions of turmeric inhibited the growth of pathogenic Gram-positive (*S. aureus* and *Staphylococcus epidermidis*) and Gram-negative (*E. coli*, *P. aeruginosa* and *S. typhimurium*) bacteria (Singh et al. 2002). The EO fraction was more effective against Gram-positive compared to Gram-negative strains, and was comparable to standard antibiotics gentamycin, ampicillin, doxycycline and erythromycin in these strains (Singh et al. 2002). A recent study by De et al. (2009) demonstrated that curcumin inhibited the growth of different clinical isolates of *H. pylori* with MICs ranging from 5 to 50 μg/mL. The gingerols, another phynylpropanoids from *Zingiber officinalis* (zinger), possess antifungal and antibacterial properties (Park et al. 2008). Ginger extract containing gingerol inhibited the growth of *H. pylori* with MICs ranging from 0.8 to 12.5 μg/mL (Mahady et al. 2003).

Constituents of EO differ in their antimicrobial activity against bacteria and fungi. Investigating the antimicrobial properties (18 bacterial species and 12 fungi) of five EO constituents (cineole, citral, geraniol, linalool and menthol), Pattnaik et al. (1997) showed that linalool had the most antibacterial activity and inhibited 17 bacteria, followed by cineole, geraniol (each of which inhibited 16 bacteria), menthol and citral aromatic compounds, which inhibited 15 and 14 bacteria, respectively. However, the antifungal activities of these EO constituents did not follow the pattern of antibacterial activities. Citral and geraniol oils were the most effective against fungi (inhibiting all 12 fungi), followed by linalool (inhibiting 10 fungi), cineole and menthol (each of which inhibited 7 fungi) compounds (Pattnaik et al. 1997).

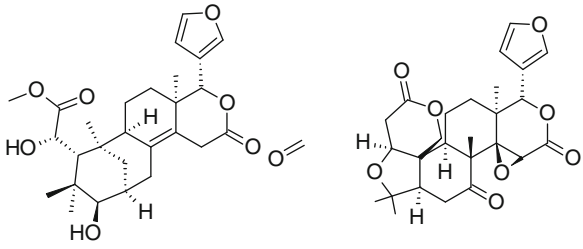
It has been suggested that the pH of EO in culture media may modify antimicrobial properties. For example, anise oil had higher antifungal activity at pH 4.8 than at 6.8, while the oil of *Cedrus deudorawas* was most active at pH 9 (Janssen et al. 1987). The structure and stereochemistry of the essential oils have profound influences on the antimicrobial activities. Alkenyl substituents incorporated into nonphenolic ring structures of essential oils such limonene showed increased antibacterial activities compared with alkyl substituents such as *p*-cymene with alkylation showing more inhibitory effect on Gram-negative bacteria (Dorman and Deans 2000). From stereochemistry of EO, it has been reported that α-isomers such as α-pinene are less active relative to β-isomers such as geraniol and nerol; *cis*-isomers are inactive contrary to *trans*-isomers; compounds with methyl-isopropyl cyclohexane rings are the most active; or unsaturation of the cyclohexane ring further increases the antibacterial activity, e.g. terpinolene, terpineol and terpineolene (Hinou et al. 1989; Dorman and Deans 2000). However, Griffin et al. (1999) reported that the specificity and level of antimicrobial activity of terpenoids were not always characterized by the functional groups, but were associated with hydrogen-bonding parameters, and for Gram-negative organisms a combination of hydrogen-bonding parameters and molecular size parameters. The antimicrobial properties of EO from different sources have been discussed in details elsewhere (Chap. 5).

1.8 Limonoids (Tetranortepenoids)

Chemically, limonoids are unique secondary metabolites, characterized by a tetranortriterpenoid skeleton with a furan ring (Fig. 1.8). They are commonly isolated from Citrous and Maliaceae plants (Hallur et al. 2002; Rahman et al. 2009; Vikram et al. 2010). Besides their health promoting effects, various limonoids have been shown to possess antibacterial, antifungal and antiviral effects (Govindachari et al. 2000; Battinelli et al. 2003; Atawodi and Atawodi 2009).

Various limonoid compounds such as mahmoodin, azadirone, epoxyazadiradione, nimbin, gedunin, azadiradione, deacetylnimbin and 17-hydroxyazadiradione, isolated from various parts of *Azadirachta indica* (Meliaceae family) have been reported to have antimicrobial activities (Siddiqui et al. 1992; Govindachari et al. 2000; Atawodi and Atawodi 2009). Rahman et al. (2009) tested two limonoids isolated from the seeds of *Swietenia mahagoni* (Meliaceae family), swietenolide and 2-hydroxy-3-O-tigloylswietenolide against various multiple-drug-resistant bacterial strains including Gram-positive (*S. aureus, S. pneumoniae* and *Haemophilus influenzae*) and Gram-negative (*E. coli, Klebsiella pneumoniae, Salmonella typhi,* and *Salmonella paratyphi*) strains. The most potent activity of swietenolide was observed against *H. influenzae*, *S. typhi*, and *S. paratyphi*, whereas 2-hydroxy-3-O-tigloylswietenolide was most active against *S. pneumoniae, S. typhi,* and *S. paratyphi*. The lowest activity was observed against *K. pneumoniae* for both compounds. The limonoids compounds may exhibit antibacterial properties against pathogenic bacteria by disrupting the quorum sensing system and biofilm production. Vikram et al. (2010) demonstrated limonin, nomilin, obacunone, deacetyl nomilin and limonin 17-O-β-D-glucopyranoside purified from seeds of grapefruits to possess the anti-quorum sensing activity and inhibitory effect on biofilm formation of pathogenic *E. coli* O157:H7 with obacunone exhibiting strong antagonistic activity.

Limonoids also have significant antiviral activity. Limonin and nomilin showed inhibitory effects on HIV-1 replication in peripheral blood mononuclear cells and monocytes/macrophages, which was not cytotoxic at the active concentrations (Battinelli et al. 2003). The antiviral activity was not much influenced by structural differences by limonin and nomilin in this study (Battinelli et al. 2003).



Swietenolide Limonin

Fig. 1.8 Chemical structures of limonoid compounds

Parida et al. (2002) demonstrated in an *in vivo* study that azadirachtin obtained from *A. indica* inhibited dengue virus type-2 replication as confirmed by the absence of dengue-related clinical symptoms in sucking mice and absence of virus specific 511 bp amplicon.

1.9 Polyacetylenes

More than 700 polyacetylene compounds have been characterized from plants, which are mainly prominent in the Asteraceae, Apiaceae and Campanulaceae including many medicinal plants from various parts of the world (Hudson 1989). Food plants of the Apiaceae plant family such as carrots, celery, parsley, fennel and parsnip contain a group of bioactive aliphatic C17-polyacetylenes including falcarinol, falcarindiol, panaxydiol, and polyacetylene 8-O-methylfalcarindiol (Zidorn et al. 2005; Christensen and Brandt 2006).

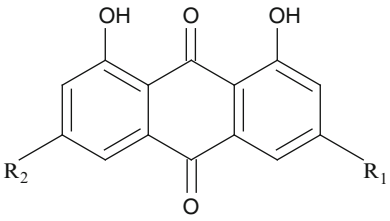
Avato et al. (1997) investigated the different polyacetylene compounds from the aerial organs of *Bellis perennis* L. Of the major constituents, methyl deca-4,6-diynoate and deca-4,6-diynoic acid, and their structural analogues, i.e. deca-4,6-diyne, dimethyl octa-3,5-diyne-1,8-dioate and deca-4,6-diyne-1,10-dioic acid, deca-4,6-diynoic acid and deca-4,6-diyne-1,10-dioic acid showed antimicrobial activity against Gram-positive and Gram-negative bacteria, respectively.

Polyacetylene carboxylic acids, 13(*E*),17-octadecadiene-9,11-diynoic acid (13,14-dihydrooropheic acid, and the known 17-octadecene-9,11,13-triynoic acid (oropheic acid, isolated from the stem bark of *Mitrephora celebica* demonstrated significant activity against MRSA and *Mycobacterium smegmatis* (Zgoda et al. 2001). Similarly, pentayne diol, a polyacetylene which was isolated from *Bidens pilosa* (a traditional medicinal herbs) showed highly potent and extensive inhibitory activities against several Gram-positive and Gram-negative pathogenic bacterial species, including MRSA, and vancomycin-resistant *Enterococcus faecalis* and *C. albicans* (Tobinaga et al. 2009). In a recent finding, a polyacetylene compound from *Carlina acaulis*, i.e. carlina oxide exhibited strong antibacterial activity against two MRSA strains, *Streptococcus pyogenes, P. aeruginosa, C. albicans*, and *C. glabrata* with less toxicity to human HeLa cells (Herrmann et al. 2011).

1.10 Anthranoids

Anthranoid compounds are widely distributed in various plants particularly in *Aloe*, *Cassia, Rheum*, *Cassia* and *Frangula*, which are traditionally used in ethnomedicine for laxative and cathartic action (Paneitz and Westendorf 1999). Naturally occurring anthranoids can be chemically described as dihydroxyanthraquinones, -dianthrones and -anthrones, often present in plants as glycones (Table 1.2; Paneitz

Table 1.2 Chemical structures of some anthranoids (Paneitz and Westendorf 1999)



Anthranoids	R1	R2
Emodin	CH_3	OH
Rhein	COOH	H
Aloe-emodin	CH_2OH	H
Physcion	CH_3	OCH_3
Chrysophanol	CH_3	H

and Westendorf 1999). Different anthranoids such as aloe-emodin, rhein, emodin, physcion and chrysophanol occur in *Rheum* species.

Anthranoids have shown antimicrobial properties in different studies. The anthranoid compounds from the rhizome of *Rheum emodi* exhibited antibacterial and antifungal activities (Babu et al. 2003). The antimicrobial effects of the three anthraquinones on *S. aureus* found to be in the order of rhein > emodin > 1,8-dihydroxyanthraquinone (Wu et al. 2006). Similarly, Wang et al. (2010) demonstrated that the sequence of antimicrobial activity against *Bifidobacterium adolescentis* of the five hydroxyanthraquinones was rhein > emodin > aloe-emodin > chrysophanol > physician. They also suggested the influence of substituent groups on phenyl ring in hydroxyanthraquinones against *B. adolescentis* activity might be related with the polarity and the sequence was carboxyl > hydroxyl > hydroxylmethyl > methyl and methoxyl. Prenylated anthranoids from leaves of *Harungana madagascariensis* have shown to inhibit *Bacillus megaterum* (Kouam et al. 2007). Additionally, the effect of emodin with antibiotics (ampicillin and oxacillin) was found to be synergistic or partially synergistic against MRSA, where emodin reduced the MICs of the antibiotics (Lee et al. 2010). However, some of the anthranoids have potent mutagenic effect (Paneitz and Westendorf 1999), which is required to consider when evaluating the antimicrobial properties of these compounds.

1.11 Conclusions and Future Prospects

There is considerable evidence that a number of phytochemicals have potential to become useful antimicrobial agents that could be employed as preventative or treatment therapies against microbial and viral diseases. Although, there are some encouraging effects *in vivo* to inhibit pathogenic microbes without affecting beneficial bacteria in the gastrointestinal tracts, more studies would be required for the

safety and efficacy of these phytochemicals to establish whether they could offer therapeutic benefits over conventional therapies.

Besides, the combination of some antimicrobial drugs and phytochemicals may act as better antimicrobial agents than antimicrobial drugs alone. For example, the application of dual combinations demonstrated synergy between streptomycin and gallic acid, ferulic acid, chlorogenic acid, allylisothiocyanate and 2-phenylethylisothiocyanate against the Gram-negative bacteria. Moreover, they can act synergistically with less efficient antibiotics to control bacterial growth (Saavedra et al. 2010). 3,4-dihydroxyphenylacetic acid and 3-hydroxyphenylacetic acid increased the susceptibility of *S. enterica* subsp. *enterica* serovar Typhimurium strains for novobiocin. In addition, organic acids present in berries, such as malic acid, sorbic acid, and benzoic acid, were shown to be efficient permeabilizers of *Salmonella* as shown by an increase in the 1-N-phenylnaphthylamine uptake assay and by lipopolrsaccharide release (Alakomi et al. 2007). Cinnamon essential oil and its major component (trans-cinnamaldehyde) enhanced the antibacterial activity of clindamycin against a toxicogenic strain of *Clostridium difficile* (Shahverdi et al. 2007). In addition, the enhancement activity of different essential oils (*Mentha longifolia* L. and *Mentha spicata* L.) and different monoterpenes (piperitone, carvone and menthone) on the antibacterial activity of nitrofurantoin has been reported (Rafii and Shahverdi 2007; Shahverdi et al. 2004). The antibacterial activity of cefixime, cephotaxime, vancomycin and tetracycline was also increased by curcumin (Moghaddam et al. 2009). Allicin has a synergistic effect with amphotericin B against *C. albicans* via enhancing the phospholipid peroxidation reaction *in vitro* and *in vivo*, which suggests that allicin could reduce the amphotericin B dose to lessen side effects (An et al. 2009). Due to the growing use of phytochemicals and other dietary phytochemical-rich supplements, it is required to understand whether problems might arise from using these preparations in combination with conventional drugs. There is lack of comprehensive studies that can establish the consequences of phytochemicals-drug interactions. However, all these evidence also suggest that intake of phytochemicals rich foods could be considered in future research while antimicrobial agents are applied to the body.

Plant genomes contain 20,000–60,000 genes, and about 15–25% of these genes encode enzymes for secondary metabolism (Bevan et al. 1998; Somerville and Somerville 1999). The genome of a plant species encodes only a small fraction of all the enzymes that are required to synthesize the entire set of secondary metabolites found throughout the plant kingdom (Pichersky and Gang 2000). Identification of particular genes for target phytochemicals and the genetic engineering techniques could allow expressing the biosynthetic pathways of some phytochemical synthesis in organisms such as *E. coli*, *B. subtilis* or *S. cerevisiae*. For example, Miyahisa et al. (2006) reported that introduction of four genes for a phenylalanine ammonia-lyase, cinnamate/coumarate:CoA ligase, chalcone synthase, and chalcone isomerase, in addition to the acetyl-CoA carboxylase, in *E. coli* cells resulted in efficient production of (2S)-naringenin from tyrosine and (2S)-pinocembrin from phenylalanine. Finally, the possibility of using phytochemicals as antimicrobial compounds would be a paradigm shift towards the potential health benefits and safety overcoming the problem of microbial resistance to drugs.

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Chapter 2
Antimicrobial Properties of Flavonoids

Luisa Pistelli and Irene Giorgi

Abstract Flavonoids are a class of plant constituents that have received increasing interest over the last decades. This chapter deals with the antimicrobial activity of some natural flavonoids or extracts rich in these constituents reported in the literature during the last 5 years.

An introduction explains briefly the chemical structure of this class of natural compounds, their biosynthesis, plant sources and health benefits. Then the most significant articles from the scientific literature are reported, divided into two sections: studies on flavonoids with antibacterial and antifungal activities, respectively. In each paragraph the papers are listed according to the chemical complexity of the flavonoid structures, from the simplest to the most complex ones, both aglicones and glycosides and often gathering together the articles according to the main microbial target. A paragraph on the antimicrobial activity of combination of different flavonoids or between flavonoids and antibiotics (synergic effect) is also present. For many of the flavonoids cited the MIC values of their activity were also reported.

The chemical structure of the majority of the compounds cited in the chapter are pictured in figures; tables have been compiled summarising the most important information reported in this chapter.

Keywords Flavonoids • Antibacterial activity • Antifungal activity • MRSA strains • Synergistic effect

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2.1 Introduction

Flavonoids are members of a class of natural compounds that have received increasing interest over the last decades. They form the largest group of natural products known so far, where thousands of flavonoids have been isolated and identified, majority of which are from food plants. They occur virtually in all parts of the plant but, as for all secondary metabolites, quantitative distribution varies among different organs of the plant and within different populations of the same plant species. Many elements such as genetic factors, maturity, climate, position of the plant, degree of exposure to light and agricultural practices contribute to these differences, but the strong tendency for taxonomically related plants to produce similar types of flavonoids indicates that genetic factors are the most important ones (Havsteen 2002).

Concerning flavonoids, up to now many physiological functions for plants have been found. A well-known physiological function of anthocyanins, flavones and flavonols is the recruitment of pollinators and seed dispersers. Many flavonoids can act as ovoposition stimulants to the insects that lay their eggs on plants; these compounds can act also as feeding attractants because many insect larvae eat leaves and other vegetal food. On the contrary, in some plants flavonoids are feeding deterrents against harmful insects. (Harborne and Baxter 1999; Havsteen 2002).

Allelopathy is a biological phenomenon by which an organism produces one or more biochemicals that influence, positively or negatively, the growth, survival, and reproduction of other organisms. In plants one can find allelopathy as interactions between higher plant and higher plant, or between microorganism and higher plant. In this second case the compounds produced from plants are called prohibitins or phytoalexins. Flavonoids have been reported as allelochemicals in both cases (Ivashina 2003). Flavonoids have not been found to be directly involved in photosynthesis, but they play a role in gene regulation and growth metabolism. Furthermore, one of their most important functions is to be UV protectors, as shown in survey of some plants. As "early" steps in the biosynthesis of flavones, flavanones, and flavonols are found even in bryophytes (mosses), it has been suggested that these compounds may have evolved first to provide chemical messengers and then as UV sunscreens (Stafford 1991).

2.2 Chemical Structures and Biosynthesis of Flavonoids

Flavonoids are a group of low-molecular-weight polyphenolic substances. Their chemical structure is built upon a C_6–C_3–C_6 skeleton in which the three-carbon bridge is usually cyclised with oxygen. Several classes are distinguished according to the degree of unsaturation and oxidation of the three-carbon segment. Under each class a large number of compounds have been identified in plants that differ in the position, number and nature of substituents on the rings. Flavan skeleton consists of three rings referred to as the A, B, and C (Fig. 2.1). In flavonoids, the benzene ring

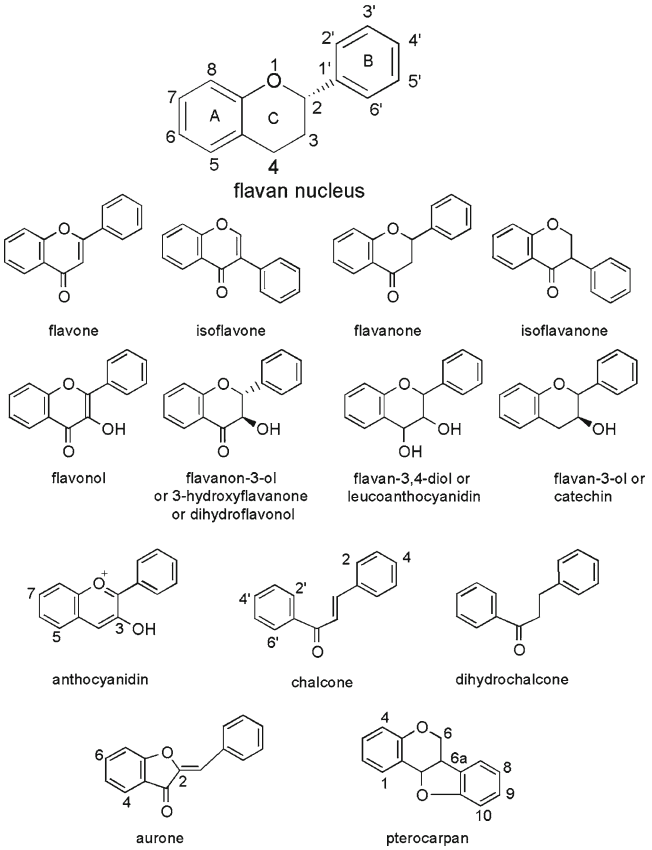


Fig. 2.1 Skeleton structures of the principal classes of flavonoids

A is condensed with the ring C, which in the 2-position carries a phenyl ring (B) as a substituent. In isoflavonoids, ring B is substituted with ring C in position 3 instead of position 2. Ring C may be a heterocyclic pyran, as in flavanols (catechins) and anthocyanidins; or pyrone, as in flavonols, flavones, and flavanones. If ring C is a furanone ring, compounds take the name of aurones, if rings A and B are connected by an 1-oxypropene or 1-oxypropane fragment, the compounds are called chalcone or dihydrocalchone, respectively. Four condensed rings produce a pterocarpane structure (Fig. 2.1).

Flavonoids are often named by trivial names, generally related to the name of the plant they were isolated from for the first time. The range of the trivial names is immense and can cause confusion, but fortunately, excellent summaries have been compiled (Swain 1976; Wollenweber and Dietz 1981).

For the reason that flavonoids are responsible for the major yellow, red, blue, and purple pigments in plants, a great deal of attention has been paid to flavonoids over the years. A wealth of information has been collected on the structures, chemical

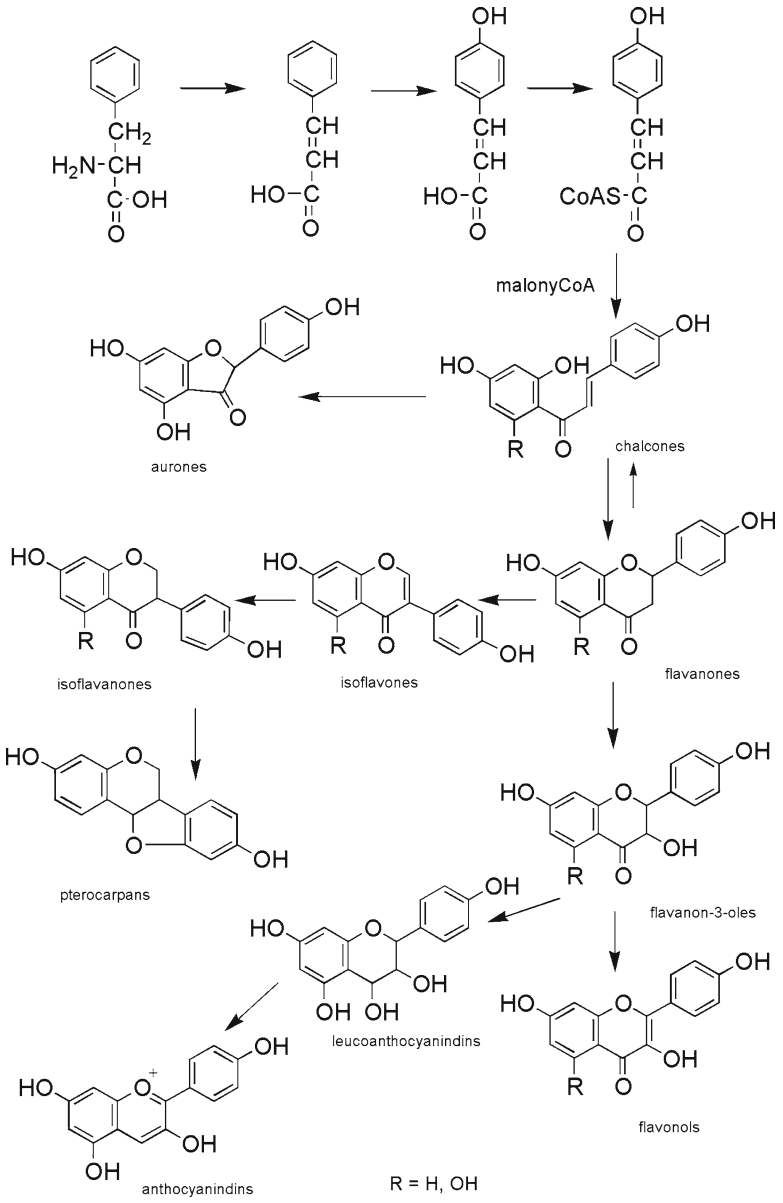


Fig. 2.2 Biosynthetic pathway of the most important classes of flavonoids

activities, and biosynthesis of these compounds, starting from the first description of acid and base effects on plant pigments by Robert Boyle in 1664 and continues to the present days. Regarding their biosynthesis, flavonoids are derived from phenyl- and malonyl-coenzyme A and it appears that branches in this pathway have evolved many times or have been lost from specific plant lineages over the course of evolution

(Winkel-Shirley 2001). In fact, flavonoids are considered as chemotaxonomic markers for the classification of plants. They are formed from the combination of derivatives synthesized from phenylalanine (via the shikimic acid pathway) and acetic acid. Phenylalanine is transformed into trans-cinnamic acid, which is then hydroxylated to *p*-coumaric acid (C-9) that condenses with three malonyl-CoA units to form a C-15 chalcone. Various subsequent ring closures, reductions and dehydrations give rise to different classes of flavonoids (Fig. 2.2). Final modifications involve the addition of further hydroxyl, methoxyl, methyl, sulphate, prenyl, methylanedioxyl, isoprenyl or other groups. Additional structural complexity is introduced by the common occurrence of flavonoids as glycosides. The glycosylation of a phenolic or alcoholic hydroxyl group of a flavonoid aglycone can occur through the hemiacetal bond (O-glycosides) or straight attachment to the C-1 of the sugar via a carbon-carbon bond (C-glycosides). A few monosaccarides, such as D-glucose and L-rhamnose, which are the most common, and the other less frequent, such as galactose, xylose and arabinose, or combination of them (di- and trisaccharides) can bind at different positions on the flavonoid aglycone; so the enormous number of flavonoids found in nature is also due to the innumerable combinations between flavonoid aglycones and sugar units (Andersen and Markham 2006).

2.3 Natural Sources and Health Benefits of Flavonoids

Several subgroups of flavonoids have been found in higher plants: chalcones, flavones, flavonols, flavandiols, anthocyanins, proanthocyanidins; the aurones (see Fig. 2.1) are widespread, but not ubiquitous. Particular classes of flavonoids, such as isoflavonoids (see Fig. 2.1), have been found in legumes and in a small number of non-legume plants. Flavonols (see Fig. 2.1) are the most abundant flavonoids in foods, of which quercetin, kaempferol, and myricetin are the three most common. Flavanones (see Fig. 2.1) are mainly found in citrus fruits and flavones are most commonly found in celery. Catechins (see Fig. 2.1) are present in large quantities in green and black teas, and in red wine; whereas, anthocyanins (see Fig. 2.1) are found in strawberries and other berries. Isoflavones are almost exclusively found in legumes. Flavonoids are almost unavoidable components in human and animal diet as they abundant in fruits, vegetables, seeds, nuts, grains, spices (USDA Database 2007). As flavonoids cannot be synthesised by humans and animals, those found in animals are considered to originate from the plants that animals have eaten. In humans, the dietary sources can differ significantly depending on the country of origin. Flavonoid intake is also influenced by factors such as feeding habits, preferences and cultural differences; accurate estimation of the average dietary intake of flavonoids is difficult, because of the wide varieties of available flavonoids and their extensive distribution in various foods, and also the diverse consumption of humans. Thus, the measurement of the dietary intake of flavonoids depends entirely on the criteria of the survey, the method used, and the reference compounds selected for analysis. So, the dietary intake of flavonoids, that has been estimated to vary from

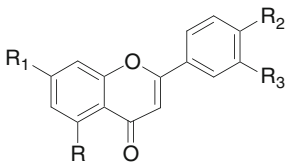
100 to 1,000 mg/day, can be easily increased (Aherne and O'Brien 2002). Apart from plants, other very important sources of flavonoids are propolis and honey, which are also used in many herbal and insect preparations in traditional medicine. In more recent years some flavonoids have been reported in the scientific literature to possess a variety of biological activity. They may act as antioxidants to inhibit free-radical mediated cytotoxicity and lipid peroxidation, or as antiproliferative agents to inhibit tumor growth. They have also been studied as weak estrogen agonists or antagonists to modulate endogenous hormone activity. Hence, flavonoids may confer protection against chronic diseases such as atherosclerosis and cancer, and may assist in the treatment of menopausal symptoms. Thus, flavonoids have been referred to as semiessential food components and are present in a number of diet supplements. Flavonoids have received close attention as potential natural source of new drugs because of other numerous biological properties in man; in particular their activity as inhibitors of enzymes (such as NADH-oxidase, xanthine oxidase, adenosine deaminase, a number of kinases, yaluronidase); as platelet adhesion inhibitors and procoagulant agents; as smooth muscle antispasmolitic agents and anti-inflammatory agents has been found (Harborne and Williams 2000; Havsteen 2002). So a number of medical applications have been studied including hypertension, inflammation, rheumatic diseases, diabetes mellitus, allergy and asthma, cancer, cardiovascular diseases, gastrointestinal ulcers, hypercholesterolemia, bacterial and viral infection, HIV, wound healing (Havsteen 2002).

2.4 Flavonoids as Antibacterial

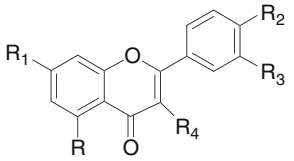
In the past 60 years, antibiotics have been crucial in the fight against infectious diseases caused by bacteria and other microbes. Antimicrobial chemotherapy has been a leading cause of rise in average life expectancy in the Twentieth Century in developed countries, but infectious diseases are again the main cause of premature deaths in developing countries. Mass population movements have made the risk higher of diffusion of noxious bacteria. Furthermore, nowadays diseases can be easily and quickly carried from one continent to another through international air travel; hence, no country is safe from the threat of infectious diseases. This is happening at a time when the number of drugs available is being progressively diminished due to the increasing resistance of microbes to antimicrobial drugs. In fact diseases caused by microbes that have become resistant to antibiotic drug therapy are an increasing public health problem. One part of the problem is that bacteria and other microbes that cause infections are remarkably resilient and have developed several ways of resisting antibiotics and other antimicrobial drugs. Another part of the problem is due to the increasing use, and misuse, of existing antibiotics in human and veterinary medicine and in agriculture. All these problems have led to increase research and development for new antibiotics in order to maintain a pool of effective drugs at all times. Flavonoids are a class of compounds intensively studied for this purpose.

Plants produce a variety of chemicals to survive attacks by microbial invasion (Grayer and Harborne 1994). These metabolites are either preformed in the plant (prohibitins) or induced after infection (phytoalexins). Since phytoalexins can also be induced by abiotic factors such as UV irradiation, they have been defined as antibiotics formed in plants via a metabolic sequence induced either biotically or in response to chemical or environmental factors. Many of these substances have been identified as flavonoids (Cowan 1999). Extraction of flavonoids and identification of their antimicrobial activity is useful not only for finding new drugs but also for obtaining natural products useful as food additives to improve the shelf life and safety of foods. In fact, an aliment can be deteriorated by spoilage bacteria, that cause and develop unpleasant odours, taste and texture, whereas foodborne pathogenic bacteria may cause diseases with flu-like symptoms such as nausea, vomiting, diarrhoea, and/or fever. Food additives with antimicrobial activity can be used to overcome such problems, but consumers tend to reject the present use of additives obtained by chemical synthesis; flavonoids, as additives derived from natural products, can be a valid and preferred alternative (Cowan 1999).

In recent years a number of articles on the antibacterial activity of flavonoids have appeared in the literature. Pretorius (2003) published a review on flavonoids as anti-infective agents reporting the main studies on flavonoids active against human and plant pathogens. The literature till 2005 about the same matter has been reviewed by Cushnie and Lumb (2005) who, in a review article also reported the problems regarding the discrepancies of the activity results in the field of antibacterial flavonoid research. Such inconsistencies may be due to variation within each assay. Even though the National Committee for Clinical Laboratory Standards published a set of guidelines for standard agar dilution, broth macro and microdilution methods for reducing the number of conflicting reports of flavonoid antibacterial activity (National Committee for Clinical Laboratory Standards 2000), it will still be necessary to consider additional variables that may lead to false results. In fact the inhibitory effect of extracts and pure compounds was very dependent on experimental conditions. Inocula concentration, nutrient amount, the solvent used and the final dilution of the antimicrobial compounds have a great influence on the assay. In consequence, some extracts reduced the growth of microorganisms but were not sufficiently effective in completely inhibiting microbial activity, making comparison with the literature more difficult since a universal consensus about methodology has not yet been established. In many experiments, minimum inhibitory concentration (MIC) has been confirmed as a good method to evaluate the antimicrobial activity of compounds with low solubility in water. Their precipitation, which can result in a reduction of antimicrobial activity, can be avoided by addition of an appropriate concentration of agar. Therefore, the method previously described by Mann and Markham (1998) to determine the MIC of essential oils is also useful for other compounds with poor water solubility including flavonoids. Recently, Klančnik et al. (2010) suggested an evaluation of the antibacterial activity of a plant extract using the microdilution method as a fast screening method for MIC determination and the macrodilution method at selected MICs to confirm the antibacterial activity of plant extracts.



Compound		R	R_1	R_2	R_3
1	Flavone	H	H	H	H
2	Apigenin	OH	OH	OH	H
3	Luteolin	OH	OH	OH	OH
4	4'Methoxyflavone	H	H	OCH_3	H
5	Genkwanin	OH	OCH_3	OH	H
6		OH	OCH_3	OCH_3	H
7		OCH_3	OCH_3	OCH_3	H



Compound		R	R_1	R_2	R_3	R_4
8	Kaempferol	OH	OH	OH	H	OH
9	Quercetin	OH	OH	OH	OH	OH
10		OCH_3	OH	OH	OCH_3	OH
11	Rhamnazin	OH	OCH_3	OH	OCH_3	OH
12	Rhamnocitrin	OH	OCH_3	OH	H	OH
13	Galangin	OH	OH	H	H	OH
14		OCH_3	OCH_3	OCH_3	H	OCH_3
15		OH	OCH_3	OH	OCH_3	OSO_3H
16		OH	OCH_3	OH	H	OSO_3H

Fig. 2.3 Chemical structure of the flavones and flavonoles **1**–**16**

As the scientific literature reported exhaustive reviews about antimicrobial activity of flavonoids till 2005 (Cushnie and Lamb 2005; Pretorius 2003), in this chapter we have covered the most recent literature since that date, listing papers that described flavonoid structures from the simplest to the more complex ones, both aglicones and glycosides, and sometimes gathering the articles according to the main microbial target. Most of compounds cited in the following paragraphs, divided according their chemical structure subclass (flavones and flavonoles, flavanones, flavanes and so on), are pictured in Figs. 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 2.10, and 2.11.

The antibacterial activities of flavonoids reported in the literature have been discussed in three principal sections in this chapter: (1) the antibacterial activity of flavonoids that have been extracted, isolated, identified and assayed; (2) the antimicrobial activity of extracts containing high concentrations of flavonoids, and (3) antimicrobial activity of combination of flavonoids or flavonoids and antibiotics

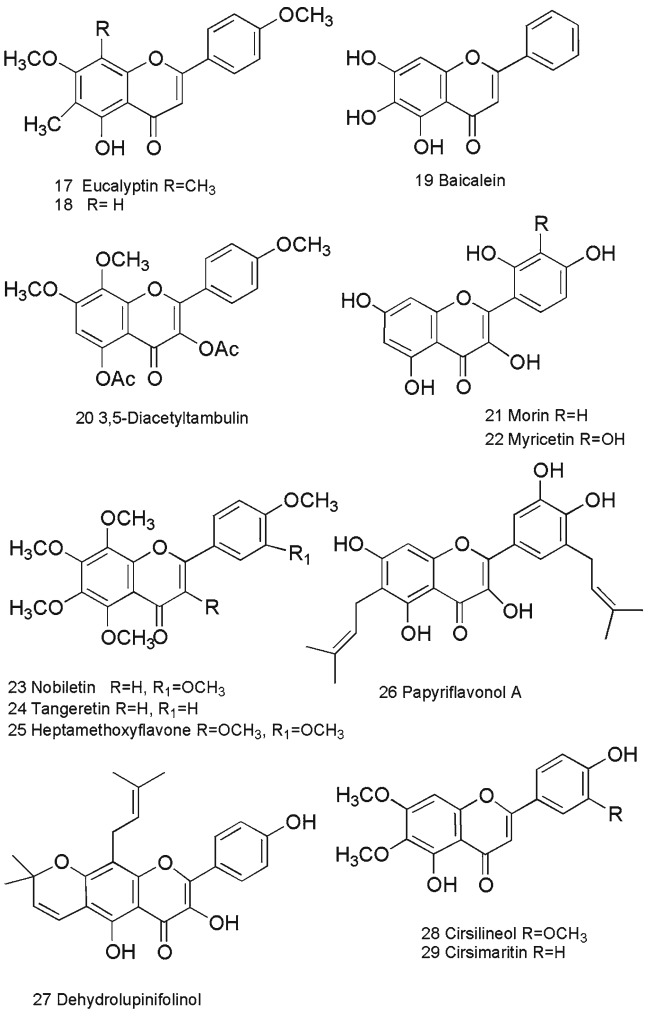


Fig. 2.4 Chemical structure of the flavones and flavonoles **17**–**29**

(synergistic effect). In Table 2.1 we have listed the most significant compounds cited, their plant sources and main bacterial targets.

2.4.1 Antibacterial Activity of Isolated Flavonoids

Bylka et al. (2004) published the activity of 4′-methoxy-5,7-dihydroxyflavone 6-C-glucoside (isocytisoside) **39** (Fig. 2.5) isolated from *Aquilegia vulgaris* L. (Ranunculaceae) against different Gram-positive, Gram-negative bacteria and also fungi for the first time. This compound was characterised by high antimicrobial

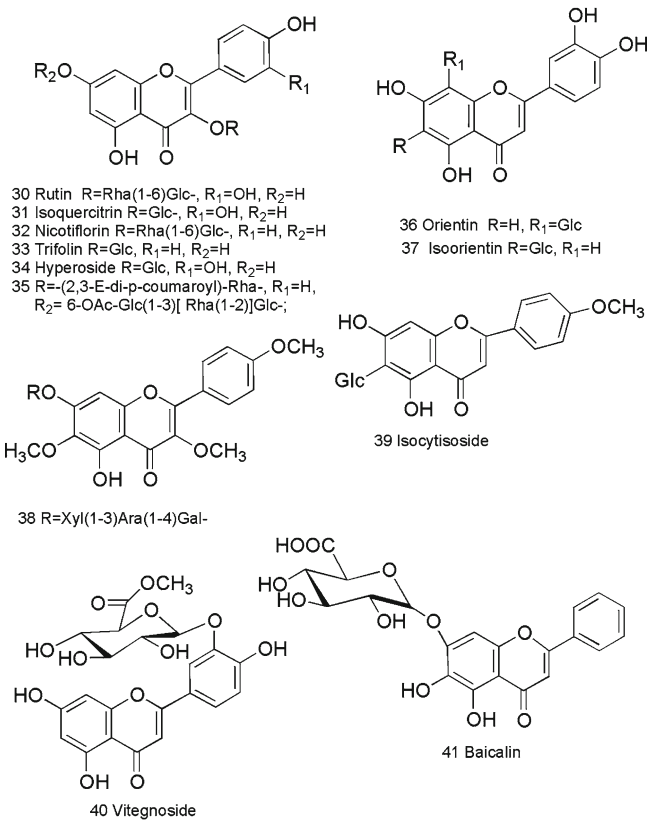


Fig. 2.5 Chemical structure of the flavone and flavonol glycosides **30–41**

activity and its MIC values ranged from 15.6 to 250 μg/ml. Gentamicin was used as a positive control. Compound **39** was particularly active against Gram-positive bacteria, and in its lowest concentrations (MIC 15.6 μg/ml) it inhibited the growth of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Aspergillus niger* (Bylka et al. 2004).

Seven antibacterial flavonoids, apigenin **2** (Fig. 2.3), genkwanin **5** (Fig. 2.3), 5-hydroxy-7,4′-dimethoxyflavone **6** (Fig. 2.3), rhamnocitrin **12** (Fig. 2.3), kaempferol **8** (Fig. 2.3), quercetin-5,3′-dimethylether **10** (Fig. 2.3), rhamnazin **11** (Fig. 2.3), were isolated by bioassay-guided fractionation from leave extract of *Combretum erythrophyllum* (Burch.) Sond. (Combretaceae). All compounds showed good activity against *Vibrio cholerae* and *Enterococcus faecalis*, with MIC values in the range of 25–50 μg/ml. Rhamnocitrin and quercetin-5,3′-dimethylether also inhibited *Micrococcus luteus* and *Shigella sonei* at 25 μg/ml. With the exception of 5-hydroxy-7,4′-dimethoxy-flavone, the flavonoids studied were not toxic towards human lymphocytes. The levels of antibacterial activity of these compounds were not high enough to justify the use of *Combretum erythrophyllum* in treating infections caused by the organisms tested, but leave extracts of this plant could be used in the

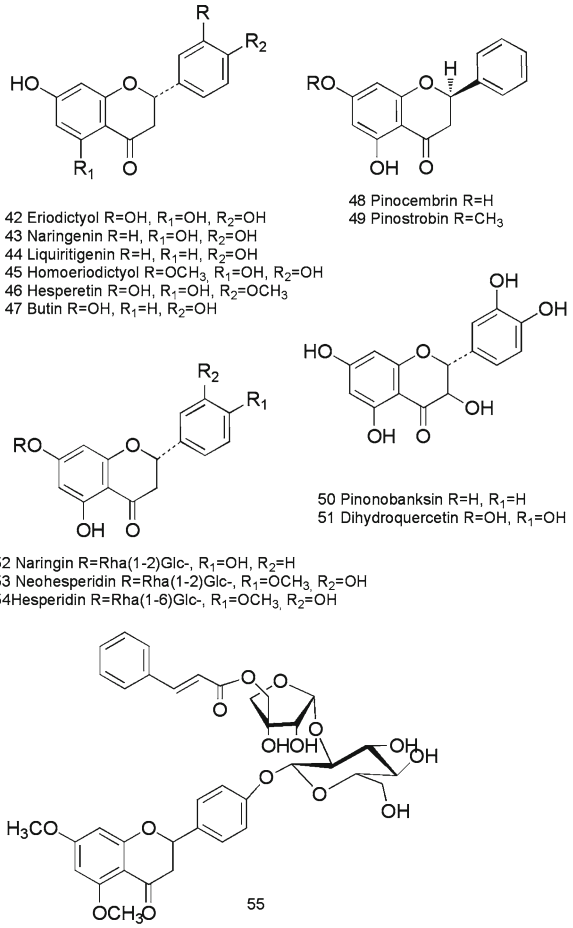


Fig. 2.6 Chemical structure of the flavanones **42–55**

purification of water contaminated by *Vibrio cholerae* especially in rural areas (Martini et al. 2004).

The methanol extract of *Marrubium globosum* Montbr. et Auch. ex Benth. ssp. *libanoticum* Boiss. (Lamiaceae), an original plant endemic to Turkey, showed a strong activity against all the bacterial strains tested by Rigano et al. (2007) with MICs between 8 and 128 μg/ml; even the particularly resistant *Pseudomonas aeruginosa* and *Staphylococcus aureus* were also inhibited. Rutin (quercetin 3-O-rutinoside) **30** (Fig. 2.5) resulted the component of extract with the highest antibacterial activity: all the bacteria were affected, especially *Staphylococcus epidermidis* and *Enterococcus faecalis* (MIC 8 μg/ml). Other active compounds were a naringenin (**43**, Fig. 2.6) derivative (naringenin 7-O-Glc) and isoquercitrin **31** (Fig. 2.5) (quercetin 3-O-Glc). Since rutin and isoquercitrin showed very similar activities and spectra, their aglicone could be crucial for activity. As those secondary metabolites isolated from *Marrubium globosum* ssp. *libanoticum* showed an interesting antibacterial activity, this may explain the use of this species in folk

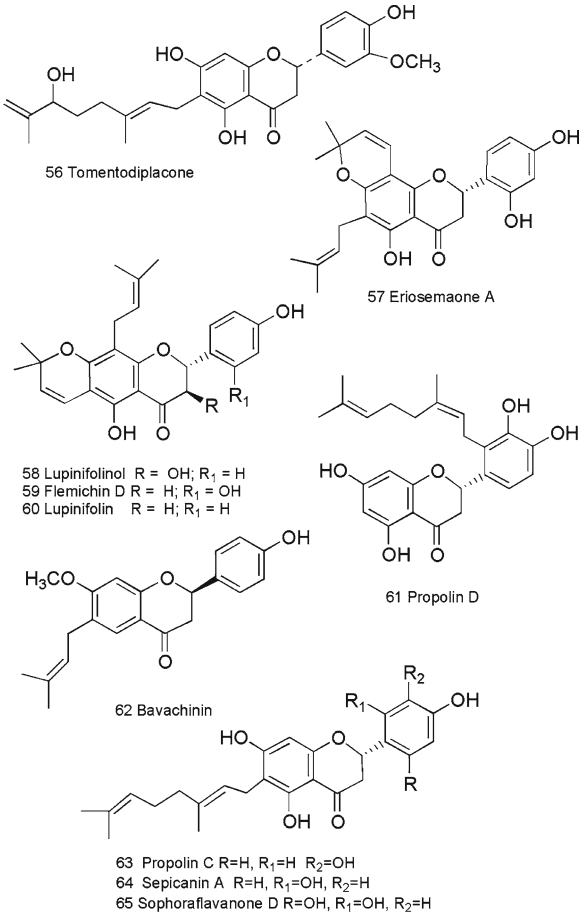


Fig. 2.7 Chemical structure of the prenyl flavanones **56–65**

medicine to treat skin and urinary tract infections as well as gastrointestinal diseases (Rigano et al. 2007).

Amorphophallus campanulatus Blume ex. Decne (Araceae) is a perennial herb with rounded tuberous root stock (corm) that is widely found in Bangladesh, India, and Africa. Recently Khan et al. (2008) isolated from this plant and tested against four Gram-positive bacteria (*Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, *Streptococcus β-haemolyticus*) and six Gram-negative bacteria (*Escherichia coli*, *Shigella dysenteriae*, *Shigella sonnei*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Salmonella typhi*) 3,5-diacetyltambulin **20** (Fig. 2.4), showing significant antibacterial activity in comparison with kanamycin used as a reference standard (30 μg/disc). The MIC values of **20** against Gram-positive bacteria ranged from 8 to 16 μg/ml and against Gram-negative bacteria ranged from 16 to 64 μg/ml. A moderate cytotoxicity was evidenced in brine shrimp nauplii assay (Khan et al. 2008).

Ethyl acetate fraction from *Argyreia speciosa* (Burm.f) Boj. (Convolvulaceae) and its isolates, quercetin 3′,7 di-O-methyl-3-sulphate **15** (Fig. 2.3) and kaempferol

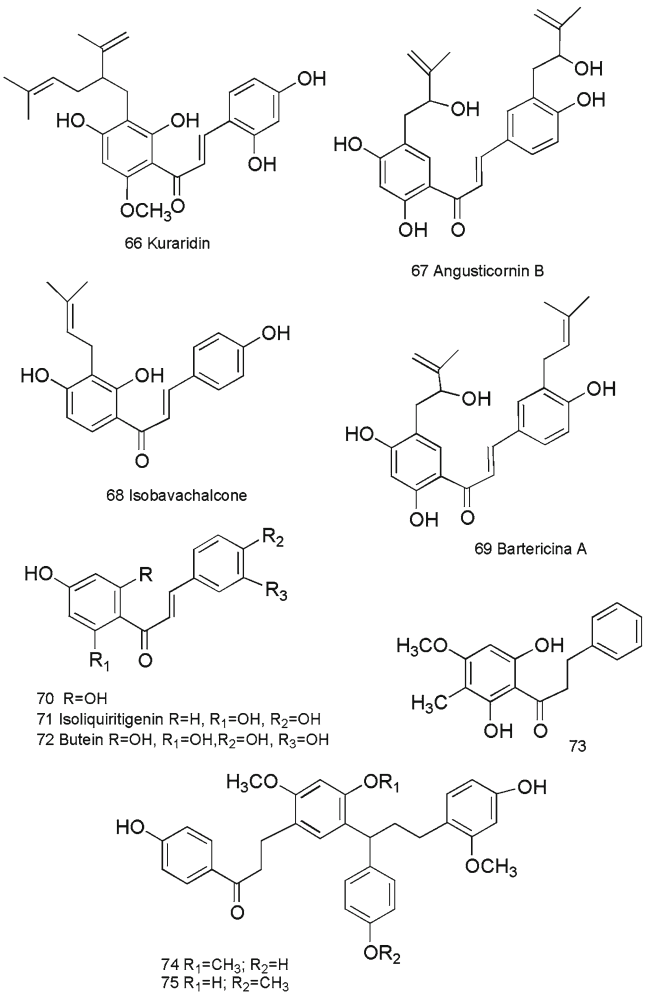


Fig. 2.8 Chemical structure of the calcones **66–75**

7-O-methyl-3-sulphate **16** (Fig. 2.3), showed significant activity against *Micobacterium tubercolosis* (MICs from 25 to 50 μg/ml) and against *Klebsiella pneumonie* (MICs from 2 to 4 μg/ml). Synergistic effects of the active fractions and flavonoid sulphates with commercially available antitubercular drugs and antibiotics against *Micobacterium tuberculosis* and other organisms were highlighted. The active compounds from *Argyreia speciosa*, a climbing shrub with woody tomentose stem, used in Ayurvedic medicine for stomach complaints, sores on foot, small pox, syphilis, dysentery, and diarrhoea, could be useful also for the treatment of pulmonary tuberculosis and pneumococcal infections (Habbu et al. 2009).

In a recent article of Santas at al. (2010) the well known flavonoid quercetin **9** (Fig. 2.3) showed a higher inhibition than kaempferol **8** (Fig. 2.3) in the disc diffusion

Fig. 2.9 Chemical structure of the isoflavones **76–86**

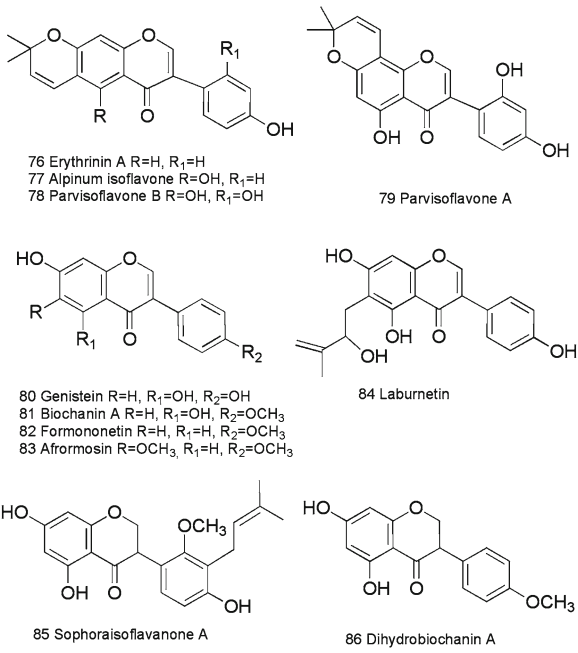
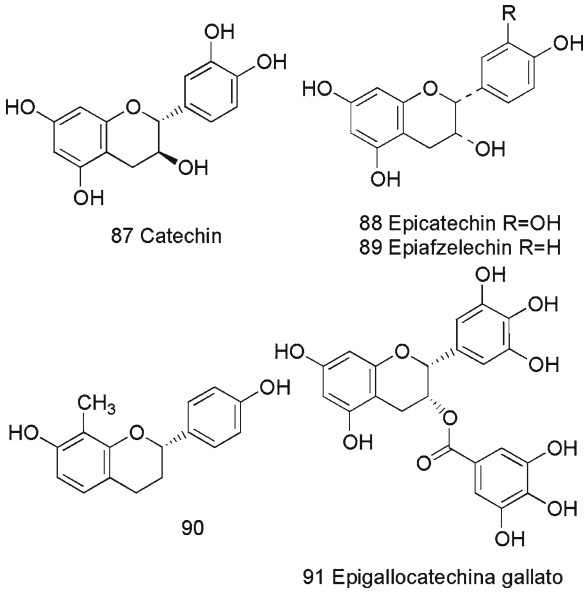


Fig. 2.10 Chemical structure of catechins **87–91**



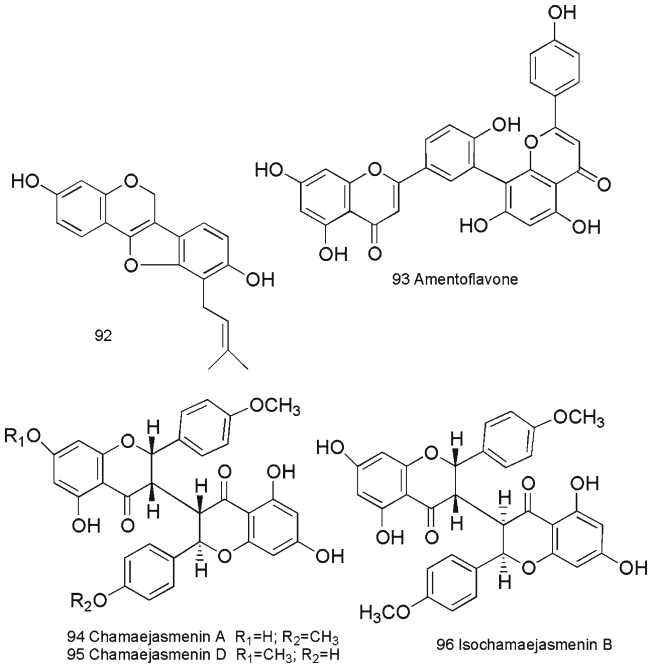


Fig. 2.11 Chemical structure of compounds **92–96**

assay against all strains of bacteria tested (from 9.8 ± 0.6 to 15.0 ± 1.0 mm); whereas, kaempferol was only effective against the Gram-positive bacteria *Staphylococcus aureus* and *Micrococcus luteus* (9.3 ± 1.2 and 10.3 ± 0.6 mm, respectively). By contrast, kaempferol was more effective than quercetin in inhibiting bacterial growth of *Bacillus cereus, Listeria monocytogenes* and *Pseudomonas aeruginosa* in micro-well dilution assay and resulted as effective as quercetin in inhibiting the growth of *Staphylococcus aureus* and *Micrococcus luteus* with MIC values of 40 μg/ml. Experimentally, kaempferol has low solubility in aqueous media which causes a loss of inhibition in the disc assay in comparison with its effect at appropriate dilution in the broth media. Although the disc diffusion assay is commonly used as a preliminary approach to test antimicrobial potential (Kloucek et al. 2005), these results demonstrate the importance of performing at least two different assays as weak inhibition can be observed in the disc assay for components with low solubility, while they can be efficient antimicrobial agents when appropriately diluted in the media (Santas et al. 2010).

Also the antibacterial activity of flavonol galangin **13** (Fig. 2.3) was assessed against 17 strains of 4-quinolone resistant *Staphylococcus aureus* using an agar dilution assay. It was discovered that the flavonol **13** had MICs of approximately 50 μg/ml against 16 of these strains, including those which exhibited 250- and 500-fold increases in norfloxacin resistance (Cushnie and Lamb 2006).

Feijoa sellowiana Berg. (Myrtaceae) produces fruits widely used for human consumption, much appreciated for its good nutritional characteristics and for its

Table 2.1 List of antibacterial flavonoids cited in this chapter

Compound no.	Name	Plant sources	Bacterial strains	References
1	Flavone	*Feijoa sellowiana*	*Pseudomonas aeruginosa* *Proteus mirabilis* *P. vulgaris* *Helicobacter pylori*	Basile et al. (2010)
2	Apigenin	*Combretum erythrophyllum*	*Vibrio cholerae* *Enterococcus faecalis*	Martini et al. (2004)
		Launaea resedifolia	*Staphylococcus aureous* *Proteus vulgaris* *P. mirabilis* *Klebsiella pneumonia* *K. oxytoca* *Morganella morgani* *Streptococcus Sp.* *Enterobacter Sp.* *Seratia Sp.*	Moussaoui et al. (2010)
		Mentha longifolia	*Escherichia coli* *Bacillus ceruleus* *B. subtilis* *Staphylococcus aureus* *Pseudomonas aeruginosa*	Akroum et al. (2009)
3	Luteolin	–	*Chlamydia pneumoniae*	Törmäkangas et al. (2005)
		Bauhinia sirindhorniae	*Bacillus subtilis*	Athikomkulchai et al. (2005)
		Ficus chlamydocarpa	*Mycobacterium smegmatis* *M. tuberculosis* *Streptococcus faecalis* *Staphylococcus aureus* *Bacillus cereus*	Kuete et al. (2008)

			B. stearothermophilus *B. subtilis* *Citrobacter freundii* *Enterobacter cloacae* *Escherichia coli* *Klebsiella pneumoniae* *Morganella morganii* *Proteus mirabilis* *Pseudomonas aeruginosa* *Shigella dysenteriae* *Salmonella typhi*	
5	Genkwanin	*Combretum erythrophyllum*	*Vibrio cholerae* *Enterococcus faecalis*	Martini et al. (2004)
6	5-Hydroxy-7,4′-dimethoxyflavone	*Combretum erythrophyllum*	*Vibrio cholerae* *Enterococcus faecalis*	Martini et al. (2004)
8	Kaempferol	*Combretum erythrophyllum*	*Vibrio cholerae* *Enterococcus faecalis*	Martini et al. (2004)
		–	*Staphylococcus aureus* *Micrococcus luteus* *Bacillus cereus* *Listeria monocytogenes* *Pseudomonas aeruginosa*	Santas et al. (2010)
		Impatiens balsamina	*Propionibacterium acnes*	Lim et al. (2007)
9	Quercetin	–	*Staphylococcus aureus* *Micrococcus luteus* *Bacillus cereus* *Listeria monocytogenes* *Pseudomonas aeruginosa*	Santas et al. (2010)

(continued)

Table 2.1 (continued)

Compound no.	Name	Plant sources	Bacterial strains	References
		Psidium guajava	*Salmonella enteritidis* *Bacillus cereus*	Arima and Danno (2002),
			Listeria monocytogenes	Rodríguez Vaquero et al. (2007), Rodríguez Vaquero and Manca de Nadra (2008)
		–	*Escherichia coli* *Streptococcus mutans*	Wood (2007)
		Impatiens balsmina	*Propionibacterium acnes*	Lim et al. (2007)
			Staphylococcus aureus *MRSA* *S. epidermidis* *Streptococcus pneumoniae* *S. pyogenes* *Pseudomonas aeruginosa* *Enterococcus faecalis* *Escherichia coli*	Hirai et al. (2010)
10	Quercetin-5,3′-dimethylether	*Combretum erythrophyllum*	*Vibrio cholerae* *Enterococcus faecalis* *Micrococcus luteus* *Shigella sonei*	Martini et al. (2004)
11	Rhamnazin	*Combretum erythrophyllum*	*Vibrio cholerae* *Enterococcus faecalis*	Martini et al. (2004)
12	Rhamnocitrin	*Combretum erythrophyllum*	*Vibrio cholerae* *Enterococcus faecalis* *Micrococcus luteus* *Shigella sonei*	Martini et al. (2004)

13	Galangin	–	*Staphylococcus aureus*	Cushnie and Lamb (2006), Lee et al. (2008)
15	–	*Argyreia speciosa*	*Micobacterium tubercolosis* *Klebsiella pneumonie*	Habbu et al. (2009)
16	–	*Argyreia speciosa*	*Micobacterium tubercolosis* *Klebsiella pneumonie*	Habbu et al. (2009)
17	Eucalyptin	*Eucalyptus maculata*	*MRSA* *Staphylococcus aureus* *Bacillus cereus* *Enterococcus faecalis* *Alicyclobacillus acidoterrestris* *Propionibacterium acnes*	Takahashi et al. (2004)
18	8-Desmethyl-eucalyptin	*Eucalyptus maculata*	*MRSA* *Staphylococcus aureus* *Bacillus cereus* *Enterococcus faecalis* *Alicyclobacillus acidoterrestris* *Propionibacterium acnes*	Takahashi et al. (2004)
20	3,5-Diacetyltambulin	*Amorphophallus campanulatus*	*Bacillus subtilis* *B. megaterium* *Staphylococcus aureus* *Streptococcus β-haemolyticus* *Escherichia coli* *Shigella dysenteriae* *S. sonnei* *S. flexneri* *Pseudomonus aeruginosa* *Salmonella typhi*	Khan et al. (2008)
21	Morin	–	*Staphylococcus aureus* *Escherichia coli*	Alvarez et al. (2006)

(continued)

Table 2.1 (continued)

Compound no.	Name	Plant sources	Bacterial strains	References
22	Myrecetin	–	*Klebsiella pneumoniae*	Lin et al. (2005)
26	Papyriflavonol A	*Broussnetia papyrifera*	*Escherichia coli* *Salmonella typhimurium* *Staphylococcus epidermis* *Staphylococcus aureus*	Sohn et al. (2004)
27	Dehydrolupinifolinol	*Eriosema chinense*	*Micobacterium tuberculosis*	Sutthivaiyakit et al. (2009)
28	Cirsilineol	*Hyptis fasciculata*	*Helicobacter pylor*	Isobe et al. (2006)
29	Cirsimaritin	*Hyptis fasciculata*	*Helicobacter pylor*	Isobe et al. (2006)
30	Rutin	*Marrubium globosum.*	*Pseudomonas aeruginosa* *Staphylococcus aureus* *S. epidermidis* *Enterococcus faecalis*	Rigano et al. (2007)
		Galium fissurense *Viscum album.* *Cirsium hypoleucum*	*Pseudomonas aeruginosa* *Acinetobacter baumannii* *Staphylococcus aureus*	Orhan et al. (2010)
			Listeria monocytogenes	Rodríguez Vaquero et al. (2007)
			Escherichia coli	Rodríguez Vaquero and Manca de Nadra (2008)
			Streptococcus mutans	Wood (2007)
		Pelargonium radula	*Staphylococcus aureus* *Proteus rettgeri*	Pepeljnjak et al. (2005)
31	Isoquercitrin	*Marrubium globosum* ssp. *libanoticum.*	*Pseudomonas aeruginosa* *Staphylococcus aureus* *S. epidermidis* *Enterococcus faecalis*	Rigano et al. (2007)
		Pelargonium radula	*Staphylococcus aureus* *Proteus rettgeri*	Pepeljnjak et al. (2005)

32	Nicotinflorin	*Galium fissurense* *Viscum album* *Cirsium hypoleucum*	*Pseudomonas aeruginosa* *Acinetobacter baumannii* *Staphylococcus aureus*	Orhan et al. (2010)
46	Hesperetin	*Citrus bergamia*	*Escherichia coli* *Pseudomonas putida* *Salmonella enterica*	Mandatari et al. (2007)
39	Neoeriocitrin	*Citrus bergamia*	*Escherichia coli* *Pseudomonas putida* *Salmonella enterica*	Mandatari et al. (2007)
41	Baicalin	*Scutellaria baicalensis*	*Chlamydia trachomatis*	Hao et al. (2009, 2010)
42	Eriodictyol	*Bauhinia sirindhorniae*	*Bacillus subtilis*	Athikomkulchai et al. (2005)
		Citrus bergamia	*Escherichia coli* *Pseudomonas putida* *Salmonella enterica*	Mandatari et al. (2007)
43	Naringenin	–	*Streptococcus mutans*	Gustafsson and Krasse (1958), Wood (2007)
		Bauhinia sirindhorniae	*Bacillus subtilis*	Athikomkulchai et al. (2005)
		Citrus paradisi	*Borrelia burgdorferi sensu lato*	Brorson and Brorson (2007)
		Citrus bergamia	*Escherichia coli* *Pseudomonas putida* *Salmonella enterica*	Mandatari et al. (2007)
44	Liquiritigenin	*Butea monosperma*	*Micobacterium tuberculosis*	Chokchaisiri et al. (2009)
46	Hesperitin	*Citrus paradisi*	*Borrelia burgdorferi sensu lato*	Brorson and Brorson (2007)
47	Butin	*Butea monosperma*	*Micobacterium tuberculosis*	Chokchaisiri et al. (2009)
48	Pinocembrin	–	*Staphylococcus aureus* *S. epidermidis* *Bacillus subtilis* *Escherichia coli* *Pseudomonas aeruginosa*	Mohammadzadeh et al. (2007), Chaillou and Nazareno (2009)

(continued)

Table 2.1 (continued)

Compound no.	Name	Plant sources	Bacterial strains	References
49	Pinostrobin	–	*Staphylococcus aureus* *S. epidermidis* *Bacillus subtilis* *Escherichia coli* *Pseudomonas aeruginosa*	Mohammadzadeh et al. (2007)
50	Pinobanksin	–	*Staphylococcus aureus* *S. epidermidis* *Bacillus subtilis* *Escherichia coli* *Pseudomonas aeruginosa*	Mohammadzadeh et al. (2007)
52	Naringin	–	*Streptococcus mutans*	Wood (2007)
		Citrus bergamia	*Escherichia coli* *Pseudomonas putida* *Salmonella enterica*	Mandatari et al. (2007)
53	Neohesperidin	*Citrus bergamia*	*Escherichia coli* *Pseudomonas putida* *Salmonella enterica*	Mandatari et al. (2007)
55	–	*Galium fissurense,.* *Viscum album* ssp. *album,*	*Pseudomonas aeruginosa* *Acinetobacter baumannii* *Staphylococcus aureus*	Orhan et al. (2010)
56	Tomentodiplacone	*Paulownia tomentosa*	*Bacillus cereus* *B. subtilis* *Enterococcus faecalis* *Listeria monocytogenes* *Staphylococcus aureus* *S. epidermidis*	Tsuchiya and Iinuma (2000)
		Hyptis fasciculata	*Helicobacter pylor*	Isobe et al. (2006)

57	Eriosemaone A	*Eriosema chinense*	*Micobacterium tuberculosis*	Sutthivaiyakit et al. (2009)
		Hyptis fasciculata	*Helicobacter pylor*	Isobe et al. (2006)
58	Lupinifolinol	*Eriosema chinense*	*Micobacterium tuberculosis*	Sutthivaiyakit et al. (2009)
59	Flemichin D	*Eriosema chinense*	*Micobacterium tuberculosis*	Sutthivaiyakit et al. (2009)
60	Lupinifolin	*Eriosema chinense*	*Micobacterium tuberculosis*	Sutthivaiyakit et al. (2009)
		Ficus chlamydocarpa	*Mycobacterium smegmatis*	Kuete et al. (2008)
			M. tuberculosis	
			Streptococcus faecalis	
			Staphylococcus aureus	
			Bacillus cereus	
			B. stearothermophilus	
			B. subtilis	
			Citrobacter freundii	
			Enterobacter cloacae	
			Escherichia coli	
			Klebsiella pneumoniae	
			Morganella morganii	
			Proteus mirabilis	
			Pseudomonas aeruginosa	
			Shigella dysenteriae	
			Salmonella typhi	
61	Propolin D	–	*MRSA*	Raghukumar et al. (2010)
62	Bavachinin	–	*Staphylococcus epidermis*	Yin et al. (2004)
			Staphylococcus aureus	
63	Propolin C	–	*MRSA*	Raghukumar et al. (2010)
64	Sepicanin	*Artocarpus sepicanus*	*MRSA*	Radwan et al. (2009)
65	Sophoraflavanone D	*Echinosophora koreensis*	*Escherichia coli*	Sohn et al. (2004)
			Salmonella typhimurium	
			Staphylococcus epidermis	
			Staphylococcus aureus	

(continued)

Table 2.1 (continued)

Compound no.	Name	Plant sources	Bacterial strains	References
66	Kuraridin	*Sophora flavescens*	*Escherichia coli* *Salmonella typhimurium* *Staphylococcus epidermis* *Staphylococcus aureus*	Sohn et al. (2004)
68	Isobavachalcone	–	*Staphylococcus epidermis* *Staphylococcus aureus*	Yin et al. (2004)
		Dorstenia barteri	*Bacillus cereus* *B. megaterium* *B. stearothermophilus* *B. subtilis* *Staphylococcus aureus* *Streptococcus faecalis* *Escherichia coli* *Shigella dysenteriae* *Proteus vulgaris* *P. mirabilis* *Shigella flexneri* *Klebsiella pneumoniae* *Pseudomonas aeruginosa* *Salmonella typhi* *Morganella morganii* *Enterobacter aerogens* *E. cloacae* *Citrobacter freundii*	Mbaveng et al. (2008)
		–	*Mycobacterium tubercolosis,* *M. smegmatis*	Kuete et al. (2010)
69	Bartericin A	*Dorstenia angusticornis*	*Candida* spp.	Kuete et al. (2007)
70	–	*Galenia africana* var. *africana*	*Mycobacterium smegmatis* *M. tuberculosis*	Mativandlela et al. (2009)

71	Isoliquiritigenin	*Bauhinia sirindhorniae*	*Bacillus subtilis*	Athikomkulchai et al. (2005)
		Butea monosperma	*Micobacterium tuberculosis*	Chokchaisiri et al. (2009)
72	Butein	*Butea monosperma*	*Micobacterium tuberculosis*	Chokchaisiri et al. (2009)
73	–	*Eucalyptus maculata*	*MRSA*	Takahashi et al. (2004)
			Staphylococcus aureus	
			Bacillus cereus	
			Enterococcus faecalis	
			Alicyclobacillus acidoterrestris	
			Propionibacterium acnes	
74	–	*Dracaena cochinchinensis*	*Helicobacter pylor*	Zhu et al. (2007)
75	–	*Dracaena cochinchinensis*	*Helicobacter pylor*	Zhu et al. (2007)
76	Erythrinin A		*Staphylococcus epidermis*	Yin et al. (2004)
			Staphylococcus aureus	
77	Alpinum isoflavone	*Ficus chlamydocarpa*	*Mycobacterium smegmatis*	Kuete et al. (2008)
			M. tuberculosis	
			Streptococcus faecalis	
			Staphylococcus aureus	
			Bacillus cereus	
			B. stearothermophilus	
			B. subtilis	
			Citrobacter freundii	
			Enterobacter cloacae	
			Escherichia coli	
			Klebsiella pneumoniae	
			Morganella morganii	
			Proteus mirabilis	
			Pseudomonas aeruginosa	
			Shigella dysenteriae	
			Salmonella typhi	

(continued)

Table 2.1 (continued)

Compound no.	Name	Plant sources	Bacterial strains	References
80	Genistein	*Ficus chlamydocarpa*	*Mycobacterium smegmatis* *M. tuberculosis* *Streptococcus faecalis* *Staphylococcus aureus* *Bacillus cereus* *B. stearothermophilus* *B. subtilis* *Citrobacter freundii* *Enterobacter cloacae* *Escherichia coli* *Klebsiella pneumoniae* *Morganella morganii* *Proteus mirabilis* *Pseudomonas aeruginosa* *Shigella dysenteriae* *Salmonella typhi*	Kuete et al. (2008)
82	Formononetin	*Butea monosperma*	*Micobacterium tuberculosis*	Chokchaisiri et al. (2009)
83	Afrormosin	*Butea monosperma*	*Micobacterium tuberculosis*	Chokchaisiri et al. (2009)
84	Laburnetin	*Ficus chlamydocarpa*	*Mycobacterium smegmatis* *M. tuberculosis* *Streptococcus faecalis* *Staphylococcus aureus* *Bacillus cereus* *B. stearothermophilus* *B. subtilis* *Citrobacter freundii* *Enterobacter cloacae*	Kuete et al. (2008)

			Escherichia coli *Klebsiella pneumoniae* *Morganella morganii* *Proteus mirabilis* *Pseudomonas aeruginosa* *Shigella dysenteriae* *Salmonella typhi*	
85	Sophoraisoflavanone A	*Echinosophora koreensis*	*Escherichia coli* *Salmonella typhimurium* *Staphylococcus epidermis* *Staphylococcus aureus*	Sohn et al. (2004)
87	Catechin	*Schotia latifoglia*	*Staphylococcus aureus* *Bacillus subtilis* *Escherichia coli* *Pseudomonas aeruginosa*	Esquenazi et al. (2002), Masika et al. (2004)
88	Epicatechin	*Schotia latifoglia*	*Staphylococcus aureus* *Bacillus subtilis* *Escherichia coli* *Pseudomonas aeruginosa*	Esquenazi et al. (2002), Masika et al. (2004)
		Mangifera indica	*Lactobacillus* sp. *Escherichia coli* *Azospirillium lipoferum* *Bacillus* sp.	Kanwal et al. (2009)

(continued)

Table 2.1 (continued)

Compound no.	Name	Plant sources	Bacterial strains	References
89	Epiafzelechin	*Ficus chlamydocarpa*	*Mycobacterium smegmatis* *M. tuberculosis* *Streptococcus faecalis* *Staphylococcus aureus* *Bacillus cereus* *B. stearothermophilus* *B. subtilis* *Citrobacter freundii* *Enterobacter cloacae* *Escherichia coli* *Klebsiella pneumoniae* *Morganella morganii* *Proteus mirabilis* *Pseudomonas aeruginosa* *Shigella dysenteriae* *Salmonella typhi*	Kuete et al. (2008)
90	–	*Dracaena cochinchinensis*	*Helicobacter pylor*	Zhu et al. (2007)
92	–	*Erythrina poeppigiana*	*MRSA*	Tanaka et al. (2004)

pleasant flavour and aroma. Recently, Basile and coworkers (2010) isolated and identified flavone **1** (Fig. 2.3) as the active component involved in the antibacterial activity of the extract of *Feijoa sellowiana* fruits, through activity-guided fractionation procedures. Flavone **1** showed high activity against the nine standard bacterial strains tested, in particular against *Pseudomonas aeruginosa, Proteus mirabilis*, and *Proteus vulgaris* (MICs 1.95 μg/ml for standard strains and 3.9 μg/ml for clinical isolates of the three bacterial species), showing also good minimal bactericidal concentration values. Flavone was also significantly more active against *Helicobacter pylori* than metronidazole (Basile et al. 2010).

A recent study from Moussaoui et al. (2010) has reported that two glycoside derivatives of apigenin (apigenin 7-O-β-glucoside and apigenin 7-O-β-glucuronide) extracted from *Launaea resedifolia* (O.K.) (Asteraceae), a perennial herb widely distributed in the arid regions of the Mediterranean area, prevented the growth of a number of microorganisms (*Staphylococcus aureous, Proteus vulgaris, Proteus mirabilis, Klebsiella pneumonia, Klebsiella oxytoca, Morganella morgani, Streptococcus spp, Enterobacter spp., Seratia spp.*) in a dose-dependent manner. In fact the medium diameter of the inhibition zone increased proportionally to the increasing concentration of flavonoids (Moussaoui et al. 2010).

Six flavonoid glycosides (5,7-dimethoxyflavanone-4′-O-β-D-glucopyranoside, 5,7-dimethoxyflavanone-4′-O-[2″-O-(5‴-O-trans-cinnamoyl)-β-D-apiofuranosyl]-β-D-glucopyranoside **55** (Fig. 2.6), 5,7,3′-trihydroxyflavanone-4′-O-β-D-glucopyranoside, naringenin-7-O-glucoside, rutin **30** (Fig. 2.5), and nicotiflorin **32** (Fig. 2.5), were assayed as antibacterial, antifungal and antiviral compounds, using the microdilution broth method. These compounds were isolated from three medicinal plants, *Galium fissurense* Ehrend.&Schönb.-Tem. (Rubiaceae), *Viscum album* L. ssp. *Album* (Viscaceae), and *Cirsium hypoleucum* DC. (Compositae), which are used in Turkish folk medicine for a variety of purposes. All the tested compounds (MICs 32–128 μg/ml) showed interesting antimicrobial activity against isolated strains of *Pseudomonas aeruginosa*, *Acinetobacter baumannii, Staphylococcus aureus*. All the compounds also had a good antifungal activity against *Candida krusei*, while only some of them demonstrated also very good antiviral activity (Orhan et al. 2010).

Psidium guajava L. (Mirtaceae), traditionally called guava, is one of the potential plants to be used in foods as a preservative. Guava, that is native to tropical America, is now cultivated in many tropical and subtropical countries for its edible fruits and used as an ingredient in many food recipes and desserts. Flavonoids extracted from guava leaves including morin-3-O-lyxoside, morin-3-O-arabinoside, quercetin **9** (Fig. 2.3) and quercetin-3-O-arabinoside were reported in 2002 to have antibacterial action against *Salmonella enteritidis* and *Bacillus cereus* (MICs 200–300 μg/ml) (Arima and Danno 2002). A more recent study has shown that the same flavonoids were able to inhibit a number of spoilage and foodborne pathogenic bacteria with different degrees of inhibition (with MICs ranging from 40 to 150 μg/ml). MICs of the flavonoids for every tested bacterium showed no significant difference from those of oxytetracycline (Rattanachaikunsopon and Phumkhachorn 2010). The findings presented showed that the flavonoids isolated from guava leaves might be

potential biologically active compounds as preservatives to improve the shelf life and the safety of foods.

Listeria monocytogenes has been recognised as an emerging foodborne pathogen and has become a major concern for the food-processing industry and for health authorities over the last decades. It is found in soil, water, dairy products, including soft cheeses, and in raw and undercooked meat, poultry, seafood and related products. It is a common bacterium in the environment and animals, and may be transferred to food and consequently the human gastrointestinal tract via raw food and contaminated dairy products. Despite efforts to eradicate the organism from foods, *Listeria monocytogenes* contamination continues to occur. This organism may cause meningitis, sepsis or abortion, but in practice only pregnant women and people with immune defects are in danger of infection. Rodríguez Vaquero et al. (2007) investigated and compared the anti-microbial properties of pure flavonoid and non-flavonoid phenolic compounds and total polyphenols of three Argentinean wines varieties: Cabernet Sauvignon, Malbec and Merlot against *Listeria monocytogenes*. The flavonoids rutin **30** (Fig. 2.5) and quercetin **9** (Fig. 2.3) were the compounds with higher inhibitory activities by agar diffusion assay. Rutin reached the strongest effect only at 25 μg/ml (Rodríguez Vaquero et al. 2007). The same authors reported in 2008 a similar study on the activity of the same wines and of compounds present in them on *Escherichia coli*. All flavonoids assayed were active against this bacterium with quercetin as the most effective one. A comparison of the inhibitory effect of quercetin on *Listeria monocytogenes* and *Escherichia coli* showed that this compound was more effective on *Escherichia coli* producing higher cellular death of the inocula with a lower quercetin concentration (200 μg/ml). On the other hand, rutin was more effective on *Listeria monocytogenes* than *Escherichia coli*. This behaviour could be related to the presence/absence of the carbohydrate moiety at the C-3 position (Rodríguez Vaquero and Manca de Nadra 2008). In both the latter studies, all wine samples produced bacterial death. Knowledge of the antibacterial effect of different wine varieties could be the basis for demonstrating whether the wine consumption with a meal may collaborate in health protection against some foodborne organisms.

Dental decay is a multifactorial disease associated with the presence of cariogenic bacteria which are embedded in the dental plaque biofilm. Recent evidence suggests that certain bioflavonoids reduce dental caries and cariogenic bacteria incidence. Some studies demonstrated that isoflavones (Sato et al. 2003), flavones, isoprenylflavones (Sato et al. 1996) and flavanones (Tsuchiya et al. 1994) inhibited cariogenic (*Streptococcus mutans*) and other oral *Streptococcus*, *Actinomyces*, and *Lactobacillus* species. Naringenin **43** (Fig. 2.6) significantly reduced experimental caries in hamsters (Gustafsson and Krasse 1958) and it has been suggested this was an inhibitory bacterial growth effect (Gustafsson 1952). More recently, the flavonoid compound quercetin-3-O-arabinopyranoside (guaijaverin) isolated from *Psidium guajava* L. (Myrtaceae) demonstrated high potential antiplaque activity by inhibiting the growth of *Streptococcus mutans*. The anti- *Streptococcus mutans* activity of the guaijaverin was found to be bacteriostatic, both heat and acid stable but alkali labile, with MICs of 2–4 mg/ml (Prabu et al. 2006). Another study evaluated two

separate, but related, dietary trials, consisting of a high-sucrose diet and dietary supplementation of naringenin **43** (Fig. 2.6) or rutin **30** (Fig. 2.5), quercetin **9** (Fig. 2.3), and naringin **52** (Fig. 2.6), on dental caries formation in 40 different male albino rats for periods of 42 days (Wood 2007). The good results of the trials for the selected bioflavonoids confirmed that these compounds act against oral bacteria and could be an alternative mean of reducing dental caries (Wood 2007).

Chlamydial infections are very common throughout the world. There is a tendency for all chlamydial species to cause persistent infections, associated with several chronic diseases such as blinding trachoma, infertility and coronary heart disease. At present, no efficient treatment for the eradication of chronic chlamydial infections exists. Luteolin **3** (Fig. 2.3) was found to be effective in suppressing lung inflammatory response and decreasing the presence of infectious chlamydia in lung tissue in a murine model (Törmäkangas et al. 2005). Many natural flavonoids and other natural and structurally similar synthetic compounds (total 57 compounds) were screened for their antichlamydial activity in human cell lines by Alvesalo et al. (2006). A number of flavonoids showed good activity and interesting structure-activity relationships were deduced. Flavones and flavonols have the same basic structure, and both groups contain both active and inactive compounds. Among flavones and flavonols, structure is related to activity in such a way that all the compounds with 50% or less inhibition contain one or more sugar moieties as substituents, whereas none of the more active compounds (over 70% inhibition) contain sugars. This basic structure of flavones and flavonols without any sugars seems to be highly active against *Chlamidia pneumoniae* compared to the similar basic structure of flavanones or isoflavones. The same phenomenon can also be observed among synthetic flavonoids, a highly active group of compounds, where three out of four compounds are derived from flavone (Alvesalo et al. 2006). Baicalin **41** (Fig. 2.5) is a flavonoid isolated from *Scutellaria baicalensis* Georgi (Lamiaceae) and is known to have an effect on multiple biological functions, including antimicrobial activities. Baicalin has emerged as a promising agent for the therapy of infectious diseases due to the increasing number of pathogenic microbial strains resistant to several antibiotics. In two separate studies Hao et al. (2009, 2010) tested *in vitro* the ability of baicalin to block *Chlamydia trachomatis* infection. It was found that baicalin markedly inhibited *Chlamydia* infection when added to infected cells at concentration of 0.48 mg/ml. Furthermore, the authors explored the correlation between baicalin and *Chlamydia* protease-like activity factor (CPAF) in the cells infected suggesting that CPAF is a primary target of baicalin; so this compound can be considered a potential agent for treating infectious diseases caused by *Chlamydia trachomatis* (Hao et al. 2009, 2010).

In recent years, some important papers have reported that prenylated flavonoids are interesting active molecules against a number of bacteria. Sohn et al. (2004) evaluated the antimicrobial activity of 18 prenylated flavonoids, purified from five different medicinal plants, using the broth microdilution methods against four bacterial (*Escherichia coli, Salmonella typhimurium, Staphylococcus epidermis* and *Staphylococcus aureus*) and two fungal microorganisms (*Candida albicans* and *Saccaromyces cerevisiae*). Most of the prenylated flavonoids showed good to strong

antibacterial and/or antifungal activities. Among them, papyriflavonol A **26** (Fig. 2.4) (extracted from the root bark of *Broussnetia papyrifera* (L.) Vent. (Moraceae)), kuraridin **66** (Fig. 2.8) (extracted from the root of *Sophora flavescens* Ait (Fabaceae)), sophoraflavanone D **65** (Fig. 2.7) and sophoraisoflavanone A **85** (Fig. 2.9) (both extracted from the root of *Echinosophora koreensis* Nakai (Fabaceae)) could be applied as anti-infective agents since they also showed low cytotoxicity measured in HepG2 cells (Sohn et al. 2004). The activity of prenylated flavonoids against Gram-positive bacteria has been further confirmed by Šmejkal et al. (2008). Eight *C*-6-geranylflavonoids were isolated from an ethanol extract of *Paulownia tomentosa* fruits. Among them tomentodiplacone **56** (Fig. 2.7) and six derivative compounds were active against *Bacillus cereus, Bacillus subtilis, Enterococcus faecalis, Listeria monocytogenes, Staphylococcus aureus* and *Staphylococcus epidermidis* showing MICs from 2 to 32 μg/ml. None of the compounds tested was active against Gram-negative bacteria *Escherichia coli, Pseudomonas aeruginosa* or *Salmonella enteritidis*. This is probably due to the lipophilic character of the compounds tested because of the presence of the geranyl side chain. Some of the flavanones were tested in a liposome membrane model, and it has been shown that the presence of a lipophilic substituent on the flavonoid skeleton increases the alteration of membrane fluidity (Tsuchiya and Iinuma 2000). The resistance of Gram-negative bacteria to the isolated compounds is probably caused by the more complex structure and hydrophilic nature of their cell walls (Šmejkal et al. 2008). Three new prenylisoflavonoids, corylifols A–C and 13 known prenylisoflavone derivatives, isolated from the seeds of *Psoralea corylifolia* L. (Fabaceae) by Yin et al. (2004) were tested on antibacterial assays. Some of the prenylflavone derivatives resulted markedly potent against two pathogenic bacteria *Staphylococcus epidermis* and *Staphylococcus aureus*. In fact, nine of them showed significant antibacterial activity against the tested microbes with MICs ranging from 9 to 73 μM. The antimicrobial activities of bavachinin **62** (Fig. 2.7), isobavachalcone **68** (Fig. 2.8) and erythrinin A **76** (Fig. 2.9) were even stronger than that of two well-known natural antimicrobial agents, bakuchiol and magnolol. About structure–activity relationships, the presence of a prenyl group at the A-ring in the chalcone derivatives made the compounds active as in compound **68**; on the contrary, if the prenyl group at A-ring is oxygenated or/and further cyclized, the compounds become inactive. Compound **63**, with a 7-methoxy group, showed stronger activity than the analogues with a 7-hydroxy group (Yin et al. 2004).

Tubercolosis was once thought to be under control, but actually it now kills hundred of thousands of people every year, especially those already suffering from AIDS. The activity against *Micobacterium tuberculosis* of some prenylated flavonoids, as eriosemaone A **57** (Fig. 2.7)**,** lupinifolinol **58** (Fig. 2.7), flemichin D **59** (Fig. 2.7), dehydrolupinifolinol **27** (Fig. 2.4) and lupinifolin **60** (Fig. 2.7) was recently reported. These compounds were isolated from hexane and dichloromethane extracts of the roots of *Eriosema chinense* Vogel (Fabaceae-Papilionoideae), a small plant that is the only member of its genus found in Thailand. Lupinifolinol showed good antimycobacterial activity, with a MIC value of 25 μg/ml and the other compounds were also more active, all exhibiting a MIC value of 12.5 μg/ml (Sutthivaiyakit et al. 2009).

The study on antibacterial activity of the isolated compounds from *Bauhinia sirindhorniae* K. & S.S. Larsen (Fabaceae-Caesalpinioideae), a tendrilled liana, indigenous to northeastern Thailand, revealed that isoliquiritigenin **71** (Fig. 2.8), isoliquiritigenin 4-methyl ether and eriodictyol **42** (Fig. 2.6) showed activity against *Bacillus subtilis* with MICs of 100, 200, 50 μg/ml respectively and minimal bactericidal concentrations (MBCs) of 100, 200, >200 μg/ml, respectively. Anti-*Staphylococcus aureus* activity of these three compounds was found to be about 200 μg/ml in term of MICs and MBCs. Furthermore, naringenin **43** (Fig. 2.6) and luteolin **3** (Fig. 2.3) exhibited activity against *Bacillus subtilis* at the same concentrations (Athikomkulchai et al. 2005).

Ficus chlamydocarpa Mildbraed & Burret and *Ficus cordata* Thunberg (Moraceae) are used traditionally in Cameroon in the treatment of filaris, diarrhoeal infections and tuberculosis. The methanol extracts from these sources as well as the isolated flavonoids alpinum isoflavone **77** (Fig. 2.9), genistein **80** (Fig. 2.9), laburnetin **84** (Fig. 2.9), luteolin **3** (Fig. 2.3) and epiafzelechin **89** (Fig. 2.10) demonstrated activity against some of the bacteria used in this study: mycobacteria (*Mycobacterium smegmatis, Mycobacterium tuberculosis)*, Gram-positive (*Streptococcus faecalis, Staphylococcus aureus, Bacillus cereus, Bacillus stearothermophilus, Bacillus subtilis)* and Gram-negative (*Citrobacter freundii*, *Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Morganella morganii, Proteus mirabilis, Pseudomonas aeruginosa, Shigella dysenteriae, Salmonella typhi)* bacterial species. Microplate dilution and radiometric respiratory methods were used to determine the susceptibility of the samples against mycobacteria*;* the micro-dilution method was used for the determination of MIC and the minimal microbicidal concentration (MMC). The best results were obtained with compounds **84**, **3** and **89** and with an extract of *Ficus cordata* (MICs 0.61–156.25 μg/ml), so that the authors concluded that the studied plant extracts, as well as some of the isolated compounds might be potential sources of new antimicrobial drugs (Kuete et al. 2008).

Flavonoid-rich fractions derived from bergamot peel, a by-product of the *Citrus bergamia* Risso (Rutaceae) fruit processing industry were found to be active against all the Gram-negative bacteria tested (*Escherichia coli, Pseudomonas putida, Salmonella enterica*). Pure flavonoids, such as neohesperidin **53** (Fig. 2.6), hesperetin **46** (Fig. 2.6), neoeriocitrin **39** (Fig. 2.5), eriodictyol **42** (Fig. 2.6), naringin **52** (Fig. 2.6) and naringenin **43** (Fig. 2.6), isolated from *Citrus* extracts, were found to be active in the range of 200–800 μg/ml. The enzyme preparation Pectinase 62 L efficiently converted common glycosides into their aglycones from bergamot extracts, and this deglycosylation increased the antimicrobial potency of *Citrus* flavonoids. Furthermore, the interactions among three bergamot flavonoids were also evaluated and combinations of eriodictyol, naringenin and hesperetin showed both synergistic and indifferent interactions depending on the test indicator organism (Mandatari et al. 2007).

Helicobacter pylori, a spiral Gram-negative bacterium, is closely associated with human gastritis, peptic ulcer, and gastric cancer (Goodwin et al. 1989). A variety of drugs with susceptibility for *Helicobacter pylori*, such as antibiotics (e.g., amoxicillin), bactericidal agents (e.g., bismuth salt), and antiprotozoal

compounds (e.g., metronidazole), were found to be effective in the clinic, but proven to be also problematic because of their adverse effects and acquired resistance (Harris 1998). Discovery and development of alternative anti-*Helicobacter pylori* therapeutics are therefore of great importance.

The genus *Dracaena* (Agavaceae), containing about 60 species, is distributed from the Old World tropic region to Canary Island. Chemical studies on the constituents of *D. cochinchinensis* (Lour.) S.C. Chen led to the isolation, from stem ethanol extract, of two new calcone derivatives (compounds **74** and **75**, Fig. 2.8), and of 4′,7-dihydroxy-8-methylflavan **90** (Fig. 2.10), that were effective against *Helicobacter pylori* with MIC values of 29.5, 29.5, and 31.3 μM, respectively. The *H. pylori* strain used (ATCC 43504) resulted very sensitive to amoxicillin but resistant to metronidazole. The three compounds exhibited inhibitory effects on the growth of the bacterium at much higher concentrations compared to amoxicillin; however, it should be noted that these compounds were more effective than metronidazole, with MIC values comparable to the synthetic agents NE-2001 and TG44 (Zhu et al. 2007).

Hyptis fasciculata Benth (Labiatae) is native to Brazil, Argentina, and Uruguay, and is used as an expectorant and sudorific remedy. From methanol extract of the aerial parts of this plant two flavonoids, cirsilineol **28** (Fig. 2.4) and cirsimaritin **29** (Fig. 2.4) were isolated and exhibited potent anti-*Helicobacter pylori* activity. To obtain structure-activity relationships the anti-*Helicobacter pylori* activity of several related flavones was tested, having the groups OH and OCH_3 in various positions of the A and C rings. All the other flavones demonstrated less or absent activity compared to compounds **28** and **29**. From the biological results the authors deduced that flavones with potent activity had mainly adjacent dimethoxy-groups, especially in positions 6 and 7, and simultaneously some hydroxy groups in the molecule (Isobe et al. 2006).

Dorstenia is a genus of about 105 species in the Moraceae plant family occurring in the tropics around the world. The methanol extract of the twigs of *D. angusticornis* Engl., and two flavonoids isolated, namely angusticornin B **67** (Fig. 2.8) and bartericin A **69** (Fig. 2.8), inhibited the growth of 22 microbial cultures belonging to three *Candida* species, 6 Gram-positive and 13 Gram-negative bacterial species (Kuete et al. 2007). This research has demonstrated for the first time the antimicrobial potency of both crude extract and flavonoids from *Dorstenia angusticornis* and has indicated that this plant represents a potential source of antimicrobial drug in the treatment of infectious diseases.

Isobavachalcone **68** (Fig. 2.8) and four other flavonoid compounds extracted from the twigs of *Dorstenia barteri* Bureau *var. multiradiata* were assayed in a recent study against 22 strains of microorganisms, namely *Bacillus cereus*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis* (Gram-positive bacteria), *Escherichia coli*, *Shigella dysenteriae*, *Proteus vulgaris*, *Proteus mirabilis*, *Shigella flexneri*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Morganella morganii*, *Enterobacter aerogens*, *Citrobacter freundii*, *Enterobacter cloacae* (Gram-negative bacteria), and four species of fungi *Candida albicans*, *Candida glabrata*, *Microsporum audorium*,

Trichophyton rubrum by the disc diffusion test. Isobavachalcone was considered a very interesting antibiotic compound because it prevented the growth of all the microbial and fungi species tested and was more active than the reference antibiotics on 15 of the 22 tested microorganisms. The lowest MIC value (0.3 μg/ml) obtained was about fourfold lower than that of the reference antibiotics (gentamycin for bacteria, nystatin for yeasts). Regarding structure-activity of compound **68**, the addition of a second 3-isoprenyl group significantly decreased the antimicrobial activity, so the authors concluded that the number and the position of the isoprenyl substituents may influence the antimicrobial activity of chalcones (Mbaveng et al. 2008). The same group of researchers deepened their work on the antibacterial properties of extract and flavonoids of *D. barteri* by targeting tubercolosis (*Mycobacterium smegmatis* and *Mycobacterium tuberculosis*) and gonorrhoea (*Neisseria gonorrhoeae*) agents. The bacteriological data indicate that the crude extract and the isobavachalcone exhibited a killing effect on *Mycobacterium tuberculosis*. MIC values below 10 μg/ml were recorded with isobavachalcone on all the tested *Neisseria gonorrhoeae* strains, meanwhile, good activity (MIC <10 μg/ml) was also recorded with the extract (Kuete et al. 2010). The results of these two studies reinforce the role of this plant and its components as anti-infective agents.

Investigation of the flowers of *Butea monosperma* (Lam.) Taub. (Fabaceae), a tree growing wildly in many parts of India, used by the rural and tribal people in curing various disorders (Patil et al. 2006) resulted in the isolation of 12 flavonoids. These included a new dihydrochalcone, dihydromonospermoside, together with three known chalcones, butein **72** (Fig. 2.8), monospermoside (butein-3-O-Glc) and isoliquiritigenin **71** (Fig. 2.8), one flavone, 7,3′,4′-trihydroxyflavone, four flavanones, butin **47** (Fig. 2.6), butrin (butin-7-O-Glc), isomonospermoside (butin-3′-O-Glc) and liquiritigenin **44** (Fig. 2.6), and three isoflavones, formononetin **82** (Fig. 2.9), afrormosin **83** (Fig. 2.9) and formononetin-7-O-Glc. All the isolated flavonoids were subjected to evaluation against *Mycobacterium tuberculosis*, where the chalcone butein **72** exhibited the highest activity with MIC of 12.5 μg/ml. The activity of the other flavonoids was between 25 and 100 μg/ml. Relationships between the flavonoid structures and their antimycobacterial activity could not be deduced. However, the assay results indicated that the presence of glucosidic moiety was harmfull for the activity of the flavonoid aglycone and that the absence of an α,β-olefinic system decreased biological activity (Chokchaisiri et al. 2009).

Schotia latifolia Jacq. (Cesalpinaceae) is tree of a small genus which occurs only in southern Africa. The seeds are edible and have been used for food historically by both indigenous African peoples and the European settlers and farmers (Aubrey 2007). Epicatechin **88** (Fig. 2.10) and catechin **87** (Fig. 2.10) were isolated by Masika et al. (2004) from the bark of *S. latifolia* through bioactivity-guided fractionation of its extracts. The compounds, that have been reported by Esquenazi et al. (2002) to possess antimicrobial activity against *Staphylococcus aureus* and acyclovir-resistant *Herpes simplex* virus type 1, showed MIC ranging from 62.5 to 250 μg/ml against other microorganisms like Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria (Masika et al. 2004).

Among edible plants with antibacterial properties, mango (*Mangifera indica* L., Anacardiaceae) is an economically important tropical fruit having a very good taste and high nutritional value. A recent study investigated the antibacterial activity of epicatechin **88** (Fig. 2.10), epicatechin-3-O-Glc and some other flavonoids, extracted from mango leaves, against four bacterial species, namely *Lactobacillus* spp., *Escherichia coli*, *Azospirillium lipoferum* and *Bacillus* spp. All the isolated flavonoids significantly reduced the growth of all the five tested bacterial species; among them epicatechin showed the greatest antibacterial activity reducing the bacterial growth by 45–99.9% at a concentration of 100 ppm. *Azospirillium lipoferum* and *Bacillus* sp. showed the highest susceptibility to this compound (Kanwal et al. 2009).

The antimicrobial activities of eucalyptus leaf extracts (26 species) against nine different species of pathogenic microorganisms that cause food poisoning, acne and athlete's foot were reported (Takahashi et al. 2004). Three flavonoids 2′,6′-dihydroxy-3′-methyl-4′-methoxy-dihydrochalcone **73** (Fig. 2.8), eucalyptin **17** (Fig. 2.4) and 8-desmethyl-eucalyptin **18** (Fig. 2.4), isolated from *Eucalyptus maculata* Hook (Myrtaceae) leaf extracts, exhibited significant inhibitory activities against six Gram-positive bacteria (*Staphylococcus aureus,* methicillin-resistant *Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis, Alicyclobacillus acidoterrestris, Propionibacterium acnes*) and a fungus (*Trichophyton mentagrophytes*) with MIC ranging from 1.0 to 31 μg/ml (Takahashi et al. 2004).

Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) in already hospitalised patients or with week immune systems pose a serious problem all over the world because MRSA strains are resistant to numerous antibiotics and can be transmitted from patient to patient via transiently colonised hands of hospital personnel (Mulligan et al. 1993). Vancomycin is the most used antibiotic for MRSA infections, but some problems can occur, such as side effects or infections from vancomycin resistant enterococci (Schouten et al. 2000). For these reasons, use of flavonoids alone or in combination with traditional antibiotics has been considered as an alternative for treating MRSA infections (Pathak et al. 1991).

In a study by Drewes and van Vuuren (2008) the extraction and the isolation of 3-methoxyquercetin, 2′-hydroxy-6′-methoxychalcone, 2′-hydroxy-4′,6′-dibenzyloxychalcone and 5,7-dibenzyloxyflavanone from the yellow flowers of *Helichrysum gymnocomum* DC (Asteraceae) were described. The presence of dibenzyloxy derivatives was unexpected because their existence in nature had not yet been recorded. The antimicrobial test results revealed high activity of these compounds against several pathogens with MIC values below 64 μg/ml against a selection of pathogens (including *Staphylococcus aureus* and the *S. aureus* methicillin and gentamycin resistant strains). The authors hypothesised that the presence of the benzyloxy group could affect antimicrobial activity. (Drewes and van Vuuren 2008). From the roots of *Erythrina poeppigiana* (Walp.) O.F. Cook (Fabaceae), a tree widely distributed in Central and South America, 3,9-dihyroxy-10-γ,γ-dimethylallyl-6a,11a-dehydropterocarpan **92** (Fig. 2.11), was isolated by Tanaka et al. (2004): it inhibited bacterial growth of MRSA at a MIC of 12.5 μg/ml against 13 MRSA strains. Inhibitory activity resulted based on bactericidal action and the biological studies reported in the article suggest that this compound exhibits

anti-MRSA activity by interfering with incorporation of metabolites and nutrients into bacterial cells or by affecting the nucleic acids of MRSA cells (Tanaka et al. 2004). Therefore, compound **92** could be studied as a potent phytotherapeutic agent for treating MRSA infections. Bioassay-guided fractionation of the ethanol extract from leaves of *Artocarpus sepicanus* Diels (Moraceae), a tree growing in tropical and subtropical Asia, led to the isolation of a new geranyl flavanone named sepicanin A **64** (Fig. 2.7). Antimicrobial test indicated the compound displayed a significant selective antibacterial activity against MRSA with MIC values of 2.9 μM. Moreover, compound **64** was potently bactericidal having a minimum bactericidal concentration of 2.9 μg/ml (Radwan et al. 2009). More prenyl flavanones active as anti-MRSA were recently isolated from propolis (see Sect. 2.4.2) collected in 2006 from Guadalcanal Province (The Solomon Islands) (Raghukumar et al. 2010). Among them propolin C **63** (Fig. 2.7), and propolin D **61** (Fig. 2.7) were screened, using an agar dilution assay, against 15 MRSA clinical isolates and showed good activity against all MRSA isolates and the methicillin-susceptible *Staphylococcus aureus* strain (MIC values in the range of 8–32 and 8–16 μg/ml respectively (Raghukumar et al. 2010). Otsuka et al. (2008) in a study isolated and identified kaempferol 3-O-(2″,4″-di-*E*-*p*-coumaroyl)-rhamnoside and kaempferol 3-O-(2″-*Z*-*p*-coumaroyl-4″-*E*-*p*-coumaroyl)-rhamnoside from *Laurus nobilis* L. (Lauraceae) leaves and measured their MICs (1–2 μg/ml) against several MRSA strains. The anti-MRSA activity of the two pure compounds was much higher than that of chemotherapeutics such as oxacillin, ciprofloxacin, norfloxacin, erythromycin and tetracycline and a little weaker than that of vancomycin, the clinical first-choice drug for MRSA infections. Furthermore, these compounds had a very good activity (MIC 0.5 μg/ml) against a methicillin-sensitive *Staphylococcus aureus* showing to be effective on both methicillin-sensitive and methicillin-resistent *Staphylococcus aureus* at similar concentrations. It was noticed that the compounds studied were also moderately effective on vancomycin-resistant enterococci, hence, the authors proposed these molecules as leads to develop anti vancomycin-resistant enterococci agents (Otsuka et al. 2008). Regarding structure-activity relationships, the 4′-hydroxyl substitution of the A ring, the 5,7-dihydroxyl substitutions in the B ring and the 3-*O*-acylated rhamnose were judged important for the anti-MRSA activity. The authors also proposed that the cumaroyl chains are important for the flavonoids to enter into MRSA cells (Otsuka et al. 2008). In addition, 3-O-methylquercetin isolated from *Carex folliculate* L. (northern long sedge) (Cyperaceae), a forage widely prevalent in the northern United States, inhibited MRSA bacterial growth, with an IC_{50} value of 6.5 μM (Li et al. 2009).

In this paragraph we have shown the most important studies on isolated flavonoids as potential antimicrobial agents published in the literature in the last 5 years. The very high increase of the number of these studies with respect of the previous periods indicates the urgent need to discover and develop alternative therapies to current antibiotics. Flavonoids are natural products with different scaffold from known antibiotics that would have less tendency to generate resistance. Hence, to isolate and identify new active flavonoids is very important not only to understand the antimicrobic properties of herbal remedies, but also to identify new molecules as lead to obtain new and more potent antibiotics.

2.4.2 *Antibacterial Activity of Extracts Containing High Concentrations of Flavonoids*

Propolis or bee glue is a complex resinous mixture of different plant exudates, which is gathered, modified and used by honeybees as a general purpose sealer and draught excluder in their hives.

More than 160 constituents have been identified in different propolis samples. It usually consists of waxes, resins, water, inorganic compounds, phenolics and essential oils. Due to its several biological and pharmacological activities, it has always been used in folk medicine, and is recently incorporated in health drink and food to improve health and prevent diseases such as inflammation, heart disease, diabetes, aging and cancer. It has been suggested that the presence of a large number of flavonoids, aromatic acids and phenolic compounds are responsible for the most biological and pharmacological activities of propolis. In general, propolis composition is directly related to that of bud exudates collected by honey bees from various trees: poplar, birch, beech, horse chestnut, alder and various conifers. Many articles relating to the antimicrobial activity of propolis are present in the literature. Among them the work of Mohammadzadeh et al. (2007) describes the inhibition in the growth of a number of bacteria and fungi (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus niger*) by propolis ethanol extract. The highest antibacterial activity was found against Gram-positive bacteria (MICs 125 μg/ml) that can probably attributed to the presence of high levels of caffeate esters and flavonoid compounds. Furthermore, the high antifungal activity showed by this extract was related to the presence of large amount of flavanones such as pinocembrin **48** (Fig. 2.6), pinostrobin **49** (Fig. 2.6), pinobanksin **50** (Fig. 2.6) and pinobanksin-3-acetate (Mohammadzadeh et al. 2007). Similarly, in a study by Yaghoubi et al. (2007) analogous activities in several Iranian propolis samples were attributed to the above cited compounds. Activity against *Staphylococcus aureus* was also determined for propolis samples collected from Santiago del Estero, Argentina. The highest antibacterial activities were measured in propolis from Banda and Capital departments. These samples contained noticeable concentrations of pinocembrin **48** which, as already referred to, it is one of the most effective flavonoids against bacteria. Antimicrobial activity correlated better with the pinocembrin content than with the total polyphenol content (Chaillou and Nazareno 2009).

As reported in Sect. 2.4.1, some isolated flavonoids showed an interesting anti-cariogenic properties. Also extracts with significant levels of flavonoidic compounds exhibited the same activity. For example, fractions rich in flavonoids from Brasilian propolis with anti-caries activity have been studied (Hayacibara et al. 2005) and the antiplaque activity of plant extracts containing a high amount of flavonoids was reported (Tsai et al. 2007; Kamrani et al. 2007). An inhibitory effect against *Streptococcus sobrinus* of methanol extract (MIC 4 mg/ml) and of aqueous extract (MIC 16 mg/ml) of rosemary (*Rosmarinus officinalis* L., Lamiaceae) has been reported (Tsai et al. 2007). Similarly, the ethanol extracts of *Pistacia vera* L. hull

inhibited the growth and the acid production of the bacteria involved in the dental caries; in particular, it was active against *Streptococcus mutans* at concentrations of 2%, 6% and 10% w/v (Kamrani et al. 2007). In a recent review by Ferrazzano et al. (2009) the potential anti-cariogenic actions of cocoa, coffee and tea beverages, which are a rich source of dietary flavonoids, and the role of their flavonoids in this activity is reported. The authors concluded that the studies carried out in last few decades have supported the antibacterial role of polyphenols from cocoa, coffee and tea, but at the present time their potential use in the control of bacteria responsible of cariogenesis is still under scrutiny. A relatively larger body of evidence has been accumulated on the effects of tea (particularly the green tea) on plaque formation; whereas, the data on cocoa and coffee are at a preliminary stage. So tea can be considered as a functional food for oral health: drinking tea counteracts the negative effects of *Streptococcus* spp. on teeth health (Ferrazzano et al. 2009). Further research on anti-cariogenic activity of these beverages could make it possible for its use in some commercial applications, since they are relatively safe, have taste and odor that is largely appreciated and could be used at a reasonable cost in the preparation of anti-cariogenic products.

Antimicrobial activity of tea catechin **87** (Fig. 2.10), epicatechin **88** (Fig. 2.10), other catechins and theaflavins has been recently reviewed by Friedman (2007). This review summarises the information reported in the literature on inhibitory activities of tea flavonoids and teas against pathogenic bacteria, bacterial toxins produced by some of these bacteria, and pathogenic and phytopathogenic viruses and fungi. Synergistic, mechanistic, and bioavailability aspects of the antimicrobial effect are also covered (Friedman 2007).

The American cranberry (*Vaccinium macrocarpon* Ait (Ericaceae)) was used by North-American Indians to fight urinary tract infections and other bacterial diseases. Proanthocyanidins, present in cranberries as oligomers of catechin and epicatechin, inhibit the docking of bacteria on tissues *in vitro* an anti-adhesive mechanism of cranberry-proanthocyanidins (Howell et al. 2001). The efficacy of cranberry juice and extracts as a prophylactic agent against recurrent urinary infections is well documented in women (Stothers 2002). The anti-adhesion effect of cranberry-proanthocyandins can also be applied for treatment of other common diseases of bacterial pathogenesis, e.g. *Helicobacter pylori*-associated gastritis and dental caries/periodontal disease (Nowack 2007).

Lyme borreliosis, caused by *Borrelia burgdorferi sensu lato*, may lead to long-term tissue infection, which may be difficult to cure. In fact this spirochete can convert (and reconvert) to cystic forms, so the infection sometimes is persistent and reactivating. Therefore, it is important to use an antibiotic capable of eradicating all germative forms (not only the motile form) of the bacterium to obtain a proper treatment for Lyme borreliosis. Grapefruit-seed extract contains bioactive flavonoids (e.g., hesperitin **46**, Fig. 2.6 and naringenin **43,** Fig. 2.6) and has been shown to possess anti-microbiological effect against bacteria and fungus (Heggers et al. 2002). To test the hypothesis that motile and cystic forms of *Borrelia burgdorferi sensu lato* can be susceptible to grapefruit-seed extract, a study was conducted by Brorson and Brorson (2007). The highest grapefruit-seed extract concentrations

(0.165–0.0052%) caused a complete disappearence of bacteria and cysts; at lower extract levels the membranes showed herniation and disruption. Grapefruit-seed extract was very active even for very short incubation times, so the author indicated this extract (possibly in combination with antibiotics) as an efficient tool in the treatment of resistant Lyme borreliosis (Brorson and Brorson 2007).

Rutin **30** (Fig. 2.5) and isoquercitrin **31** (Fig. 2.5) were the main components of two fractions from the ethanolic extract of *Pelargonium radula* (Cav.) L'Hérit (Geraniaceae) leaves. Antimicrobial activities of the two fractions were tested against 11 species of bacteria and 11 of fungi. Both flavonoidic fractions inhibited the growth of many bacterial strains tested. In particular, both fractions demonstrated strong inhibitory activity against *Staphylococcus aureus*, *Proteus rettgeri, Candida tropicalis* and *Microsporum gypseum* (active concentration less than 7%). Only isoquercitrin-rich fraction strongly inhibited *Staphylococcus* sp. (coagulase-negative) and *Candida lusitaniae*, while rutin-rich fraction was active only against *Fusarium graminearum*. Although flavonoids are not the main active principle of *Pelargonium radula*, these results demonstrated that the main flavonoids, isoquercitrin and rutin, contribute significantly to the intensity and range of the overall antimicrobial activity of the plant (Pepeljnjak et al. 2005).

Murta (*Ugni molinae* Turcz. (Myrtaceae)) is a wild shrub growing in the coast and pre-Andean mountains of South Chile. Murta fruits are also highly appreciated due to the pleasant flavor and aroma. The infusions have been traditionally used to lessen urinary tract pain, and as astringents, stimulants and phytoestrogenics. A recent study reported that the aqueous extracts from Murta leaves decreased the growth of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*, and showed no activity against beneficial, probiotic bacteria. Myricetin glucoside and quercetin glucoside/glucuronide/dirhamnoside were hypothesised to contribute to the antimicrobial activity of the extracts, and a significant correlation between the polyphenol content and antimicrobial activity on harmful bacteria was found (Shene et al. 2009).

2.4.3 Antibacterial Activity of a Combination of Flavonoids or a Combination of Flavonoids and Antibiotics (Synergistic Effect)

The development of antibiotic resistance in bacteria can be natural (intrinsic) or acquired and this can be transmitted within the same or different species of bacteria. A spontaneous gene mutation gives rise to natural resistance; whereas, the acquired resistance is achieved through the transfer of DNA fragments like transposons from one bacterium to another. One strategy employed to overcome this resistance is the use of a combination of drugs that give synergism. Synergism is a positive interaction created when two agents combined exert an effect that is greater than the sum of their individual effects. For example, the most successful strategy that has been adopted to overcome resistance to penicillinase is by administering inhibitors of

β-lactamases as clavulinic acid with antibiotics as sulbactam and tazobactam (Hemaiswarya et al. 2008). Combination therapy can be used also to expand the antimicrobial spectrum, to obtain synergistic antimicrobial activity that permit to lower dosages of drugs and to minimize toxicity. It could be an alternative to monotherapy for patients difficult to treat by standard treatment (Aiyegoro and Okoh 2009). Flavonoids are studied for their possible synergistic effect in combination among them and with classical antibiotics and may be important tools in antibacterial strategies.

Extended-spectrum-lactamases (ESBLs) are plasmid-mediated class A enzymes commonly found in the family Enterobacteriaceae, mainly in *Klebsiella pneumoniae*. In a study by Lin et al. (2005), the flavonol myricetin **22** (Fig. 2.4) inhibited ESBL-producing *Klebsiella pneumoniae* at a high minimum inhibitory concentration, but exhibited significant synergic activity against the same bacteria in separate combination with amoxicillin/clavulanate, ampicillin/sulbactam and cefoxitin. Because of low toxicity of flavonoids, the authors concluded that the combination of antibiotics and flavonoids could be a new strategy for developing therapies for infections caused by ESBL-producing bacteria (Lin et al. 2005).

In an interesting paper (Alvarez et al. 2006), the bacteriostatic action of flavonoids quercetin **9** (Fig. 2.3), morin **21** (Fig. 2.4) and rutin **30** (Fig. 2.5) and combinations of two of them against *Staphylococcus aureus* and *Escherichia coli* was studied. The combinations improved the bacteriostatic action of a single flavonoid. In fact a noticeable diminution of MIC against *Escherichia coli* in the presence of a second flavonoid demonstrated synergism between the compounds studied. The combinations of different flavonoids, on the contrary, were not effective in improving activity against a Gram-positive bacterium such as *Staphylococcus aureus*. These results may be due to a different transport mechanism through the bacterial cell walls of the flavonoids considered (Alvarez et al. 2006).

Aerial parts of *Galenia africana* L. var. *africana* (Aizoaceae) are being used in South Africa to treat venereal sores, asthma, coughs, wounds, eye infections, tuberculosis, and skin diseases. Indigenous tribes chew the leaves to relieve toothache. The bioassay-guided fractionation of the ethanol extract of the leaves of this plant led to the isolation of some flavonoids; among them, 5,7,2′-trihydroxyflavanone and (*E*)-2′,4′-dihydroxychalcone **70** (Fig. 2.8) exhibited moderate antituberculosis activity against *Mycobacterium smegmatis* and *M. tuberculosis*. But a combination of 5,7,2′-trihydroxyflavanone and antituberculosis drug isoniazide reduced their original MICs 16-fold, demonstrating significant synergistic activity (Mativandlela et al. 2009).

In another study, the *in vitro* activities of antibiotic and phytochemical combinations against *Pseudomonas aeruginosa* were tested by the fractional inhibitory concentration method, derived from the MICs of the agents in combination. The antimicrobial activity of phytochemicals (protocatechuic acid, quercetin **9** (Fig. 2.3), caffeic acid), alone and in combination with antibiotics, was evaluated using the checkerboard assay and time-kill curve methods. There was synergism between gentamicin and caffeic acid, and sulfadiazine and the three phytochemicals under investigation. The MIC of sulfadiazine was 256 μg/ml, and that of gentamicin 2 μg/ml. When gentamicin was combined with one-quarter the MIC of caffeic

acid, the MIC of gentamicin was reduced fourfold. When sulfadiazine was tested with one-quarter the MIC of protocatechuic acid, quercetin, and caffeic acid, the MIC was reduced fourfold in combination with each of the drugs. These results indicate the potential efficacy of phytochemicals in combination with antibiotics for enhancing total biological activity (Sakharkar et al. 2009).

In the ethanol extracts of *Mentha longifolia* (Lamiaceae) were identified five flavonoids: luteolin-7-O-glucoside, luteolin-7,3′-O-diglucoside, apigenin **2** (Fig. 2.3), quercetin-3-O-glucoside and kaempferol-3-O-glucoside. These molecules were assayed against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus ceruleus*, *Pseudomonas aeruginosa* and *Bacillus subtilis* by the diffusion on agar method and gave an inhibition with MICs varying between 0.095 and 0.025 mg/ml. Only the luteolin-7-O-glucoside and the luteolin-7,3′-O-diglucoside did not have an action on the growth of *Escherichia coli* and *Pseudomonas aeruginosa.* Quercetin-3-O-glucoside inhibited the growth of the five species and gave for each one the lowest MIC. But, the synergism among the three most active flavonoids (apigenin, quercetin-3-O-glucoside and kaempferol-3-O-glucoside) gave the best antimicrobial activity with inhibition of 50 μg/ml for *Escherichia coli*, 70 μg/ml for S*taphylococcus aureus*, 45 μg/ml for *Bacillus cereus*, 40 μg/ml for *Pseudomonas aeruginosa* and 10 μg/ml for *Bacillus subtilis* (Akroum et al. 2009).

The Chinese herb *Alpinia officinarum* H. (Zingiberaceae) has been used in the Orient for several hundreds of years as a spice and to treat various infectious diseases; one of its active constituents is galangin **13** (Fig. 2.3). A recent study investigated the antimicrobial activity of galangin and gentamicin against 16 MRSA strains and a methicillin-susceptible *Staphylococcus aureus* MSSA strain. The MICs of galangin were in the range of 62.5–125 μg/ml, and the MICs of gentamicin ranged from 1.9 μg/ml to 2,000 μ/ml. Furthermore, gentamicin-galangin combinations against three MRSA strains and the MSSA strain markedly lowered the MICs, showing a marked synergism (Lee et al. 2008). Therefore, the mixture of gentamicin and galangin could be used in combination against *Staphylococcus aureus* infection.

Propionibacterium acnes is a non-spore forming, Gram-positive anaerobic bacterium, a major etiologic agent of acne vulgaris. Topical antibiotics reduce the population of *P. acnes* and exert anti-inflammatory activity, but have the major disadvantage of dramatically increasing bacterial resistance. The *in vitro* antibacterial activity against antibiotic-resistant *P. acnes* of kaempferol **8** (Fig. 2.3) (isolated from the methanol extract of the flowers of *Impatiens balsamina* L., Balsaminaceae) and quercetin **9** (Fig. 2.3), alone and in combination with erythromycin or clindamycin was investigated by Lim et al. in 2007. All the combinations except that of kaempferol with quercetin were synergic. The combination of kaempferol and quercetin showed an additive effect. The two compounds have a similar structure, suggesting that they probably inhibit the growth of *P. acnes* by the same action mechanism. The results showed that the amount (4 μg/ml) of clindamycin combined with kaempferol or quercetin required to inhibit antibiotic-resistant *P. acnes* was less than an eighth of the amount (32 μg/ml) required using clindamycin alone. In this case, the amounts of kaempferol and quercetin required to inhibit antibiotic-resistant *P. acnes* were also reduced from 64 to 4 μg/ml and from 64 to 8 μg/ml,

respectively. In the case of combining erythromycin with kaempferol or quercetin, the amount of erythromycin was reduced to a fourth of the amount required when used alone and the amounts of kaempferol and quercetin required were both 1 μg/ml. The combination of clindamycin with kaempferol or quercetin showed the most synergistic effect, so they could be useful to develop tools to treat acne (Lim et al. 2007).

The antibacterial effects of tea polyphenols extracted from Korean green tea (*Camellia sinensis* (L.) O. Kuntze (Theaceae)) against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) were evaluated (Cho et al. 2008). Characterisation of the MIC of oxacillin for 30 *Staphylococcus aureus* strains isolated from patients treated with oxacillin identified 13 strains with an oxacillin MIC ≥ 4 μg/ml as MRSA (range: 8–512 μg/ml), while 17 strains were methicillin-susceptible *Staphylococcus aureus* (MSSA; range: 0.25–0.5 μg/ml). The MICs of tea polyphenols ranged from 50 to 180 μg/ml for both the MSSA and the MRSA strains. The MICs of oxacillin for each of the 13 MRSA strains were reduced between 8- and 128-fold when these strains were coincubated with sub-MIC (≤0.5 × MIC) levels of tea polyphenols, demonstrating that this combination was synergistic for all of the clinical MRSA isolates (Cho et al. 2008).

Antibacterial activity of quercetin **9** (Fig. 2.3) was closely studied very recently against Gram-positive and Gram-negative bacteria and was found to exert selective antibacterial activity against *Staphylococcus aureus*, including MRSA, and *Staphylococcus epidermidis* (Hirai et al. 2010). Other tested Gram-positive cocci and Gram-negative rods, such as *Streptococcus pneumoniae, Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Enterococcus faecalis,* and *Escherichia coli*, did not show susceptibility to any concentration of quercetin up to 50 μM. On the contrary, some clinical MRSA strains showed remarkable susceptibility to quercetin. Furthermore, in combination with antibiotics, such as oxacillin, ampicillin, vancomycin, gentamicin, and erythromycin, quercetin showed markedly enhanced antibacterial activity against MRSA. Such combination therapy may prove to be a promising strategy for overcoming the problems encountered in the treatment of MRSA infections. The results of this study indicate that inhibitors of bacterial cell wall synthesis, such as oxacillin, erythromycin, ampicillin and vancomycin, brought about a remarkable combination effect on antibacterial activity, while minocycline and rifampicin, which are protein and nucleic acid synthesis inhibitors, showed no combination effect with quercetin. Thus, the authors suggested that inhibition of bacterial cell wall synthesis may play a role in the enhancement of quercetin antibacterial activity when used in combination with other antibiotics (Hirai et al. 2010).

2.5 Flavonoids as Antifungal

A fungus is a living organism that can cause infection when it grows in the human body. Some fungi, such as *Candida*, are usually found in the bodies of healthy people and cause little or no harm. Other fungi, such as *Aspergillus* and *Cryptococcus*, are

found in the air. Infection can occur when the fungus is either inhaled into the lungs or comes into contact with post-operative wounds. For immuno-depressed patients, fungal infections can become severe and must be treated quickly. For example, cancer patients tend to have weakened immune systems as a result of chemotherapy or the disease. Once they are infected, their weak immune system allows the fungus to grow quickly. Because of this risk, some cancer patients even with no obvious fungal infection are treated with antifungal therapy to prevent infection from developing (Kaplan et al. 2009). There are three classes of drugs typically used to treat fungal infections: polyenes, azoles, and echinocandins (Li and Calderone 2008). In these last years, the incidence of opportunistic fungal infections in immuno-compromised patients, such as those undergoing treatment with immunosuppressive drugs, intensive chemotherapy, AIDS patients and neonates, is increasing at an alarming rate. These mycoses are very difficult to eradicate so the search for new effective compounds is very important (Gullo 2009).

Fungi are also important plant pathogens causing disease in cultivated plants, hence, reducing yield and quality in agricultural cultivation, thereby, causing considerable economic loss for producers. Plant pathogens are estimated to cause yield reduction in crops of almost 20% worldwide (Oerke et al. 1994). Moreover, food quality has to be guaranteed by controlling fungi that produce mycotoxins (Hadacek and Greger 2000). Some of the species causing plant diseases are *Fusarium oxysporium*, *Corynespora cassiicola* and *Cochliobolus miyabeanus. Aspergillus* and *Penicillum* species are responsible for contamination and spoilage of food and production of micotoxin as well. The use of fungicides could be strategy for reducing all these problems. Synthetic fungicides are used to control plant diseases in agriculture, but they can cause environmental problems. Furthermore, fungi can develop resistance and this fact also limits their application. Besides, synthetic compounds used in foods as additives are not welcomed by consumers, while natural additives are seen as a benefit for both quality and safety. So the demand for antifungal molecules of natural origin by the food industry and agriculture is increasing.

As a number of flavonoids have been recognised as plant defensive agents, their antifungal activity has been proposed for use against fungal pathogens in humans, in agriculture and for foodstuff protection. Although the action mechanism of such compounds against fungi is still unknown, their efficacy, availability at low cost, and low toxicity to humans give flavonoids potential as natural fungicides.

Until 2003 few papers were published in the literature about the antifungal activity of flavonoids on human and plant fungal pathogens and reported in a couple of reviews (Pretorius 2003; Cushnie and Lamb 2005). In last few years interest in these topics is growing and the numbers of studies have significantly increased. In Table 2.2 we have listed the most significant compounds cited, their plant sources and main fungal targets.

Many researchers have studied in particular flavonoid activity against *Candida albicans,* which is a pleomorphic fungus that can exist either as a commensal or an opportunistic pathogen and is capable of causing infection. In immuno-compromised individuals, the yeast can pass through the mucosa and invade different tissues, resulting in significant damage and even mortality. *C. albicans* secretes various

Table 2.2 List of antifungal flavonoids cited in this chapter

Compound no.	Name	Plant sources	Fungi	References
2	Apigenin	–	*Candida albicans*	Yordanov et al. (2008)
3	Luteolin	*Ficus sarmentosa*	*Fusarium graminearum*	Wang et al. (2010)
			Septoria zeicola	
4	–	*Psoralea corylifolia*	*Trichophyton rubrum*	Prasad et al. (2004)
			Trichophyton mentagrophytes	
			Epidermophyton floccosum	
			Microsporum gypseum	
7	–	*Kaempferia parviflora*	*Candida albicans*	Yenjai et al. (2004)
8	Kaemferol	–	*Candida albicans*	Yordanov et al. (2008)
9	Quercetin	–	*Aspergillus carbonarius*	Romero et al. (2009)
14	–	*Kaempferia parviflora*	*Candida albicans*	Yenjai et al. (2004)
19	Baicalein	*Scutellaria baicalensis*	*Candida albicans*	Cao et al. (2008)
		–	*Cryptococcus gatti*	McNulty et al. (2009)
23	Nobiletin	–	*Penicillium digitatum*	Ortuño et al. (2006)
30	Rutin	*Solanum palinchantum*	*Aspergillus ochraceus*	Pereira et al. (2008)
		Grapes	*Aspergillus carbonarius*	Romero et al. (2009)
33	Trifolin	*Camptotheca acuminate*	*Alternaria alternata*	Li et al. (2005)
			Epicoccum nigrum	
			Pestalotia guepinii	
			Drechslera sp	
			Fusarium avenaceum	
34	Hyperoside	*Camptotheca acuminate*	*Alternaria alternata*	Li et al. (2005)
			Epicoccum nigrum	
			Pestalotia guepinii	
			Drechslera sp	
			Fusarium avenaceum	

(continued)

Table 2.2 (continued)

Compound no.	Name	Plant sources	Fungi	References
35	–	*Saussurea lappa*	*Aspergillus niger* *Aspergillus ochraceus* *Aspergillus versicolor* *Aspergillus flavus* *Penicilium ochrochloron* *Penicillium funiculosum* *Trichoderma viride*	Rao et al. (2007)
36	Orientin	*Piper solmsianum*	*Microsporum canis* *Microsporum gypseum* *Trichophyton mentagrophytes* *Trichophyton rubrum* *Epidermophyton flocosum*	De Campos et al. (2005)
38	–	*Butea monosperma*	*Fusarium digitatum* *Penicillum digitatum*	Yadava and Tiwari (2007)
40	Vitegnoside	*Vitex negundo*	*Cryptococcus neoformans* *Trichophyton mentagrophytes*	Sathiamoorthy et al. (2007)
42	Eriodictyol	*Ficus sarmentosa*	*Fusarium graminearum* *Septoria zeicola*	Wang et al. (2010)
44	Liquiritigenin	*Pterocarpus indicus*	*Fusarium oxyxporum* *Cochliobolus myadensis* *Corynespora cassiicola* *Tricoderma harzianum* *Penicillum italicum* *Aspergillum niger*	Kusuma et al. (2005)
45	Homoeriodictyol	*Ficus sarmentosa*	*Fusarium graminearum* *Septoria zeicola*	Wang et al. (2010)
48	Pinocembrin	*Swartzia apetala*	*Candida* sp	de Araujo et al. (2009)
51	Dihidroquercetin	*Ficus sarmentosa*	*Fusarium graminearum* *Septoria zeicola*	Wang et al. (2010)

52	Naringin	–	*Penicillium digitatum*	Ortuño et al. (2006)
54	Hesperidin	*Citrus sinensis* *Citrus paradisi*	*Penicillium digitatum*	Ortuño et al. (2006)
67	Angusticornin B	*Dorstenia angusticornis*	*Candida* sp	Kuete et al. (2007)
68	Isobavacalchone	*Dorstenia barteri*	*Candida albicans* *Candida glabrata*	Mbaveng et al. (2008)
71	Isoliquiritigenin	*Pterocarpus indicus*	*Fusarium oxyxporum* *Cochliobolus myadensis* *Corynespora cassiicola* *Tricoderma harzianum* *Penicillum italicum* *Aspergillum niger*	Kusuma et al. (2005)
78	Parvisoflavone B	*Eriophorum scheuchzeri*	*Cladosporium cucumerinum* *Candida albicans*	Maver et al. (2005)
79	Parvisoflavone A	*Eriophorum scheuchzeri*	*Cladosporium cucumerinum* *Candida albicans*	Maver et al. (2005)
81	Biochanin A	*Swartzia polypbylla*	*Trichophyton mentagrophytes* *Microsporum gypseum*	Rojas et al. (2006)
86	Dihydrobiochanin A	*Swartzia polypbylla*	*Trichophyton mentagrophytes* *Microsporum gypseum*	Rojas et al. (2006)
93	Amentoflavone	*Cupressocyparis leylandii*	*Altemana altemate* *Cladosporium oxysporum* *Fusarium culmorum*	Prasad et al. (2004)
		Taxus baccata *Ginkgo biloba*	*Candida albicans* *Saccharomyces cerevisiae*	Jung et al. (2006)
		Selaginella tamariscina	*Trichosporon beigelii*	
94	Chamaejasmenin A	*Stellera chamaejasme*	–	Yang et al. (2005)
95	Chamaejasmenin D	*Stellera chamaejasme*	–	Yang et al. (2005)
96	Isochamaejasmenin B	*Stellera chamaejasme*	–	Yang et al. (2005)

proteolytic and lipolytic enzymes that facilitate its penetration into host cells. Apigenin **2** (Fig. 2.3) and kaempferol **8** (Fig. 2.3) were tested in mice using a model of systemic *C. albicans* infection, demonstrating a strong decrease in the number of isolated live *Candida* cells on day 5 and day 10, with the best effect established for kaempferol. This effect might be related to inhibition of extracellular enzyme activity of *C. albicans* in systemic infection. Furthermore, the demonstrated beneficial effect of kaempferol in cutaneous *C. albicans* infection requires further investigations on its mechanism of action (Yordanov et al. 2008).

Pereira et al. (2008) reported isolation of rutin **30** (Fig. 2.5) from *Solanum palinacanthum* Dunal (Solanaceae), a perennial herb common in pastures and roadsides in Brazil, where it has been used to treat skin diseases. Rutin showed low antibacterial activity by broth microdilution assays, but the MIC value against *Aspergillus ochraceus* (35 μg/ml) was similar to that of benzalkonium chloride, the only fungicide used in Brazil to control such fungus in coffee beans.

The effect of phenolic compounds, such as quercetin **9** (Fig. 2.3) and rutin **30** (Fig. 2.5), naturally occurring in grapes, were evaluated on *Aspergillus carboarius* growth and Ocratoxin A production in a study by Romero et al. (2009). A significant decrease in production of toxin and a less effect on *A. carbonarius* growth was observed with the two flavonoids studied in comparison with the control. The successful inhibition of both mycelial growth and release of ochratoxin A by these compounds normally present in grapes indicates the possibility of their use as plant fungicides, especially against the growth of ochratoxigenic *Aspergilli* (Romero et al. 2009).

Saussurea lappa C.B. Clarke, syn. *Saussurea costus* (Falc) Lipsch (Asteraceae) is a Himalayan species that occurs at elevations from 2,700 to 4,000 m in Kashmir. Four new flavonoids were isolated from ethanol extract of the roots and assayed against *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus versicolor*, *Aspergillus flavus*, *Penicilium ochrochloron*, *Penicillium funiculosum*, *Trichoderma viride*. The most active compound was kaempferol glycoside **35** (Fig. 2.3) with MICs ranging from 0.3 to 1.2 nmol/ml. Commercial fungicide miconazole was used as a control (MICs 1.0–4.0 nmol/ml) (Rao et al. 2007).

Leaf spots and root rots are major fungal diseases in *Camptotheca acuminate* Decne (Cornaceae) that limit cultivation of this tree from which camptothecin, a promising anticancer and antiviral alkaloid, is extracted (Li et al. 2005). Flavonoids trifolin **33** (Fig. 2.5) and hyperoside **34** (Fig. 2.5), isolated from this same plant effectively controlled fungal pathogens *in vitro*, including *Alternaria alternata, Epicoccum nigrum, Pestalotia guepinii, Drechslera sp*., and *Fusarium avenaceum.* They inhibited mycelial growth by approximately 50% (EC_{50}) at 100 or 150 μg/ml and has been considered as leads for the development of new fungicides. But their antifungal activity in the plant is very low, and authors concluded that these flavonoids can not be used to control fungal pathogens in *Camptotheca* plantations and to enhance camptothecin production (Li et al. 2005).

Among local people in the northeast of Thailand, the rhizomes of *Kaempferia parviflora* (Zingiberaceae) have been known as a health-promoting herb, which is also frequently used for the treatment of colic disorder, peptic and duodenal ulcers

and as a tonic drink made commercially available (Chomchalow et al. 2003). Yenjai et al. (2004) isolated two flavone derivatives, compounds **14** (Fig. 2.3) and **7** (Fig. 2.3), having interesting antifungal activity against *Candida albicans* with respective IC_{50} values of 39.71 and 17.63 μg/ml. These flavonoids did not show cytotoxicity against a number of human cell lines. This information suggests that the rhizomes of *Kaempferia parviflora* may be a safe ingredient in traditional medicine preparations.

Biomaterials such as stents, shunts, prostheses, implants, endotracheal tubes, pacemakers and various types of catheters have all been shown to facilitate *C. albicans* colonisation and biofilm formation. Biofilm-associated infections are difficult to treat because of their low susceptibilities to antimicrobial therapy. It is reported that *C. albicans* biofilms are resistant to a variety of clinical antifungal agents, including amphotericin B and fluconazole (Ramage et al. 2005). Baicalein **19** (Fig. 2.4) is a major component of *Scutellaria baicalensis* Georgi (Lamiaceae), which is a Chinese herb and described in the Chinese Pharmacopoeia as a medicine. In a study by Cao et al. (2008) *in vitro* activity of baicalein against *C. albicans* biofilms was evaluated. Biofilm production was significantly inhibited by baicalein addition at any time up to 24 h, with 89% inhibition at 0 h and 52% inhibition at 24 h of the incubation period. This finding could be useful for the development of new strategies to reduce the incidence of device-associated infections and in preventing biofilm formation on surfaces and biomaterials (Cao et al. 2008). Furthermore, baicalein has a potent broad-spectrum antifungal properties while galangin exhibited potent and selective activity to *Cryptococcus gatti* ($IC_{50} = 0.5$ μg/ml, amphotericin B $IC_{50} \leq 0.25$ μg/ml). The results also showed significant overlap between cytochrome inhibitory and antifungal activity, indicating that a cytochrome target may be involved (McNulty et al. 2009).

In 2004, Prasad et al. reported that 4′-methoxy flavone **4** (Fig. 2.3) is the active compound of the methanolic extract of seeds of *Psoralea corylifolia* L. (Fabaceae) with significant antifungal activity against *Trichophyton rubrum*, *Trichophyton mentagrophytes, Epidermophyton floccosum* and *Microsporum gypseum*. The compound was submitted to the tube dilution method showing MICs of 62.5 μg/ml for *Trichophyton mentagrophytes* and *Trichophyton rubrum,* and 125 μg/ml for the rest of the susceptible species. It is interesting to note that the active flavonoid is a lipophilic compound possessing a methoxy group in the *para* position of B-phenyl ring. This function is considered to be essential for antifungal activity of the flavone because it could make easier the penetration of the molecule in fungal membranes (Prasad et al. 2004).

Also the biflavone amentoflavone **93** (Fig. 2.11) is an excellent candidate as an antifungal agent. It has been isolated from three different plants, including *Cupressocyparis leylandii* (Dallim. & A.B. Jacks.) (Cupressaceae), *Taxus baccata* L. (Coniferae), and *Ginkgo biloba* L. (Ginkgoaceae) and exhibited antifungal activity on phytopathogens such as *Altemana altemate*, *Cladosporium oxysporum*, *Fusarium culmorum*, and *Fusarium avenaceum* (Krauze-Baranowska et al. 1999; Krauze-Baranowska and Wiwart 2003). Recently, it has been considered as a candidate molecule for treating human infectious diseases caused by

pathogenic fungi. In fact compound **93**, isolated from *Selaginella tamariscina* (Beauv.) (Selaginellaceae), a traditional Chinese herb used for the therapy of chronic trachiitis and several forms of cancers in the Orient, exhibited a potent antifungal activity (MICs 5–10 μg/ml) against *Candida albicans, Saccharomyces cerevisiae* and *Trichosporon beigelii* without hemolytic activity on human erythrocytes (Jung et al. 2006).

A new screening bioassay detecting deformation of mycelia germinated from the conidia of *Pyricularia oryzae*, a phytopathogenic fungus, was developed for quantitative application in screening antimitotic and antifungal agents by Kobayashi and coworkers (Kobayashi et al. 1996). This bioassay method is quick, easy to perform, and has been efficiently used in the screening of antimitotic, antineoplastic, and antifungal agents, such as rhizoxin and fusarielin A from fungal metabolites. *Stellera chamaejasme* L. (Thymeleaceae), has traditionally been used to make paper in Tibet. It is a toxic plant widely distributed in the north and southwest of China, also used in veterinary medicine to purge livestock from worms and other intestinal parasites and in traditional Chinese medicine for the treatment of scabies, tinea, stubborn skin ulcers, chronic tracheitis, and tuberculosis (Xu et al. 2001). Diflavones chamaejasmenin A **94** (Fig. 2.11), chamaejasmenin D **95** (Fig. 2.11), and isochamaejasmenin B **96** (Fig. 2.11) extracted by Yang et al. (2005) from the roots of this plant demonstrated potent antimitotic and antifungal activity with MIC values of 3.12, 6.25, and 6.25 μg/ml, respectively. The positive control, rhizoxin, had a MIC value of 1.25 μg/ml. This activity suggested that the biflavanones **94–96** might be the principal antimitotic and antifungal components in *Stellera chamaejasme* (Yang et al. 2005).

Piper solmsianum C. DC. var. *solmsianum* (Piperaceae) is a shrub commonly found in areas with wet tropical soils. It grows in Brasil where it is known popularly as "pariparoba". From an ethnopharmacological point of view, the pungent and aromatic fruits of some species of *Piper* are used as spices and most of them find wide application in traditional medicine as insecticides, antivirals, antimicrobials and particularly antifungals. These biological properties have been attributed to the presence of lignans and/or amides, such as alkyl or olefinic isobutylamides, flavonoids, kawa-lactones, butenolides and cyclohexane epoxides (Sengupta 1987). In 2005, it was demonstrated that flavonoid orientin **36** (Fig. 2.5), isolated from *Piper solmsianum*, possessed a pronounced activity against *Microsporum canis*, *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Epidermophyton flocosum* (MICs between 7 and 9 μg/ml) with potency as high as the standard antifungal drug (ketoconazole) using the agar dilution method (De Campos et al. 2005).

Sathiamoorthy et al.(2007) found significant antifungal activity in the ethanol extract of leaves of *Vitex negundo* Linn. (Lamiaceae) against *Cryptococcus neoformans* and *Trichophyton mentagrophytes*. *Vitex negundo* is a large shrub grown throughout India and commonly used in traditional medicine having a variety of biological activities. In the same study (Sathiamoorthy et al. 2007) is described the isolation and evaluation of antifungal activity of three known flavonoids 5′-hydroxy-3′, 4′, 3, 6, 7-pentamethoxyflavone, luteolin **3** (Fig. 2.3), and isoorientin

37 (Fig. 2.5) and of a new flavone glycoside **40** (Fig. 2.5)**,** named vitegnoside. Compound **40** showed promising activity against *Trichophyton mentagrophytes* and *Cryptococcus neoformans* (MICs 6.25 μg/ml) compared to the standard antifungal drug fluconazole, having MIC of 2 μg/ml against *Trichophyton mentagrophytes* (Sathiamoorthy et al. 2007).

A new flavone glucoside was isolated in 2007 from the methanol fraction of *Butea monosperma* O. Kuntze (Fabaceae) flowers, a plant used in India for many diseases. The isolated compound was identified as 5,7-dihydroxy-3,6,4′-trimethoxyflavone-7-O-xylapyranosyl-(1–3)-O-arabinopyranosyl-(1–4)-O-galactopyranoside **38** (Fig. 2.5) and showed strong activity against *Fusarium digitatum* and *Penicillum digitatum* at the same concentration of the standard antifungal agent, griseofulvin (Yadava and Tiwari 2007).

Citrus peel is rich in flavanone glycosides and polymethoxyflavones. Ortuño et al. (2006) reported their content in the mature fruits of several varieties of *Citrus paradisi* Mac Fayden (grapefruit) and *Citrus sinensis* (L.) Osbeck (Rutaceae) (orange). The presence of three polymethoxyflavones, nobiletin **23** (Fig. 2.4), eptamethoxyflavone **25** (Fig. 2.4) and tangeretin **24** (Fig. 2.4) was ascertained in all the grapefruit varieties analysed. Also flavanones hesperidin **54** (Fig. 2.6) and naringin **52** (Fig. 2.6) were detected. Only nobiletin, hesperidin and naringin acted as antifungal agents against *Penicillium digitatum*, because, when added to a potato dextrose agar medium, they reduced its radial growth. The polymethoxyflavone nobiletin was the most effective compound, followed by the flavanones hesperidin and naringin; in fact the growth of the fungus 100 h after culture starting was inhibited by 75%, 38% and 25%, respectively, compared with the growths observed in the corresponding controls (Ortuño et al. 2006). Also another recent paper (Salas et al. 2011) reports the antifungal activity of isolated flavonoids from immature aborted fruits of the *Citrus* species, such as naringin **52** (Fig. 2.6), hesperidin **54** (Fig. 2.6) and neohesperidin **53** (Fig. 2.6), on four fungi often found as food contaminants: *Aspergillus parasiticus*, *Aspergillus flavus*, *Fusarium semitectum* and *Penicillium expansum*. All the flavonoids, at 0.25 mM, showed the capacity to alter the growth of fungi but at this concentration total inhibition was not achieved for any of the moulds. Although all the flavonoids showed antifungal activity, the intensity of this activity depended on the type of fungus and compound used. The possible use of these flavanones obtained as byproducts at low cost from the residues of the citrus industries as natural fungicidal agents opens an interesting option for these industries (Salas et al. 2011).

Recently, the methanol extract of stems and leaves of *Ficus sarmentosa* var. *henryi* (King) Corner (Moreaceae), a scramble and liana shrub widely distributed in Taiwan, Zhejiang and Sichuan provinces, China, demonstrated potent inhibitory activities against 6 crop pathogenic fungi, *Fusarium graminearum*, *Pumpkin fusarium*, *Curvularia lunata, Septoria zeicola, Botrytis cinerea*, and *Rhizoctonia solani* (Wang et al. 2010). Four flavonoids were isolated from this extract by activity-guided fractionation and they were identified as eriodictyol **42** (Fig. 2.6), homoeriodictyol **45** (Fig. 2.6), dihydroquercetin **51** (Fig. 2.6), and luteolin **3** (Fig. 2.3) and displayed excellent inhibitory activity against *F. graminearum* and *S. zeicola*. Among them,

luteolin showed the strongest inhibitory activity with IC50 values of 56.38 and 81.48 μg/ml, respectively (Wang et al. 2010).

Liquiritigenin **44** (Fig. 2.6) and isoliquiritigenin **71** (Fig. 2.8) are considered characteristic constituents of the leguminosae family. These two flavonoids, isolated from Amboyna wood (*Pterocarpus indicus* Willd., Leguminosae) by Kusuma et al. (2005), were reported to have a significant effect on mycelial growth of fungal pathogens. A low concentration of both compounds (MICs 20–40 μM and 16–33 μM, respectively) was enough to inhibit the growth of *Fusarium oxyxporum*, *Cochliobolus myadensis*, *Corynespora cassiicola*, *Tricoderma harzianum* and *Penicillum italicum*, while higher concentrations were needed for *Aspergillum niger* (MICs 320 and 265 μM, respectively). Antifungal activities resulted concentration dependent (Kusuma et al. 2005). As other plant species are sources of liquiritigenin and isoliquiritigenin, such as *Dalbergia sericea, Glycirrhiza inflate, Crinum bulbispermium, Siofranchetia sinensis*, production of these potentially useful compounds with biotechnological methods could be possible.

Swartzia apetala Raddi (Fabaceae) var. *glabra* is known in Brazil as "arruda rajada" and has been used in civil construction and hydraulic work on the basis of its large durability. From this source pinocembrin **48** (Fig. 2.6) was isolated and evaluated by the agar diffusion method against nine yeasts of the *Candida* genus. Compound **48** showed activity against most of yeasts analyzed with inhibition zone ranging from 5 to 15 mm (de Araujo et al. 2009). *Swartzia polyphylla* DC. (Leguminosae) is a tree found in the Amazonian region of Peru where the wood is used in construction. Isoflavones biochanin A **81** (Fig. 2.9) and dihydrobiochanin A **86** (Fig. 2.9), isolated from a methanol extract of *Swartzia polyphylla* bark, demonstrated at a concentration of 1 μg/ml, an antifungal activity against *Trichophyton mentagrophytes* and *Microsporum gypseum* similar to Amphotericin B and Itraconazol (1.6 μg/ml), used as a positive control, tested by the agar-well diffusion assay (Rojas et al. 2006). Several other *Swartzia* species from Africa and South America have been investigated, and have yielded molluscicidal steroidal saponins and pterocarpans with potential antifungal activity (Abdel-Kader et al. 2000; Borel and Hostettmann 1987: Borel et al. 1987; Magalhães et al. 2003).

Also other isoflavonoids as parvisoflavones A **79** (Fig. 2.9) and B **78** (Fig. 2.9), isolated from the aerial parts of *Eriophorum scheuchzeri* Hoppe (Cyperaceae) showed good antifungal activity against *Cladosporium cucumerinum* and *Candida albicans*, by bioautography on thin-layer chromatograms and by the agar overlay method, comparable with the reference compound miconazole. Both compounds **74** and **75** showed good activity, so the position of the dimethylpyran ring does not seem to influence this activity (Maver et al. 2005). The antimicrobial activity of the crude extract of the twigs of *Dorstenia barteri* as well as that of four flavonoids isolated from this extract was studied by Mbaveng et al. (2008). Isobavacalchone **68** (Fig. 2.8) was the most active compound showing an MIC value of 0.3 μg/ml against *C. albicans* and *C. glabrata* as reported also in the antibacterial section (Mbaveng et al. 2008). The susceptibility of *C. albicans* to some catechins and the synergism of the combination of catechins and antimycotics were evaluated by a Japanese group in 2004.

The antifungal activity of the catechins studied was pH dependent, in fact the concentration of epigallocatechin gallate **91** (Fig. 2.10) causing 90% growth inhibition of tested strains of *C. albicans* was 2,000 μg/ml at pH 6.0, 500–1,000 μg/ml at pH 6.5 and 15.6–250 μg/ml at pH 7.0. Other results reported in the study indicated that epigallocatechin gallate enhances the antifungal effect of amphotericin B or fluconazole against antimycotic-susceptible and -resistant *C. albicans* (Hirasawa and Takada 2004).

2.6 Conclusion

Antibacterial and antifungal activities of many natural flavonoids, isolated mainly from plant, have been covered in this chapter, starting from 2005 till now. A number of flavonoids have shown very interesting biological results, so they could become a useful alternative to synthetic antibiotics. In fact, antimicrobial activity is very promising when MIC is below 100 μg/ml for plant extracts and 10 μg/ml for isolated compounds (Rios and Recio 2005). From a survey of the chapter, many flavonoids or flavonoids-rich extracts possess the above cited requirements. For example flavone **1** could be included in antimicrobial, antifungal, and anti-*Helicobacter pylori* regimens for its high activity at low concentration. Isobavachalcone **68** and the crude extract of *Dorstenia barteri* are very interesting for their antibacterial properties, particularly strong against *Mycobacterium tubercolosis* and *Neisseria gonorrhoeae*. Kaemferol glycoside derivatives from *Laurus nobilis* showed a very important activity against MRSA strains and could represent a valid alternative to the actually used synthetic antibiotics and are worth being considered as clinically significant antibacterial and antifungal compounds or as food preservative. Among flavonoids with high antifungal activity, biochanin A **81** and dihydrobiochnin A **86** could represent an alternative to synthetic drugs.

Flavonoids are also important for their possible synergistic effect in combination among them or with classical antibiotics and may be an effective tool to overcome some of the problems arising in antimicrobial therapy. Up to now the number of known natural compounds acting in synergy with synthetic drugs against fungal and bacterial species is small, but many studies on flavonoids with this kind of synergistic activity are in progress.

From this survey of the cited literature we can notice that problems concerning the standardization of antimicrobial assays are not completely solved. Often, incomparability of results obtained by the different researchers using different methods greatly diminishes quality data and makes compilation of databases useless.

In conclusion, flavonoids and plant extract rich in flavonoidic compounds as antimicrobial agents present two main positive features: the first is their natural origin, which means more safety for consumers, and the second is that they are considered to be low risk for resistance development by pathogenic microorganisms; thus they can be considered a valuable support in the treatment of infections and may contribute to the development of new and safe antimicrobial agents.

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Chapter 3
Antiviral Properties of Phytochemicals

Ai-Lin Liu and Guan-Hua Du

Abstract In recent years, significant progress has been achieved for the development of novel anti-viral drugs. These newly developed drugs belong to three groups of compounds, nucleoside analogues, thymidine kinase-dependent nucleotide analogues and specific viral enzyme inhibitors. It has been found that the natural products, like plant-derived compounds (phytochemicals) as well as traditional medicines, like traditional Chinese medicines (TCM), Ayurvedic medicines and so on, are the important sources for potential and novel anti-viral drugs. In this chapter, the history of natural products as antiviral drugs, the approaches to discover potential lead compounds, and the anti-viral properties of phytochemicals with different action mechanisms are discussed. The key conclusion is that natural products are most important sources for novel anti-viral drugs.

Keywords Phytochemicals • Polysaccharides • Flavonoids • Organic acids • Alkaloids • Saponins • Essential oils • Stilbenes • Antiviral activity assay

Abbreviations

ADV	Adenovirus
AIDS	Acquired immunodeficiency syndrome
BHV	Bovine herpes virus
CB4	Coxsackie B4
CMV	Cytomegalovirus
Cox B3	Coxsackie B3
CPE	Cytopathic effect

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DENV-2	Dengue virus type-2
DHBV	Duck hepatitis B virus
EBV	Epstein-barr viurs
EC_{50}	50% effective concentration
ELISA	Enzyme linked immunosorbent assay
EV71	Enterovirus 71
Flu A	Influenza A
HA	Haemoglutinin
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HRV2	Human rhinovirus 2
HRV3	Human rhinovirus 3
HSV	Herpes simplex virus
HSV-1	Herpesvirus type 1
HSV-2	Herpesvirus type 2
HTS	High throughput screening
IC_{50}	50% inhibiting concentration
IVA	Influenza virus A
LD_{50}	Half lethal dose
MES	2-N-Morpholino-ethanesulfonic acid
MDCK	Madin-Darby canine kidney
MOI	Multiplicity of infection
MoMLV	Moloney murine leukemia virus
MUNANA	2′-(4-methylumbelliferyl)-α-D-acetylneuraminic acid
MTT	3-[4,5-Dimethyl-thiazol-2-yl] -2,5-diphenyl tetrazolium bromide
NA	Neuraminidase
PAGE	Polyacrylamide gel electrophoresis
PI-3	Para influenza 3
PRRS virus	Porcine reproductive and respiratory syndrome virus
RBC	Red blood cells
RSV	Respiratory syncytial virus
RT	Reverse transcriptase
RT-PCR	Real time polymerase chain reaction
SAR	Structure-activity relationship
SARS	Severe acute respiratory syndrome
SARS-CoV	Severe Acute Respiratory Syndrome associated coronavirus
SI	Selective index
SV40	Simian vacuolating virus 40 or Simian virus 40
TCM	Traditional Chinese medicine
VHSV	Viral haemorrhagic septicaemia virus
VSV	Vesicular stomatitis virus
VZV	Varicella-zoster virus

3.1 Introduction

A virus is a small, infectious agent, which contains little more than bundles of gene strands of gene strands of either RNA or DNA, and may be surrounded bya shell of protein. Unlike bacterial cells, which are free-living entities, viruses utilize the host cell environment to propagate new viruses. The newly made viruses then leave the host cell, sometimes killing it in the process, and proceed to infect other cells within the host. Virus infection can cause a mild illness as a cold or a deadly illness as the bloody African fever. The viruses that cause Lassa fever and Ebola fever and the retrovirus that causes acquired immunodeficiency syndrome (AIDS) are examples spreading easily, threatening people's life and health sometimes swiftly, and for which there is no cure or vaccine (Jassim and Naji 2003). Viral infection has become one of the main causes of morbidity and mortality worldwide (Golean et al. 2009).

Since viruses have plenty of invasion strategies. Each strain of virus has its unique configuration of surface molecules, which work like keys in locks, enabling viruses enter into hosts by accurately fitting the molecules on their surfaces of target cells (Jassim and Naji 2003). Since viruses and hosts are intimately connected, the designing of effective anti-virals that will inhibit the viral enzymes or its replication without affecting the host cells has proved to be difficult (Golean et al. 2009; Chattopadhyay et al. 1999). The public health measures and prophylactic vaccines are still the important means, by which society controls the spread of viral infections.

With the advances in molecular biology and reverse genetics in the past two decades, many viruses have been known to have unique features in their structures or in their replication cycles, which are their potential targets. Since viral enzymes are crucial for disease progression and virus replication, inhibitors against viral enzymes have been the most desirable strategies. Most of the well-studied inhibitors against HIV, herpes simplex virus (HSV) or the influenza viruses target the host cell binding (T-20, betulinic acid, etc.), uncoating of capsid (amantadine derivatives, pleconaril), replication (reverse transcriptase inhibitors like zidovudine or abacavir, nevirapine, etc.), integrase inhibitors, DNA or RNA polymerase inhibitors (acyclovir, cidofovir, ribavirin, etc.), proteinase involved in viral polyprotein precursors and assembly/maturation inhibitors (indinavir, ritonavir and rimantadine, etc.). Based on this strategy, numerous compounds have been tested on different viruses in the past decades, but there are still less than 40 licensed anti-virals in the market (Chattopadhyay et al. 2009).

The development of viral resistance towards current antiviral agents enhances the need for new effective compounds against viral infections. Some new anti-viral compounds are currently undergoing either preclinical or clinical evaluation, and perspectives for finding new interesting antiviral drugs are promising (Chattopadhyay et al. 2009). Among these antiviral substances are several natural compounds isolated from plants used in traditional medicines.

According to statistics, natural products have become one of the main resources for new drug research and development in the past two decades, especially for the treatment of infectious diseases. Approximately 44% of the antiviral drugs approved between 1981 and 2006 were natural products, semi-synthetic natural product analogues or

synthetic compounds based on natural-product pharmacophores (Newman and Cragg 2007). Drug screening of plant extracts has led to the evaluation of their *in vitro* antiviral activities (Jassim and Naji 2003; Mukhtar et al. 2008). In addition, the application of new technologies and methods, such as high throughput screening (HTS) and molecular biology, has greatly increased the probability of finding valuable new bioactive plant extract(s) or compound(s) (Li and Vederas 2009).

In this chapter, (1) the history of natural products as antiviral drugs, (2) the main *in vitro* models used to discover and evaluate potential "lead" compounds as "antiviral substances", and (3) the anti-viral properties of phytochemicals with special action mechanisms have been reviewed.

3.2 History of Natural Products as Antiviral Drugs

The history of medicinal plants can date back to the origin of human civilization on earth (Mukhtar et al. 2008). In the early times, natural products were directly used for the treatment of diseases, including viral infection diseases. Natural products have made great contributions to human health, many active constituents from natural products have been determined and their mechanisms of action have been elucidated.

The inhibitory effects of medicinal plants extracts on the replication of several viruses were reported in the past six decades. In 1952, 288 plants for anti-influenza activity were evaluated by the Boots drug company (Nottingham, England); 12 out of these plants were found to be effective against influenza viruses in embryonated eggs (Chantrill et al. 1952). In the 1970s, the antiviral activities of extracts from grape, apple, strawberry and other fruit juices against HSV, poliovirus type 1, coxsackievirus B5 and echovirus 7 were reported (Konowalchuk and Speirs 1976, 1978a, b). In 1995, 100 British Colombian medicinal plants were also reported to have antiviral activities, 12 out of which showed significant antiviral effect against corona viruses, respiratory syncytial virus (RSV), para-influenza virus type 3 (PI3), herpesvirus type 1 (HSV-1) and retavirus (McCutcheon et al. 1995). In 1998, more than 800 common Chinese herbal medicines were detected for antiviral activities against HIV and more than 100 plants exhibited anti-HIV activities (Lou 1998). In 2005, more than 200 Chinese herbal medicines were screened for antiviral activities against Severe Acute Respiratory Syndrome associated coronavirus (SARS-CoV), 4 out of which showed potent antiviral activities (Li et al. 2005a).

In addition, herpes simplex virus type 2 (HSV-2) (Debiaggi et al. 1988), HIV (Asres and Bucar 2005; Vermani and Garg 2002), hepatitis B virus (HBV) (Huang et al. 2003, 2006; Kwon et al. 2005), dengue virus type-2 (DEN-2) (Parida et al. 2002; Reis et al. 2008) and emerging viral infections associated with poxvirus and severe acute respiratory syndrome (SARS) virus (Kotwal et al. 2005) were strongly inhibited by various plants extracts. Most of these studies utilized either water soluble or alcoholic extracts of medicinal plants, and limited efforts have been directed toward the identification of active natural ingredients exhibiting antiviral effects. Some typical examples of traditional plant medicines and their antiviral activities are shown in Table 3.1.

Table 3.1 Partial viruses inhibited by medicinal plants

Virus	Medicinal plant used	Antiviral effect	Reference
Hepatitis C virus (HCV)	*Acacia nilotica* L.	Exhibited significant activity against HCV *in vitro*	Rehman et al. (2011)
	Solanum nigrum L.	Displayed potential antiviral activity against HCV	Javed et al. (2011)
Hepatitis B virus (HBV)	*Ophioglossum pedunculosum* Desv.	Exhibit modest activity of blocking HBsAg secretion *in vitro*	Wan et al. (2011)
	Boehmeria nivea L.	Reduced HBV production in an *in vitro* and *in vivo* model	Huang et al. (2006)
	Polygonum cuspidatum Sieb. and Zucc.	Inhibited hepatitis B virus in a stable HBV-producing cell line	Chang et al. (2005)
Herpes simplex virus (HSV)	*Carissa edulis* Vahl.	Exhibited strong anti-HSV 1 and 2 activities both *in vitro* and *in vivo*	Tolo et al. (2006)
	Geranium sanguineum L.	Significantly inhibited the replication of HSV-1 and HSV-2 *in vitro*	Serkedjieva and Ivancheva (1999)
Influenza virus	*Geranium sanguineum* L.	Reduced the infectivity of various influenza virus strains *in vitro* and *in vivo*	Pantev et al. (2006) and Serkedjieva (1997)
	Elderberry	Showed an efficient, safe and cost-effective treatment for influenza *in vitro*	Zakay-Rones et al. (2004)
Poliovirus	*Guazuma ulmifolia* Lam.	Inhibited poliovirus replication, as well as, blocked the synthesis of viral antigens in infected cell cultures	Felipe et al. (2006)
Viral haemorrhagic septicaemia virus (VHSV)	*Olea europaea* L.	Inhibited viral replication *in vitro*	Micol et al. (2005)

(continued)

Table 3.1 (continued)

Virus	Medicinal plant used	Antiviral effect	Reference
Human immunodeficiency virus (HIV)	*Phyllanthus amarus* Schum. and Thonn	Inhibited HIV replication both *in vitro* and *in vivo*	Notka et al. (2004)
	Olive leaf	Inhibited acute infection and cell-to-cell transmission of HIV-1	Lee-Huang et al. (2003)
Vesicular stomatitis virus (VSV)	*Trichilia glabra* L.	Inhibited VSV *in vitro*	Cella et al. (2004)
Human adenovirus type 1 Coxsackievirus B1	Black soybean	Inhibited human adenovirus type 1 and coxsackievirus B1 *in vitro*	Yamai et al. (2003)
Dengue virus type-2 (DENV-2)	*Azadirachta indica* Juss.(Neem)	Inhibited DENV-2 both *in vitro* and *in vivo*	Parida et al. (2002)
	Uncaria tomentosa (Willd.) DC.	Displayed *in vitro* antiviral activity against DENV-2	Reis et al. (2008)
Severe Acute Respiratory Syndrome associated coronavirus (SARS-CoV)	*Lycoris radiate, Artemisia annua*	Showed potent antiviral activities against SARS-CoV *in vitro*	Li et al. (2005a)

Moreover, recent studies showed that plant extracts possess antiviral potential of against viral strains resistant to conventional antiviral agents (Serkedjieva 2003; Tolo et al. 2006), which have challenged the modern drug discovery practices. Thus, the further exploration of natural antiviral components of medicinal plants is necessary.

Although medicinal plants have been used throughout the world, however, their wide usage has been limited to China, India, Japan, Pakistan, Sri Lanka, Thailand and a number of African countries. However, developed countries are gradually accepting the usage of plant-based natural medicinal products in their healthcare systems. In England, because of the outstanding performance of TCMs on some stubborn diseases, such as skin eczema, currently the British government appears to support the research and the usage of TCMs, and therefore the international drug market for TCMs has been rapidly expanded, especially in England (Jiang 2005).

Besides, the Natural Health Product Regulations of Canada promulgated in 2004 and the performance of phase III clinical trial of Chinese medicine Danshen dripping pills in America in 2010 are also very important steps toward modernization of plant-based product usage in healthcare. These advances encourage the usage of modern technology and evidence-based scientific support toward promoting medicinal plants and the associated products (Siow et al. 2005).

3.3 Common Models for Antiviral Activity Assay

The antiviral activities of natural products, including ingredients, fractions and extracts, need to be evaluated by various anti-viral models, including *in vitro* and *in vivo* models. Besides, the analysis of assay results of natural products is also very important. In the present section, the models to evaluate the *in vitro* anti-viral activities of potential natural products have been summarized, and the evaluation methods of several representative models have been introduced. The antiviral models include molecular level models and cellular level models (Table 3.2; Chattopadhyay et al. 2009).

3.3.1 Molecular Level Models

Molecular level models are usually the first step in screening a large number of substances, including compounds or extracts, for their inhibition and action mechanisms on viral enzymes or function proteins, and therefore provide useful activity information for further strategy for drug discovery, including the related molecular level models and/or cellular level models.

3.3.1.1 Reverse Transcriptase (RT) Activity Assay

RT activity can be assayed by adding lysates to a 96-well microtiter plate which has a poly (A) nucleotide chain covalently bound to the base of each well. A mix of 5′

Table 3.2 Molecular level, and cellular level for testing anti-viral agents against common viruses

Virus	Disease	Molecular level model	Cellular level model
HSV	Herpes	DNA polymerase assay	Plaque assay (Vero, MRC-5, HFF, BHK, HEp-2 cell lines)
Influenza	Flu	NA, HA, RNA polymerase assays	Plaque assay, HA, HAI, RT-PCR (MDCK, A549 cell lines)
Hepatitis C virus (HCV)	Liver cirrhosis	Protease, RNA polymerase assay	RT- PCR, no cell line facility
HBV	Liver cirrhosis	Reverse transcriptase assay, interferons	RT- PCR (HepG2 2.2.15)
Entero virus	Diarrhoea		RT-PCR, PAGE
RSV	Common cold	RNA polymerase assay	Plaque assay, MTT assay, RT-PCR Cell-ELISA, HEp-2
HIV	AIDS	Reverse transcriptase and integrase activity assays	Primary macrophages, HFF cells
Rabies virus	Hydrophoba		McCoy cells
Polio virus	Paralysis, Aseptic meningitis	RNA polymerase assay	Plaque assay, RT-PCR (Hela cell line)

bromodeoxyuridine triphosphate (BrdUTP) is added, and the plate is incubated at 33°C for 48 h (Sivapalasingam et al. 2005).

The plate is washed to terminate the reaction and a BrdU-binding antibody conjugated to alkaline phosphatase (AP) is added and incubated at 33°C for 90 min.

Following a second wash, the AP substrate p-nitrophenyl phosphate is added and the resulting yellow color change is measured to quantify the amount of incorporated BrdUTP. Following addition of the AP substrate each plate is read at three time points: 10 min, 2–3 h, and 5–6 h or overnight at 15–24 h. The results are expressed in femtograms HIV-1 RT activity/ml plasma. The lower limit of detection is <1 fg/ml. Results are calculated using ExaVir Load Analyzer software that automatically determines the amount of RT activity in samples using a standard curve generated from an 11-point dilution of a known amount of recombinant HIV-1 RT. The results are also given in units of HIV-1 RNA equivalent copies/ml using a conversion factor derived from a Swedish cohort comprised mainly of subtype B virus. Assay precision is determined on duplicates of five samples, and assay reproducibility is determined on seven samples run on different days.

3.3.1.2 DNA Polymerase Activity Assay

Although different DNA polymerases have distinct functions and substrate affinities, their general mechanism of action is similar. Thus, they can all be studied using the same technical principle, the primer extension assay employing radioactive tags (Lope et al. 2007).

To explore the use of fluorescent primers in assaying DNA polymerase activity with *in vitro* primer extension, we can use the following 40 mer oligodeoxynucleotides as templates: an oligo dT (5′ TTTTTTTTTTTTTTTTTTTTTTT GTCGTG ACTGGGAAAAC) and a (dC.dA)n template (5′ CACACACACACACACACACA CACGTCGTGACTGGGAAAAC), where the underlined sequences are the 17-mer M13 (−40) universal primer sequence. Fluorescein-labeled M13 primer (0.5 μM; Alpha DNA, Montreal, Canada) is annealed to the two templates (0.8 μM each) in the presence of 50 mM Tris-HCl, pH 7.5, and 100 mM NaCl by heating to 90°C for 5 min, followed by cooling to 25°C over several hours, to obtain our substrate.

Since the Klenow fragment of DNA polymerase I is very well characterized, it is used as a model in the experiments. Maintaining the same conditions as in radioactive labeling primer experiments (Tuske et al. 2000; Lestienne et al. 2003), it could be assessed in different settings for Klenow activity. Briefly, DNA polymerase I (Klenow fragment) is incubated with a plant constituent, 5′-fluorescein-end-labeled primer-template substrate (50 nM), 200 μM deoxynucleotide, 10 mM Tris-HCl, pH 5, 6, 7 or 8, 5 mM dithiothreitol, 100 μg/ml bovine serum albumin, 0, 10, 100 or 200 mM $MgCl_2$ and 0, 50, 100 or 200 mM NaCl. Reaction mixtures are incubated at 37°C for 30 min. The reaction is quenched by the addition of stop solution (10 μl) (80% deionized formamide, 10 mM EDTA, pH 8, 1 mg/ml xylene cyanol, and 1 mg/ml bromophenol blue) heated to 90°C for 2 min and placed on ice. Products are resolved on denaturing polyacrylamide sequencing gels in an automated ALF DNA sequencer (GE Healthcare). Analyses of the deoxynucleotide incorporation are carried out with Allelelinks software, version 1.0 (GE Healthcare).

3.3.1.3 Neuraminidase Inhibition Assay

A standard fluorimetric assay is used to measure influenza virus NA activity. The substrate MUNANA (2′-(methylumbelliferyl)-α-D-acetylneuraminic acid) is cleaved by NA to yield a fluorescent product, which can be quantified. The reaction mixture containing the test compounds/extracts and NA enzyme or a virus suspension in 32.5 mM MES buffer with 4 mM $CaCl_2$ (pH 6.5) is incubated for 40 min at 37°C. After incubation, the reaction is terminated by the addition of 34 mM NaOH. The fluorescence is quantified at an excitation wavelength of 360 nm and emission wavelength of 450 nm. The 50% inhibitory concentration (IC_{50}) is defined as the concentration of NA inhibitor necessary to reduce the NA activity by 50% relative to that in a reaction mixture containing virus but no inhibitor (Liu et al. 2008a).

3.3.1.4 Hemagglutination Inhibition Assay

The hemagglutination assay (HA) is a quantitation of hemagglutination protein of viruses. Some viral families have surface or envelope proteins, which are able to agglutinate (stick to) human or animal red blood cells (RBC) and bind to its N-acetylneuraminic acid. The RBC will form a type of lattice in this case.

In contrast to plaque assay or LD_{50}, HA does not give any measure of viral infectivity, because no virus replication is required in this assay. It is an easy, simple and rapid method and can be applied to large amounts of samples. The detailed conditions depend on the type of virus. Some viruses bind RBCs only at certain pH values, others at certain ionic strengths.

If the test compounds inhibit virus replication and thus viral titre, the HA value will be reduced. HA inhibition assay is employed to test the effect of the test compounds in virus adsorption to target cells. Compound solutions (25 μl) with twofold serial dilution with PBS are mixed with equal volume of influenza virus solution (200HAU/25 μl). After incubation for 30 min at room temperature, 50 μl of the solution is mixed with equal volume of 1% chicken erythrocyte suspension and incubated for 30 min at room temperature (Song et al. 2005).

3.3.2 Cellular Level Models

Cellular level models usually exploit the virus's ability to infect and replicate in specific cell lines in cell culture systems. The cell culture system provides a rapid method to grow viruses at higher titres to test the antiviral activity of compounds/extracts. It is based on the observation that virus infection and multiplication results in cytopathic effect (CPE) due to either release of virus or induction of apoptosis as a result of host immune responses. Inhibition of CPE in presence of test compound could be due to inhibition of virus replication. The assay results combined with molecular level assay results will guide the later study in animal models.

3.3.2.1 Cytotoxicity in MDCK Cells

Cell viability is determined with the MTT (3-(4,5)-dimethylthiahiazo (-z-y1)-3, 5-di-phenytetrazoliumromide) method (Liu et al. 2008a). Consecutive threefold serial dilutions (100 μl) of compounds/extracts and reference compounds are added to cell monolayers in replicates. Blank medium is used as a control. After 3 days of incubation at 37°C, 12 μl of MTT solution (5 mg/ml in phosphate-buffered saline) is added to each well. The plate is further incubated at 37°C for 3 h to allow the formation of the formazan product. After removal of the medium, 100 μl of dimethyl sulfoxide (DMSO) is added to dissolve the formazan crystals. After 15 min, the contents of the wells are homogenized on a microplate shaker. The optical densities are then measured with a microplate spectrophotometer at a wavelength of 540 nm. The median cytotoxic concentration (CC_{50}) is calculated as the concentration of the tested sample that reduced the number of viable cells to 50% of the untreated control. The maximal non-cytotoxic concentration (MNCC) is defined as the maximal concentration of the sample that does not exert a cytotoxic effect and resulted in more than 90% viable cells.

3.3.2.2 CPE Reduction Assay

The antiviral activities of the test compounds/extracts are measured with the CPE inhibition assay (Liu et al. 2008b). Viral suspension (200 $TCID_{50}$/ml, 100 µl) is added to each well of a 96-well plate containing a confluent cell monolayer. After incubation at 37°C for 2 h, the virus solution is removed, and 100 µl of consecutive threefold serial dilutions of the test compounds/extracts and reference compounds are added to each well, using the MNCC as the highest concentration. An infection control without samples is also included. The plates are incubated at 37°C in a humidified CO_2 atmosphere (5% CO_2) for 24 h, after which the CPE is assessed. The virus-induced CPE is scored as follows: 0 = no CPE, 1 = 0–25% CPE, 2 = 25–50% CPE, 3 = 50–75% CPE, and 4 = 75–100% CPE. The reduction in virus multiplication is calculated as a percentage of the virus control (% virus control = CPE_{exp}/$CPE_{virus\ control} \times 100$). The IC_{50} of the CPE with respect to the virus control is estimated using the Reed–Muench method. The selective index (SI) is calculated as the ratio CC_{50}/IC_{50}.

3.3.2.3 Plaque Inhibition Assay

For plaque inhibition assays, confluent monolayer MDCK cells cultured in a 6-well tissue culture plate (1×10^5 cells/cm^2) are infected with a mixture of approximately 500 PFU/ml of virus. After 60 min for virus adsorption, the solution is removed and the cells are washed twice with pre-warmed MEM medium, and replaced with overlay medium (DMEM containing 10 µg/ml trypsin, 1% low melting agarose, without serum), containing test compounds/extracts at different concentration. After incubating cultures for 2–3 days at 37°C with 5% CO_2, monolayers are fixed with 4% formaldehyde solution for 30 min and the agarose is then removed by flowing water and stained with 1% (w/v) crystal violet solution. The plaques are counted by visual examination and percentage of plaque inhibition is calculated as relative to the control without samples. A required concentration to reduce the 50% plaque number (EC_{50}), is calculated by regression analysis of the dose–response curves generated from these data (Song et al. 2005).

3.3.2.4 Influenza Virus Quantitative RT-PCR Analysis

MDCK cells are grown at about 90% confluence and infected with influenza virus at 0.1 MOI and cultured in the presence of compounds/extracts at various concentrations. At 16 h post-infection, cells are scraped off and collected by centrifugation (500 g for 5 min). Cell pellets are washed with PBS twice. Total cellular and viral RNAs are isolated from pellets using the RNeasy mini kit (QIAGEN) following the manufacture's protocol. First-strand cDNA is synthesized from 1 µg of total RNA with Omniscript RT kit (QIAGEN) using specific primers. PCR reactions are performed with 50 µl of reaction buffer [5 µl of cDNA template, 50 pmols of

primers, 0.1 mM dNTPs, and 0.5U of EX-Taq polymerase (Takara)]. The amplification conditions are as follows: 94°C for 5 min (1 cycle), 94°C for 1 min, 55°C 40 s and 72°C 1 min 40 s (18 cycles, respectively). NP RNA is chosen for detection and the primer sequences used for the detection of viral RNA are 5′-TGC TGG ATT CTC GTT CGG TC (sense) and 5′-CCT TTA TGA CAA AGA AGA AATAAGGCG (antisense). The β-actin is used as internal control of cellular RNAs, with primer sequences of 5′-TCA CCC GAG TCC ATC ACG AT (sense) and 5′-GAA GTA CCC CAT TGA GCA CGG (antisense). The reverse transcription and PCR products are resolved on 1.0% agarose gels and stained with ethidium bromide (Song et al. 2005).

3.3.2.5 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA assays allow the detection of viral antigen or antibody, using a solid-phase assay system. Although qualitative, it offers a rapid, sensitive, and specific method for detection and gross quantitation of virus. Absolute quanitation is possible if a series of pre-determined viral titres are used to get elisa readings and matched with that unknown samples can be calculated to estimate the quantity of the virus samples (untreated or drug-treated). The method cannot differentiate between infectious and non-infectious virus particles as a result of which the drug affecting at the earlier or latter stage of viral replication or maturation may not be understood (Chattopadhyay et al. 2009).

Briefly, the untreated or drug-treated cells are infected with a known concentration of virus, adsorbed for 1 h, washed and incubated for 2–4 days depending on virus-induced CPE. Virus is harvested after freeze-thawing, centrifuged, and supernatant is diluted with the sample diluent and used for ELISA experiments. To each of the 96 well strip-plate coated with virus specific antibody 100 µl control or test sample is added followed by 1 h incubation at room temperature with 100 µl conjugate containing enzyme labeled virus-specific antibody. After washing five times 100 µl of substrate is added and incubated in dark for 10 min at room temperature. Most of the assays employ horseradish peroxidase, alkaline phosphatase, or β-D-galactosidase. Reaction is stopped with stop solution in kit (usually 5% H_2SO_4) and absorbance reading is taken photometrically at OD_{450}. Alternatively, the drug treatment and virus infection can be performed in 96-well formats, instead of preparing virus supernatants. For this, quadruplicate monolayers in 96-well microtitre plates are overlaid with log10 dilution of test compounds followed by the infection with virus. After 16–20 h incubation at 37°C, monolayers are fixed with 0.05% glutaraldehyde in PBS and assayed for protein specific for the virus on the cell surface. ELISA is performed with monoclonal antibodies (MAb) to specific protein of the corresponding virus strains and protein A horseradish peroxidase conjugate (Bio Rad, Hercules, CA). The optical densities (OD_{450}) are measured and expressed as a percentage of nondrug-treated virus-infected cells (virus control). The concentration causing 50% reduction in optical density values (EC_{50}) was evaluated from graphic plots. The selectivity index (SI) was determined from the ratio of IC_{50}:EC_{50}.

3.4 Antiviral Activities of Different Phytochemicals

The ever increasing resistance of human pathogens to current anti-infective agents is a serious medical problem, leading to the need to develop novel antibiotic prototype molecules. In the case of viruses, the search for antiviral agents involves additional difficulties, particularly due to the nature of the infectious viruses. Thus, many compounds that may cause the death of viruses are also very likely to injure the host cell that haibours them. Natural products are increasingly appreciated as leads for drug discovery and development. Screening studies have been carried out in order to find antiviral agents from natural sources, and the extracts of traditional medicinal plants, marine organisms and fungi often show antiviral activities. The evidence indicates that there may be numerous potentially useful antiviral phytochemicals in nature, waiting to be evaluated and exploited. In addition, other plants, not previously utilized medicinally, may also reveal antivirals. Compounds from medicinal plants are of interest as possible source to control viral infection, which include flavonoids, alkaloids, polysaccharides, organic acids, essential oils, and others. Natural substances offer interesting pharmacological perspectives for antiviral drug development in regard to broad-spectrum antiviral properties and novel mechanisms of action.

3.4.1 Polysaccharides

Among natural antiviral agents, recent investigations have reconsidered the interest of phyto-polysaccharides, which act as potent inhibitors of different viruses (Martinez et al. 2005). This section will illustrate a variety of antiviral polysaccharides from natural sources since 1990, with the aim of making this matter more accessible to drug development, some typical examples of such medicines and their antiviral activities are shown in Table 3.3.

The inhibitory effects of polyanionic substances on the replication of HSV and other viruses were reported almost five decades ago. Shortly after the identification of HIV as the causative agent of AIDS in 1984, heparin and other sulfated polysaccharides were found to be potent and selective inhibitors of HIV-1 replication in cell culture. Since 1988, the activity spectrum of the sulfated polysaccharides has been shown to extend to various enveloped viruses, including HSV *in vitro* (Karmakar et al. 2010; Herold et al. 1995), human cytomegalovirus (HCMV) (Baba et al. 1988), respiratory syncytial virus, influenza A and B virus (Damonte et al. 1994), DENV-2 and DENV-3 in the human hepatoma HepG2 (Talarico et al. 2005).

As potential anti-HIV drug candidates, sulfated polysaccharides offer a number of promising features. Some polysulfates show a differential inhibitory activity against different HIV strains, suggesting that marked differences exist in the target molecules with which polysulfates interact. They not only inhibit the cytopathic effect of HIV, but also prevent HIV-induced syncytium (giant cell) formation. Furthermore, experiments carried out with sulfate samples of increasing molecular weight and with sulfated cyclodextrins of different degrees of sulfation have shown that antiviral

Table 3.3 Summary of the most active polysaccharides from medicinal plants

Compounds	Anti-viral effect	Examples of plant source	Reference
Sulfated polysaccharide fraction	Exerted activity against HSV-2, HCMV, RSV, influenza A and B virus	*Nothogenia fastigiata*	Damonte et al. (1994)
A polysaccharide	Exhibited activities against HSV-1	*Sclerotium glucanicum*	Marchetti et al. (1996)
A polysaccharide	Exhibited activities against HSV	*Prunella vulgaris*	Xu et al. (1999)
A polysaccharide	Exhibited activities against HIV	*Rhizophora mucronata*	Premanathan et al. (1999b)
A polysaccharide	Exhibited activities against HSV	*Achyrocline flaccida*	Garcia et al. (1999)
Sulfated galactans	Exhibited antiviral activity against HSV-1 and HSV-2	*Bostrychia montagnei*	Duarte et al. (2001)
An acidic polysaccharides fraction (APS)	Exhibited activities against the replication of HSV-2 and VSV	*Cedrela tubiflora*	Craig et al. (2001)
Sulfated polysaccharides	Exhibited activities against DENV-2 and DENV-3	*Gymnogongrus griffithsiae* and *Cryptonemia crenulata*	Talarico et al. (2005)
Protein bound polysaccharides	Exhibited potent HSV-1 and HSV-2 antiviral activity	*Ganoderma lucidum*	Eo et al. (2000) and Liu et al. (2004)
Sulfated polysaccharide	Exhibited antiviral activity against HSV-1 and HSV-2 both *in vitro* and *in vivo*	*Porphyridium* sp.	Huheihel et al. (2002)
Polysaccharide fractions	Exhibited antiviral activity against HSV-1 and HIV-1	*Arthrospira platensis*	Rechter et al. (2006)
Polysaccharide fractions	Exhibited antiviral activity against HSV	*Grateloupia indica*	Chattopadhyay et al.(2007)
Polysaccharide extract	Displayed activity against WSN influenza A *in vivo*	*Echinacea purpurea*	Fusco et al. (2010)

activity increases with increasing molecular weight and degree of sulfation (Duarte et al. 2001; Wang et al. 2010; Karmakar et al. 2010). A sugar backbone is not strictly needed for the anti-HIV activity of polysulfates because sulfated polymers composed of a carbon–carbon backbone have also proved to be highly efficient anti-HIV agents *in vitro*. Other, yet to be defined, structural features may also play an important role. From studies on their mechanism of action, polysulfates appear to exert their anti-HIV activity by shielding off the positively charged sites in the V3 loop of the viral envelope glycoprotein (gp120), which is necessary for virus attachment to cell surface heparan sulfate, a primary binding site, before more specific binding occurs to the CD4 receptor of $CD4^+$ cells (Huheihel et al. 2002). This general mechanism also explains the broad antiviral activity of polysulfates against enveloped viruses. Variations in the viral envelope glycoprotein region may result in differences in the susceptibility of different enveloped viruses to compounds that interact with their envelope glycoproteins. The efficacy of sulfated polysaccharide against herpes simplex virus types 1 and 2 (HSV-1 and -2) were demonstrated *in vivo* (rats and rabbits) (Huheihel et al. 2002).

The proteoglycan seems to be a potential candidate for anti-HSV agents. A proteoglycan (GLPG), extracted and purified from the mycelia of *Ganoderma lucidum* by ethanol (EtOH) precipitation and DEAE-cellulose column chromatography, displayed antiviral activities against HSV-1 and type 2 (HSV-2). The study on its action mechanism suggested that GLPG inhibits viral replication by interfering with the early events of viral adsorption and entry into target cells (Liu et al. 2004).

The *in vivo* anti-viral activity has partially been demonstrated (Fusco et al. 2010). The *in vivo* experiment of polysaccharide extract from *Echinacea purpurea*, a widely consumed botanical product, indicated that mice infected with WSN influenza A and treated with *E. purpurea* polysaccharide extract had less weight loss than untreated mice but similar pulmonary viral titers. *Echinacea*-treated mice had lower systemic and pulmonary KC and IL-10 levels and lower systemic IFN-γ levels following influenza infection. These suggest that *E. purpurea* alters the clinical course of influenza infection in mice through modulation of cytokines and not direct antiviral activity.

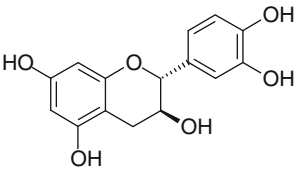
3.4.2 Flavonoids

Flavonoids are very common natural products widely existing in plant kingdom. Flavonoids, including genistein, catechins and so on, have been shown to reduce the infectivity of a variety of viruses affecting humans and animals, including adenovirus, HSV, HIV, porcine reproductive and respiratory syndrome virus, and rotavirus (Andres et al. 2009). Current results about the mechanisms of action underlying their antiviral properties suggest a combination of effects on both the virus and the host cell. Flavonoids have been reported to affect virus binding, entry, replication, viral protein translation, formation of certain virus envelope glycoprotein complexes and virus release (Andres et al. 2009). They also affect a variety of host cell signaling

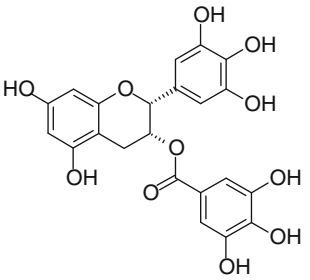
Table 3.4 Viruses inhibited by flavonoids

Flavonoids	Inhibited viruses	Model	Reference
Catechins	Adenovirus	Hep2 cells	Webster et al. (2006)
	Influenza virus	MDCK cells	Song et al. (2005)
	HIV-1	T cells	Williamson et al. (2006)
	HSV	Vero and CV1 cells	Isaacs et al. (2008)
	HPV	Human condyloma	Reuter et al. (2010)
Genistein	Adenovirus	SW480 cell	Li et al. (2000b)
	Arenaviruses	Vero cells	Vela et al. (2008)
	BHV-1	MDBK cells	Akula et al. (2002)
	Bovine viral diarrhea virus	MDBK cells	Lecot et al. (2005)
	EBV	Ramos cells	Fukuda and Longnecker (2005)
	HSV-1, HSV-2	Vero cells	Yura et al. (1993) and Lyu et al. (2005)
	Human CMV	HEL 299 cells	Evers et al. (2005)
	HHV-8	HFF cells	Sharma-Walia et al. (2004)
	HIV	Primary macrophages	Stantchev et al. (2007)
	MoMLV	XC cells	Kubo et al. (2003)
	RSV	Vero cells	Rixon et al. (2005)
	Rotavirus	MA-104 cells	Andres et al. (2007)
	SV40	CV-1 cells	Dangoria et al. (1996) and Pelkmans et al. (2002)
Quercetin	Adenovirus	BCC-1/KMC cells	Chiang et al. (2003b)
	Coronovirus	Vero cells	Yi et al. (2004)
Luteolin	Coronovirus	Vero cells	Yi et al. (2004)
	Influenza virus	MDCK cells	Liu et al. (2008a)
Apigenin	Influenza virus	MDCK cells	Liu et al. (2008a)
3-Deoxysappanchalcone	Influenza virus	MDCK cells	Liu et al. (2009)
Sappanchalcone	Influenza virus	MDCK cells	Liu et al. (2009)
Isoflavones mix	PRRS virus	Pigs (*in vivo*)	Greiner et al. (2001)
Gorvanol A	HSV-1	Vero cells	Arthan et al. (2002)
Kaempferol	HSV-1	Vero cells	Amoros et al. (1992)
5,6,7-Trimethoxyflavone	HSV-1, Human CMV, Poliovirus	Vero and MRC-5 cells	Hayashi et al. (1997)
Irisolidone	JC virus	Primary astrocytes	Kim et al. (2006)
3-Methylkaempferol	Poliovirus	Vero cells	Robin et al. (2001)
3(2H)-isoflavene	Poliovirus	HeLa R19 cells	Salvati et al. (2004)
Pedunculosumosides A and G	Human B Virus (HBV)	HepG2 2.2.15 cells	Wan et al. (2011)

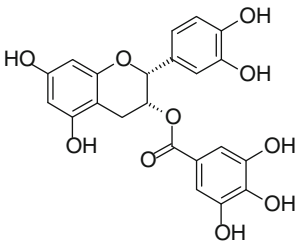
processes, including induction of gene transcription factors and secretion of cytokines (Andres et al. 2009). Although enormous promising results were from *in vitro* experiments, a few *in vivo* results can partly confirm their *in vivo* efficacy (Miki et al. 2007). Flavonoids possess antiviral properties against a wide range of viruses under both *in vitro* and *in vivo* conditions (see Table 3.4 and Fig. 3.1).



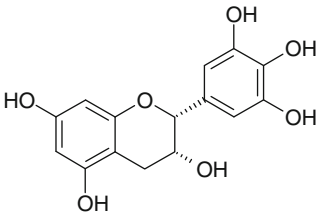
(+)-Catechin



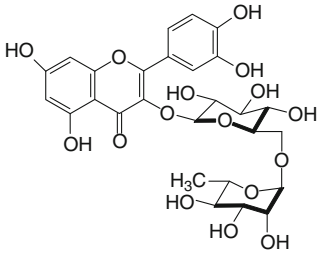
(–)-Epigallocatechin gallate (EGCG)



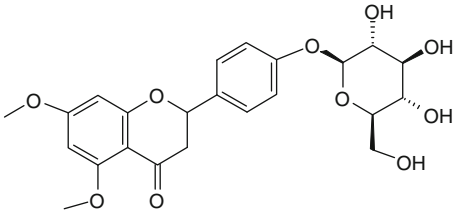
(–)-Epicatechin gallate (ECG)



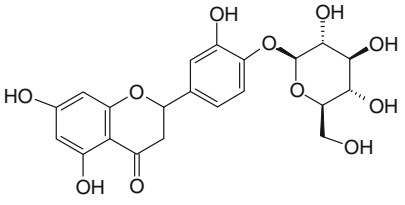
(–)-Epigallocatechin (EGC)



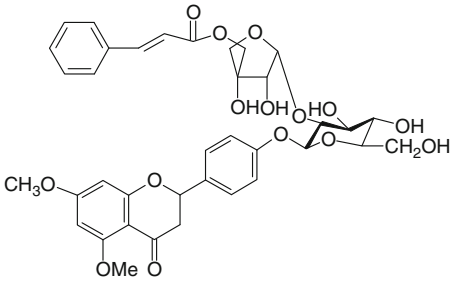
Rutin



5,7-dimethoxyflavanone-4′-O-β-d-glucopyranoside

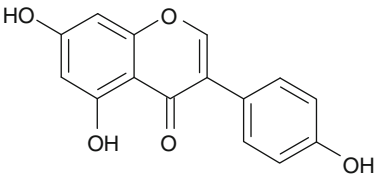


5,7,3′-trihydroxy-flavanone-4′-O-β-d-glucopyranoside

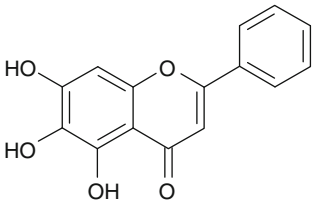


5,7-dimethoxyflavanone-4′-O-[2″-O-(5‴-O-trans-cinnamoyl)-β-d-apiofuranosyl]-β-d-glucopyranoside

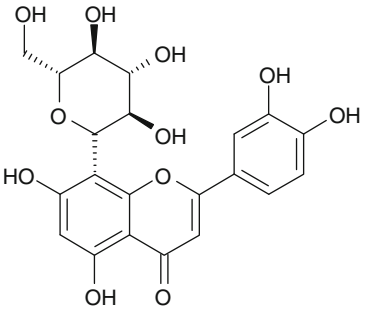
Fig. 3.1 The chemical structures of the active flavonoids from medicinal plants



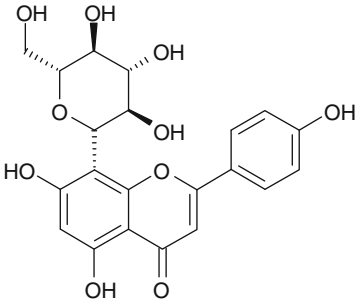
Genistein



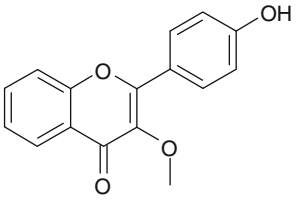
Baicalein



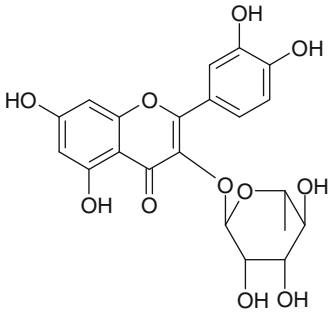
Orientin



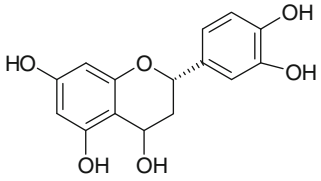
Vitexin



Chrysosplenol C



Quercetin 3-rhamnoside (Q3R)



Luteoforol

Fig. 3.1 (continued)

Tea polyphenols from green tea mainly consist of catechins ((−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin (EGC)). Their ability to inhibit several different viruses was evaluated. Among the catechins, the EGCG and ECG were found to be potent inhibitors of influenza virus replication

in MDCK cell culture and this effect was observed in all influenza virus subtypes tested, including A/H1N1, A/H3N2 and B virus (Demeule et al. 2002). However, the sensitivity in hemagglutination inhibition was widely different among three different subtypes of influenza viruses tested. Quantitative RT-PCR analysis revealed that, at high concentration, EGCG and ECG also suppressed viral RNA synthesis in MDCK cells whereas EGC failed to show similar effect (Song et al. 2005). Similarly, EGCG and ECG inhibited the neuraminidase activity more effectively than the EGC (Song et al. 2005). The results show that the 3-galloyl group of catechin skeleton plays an important role on the observed antiviral activity, whereas the 5′-OH at the trihydroxy benzyl moiety at 2-position plays a minor role (Song et al. 2005). In addition, EGCG has not only potential use as adjunctive therapy in HIV-1 infection (Williamson et al. 2006), but has greater anti-HSV activity than other green tea catechins and inactivates multiple clinical isolates of HSV-1 and HSV-2 by binding to gB, gD, or another envelope glycoprotein, it appears to be a promising candidate for use in a microbicide to reduce HSV transmission (Isaacs et al. 2008). Among green tea chatechins, EGCG was the most effective when added to the cells during the transition from the early to the late phase of adenovirus infection, suggesting that EGCG inhibits one or more late steps in virus infection (Webster et al. 2006). It is worth to mention that Veregen (80% catechins) as a prescription drug for condyloma treatment was approved by US FDA in 2006, suggesting that medicinal plants will open up new prospects for the pharmaceutical industry (Reuter et al. 2010).

The flavonoids apigenin and luteolin displayed significant *in vitro* anti-influenza viral activity, which were isolated from the traditional Chinese medicinal plant *Elsholtzia rugulosa* Hemsl. (Liu et al. 2008a). The structure-activity relationship (SAR) of flavonoids as influenza virus neuraminidase inhibitors revealed that for good inhibitory effect, the 4′-OH, 7-OH, C4=O, and C2=C3 functionalities were essential, and the presence of a glycosylation group greatly reduced NA inhibition (Liu et al. 2008b). The ability of flavonoids to inhibit the activity of NA is one of the pathways to inhibit the replication of influenza virus. These findings provide important information for the exploitation and utilization of flavonoids as NA inhibitors for influenza treatment.

It was reported that rutin, 5,7-dimethoxyflavanone-4′-O-β-d-glucopyranoside and 5,7,3′-trihydroxy-flavanone- 4′-O-β-d-glucopyranoside were active against PI-3 (Parainfluenza-3 virus), while 5,7-dimethoxyflavanone-4′-O-[2″-O- (5‴-O-trans-cinnamoyl)-β-d-apiofuranosyl]-β-d- glucopyranoside inhibited potently HSV-1 (Orhan et al. 2009).

While baicalein and genistein can block human cytomegalovirus (HCMV) replication at concentrations that were significantly lower than those producing cytotoxicity against growing or stationary phase host cells. The study of their mechanisms of action suggested that the primary mechanism of action for baicalein may be to block HCMV infection at entry while the primary mechanism of action for genistein may be to block HCMV immediate-early protein functioning (Evers et al. 2005). Flavonoids orientin and vitexin found in the flower of Chinese folk medicine *Trollius chinensis* Bunge exhibited potent or moderate antiviral activity against PI-3 (Li et al. 2002).

The flavonoid chrysosplenol C yielded from the green aerial parts of the Australian plant *Pterocaulon sphacelatum* (Labill.) Benth. and Hook. f. ex F. Muell., which is a 4′-hydroxy-3-methoxyflavone, has the ability to inhibit the replication of rhinoviruses (Semple et al. 1999). While the antiviral activity of quercetin 3-rhamnoside (Q3R) from traditional Chinese medicine *Houttuynia cordata* was evaluated using a cytopathic effect (CPE) reduction method, the assay results demonstrated that Q3R possessed strong anti-influenza A/WS/33 virus reducing the formation of a visible CPE. In addition, Q3R inhibited virus replication in the initial stage of virus infection by indirect interaction with virus particles. Therefore, these findings provide important information for the utilization of Q3R for influenza treatment (Choi et al. 2009b).

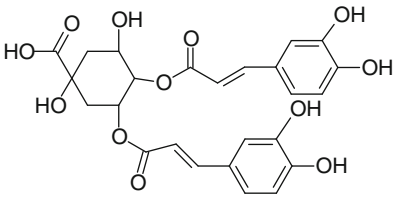
Flavonoid luteoforol isolated from *Hypericum connatum* (Guttiferae), which is used in southern Brazil in the treatment of lesions in the mouth and often related to acute herpetic gingivo-stomatitis, displayed antiviral activity against HSV-1 DNA viral strains KOS and VR733 (ATCC) (Fritz et al. 2007). Moreover, flavones are inhibitors of HIV-1 proteinase (Brinkworth et al. 1992).

Therefore, flavonoids and their structurally similar compounds possess antiviral properties against a wide range of viruses under both *in vitro* and *in vivo* conditions. These findings will provide important information for new drug design and for their exploitation and utilization for the treatment of viral infection.

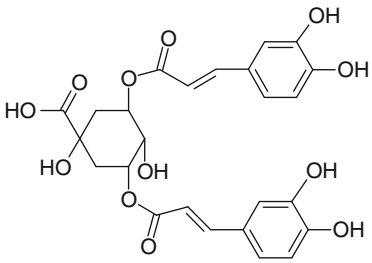
3.4.3 Organic Acids

Besides, some natural organic acids also possess antiviral activities. Three caffeoylquinic acid derivatives, namely 3,4-di-O-caffeoylquinic acid, 3,5-di-O- caffeoylquinic acid, and 3-O-caffeoylquinic acid isolated from traditional Chinese medicinal plant *Schefflera heptaphylla* were investigated for their antiviral activity against RSV (see Fig. 3.2; Li et al. 2005b). 3,4-Di-O-caffeoylquinic acid and 3,5-di-O- caffeoylquinic acid exhibited potent anti-RSV activity with IC_{50} values of 2.33 μM (1.2 μg/ml) and 1.16 μM (0.6 μg/ml), respectively, in a plaque reduction assay. The antiviral action of 3,4-di-O-caffeoylquinic acid and 3,5-di-O-caffeoylquinic acid was specific against RSV, as they had no obvious antiviral activity against influenza A (Flu A), Coxsackie B3 (Cox B3), and Herpes simplex type one (HSV-1) viruses (Li et al. 2005b). Studies indicated that the dicaffeoylquinic acids could inhibit RSV directly, extracellularly, but only at much higher concentrations than seen in standard assays. Moreover, they could not inhibit RSV attachment to host cells, and could not protect HEp-2 cells from RSV infection at lower concentrations. The data suggest that the compounds exert their anti-RSV effects via the inhibition of virus–cell fusion in the early stage, and the inhibition of cell–cell fusion at the end of the RSV replication cycle (Li et al. 2005b).

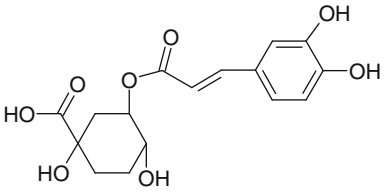
Raoulic acid is a principal ingredient of the plant *Raoulia australis* Hook. F (Fig. 3.2; Choi et al. 2009a). Antiviral assay using cytopathic effect (CPE) reduction method showed that raoulic acid possessed strong antiviral activity against



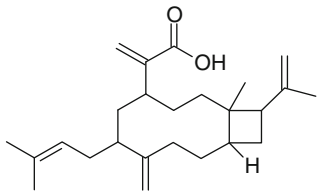
3,4-di-*O*-caffeoylquinic acid



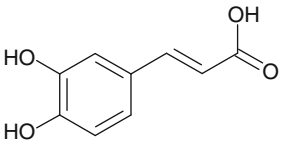
3,5-di-*O*-caffeoylquinic acid



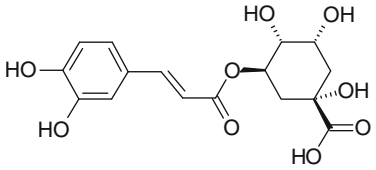
3-*O*-caffeoylquinic acid



Raoulic acid



Caffeic acid



Chlorogenic acid

Fig. 3.2 The chemical structures of the active organic acids from medicinal plants

human rhinovirus 2 (HRV2) with IC_{50} value of less than 0.1 μg/ml, human rhinovirus 3 (HRV3) with a IC_{50} value of 0.19 μg/ml, coxsackie B3 (CB3) virus with IC_{50} values of 0.33 μg/ml, coxsackie B4 (CB4) virus with IC_{50} values of 0.40 μg/ml, and enterovirus 71 (EV71) virus with IC_{50} values of less than 0.1 μg/ml. However, the compound did not possess antiviral activity against influenza A (Flu A/PR, Flu A/WS, H1N1) and B viruses.

Moreover, the antiviral activity of aqueous extract and pure compounds of *Plantago major* L., a popular traditional Chinese medicine, were studied on a series of viruses, namely herpesviruses (HSV-1, HSV-2) and adenoviruses (ADV-3, ADV-8, ADV-11). The results showed that aqueous extract of *P. major* possessed only a slight anti-herpes virus activity (Wang et al. 2009). While certain pure compounds exhibited potent antiviral activity. Among them, caffeic acid exhibited the strongest activity against HSV-1 (EC_{50} = 15.3 μg/ml, SI = 671), HSV-2 (EC_{50} = 87.3 μg/ml, SI = 118) and ADV-3 (EC_{50} = 14.2 μg/ml, SI = 727), whereas chlorogenic acid possessed the strongest anti-ADV-11 (EC_{50} = 13.3 μg/ml, SI = 301) activity (Wang et al. 2009). The study concludes that pure compounds of *P. major*, which possess antiviral activities are mainly derived from the phenolic compounds, especially

caffeic acid. Its mode of action against HSV-2 and ADV-3 was found to be at multiplication stages (postinfection of HSV-1: 0–12 h; ADV-3: 0–2 h) (Chiang et al. 2002a). In addition, chlorogenic acid and caffeic acid also showed an inhibitory effect on HBV (hepatitis B virus) replication (Fig. 3.2; Wang et al. 2009).

3.4.4 Alkaloids

There are some alkaloids which exhibit antiviral activities both *in vitro* and *in vivo*. The naphthoindozidine alkaloid, together with 7-demethoxytylophorine and 7-demethoxytylophorine N-oxide isolated from the aerial parts of *Cynanchum komarovii*, had antiviral activities against tobacco mosaic virus (An et al. 2001). While the sophoridine is one of the active constituents extracted from Chinese medicinal herb, *Sophora flavescens*, exhibited significantly antiviral activity against coxsackievirus B3 (CVB3) *in vitro* (primarily cultured myocardial cells) and *in vivo* (BALB/c mice) by regulating cytokine expression, and it is likely that sophoridine itself, not its metabolites, is mainly responsible for the antiviral activities (Zhang et al. 2006). While another alkaloid lycorine, isolated from *Lycoris radiate* possesses anti-SARS-CoV activity (Fig. 3.3; Li et al. 2005a).

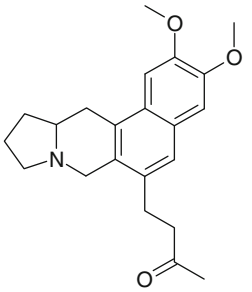
3.4.5 Saponins

Plant saponins are a group of naturally occurring triterpene or steroid glycosides which include a large number of biologically and pharmacologically active compounds. Saponins have been shown in both *in vitro* and *in vivo* experimental test systems during the last decade to possess a broad spectrum of biological and pharmacological activities. This section will summarize some of the recent advances concerning antiviral activities of saponins.

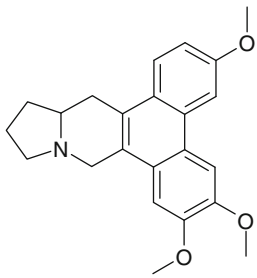
The antiviral activity of a triterpene saponin isolated from *angallis arvensis*, was studied *in vitro* against several viruses including HSV-1, adenovirus type 6, vaccinia, vesicular stomatitis and poliovirus (Amoros et al. 1987). The drug was found to inhibit the replication of HSV-1 and poliovirus type 2 as shown by inhibition of cytopathic effect and reduction of virus production. The action was not due to a virucidal effect but might involve inhibition of virus-host cell attachment. Single cycle experiments indicated that saponins interfered with both early and late events of herpes virus replication (Amoros et al. 1987).

The Tibetan herb *Potentilla anserina* L. has been widely used in China for many thousands of years to treat hepatitis-B. Bioassay-guided fractionation of the ethanol extract of the rhizomes led to the isolation of a triterpenoid saponin (TS) that was determined to be $2\alpha,3\beta,19\alpha$-trihydroxyurs-12-en-28-oic acid β-d-glucopyranosyl ester (Fig. 3.4; Zhao et al. 2008). Using models of HBV infection, this compound was evaluated for its effect on HBV antigene expression in the 2.2.15 cell line

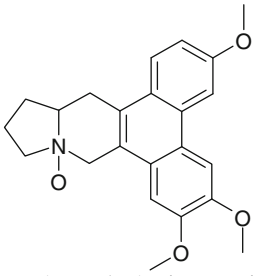
Fig. 3.3 The chemical structures of the active alkaloids from medicinal plants



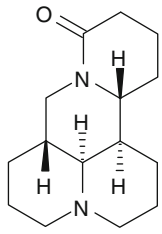
Naphthoindozidine alkaloid



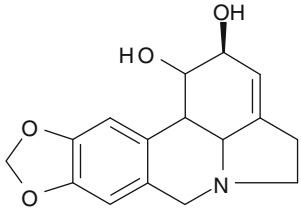
7-demethoxytylophorine



7-demethoxytylophorine N-oxide

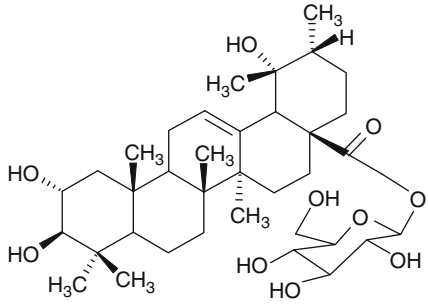


Sophoridine



Lycorine

Fig. 3.4 The chemical structure of the most active saponin from *Potentilla anserina* L.



Triterpenoid saponin (TS) (2α, 3β,19α-trihydroxyurs -12-en-28-oic acidβ-D-glucopyranosyl ester)

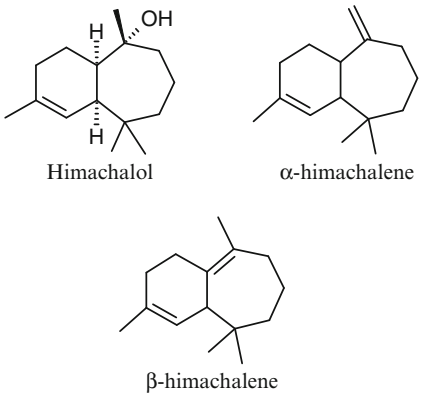


Fig. 3.5 The chemical structures of the active essential oils from medicinal plants

in vitro and anti-hepatitis B virus (HBV) activities in Peking ducklings *in vivo* (Zhao et al. 2008). Results showed that it could decrease the expression levels of HBsAg, HBeAg and HBVDNA in the 2.2.15 cell culture. The antiviral study *in vivo* on Peking ducklings also demonstrated that this compound inhibits duck hepatitis B virus (DHBV) DNA replication (Zhao et al. 2008).

3.4.6 Essential Oils

The application of essential oils as possible therapeutic agents against diseases is gaining attention due to various factors. Growing consumer preferences for inexpensive, natural and traditional medicines that are also effective on resistant bacterial or viral strains have stimulated investigations into the bioactive properties of essential oils. This section will summarize some of the recent advances concerning antiviral activities of essential oils.

The *Artemisia arborescens* L. essential oil showed significant antiviral activity against Herpes simplex virus type 1 (HSV-1) (Sinico et al. 2005). Peppermint oil, the essential oil of *Mentha piperita*, exhibited high levels of virucidal activity against HSV-1 and HSV-2, and it is also active against an acyclovir resistant strain of HSV-1 (HSV-1-ACV^{res}) (Schuhmacher et al. 2003).

The essential oil from *Santolina insularis* was also inhibited virus replication against HSV-1 and HSV-2 *in vitro* (Schnitzler et al. 2008). Its antiviral activity was principally due to direct virucidal effects (De Logu et al. 2000). Lemon balm oil, the essential oil of *Melissa officinalis*, is capable of exerting a direct antiviral effect on HSV-1 and HSV-2. Considering the lipophilic nature of essential oils, which enable it to penetrate the skin, and a high selectivity index, essential oils might be suitable for topical treatment of herpetic infections (Schnitzler et al. 2008).

The wood essential oil from *Cedrus libani* widely used as traditional medicine in Lebanon for treatment of different infection diseases are mainly consist of himachalol (22.50%), β-himachalene (21.90%), and α-himachalene (10.50%) was identified for their *in vitro* antiviral activities against HSV-1 (Fig. 3.5; Loizzo et al. 2008).

All essential oils from anise, hyssop, thyme, ginger, camomile and sandalwood possess virucidal activity against HSV-2 mainly before adsorption probably by interacting with the viral envelope (Koch et al. 2008). Camomile oil exhibited a high selectivity index and seems to be a promising candidate for topical therapeutic application as virucidal agents for treatment of herpes genitalis (Koch et al. 2008).

3.4.7 Stilbenes

In recent years, stilbenes, which widely exist in natural kingdom, have attracted much attention for their various biological activities, including antimicrobial, anti-cancer, anti-inflammation, hepatoprotective and hepatotoxic and so on (Liu et al. 2010).

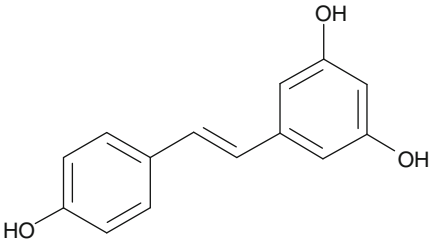
Resveratrol (3,5,4′-trihydroxystilbene) is a natural component of certain foods, such as grapes, has been shown to have anti-HSV activities *in vitro* and *in vivo* (Fig. 3.6; Docherty et al. 2004, 2005). Moreover, resveratrol was found to inhibit varicella-zoster virus (VZV) replication in a dose-dependent and reversible manner (Docherty et al. 2006). Two oligostilbenes dibalanocarpol and balanocarpol isolated from the leaves of *Hopea malibato* exhibited very modest HIV-inhibitory activity *in vitro* (Dai et al. 1998).

Besides, oligostilbenes also possess antiviral effect. Four stilbenoids, including isorhapontigenin, shegansu B, gnetupendin B and gnetin D from the lianas of *Gnetum pendulum* (Gnetaceae), exhibited significant *in vitro* anti-influenza viral activities in MDCK cells with IC_{50} values from 0.67 to 11.99 μg/ml, their action mechanism is mainly through influenza neuraminidase (NA) inhibitory effects (see Fig. 3.6; Liu et al. 2010).

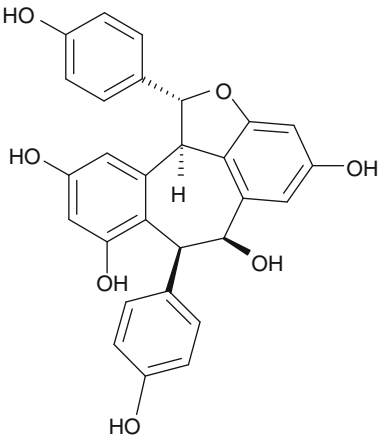
3.4.8 Others

In addition, the antiviral activity of several other kinds of natural products was also reported. Terpenoids caesalmin B and bonducellpin D from the seeds of traditional Chinese medicinal plant *Caesalpinia minax* exhibited inhibitory activities on the PI-3 virus *in vitro* by cytopathogenic effects (CPE) reduction assay (Jiang et al. 2002). Hippomanin A from *Phyllanthus urinaria* Linnea (Euphorbiaceae), which is a commonly used traditional medicinal plant in oriental countries, exhibited antiviral activity against HSV-2 but not HSV-1 infection (Fig. 3.7; Yang et al. 2007).

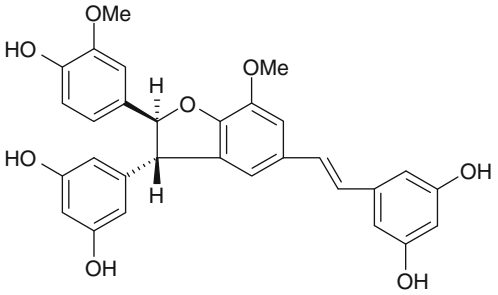
Chrysophanic acid (1,8-dihydroxy-3-methylanthraquinone), isolated from the Australian Aboriginal medicinal plant *Dianella longifolia*, has been found to inhibit the replication of poliovirus types 2 and 3 (*Picornaviridae*) *in vitro* (Fig. 3.7; Semple et al. 2001). The compound inhibited an early stage in the viral replication cycle, but did not have an irreversible virucidal effect on poliovirus particles. Chrysophanic acid did not have significant antiviral activity against five other viruses which were tested: Coxsackievirus types A21 and B4, human rhinovirus type 2 (*Picornaviridae*), and the enveloped viruses Ross River virus (*Togaviridae*) and HSV-1 (*Herpesviridae*).



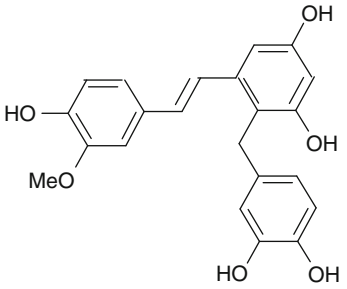
Resveratrol



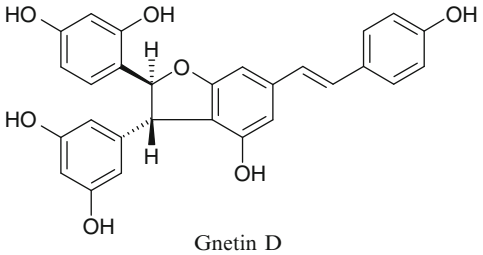
Balanocarpol



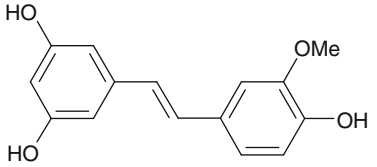
Shegansu B



Gnetupendin B



Gnetin D



Isorhapontigenin

Fig. 3.6 The chemical structures of the active stilbenes from medicinal plants

Four structurally-related anthraquinones – rhein, 1,8-dihydroxyanthraquinone, emodin (3-methyl-1,6,8-trihydroxyanthraquinone) and aloe-emodin were also tested for activity against poliovirus type 3 (Semple et al. 2001). None of the four compounds was as active as chrysophanic acid against the virus. The results suggested that two hydrophobic positions on the chrysophanic acid molecule (C-6 and the methyl group attached to C-3) were important for the compound's activity against poliovirus (Semple et al. 2001).

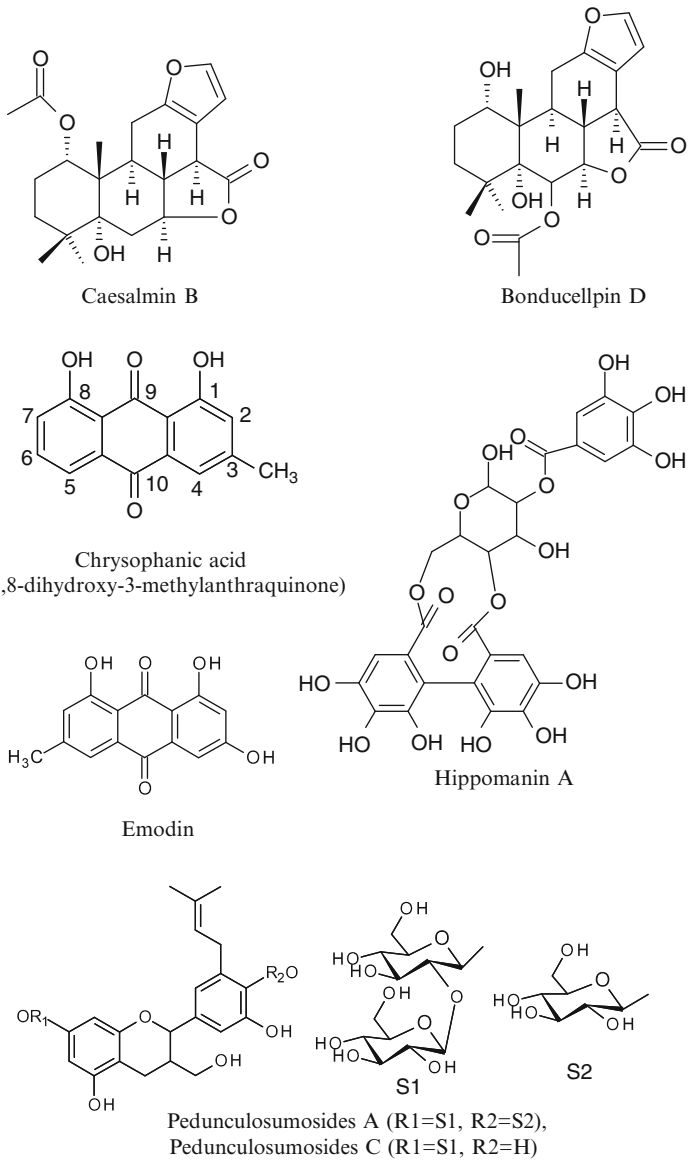


Fig. 3.7 The chemical structures of the other kinds of constituents from medicinal plants

Recently, it was reported that emodin isolated from the roots of *Rheum tanguticum* possess remarkable antiviral activity against HSV infection *in vitro* and *in vivo* (Xiong et al. 2011), and thus emodin is a promising agent in the clinical therapy of HSV infection. Besides, seven new homoflavonoid glucosides, pedunculosumosides A–G were isolated from the whole plant of *Ophioglossum pedunculosum,*

pedunculosumosides A and C exhibit modest activity of blocking HBsAg secretion with IC_{50} values of 238.0 and 70.5 μM, respectively (Wan et al. 2011).

With increasing of interest in the drug discovery from natural products, more and more antiviral activities of phytochemicals will be reported, which will greatly promote the new drug discovery and development for the treatment of viral infection disease.

3.5 Future Perspective

Not only the common viral diseases are still fatal, but new viral infections have emerged in worldwide in recent years. The currently available antivirals though effective are too expensive for most developing countries. Thus, the development of safe, effective and inexpensive antiviral drugs is among the top global priorities since many viruses are not yet curable and mortality rates are still high for example with HIV and hepatitis. Because of the tendency of viral mutation to drug-resistant forms, it is essential to continue the search for useful and novel natural antiviral agents, which can be expected to prolong the efficacy of drug therapy. Recently a lot of attention has been given to screening of various species of medicinal plants especially with antiviral activity (Asres et al. 2001; Jassim and Naji 2003; Chattopadhyay et al. 2006, 2009).

Since an obvious number of plant extracts have showed positive results, it seems reasonable to conclude that there are probably many potential antiviral agents. Further characterization of the active ingredients will reveal useful compounds. Some of these compounds belong to a wide range of different structural classes, e.g. coumarins, flavonoids, tannins, alkaloids, lignans, terpenes, naphtho- and anthraquinones, polysaccharides, proteins and peptides. There may also be novel phytochemicals. Although large numbers of new compounds have been isolated from medicinal plants, only some have been marketed as pharmaceutical products. Several compounds have been or are undergoing various phases of clinical trials. The traditional use of some of the medicinal plants for the treatment of infectious diseases of viral origin, therefore, is justified. In conclusion, active phytochemicals will provide important information for the development of new medicinal plant products in controlling the threats posed by some pathogenic viruses.

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Chapter 4
Antimicrobial Properties of Organosulfur Compounds

Osman Sagdic and Fatih Tornuk

Abstract Organosulfur compounds are defined as organic molecules containing one or more carbon-sulfur bonds. These compounds are present particularly in *Allium* and *Brassica* vegetables and are converted to a variety of other sulfur containing compounds via hydrolysis by several herbal enzymes when the intact bulbs are damaged or cut. Sulfur containing hydrolysis products constitute very diverse chemical structures and exhibit several bioactive properties as well as antimicrobial. The antimicrobial activity of organosulfur compounds has been reported against a wide spectrum of bacteria, fungi and viruses. Despite the wide antimicrobial spectrum, their pungent flavor/odor is the most considerable factor restricting their common use in foods as antimicrobial additives. However, meat products might be considered as the most suitable food materials in this respect since *Allium* and *Brassica* vegetables especially garlic and onion have been used as flavoring and preservative agents in meat origin foods. In this chapter, the chemical diversity and *in vitro* and *in food* antimicrobial activity of the organosulfur compounds of *Allium* and *Brassica* plants are summarized.

Keywords Organosulfur compounds • Garlic • Onion • *Allium* • *Brassica* • Thiosulfinates • Glucosinolates

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4.1 Introduction

Phytochemicals are defined as non-nutrient bioactive plant origin compounds naturally present in fruits, vegetables and whole grains (Liu 2004). Dietary intake of phytochemicals has been strongly linked to reduced risk of major chronic diseases such as cancer, diabetes and cardiovascular diseases (Liu 2003). Use of fruit and vegetables as health remedies traditionally is very common in a considerable part of world population (Salama and Marraiki 2010).

One group of sulfur forming in plants is known as organosulfur compounds. Organosulfur compounds, defined as organic molecules containing one or more carbon-sulfur bonds, are one of the groups present in foods as natural preservatives (Cremlyn 1996). These compounds are valued not only due to their rich and varied chemistry, but also for their many important biological properties (Polshettiwar and Kaushik 2004).

The families *Alliaceae* and *Brassicaceae,* two members of the plant kingdom, contain sulfur containing materials which possess biological properties (Stoewsand 1995). Today, it is well established that these vegetables are natural sources of a group of phytochemicals known as organosulfur compounds. *Allium* and *Brassica* vegetables especially garlic and onion have been known to be used as health remedies in ancient civilizations. Therapeutic application of these vegetables for the prevention of various diseases such as cancer and cardiovascular diseases has been well-studied (Vazquez-Prieto and Miatello 2010).

Organosulfur compounds in *Allium* and *Brassica* plants are called thiosulfinates and glucosinolates, respectively. These phytochemicals are characterized by the properties that give the plants their specific flavors and odors. Thiosulfinates and glucosinolates are also converted to various new sulfur containing materials which exhibit a variety of bioactive properties such as antimicrobial, anticarcinogenic, antitumor and pesticidal etc. via a number of biosynthetic reactions. This chapter outlines the antimicrobial properties of the organosulfur compounds present in *Allium* and *Brassica* plants and their derivatives on diverse microorganisms including bacteria, fungi and viruses.

4.2 Thiosulfinates

The major sources of organosulfur compounds are *Allium* vegetables. The *Allium* genus includes approximately 600 species, the most widely consumed of which are onions (*Allium cepa*), garlic (*Allium sativum*), leeks (*Allium porrum*), chives (*Allium schoenoprasum*), and shallots (*Allium ascalonicum*) (Bianchini and Vainio 2001; Benkeblia and Lanzotti 2007). Among the *Allium* species, onion and garlic are the oldest and most commonly cultivated plants by different cultures (Lanzotti 2006).

The earliest records of onion and garlic date back to the sixteenth century BC when several medicinal formulas based on garlic and onions as a curative agent for a variety of diseases such as heart problems, headache and tumors were mentioned

Table 4.1 Approximate composition of garlic and onion (Lawson 1996; Rahman 2003; Dini et al. 2008)

Components	Garlic (%)	Onion
Moisture	62–68	88.6–92.8%
Carbohydrates	26–30	5.2–9.0%
Proteins	1.5–2.1	0.9–1.6%
Ash	–	0.6%
γ-Glutamylcysteines	0.5–1.6	–
Lipids	0.1–0.2	Trace–0.2%
Fiber	1.5	1.7%
Total sulfur compounds	1.1–3.5	–
Sulfur	0.23–0.37	50–51 mg g^{-1}
Calcium	–	190–540 mg g^{-1}
Nitrogen	0.6–1.3	–
Minerals	0.7	–
Vitamins	0.015	100 mg g^{-1}
Saponins	0.04–0.11	–

– no data

in the Codex Ebers, the Egyptian medical papyrus (Block 1985). The Sumerians, Indians, Romans, Ancient Egyptians and Greeks are also all known to have consumed garlic and onions for thousands of years (Ahmad 1996). In China, tea made from garlic and onion has been used as a remedy for fever, headache, cholera and dysentery; garlic was utilized as an antiseptic in the treatment of gangrene in the World Wars (Corzo-Martinez et al. 2007). Garlic and onion are still used in folk medicine in all over the world. At present, these vegetables are among the most cultivated plants in the world with production amounts of 9 and 55 million tons per year, respectively (Peter 2000; FAO 2004).

4.2.1 Occurrence and Chemistry of Thiosulfinates

Not only do people consider *Allium* vegetables important because of their characteristic flavor that contributes to the savoriness of various dishes in world cuisine, they are also aware of the nutritional potential of these vegetables since they are rich in nutrients such as high levels of phosphor, potassium, zinc, vitamins A, C and D, folic acid, calcium and selenium etc. is also considered by people (Peter 2000; Rahman 2003). They are also rich sources of phenolic compounds (Vinson et al. 1998). Table 4.1 shows the approximate composition of fresh garlic and onion bulbs.

As in most fresh foods, water is the main constituent of *Allium* vegetables (Table 4.1). Although garlic and onion contain similar compounds to a certain extent, the amounts of major components in onion are rather low compared to those of garlic. Fresh garlic contains a moisture level of 62–68% (Table 4.1), while it is

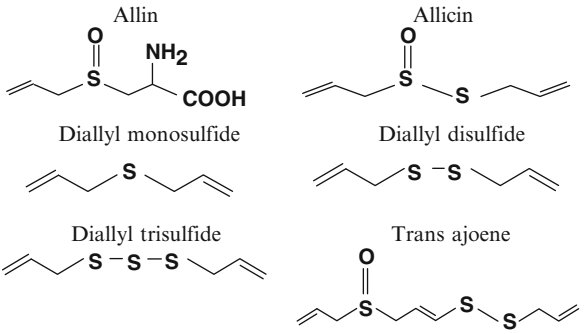

Fig. 4.1 Chemical structures of alliin and its main hydrolysis products

even higher (88.6–92.8% of fresh weight) in onion than that in garlic (Peter 2000). When considering the biological importance of these vegetables, sulfur containing compounds attract the attention. As seen Table 4.1, the proportion of total sulfur compounds in garlic is 1.1–3.5%, meanwhile its amount in onion is only one third of that found in garlic (Benkeblia 2004).

Thiosulfinates are the most studied compounds among the active constituents of *Allium* vegetables. Their main structures were first reported by Wertheim and later by Semmler in garlic and onion oils in the nineteenth century (Benkeblia and Lanzotti 2007). Block (1992) identified eight different thiosulfinates from nine *Allium* vegetables including garlic and onion by HPLC analysis. Garlic was found to be the richest source of thiosulfinates with amounts ranging from 15 μmol/g (garlic grown at low temperature, 21°C) to 53 μmol/g (elephant garlic). The thiosulfinate contents of the onion species were all <0.35 μmol/g.

Intact garlic bulbs possess certain sulfur containing compounds known as S-alk(en)yl-L-cysteine sulphoxides (ACSOs). Alliin (S-allylcysteine sulfoxide), the major ACSO of these vegetables is a colorless, odorless water soluble amino acid so long as the bulb is intact or undamaged (Corzo-Martinez et al. 2007). These are present in all *Allium* spp. constituting 1–5% of dry weight of the plant. However, when the cells are damaged, the enzyme alliinase (or alliin lyase) releases and converts alliin into certain volatile and reactive components called thiosulfinates (Block 1985). Allicin (allyl 2-propene thiosulfinate) is the most abundant thiosulfinate produced (70% of overall garlic thiosulfinates), whereas allyl methyl thiosulfinate (AMTS) is the second (18%) and additional thiosulfinates are also formed in lower concentrations (Mazza 2002). Since the thiosulfinates as well as allicin are very unstable, they readily undergo various transformations to form more stable compounds, to alkyl sulfides such as di- and trisulfides, allyl sulfides, vinyl dithiins, ajoenes and mercaptocysteines (Shi et al. 2005). These compounds can be yielded in a variety of reactions such as dehydration, rearrangement, condensation, Diels-Alder reaction, hydrolysis and pyrolysis depending on conditions and still exhibit biological activity (Lanzotti 2006). Figure 4.1 illustrates chemical structures of alliin and sulfur containing compounds forming from alliin in *Allium* vegetables. Onions also produce these sulfides but only at the level of 4 mg/100 g of fresh weight which means barely any amount compared to garlic (Shi et al. 2005).

4.2.2 Antimicrobial Properties of Thiosulfinates

Even though it is common knowledge that *Allium* vegetables contain a variety of functional components, the beneficial aspects of the *Allium* species have been mainly attributed to specific organosulfur compounds. In addition to the traditional use of *Allium* vegetables for thousands of years, scientific studies carried out on these vegetables, their extracts or specific organosulfur compounds have also proved their diverse pharmacological and biological benefits such as antimicrobial, antioxidative, antitumor and antiasthmatic (Lanzotti 2006), while effects of various *Allium* products such as garlic oil and methanol extract to inhibit rumen fermentation and methane production were also discussed (Patra and Saxena 2010). In general, cooked or waited vegetables are recommended for human consumption due to their prolonged protective effect resulting from the increased level of bioactive compounds in the vegetal tissues (Goncagul and Ayaz 2010).

Allium vegetables have been shown to have antibacterial, antifungal, antiviral and antiprotozoal activities. This activity has been attributed to thiosulfinates and to the other sulfur containing compounds present in these vegetables. It has been found that the main antimicrobial agents are breakdown products of allicin such as diallyl disulfide (DADS), diallyl trisulfide (DATS), diallyl sulfide (DAS) and ajoene, antimicrobial strength of which is higher than that of allicin (Corzo-Martinez et al. 2007). It is obvious that the enzyme alliinase plays a critical role in antimicrobial action since thiosulfinates are generated following the activation of this enzyme after the vegetable tissues are injured. Inactivation of alliinase as a result of prolonged heating causes the failure of antimicrobial activity (Lawson 1996). In general, each sulfur compound has individually been found to exhibit weaker antimicrobial activity as compared to the crude extracts of *Allium* spp. (Lawson 1998). It is also known that some proteins, saponins and phenolic compounds can contribute to antimicrobial activity (Griffiths et al. 2002).

As in other natural antimicrobial compounds of plant origin (spice essential oils, phenolics and other plant extracts etc.), these compounds may generally show their antimicrobial activity by altering the permeability of microbial cell walls and the replacing of intracellular and extracellular materials with each other. Moreover, the main mechanism involved in the antimicrobial effect is assumed to be the inhibition of thiol-containing enzymes in microorganisms by the rapid reaction of thiosulfinates with thiol groups (Ankri and Mirelman 1999).

4.2.2.1 Antibacterial Activity

The antibacterial activities of the *Allium* species have been well documented. Historically, Louis Pasteur first noted the antibacterial activity of garlic (Block et al. 1993). By the 1900s, substances showing antibacterial action were beginning to be identified. Allicin was the first organosulfur compound to be isolated and defined as an antibacterial agent in garlic (Cavallito and Bailey 1944). The antibacterial activity

of allicin is remarkably more bacteriostatic than bactericidal. Allicin in concentrations of 1:85.000 in broth is bactericidal against a wide variety of Gram-negative and Gram-positive organisms (Cavallito and Bailey 1944). Feldberg et al. (1988) suggested that the antibacterial action of allicin caused a quick and complete inhibition of RNA biosynthesis and additionally a partial inhibition of DNA and protein synthesis. In addition to allicin, other thiosulfinates have also been found to exhibit antibacterial activity. A great number of *in vitro* and *in situ* studies have been made in order to reveal the antibacterial properties of individual organosulfur compounds or various extracts of *Allium* vegetables.

It has been proven that the *Allium* species can inhibit both Gram (+) and Gram (−) bacteria as well as toxin production. Table 4.2 summarizes the *in vitro* studies made to identify antibacterial activities of *Allium* vegetables or their components. Garlic, the most studied vegetable, has been reported to be effective against strains of *Pseudomonas, Proteus, Escherichia coli, Staphylococcus aureus, Klebsiella, Salmonella, Micrococcus, Bacillus subtilis, Mycobacterium* and *Clostridium*. It may also inhibit beneficial intestinal microflora, but the potentially harmful species of the *Enterobacteriaceae* family are more sensitive to garlic compounds especially allicin (Corzo-Martinez et al. 2007). The sensitivity of *Listeria monocytogenes,* a psychrotrophic pathogen which can be found in foods, was measured against various extracts of *Allium vinelae* (wild garlic) by the disc diffusion method and methanol extract was found to be more active than ethanol and *n*-hexane extracts (Sagun et al. 2006). The efficiency of garlic shoot juice on *L. monocytogenes* was also shown (Kim et al. 2007). General lysis of the cytoplasm, lysis of the cell wall and cellular swelling were observed in bacterial cells by scanning electron microscopy in the same study. The inhibitory effect of garlic against *S. aureus, Salmonella* Typhi*, E. coli* and *L. monocytogenes* was measured using the turbidity method in another study. 80% inhibition level for *E. coli, S.* Typhi*, S. aureus* and *L. monocytogenes* were provided in the presence of garlic with levels of 3.95%, 7.0%, 5.0% and 8.8% in *in vitro* conditions, respectively (Kumar and Berwal 1998). Allicin and other *Allium* products were also considered as potential control agents for oral pathogens, causes of the dental diseases, such as *Streptococcus mutans, S. sobrainus, Actinomyces oris, Act. viscosus* and *Porphyromonas gingivalis* (Bakri and Douglas 2005; Bachrach et al. 2011).

As can be seen in Table 4.2, the *Bacillus* species are the most sensitive bacteria against *Allium* compounds or extracts while ajoene has the highest antibacterial effect. The sensitivity of *E. coli* to allicin is higher compared to other sulfur containing compounds. It can also be seen that *P. aeruginosa* is the most resistant species against all organosulfur compounds in general. However, the different minimum inhibitory concentration (MIC) levels reported by researchers may arise from extraction method, final thiosulfinate concentration in the extracts and the method applied for the assessment of the inhibitory effect (Benkeblia and Lanzotti 2007).

Studies on the antibacterial activity of thiosulfinates have mainly focused on garlic and onion. These studies showed that garlic was more effective in bacterial inhibition than onion since the sulfur compound content of garlic is four times higher compared to the level found in onion. Furthermore, both onion and garlic are

Table 4.2 *In vitro* antibacterial activity of *Allium* vegetables or their components

Antibacterial organosulfur compounds	Bacteria	MIC value (μg/mL)	Reference
Ajoene	*Bacillus cereus*	4	Naganawa et al. (1996)
	Staphylococcus aureus	16	
	Escherichia coli	116	
Z-ajoene	*B. cereus*	5	Yoshida et al. (1998)
	B. subtilis	5	
	S. aureus	20	
	E. coli	100	
	P. aeruginosa	>500	
Allicin[a]	*S. aureus*	12	Ankri and Mirelman (1999)
	E. coli	15	
Garlic oil	*P. aeruginosa*[b]	16–20	Tsao and Yin (2001)
DAMS		80–88	
DADS		64–72	
DATS		32–36	
DATTS		40893	
Garlic oil	*K. pneumoniaee*[b]	24–48	Tsao and Yin (2001)
DAMS		96–104	
DADS		72–80	
DATS		40–48	
DATTS		20–24	
A. atroviolaceum[c]	*B. subtilis*	0.31	Chehregani et al. (2007)
A. eriophyllum var. *laceratum*[c]		0.31	
A. scabriscapum[c]		0.75	
Z-10-devinylajoene	*B. cereus*	60	Yoshida et al. (1999)
	B. subtilis	50	
	S. aureus	70	
	E. coli	>100	
	P. aeruginosa	>100	
DAMS[d]	*Helicobacter pylori*	2.07–4.15	O'gara et al. (2000)
DADS[d]		100	
DATS[d]		13–25	
DATTS[d]		3–6	
Allicin[d]		6–12	
Garlic oil[e]		8–32	
Garlic powder[e]		250–500	
Z-10-devinylajoene	*B. cereus*	5	Yoshida et al. (1998)
	B. subtilis	5	
	S. aureus	30	
	E. coli	125	
	P. aeruginosa	170	

(continued)

Table 4.2 (continued)

Antibacterial organosulfur compounds	Bacteria	MIC value (μg/mL)	Reference
Fresh Garlic	*Vibrio* ssp.[f]	0.16–0.31	Kasornchandra et al. (2005)
	E. coli	0.31	

MIC Minimum inhibitory concentration, *DAMS* Diallyl monosulfide, *DADS* Diallyl disulfide, *DATS* Diallyl trisulfide, *DATTS* Diallyl tetrasulfide

[a] The results are 50% lethal dose concentrations of allicin

[b] Total numbers of clinical isolates of *P. aeruginosa* and *K. pneumoniae* were 123 and 114, respectively

[c] Aqueous extracts of the plant bulbs and flowers were used for testing the antibacterial activities of *A. atroviolaceum – A. eriophyllum* var. *laceratum* and *A. scabriscapum*, respectively

[d] Three strains of *H. pylori* were tested

[e] Eight strains of *H. pylori* were tested

[f] Seven strains of *Vibrio* ssp. were tested

the most commonly used food ingredients for flavor enhancement purposes. Meat and dairy products are especially favorable for flavoring by these materials (Sagun et al. 2006; Salem et al. 2010). In addition to food flavoring, a number of biological effects, such as the antibacterial activity of *Allium* species in food systems, have been proven.

The addition of garlic essential oil to minced beef contributed to its storage stability by decreasing native bacterial counts (APC, *Enterobacteriaceae,* coliform bacteria and *S. aureus*) (Salem et al. 2010). However, all garlic oil supplements (0.5%, 1.0% and 1.5%) demonstrated fair enhancements of sensory attributes in storage periods of 3 days, while the samples treated with 0.5% garlic oil demonstrated the lowest sensory enhancement after 6 days. Wong and Kitts (2002) showed that the pre-seasoning of steak with garlic caused a reduction in the psychrotroph population in both non-irradiated and irradiated steaks after refrigerated storage for 2 weeks.

Yin and Cheng (2003) revealed the antibacterial properties of four thiosulfinates (diallyl sulfide, DADS, s-ethyl cysteine and n-acetyl cysteine) in minced beef. APC was significantly reduced and the growth of five inoculated pathogenic bacteria, *S.* Typhimurium*, E. coli* O157:H7, *L. monocytogenes*, *S. aureus* and *Campylobacter jejuni* was inhibited by the presence of DAS and DADS in ground beef. Park et al. (2008) reported an antimicrobial effect which inhibited the growth of APC and *Enterobacteriaceae* in pork belly and loin cuts by the injection of solutions containing 5% of garlic or onion powder into the cuts and the inhibition was superior compared to sodium ascorbate injection. Aydin et al. (2007) investigated the antimicrobial effect of chopped garlic in ground beef and raw meat balls (cig kofte, a traditional Turkish raw meat product). The addition of garlic at 5% or 10% to the raw meatball mix decreased the microbial count, in terms of APC and yeast and mold counts. However, it was not as effective in ground beef as much as it was in raw meat ball mix.

Various garlic products, garlic oil, garlic powder and fresh garlic were investigated for their antibacterial efficiency in chicken sausage. The addition of fresh garlic (30 g/kg) or garlic powder (9 g/kg) significantly reduced the APC and, subsequently,

Table 4.3 *In vitro* antifungal activity of thiosulfinates

Antifungal organosulfur compounds	Fungi	MIC value (μg/mL)	Reference
Ajoene	*A. niger*	16.6	Yoshida et al. (1987)
	Candida albicans	7.6	
Ajoene	*C. albicans*	13	Naganawa et al. (1996)
	S. cerevisiae	12	
Allicin	*A. niger*	30.9	Yoshida et al. (1987)
	C. albicans	17.3	
Z-10-devinylajoene	*S. cerevisiae*	80	Yoshida et al. (1999)
Z-ajoene	*S. cerevisiae*	20	Yoshida et al. (1998)
Z-10-devinylajoene	*S. cerevisiae*	75	Yoshida et al. (1998)
Allicin	*C. albicans*[a]	0.3–0.8	Ankri and Mirelman (1999)
	C. parapsilosis	0.15	

MIC Minimum inhibitory concentration
[a]Two different isolates of *Candida albicans* were tested

the shelf-life of the product was extended to 21 days. However, the addition of garlic oil resulted in no additional reduction in APC when compared with control samples (Sallam et al. 2004).

4.2.2.2 Antifungal Activity

The importance of fungi is highlighted by their harmful effects, such as food spoilage or toxin production as well as by their beneficial properties such as food fermentation. The antifungal activity of thiosulfinates has been extensively reported by a variety of *in vitro* and *in vivo* studies. Yamada and Azuma (1977) first reported the antifungal activity of allicin against *Candida, Cryptococcus, Trichophyton, Epidermophyton* and *Microsporum*. These researchers also stated that the MIC of allicin against various pathogenic fungi was affected considerably by differences in experimental conditions, e.g., incubation time, inoculum size, type of medium and medium pH. Moreover, alike in antibacterial activity, the breakdown products of allicin, including DATS, DADS, DAS and ajoene are the major active antifungal agents of onion and garlic extracts and have a greater antifungal effect than allicin (Tansey and Appleton 1975; Corzo-Martinez et al. 2007). Table 4.3 lists the results of a number of *in vitro* studies showing the antifungal properties of thiosulfinates in *Allium* vegetables.

Several models for mode of antifungal action of thiosulfinates were suggested by various researchers. Yamada and Azuma (1977) reported that allicin in the concentration of 3.13 μg/mL inhibited swelling and germination of fungi spores in nutritious media. Barone and Tansey (1977) showed that garlic and allicin suppressed *C. albican*s cell metabolism by the inactivation of proteins, competitive inhibition of sulfhydryl compounds, or by the noncompetitive inhibition of enzyme function by oxidation. In another study, the deformation and distortion of yeast cells, cell

collapse and cytoplasmic debris were observed in cells grown in the presence of garlic aqueous extract (Ghannoum 1988). Adetumbi et al. (1986) proposed that garlic extract inhibited the protein and nucleic acid synthesis of *C. albicans* in parallel with growth inhibition, but inhibited lipid synthesis completely.

The antifungal effects of *Allium* extracts have also been reported by many researchers. Yin and Tsao (1999) found that garlic bulb extract exhibited the best inhibitory effect among the extracts of seven different *Allium* vegetables on *Aspergillus niger, A. flavus* and *A. fumigatus* with the MIC values of 35, 75 and 104 μg/mL, respectively. Benkeblia (2004) showed the dose-dependent *in vitro* inhibition of five different essential oil extracts of three types of garlic and onion on *A. niger, Penicillium cyclopium* and *Fusarium oxysporum*. Lower concentrations of onion extracts (50 and 100 mL/L) did not cause a significant inhibitory effect compared to control. Shams-Ghahfarokhi et al. (2006) also investigated the antifungal activities of aqueous extracts of garlic and onion on *Malassezia furfur* (25 strains), *C. albicans* (18 strains), and other *Candida* spp. (12 strains) as well as 35 strains of various dermatophyte species. They showed that the extracts were able to inhibit the growth of all fungi tested in a dose-dependent manner. The activity of aqueous garlic extract against *M. furfur, C. albicans*, other *Candida* spp. and the dermatophytes was 64, 8, 4 and 32–128 fold that of the aqueous onion extract, respectively.

Heated garlic as well as fresh garlic and mustard is already used in some foods as preservatives. Kim and Kyung (2003) investigated the antifungal activity of heated garlic and alliin against different strains of *C. albicans, C. utilis*, *Saccharomyces cerevisiae*, *Pichia membranifaciens, Zygosaccharomyces bisporus* and *Z. rouxii*. Alliin heated in distilled water showed an antifungal activity pattern similar to that of heated garlic, suggesting that the compound(s) thermally generated from alliin were the main antifungal compound(s) of heated garlic. The antifungal activity was increased as the time of heating increased up to 45 min at 121°C, and the activity did not change when garlic was further heated for up to 120 min.

4.2.2.3 Antiviral Activity

Relatively few studies have shown antiviral properties of thiosulfinates. Weber et al. (1992) tested the antiviral potentials of not only fresh garlic extract but also its sulfur components, allicin, allyl methyl thiosulfinate, methyl allyl thiosulfinate, ajoene, alliin, deoxyalliin, DADS, and DATS against selected viruses including, herpes simplex virus type 1, herpes simplex virus type 2, parainfluenza virus type 3, vaccinia virus, vesicular stomatitis virus, and human rhinovirus type 2. The order for virucidal activity was generally found as: ajoene≥allicin≥allyl methyl thiosulfinate≥methyl allyl thiosulfinate while no activity was observed for alliin, deoxyalliin, DADS or DATS. Ajoene, allyl alcohol and DADS were shown to be active against HIV (Human immunodeficiency virus) -infected cells (Tatarintsev et al. 1992; Shoji et al. 1993). Chen et al. (2011) studied the antiviral activity of the powders of five *Allium* plants (garlic, onion, leek, shallot and green onion) and allicin against two adenovirus isolates (ADV41 and ADV3). They found that shallots

exhibited the highest level of antiviral activity for both ADV41 and ADV3, followed by garlic and onions. Shallots exhibited the highest level of antiviral activity against ADV3 and ADV41 infection from 0 to 2 h, during the early period of virus replication.

4.2.2.4 Antiparasitic Activity

The antiparasitic effects of freshly crushed garlic have been known since ancient times. Chinese people traditionally use the alcoholic extract of garlic cloves to cure intestinal diseases. Only a few reports are available regarding the antiparasitic activity of *Allium* vegetables or their sulfur compounds. It was reported that allicin (30 μg/mL) could efficiently inhibit the growth of some such as *Giardia lamblia*, *Leishmania major, Leptomonas colosoma* and *Crithidia fasciculate* (Ankri and Mirelman 1999). Ankri et al. (1997) also showed that allicin inhibited cysteine proteinases and the cytopathic effects of *Entamoeba histolytica*. Lun et al. (1994) tested the antiparasitic activity of DATS, known as dasuasu in China, against several important protozoal parasites *in vitro*. The IC_{50} (concentration to inhibit metabolism or growth of parasites by 50%) for *Trypanosoma brucei brucei, T. b. rhodesiense, T. b. gambiense, T. evansi, T. congolense* and *T. equiperdum* was found in the range of 0.8–5.5 μg/mL. IC_{50} values were 14 and 59 μg/mL for *E. histolytica* and *G. lamblia*, respectively.

4.3 Glucosinolates

Glucosinolates (GLSs) are a class of organic compounds which are formed from glucose and an amino acid and contain sulfur and nitrogen (Du and Halkier 1998). Cruciferous or *Brassica* vegetables are rich sources of GLSs and all the members of these plants contain these organosulfur compounds naturally (Song et al. 2005; Higdon et al. 2007). However, GLSs do not only exist in crucifers; at least 500 non-cruciferous species were reported to contain one or more forms of GLSs (Fahey et al. 2001). Similar to cruciferous vegetables, many plants belonging to other plant families such as *Capparaceae* also contain GLSs (Whitmore and Naidu 2000).

Brassicaceae is a large family including 338 genera and 3,709 species (Warwick et al. 2006). Most cultivated *Brassica* species include mustards, cabbage (derived from *Brassica oleracea*, Capitata, Pekinensis, Chinensis and Acephala groups), cauliflower (derived from *B. oleracea* var. *botrytis*), Brussels sprouts (*B. oleracea* var. *gemmifera* Zenker), broccoli (derived from *B. oleracea*), kohlrabi (*B. oleracea* L. var. *gongylodes* L.), kale (*B. oleracea* L. var. a*cephala group ornamental*), horseradish (*Armoracia rusticana* G. M. Sch.), wasabi or Japanese horseradish (*Wasabia japonica* Matsum or *Wasabia tenuis* Matsum), and radishes (*Raphanus sativus* L. var. *radicula* Pers.) (Whitmore and Naidu 2000).

Table 4.4 GLSs, their degradation products and precursors (Pengelly 2004)

Glycosinolate	Degradation product	Precursor	Plant
Sinigrin	Allyl isothiocyanate	Homo-thionine	*Brassica nigra, B. juncea, B. oleracea*
Sinalbin	p-hydroxybenzyl thiocyanate	Tyrosine	*Brassica alba, Sinapis alba*
Gluconasturtiin	Phenylethyl thiocyanate	Phenylalanine	*Armoracia rusticana, Nasturtium officinalis*
Glucobrassicin	3-indolylmethyl isothiocyanate	Tryptophan	*B. oleracea, B. sativus* var. *niger*
Glucotropaeolin	Benzyl isothiocyanate	Phenylalanine	*Tropaeolum majus, Lepidium sativum*
Progoitrin	2-hydroxy-3-butenyl isothiocyanate	Tyrosine	*B. oleracea*

4.3.1 Occurrence and Chemistry of Glucosinolates

GLSs are amino acid derived secondary plant metabolites which contain a sulphate and a thioglucose (Halkier and Du 1997). These compounds are mainly found in the seeds, although they may also be present in other tissues of the plants. Various amino acids such as tyrosine, phenylalanine and tryptophan, are precursor components and are converted to GLSs by decarboxylation. Table 4.4 lists the GLSs, their degradation products and precursors.

Cruciferous plants may contain total GLSs of about 1% of dry weight but the content is rather variable. For example, broccoli sprouts or seeds may possess 70–100 μmol/g total GLSs of fresh weight. More than 120 individual GLS compounds have been identified in different plant families so far (Fahey et al. 2001). More than 80% of all GLSs identified are found in the *Brassicaceae* family (Kjaer 1976; Whitmore and Naidu 2000) although *Arabidopsis thaliana* was found to contain 20 different GLSs (Brown et al. 2003).

GLSs are classified in aliphatic, aromatic and indolyl groups on the basis of whether they are derived from aliphatic amino acids (often methionine), phenylalanine or tyrosine, or tryptophan, respectively (Halkier and Du 1997). The structural variation of GLSs results from chain elongations of amino acids before the formation of the GLS core structure and secondary modifications of the GLSs side chain (e.g. thiol oxidation, desaturation, hydroxylation, and esterification) and/or glucose moiety (esterification) (Wittstock and Halkier 2002). Fahey et al. (2001) grouped GLSs under ten different chemical structures (sulfur-containing side-chains; aliphatic, straight-chain; aliphatic, branched-chain; olefins; aliphatic, straight and branched-chain alcohols; aliphatic, straight-chain ketones; aromatic; benzoates; indole; multiply glycosylated and others).

GLSs are chemically and thermally stable to a certain extent and therefore generally enzymatic hydrolysis occurs (Polat 2010). Myrosinase is a specific class of β-thioglucosidases located in idioblasts (myrosin cells) distributed in most tissues

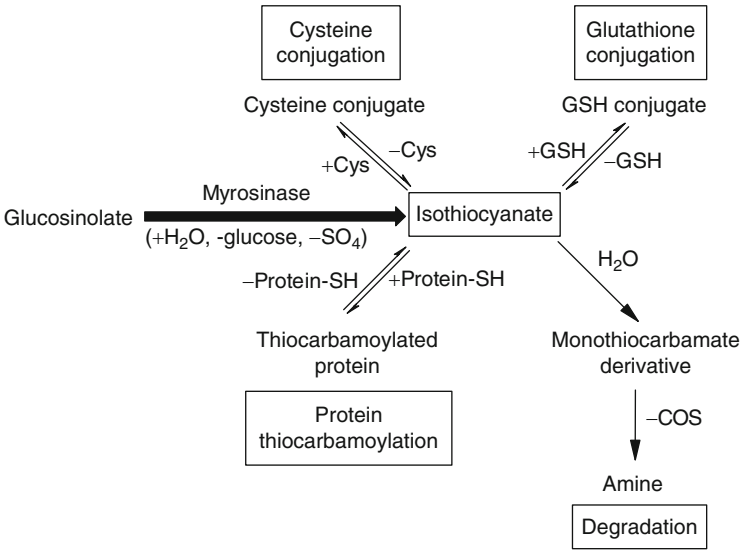

Fig. 4.2 Main hydrolysis mechanism of glucosinolates (Song et al. 2005)

of GLS producing plants (Grubb and Abbel 2006). Reaction kinetics or the enzyme myrosinase may differ depending on the plant species and an individual plant may contain multiple forms of myrosinase (James and Rossiter 1991). By the enzyme myrosinase releases in consequence of tissue damage caused by bruising, wounding, mastication, harvest, shipping and/or handling, relatively nonreactive GLSs are hydrolyzed (Fahey et al. 2001) (Fig. 4.2). The hydrolysis products are thioglucose, sulphate and an unstable derivative which is converted to several degradation products, many of which have pronounced biological effects. These degradation products include isothiocyanates (ITCs), nitriles and thiocyanates. However, epithionitriles and oxazolidine-2-thiones are also produced depending on several factors such as pH and side-chain structures (Halkier and Du 1997) (Fig. 4.2). Stable ITCs are usually formed at pH 6–7, while nitriles are the main hyrolysis products under acidic conditions. Although the majority of hydrolysis products are relatively very stable, indole GLSs such as glucobrassicin yield unstable ITCs and further hydrolysis occurs to give 3-indolemethanol, 3-indoleacetonitrile and 3,3′-diindolylmethane and subsequently condenses into dimers, trimers or tetramers (Holst and Williamson 2004; Gilbert and Senyuva 2008).

Sinigrin is one of the most common mustard oil GLSs found in many cruciferous species and a few other plant families. When plant tissues are damaged, this glucoside is hydrolyzed to release allyl thiocyanate (ATC), a volatile mustard oil (Erickson and Feeny 1974) (Fig. 4.3). Sinalbin is the predominant GLS of yellow mustard and yields mainly nonvolatile 4-hydroxybenzyl ITC responsible for the hot mouthfeel within the hydrolysis by myrosinase (Choubdar et al. 2010). Glucobrassicin and

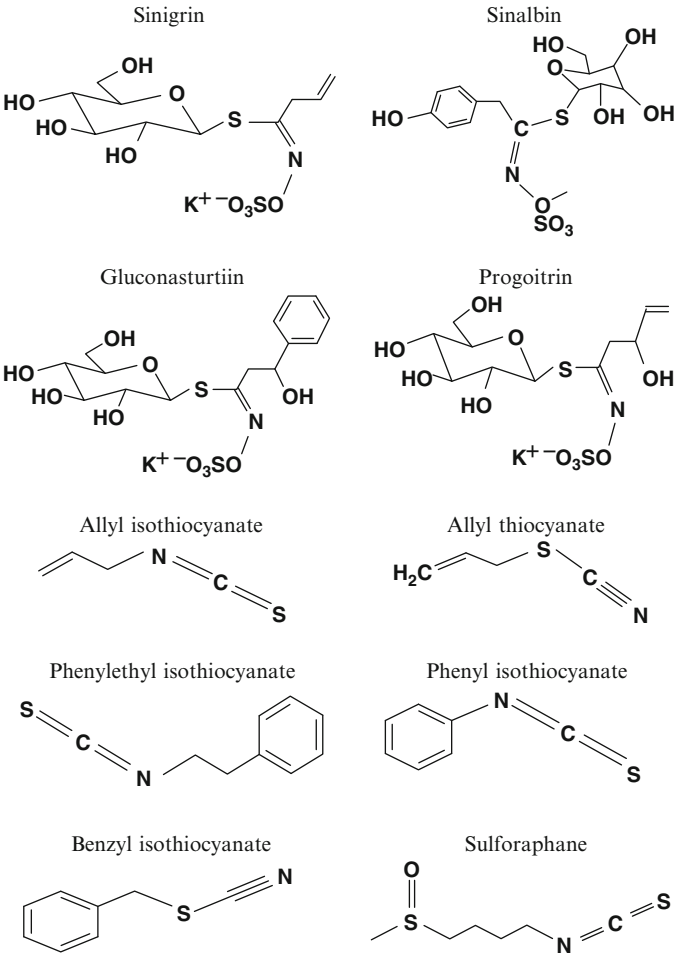

Fig. 4.3 The chemical structures of main glucosinolates and their hydrolysis products

glucoiberin are found in all types of *B. oleracea*, although *B. oleracea* can contain a diversity of GLSs (Cartea and Velasco 2008) (Fig. 4.3).

ITCs are the main hydrolysis products of GLSs. Figure 4.3 shows the chemical structures of main ITCs formed by the hydrolysis of GLSs. Cabbage species contain 4–146 ppm allyl isothiocyanate (AITC), 0–2.8 ppm and 1–6 ppm phenylethyl isothiocyanate (PEITC). The pulps and seeds of ripe papaya fruit contain 4 and 2,910 ppm benzyl isothiocyanate (BITC), respectively (Van Etten et al. 1976; Whitmore and Naidu 2000). Sulforaphane (SFN – generally known as a major ITC converted from glucoraphenine in radish) contents in *Baemuchae* and radish sprouts were found to be 172.6 ± 11.23 and 131.3 ± 4.08 l μg/g, respectively (Lim et al. 2009). BITC is produced by the hydrolysis of glucotropaeolin and the most abundant

ITC found naturally in several plants such as *Carica papaya* L., Indian cress (*Tropaeolum majus* L.) and garden cress (*Lepidium sativum* L.) (Troncoso-Rojas et al. 2005) (Fig. 4.3).

Amounts of GLSs and ITCs in *Brassica* vegetables may be influenced from various storage, processing and cooking conditions. Storage at ambient temperature or in cold conditions does not cause significant loss of GLSs while finely shredded vegetables may be exposed to a remarkable decline in GLS levels. Cooking by steaming, microwaving and stir-fry does not result in significant loss of GLSs whereas boiling shows significant losses by leaching into cooking water (Song and Thornalley 2007).

4.3.2 Antimicrobial Properties of Glucosinolates and Isothiocyanates

The biological properties of cruciferous plants such as cabbage, broccoli, cauliflower, Brussels sprouts and kale, have mainly been associated with GLSs and their breakdown products such as ITCs, nitriles, thiocyanates and other indole compounds. First of all, GLSs are the defensive compounds of these plants against herbivores, insects and other harmful organisms (Fahey et al. 2001). Over the past 30 years, a number of secondary metabolites including GLSs, and specifically their breakdown products has been considered as important bioactive tools (i.e. anticarcinogenic, antioxidant) in the diet and therapy (Aires et al. 2009). Moreover, the pesticidal activity of crucifers primarily depends on the breakdown products, while intact GLSs have generally less biological activity (Vaughn 1999). Volatile ITCs are considered as the major inhibitor allelochemicals of *Brassica* plants. For example, methyl isothiocyanate (MITC) can inhibit the growth of various weeds such as pigweed, dandelion and crabgrass (Whitmore and Naidu 2000). AITC, ATC, allyl isocyanate (AIC) and allyl cyanide (AC) are all considered as potent fumigant materials against house fly (Tsao et al. 2002). On the other hand, in view of their antimicrobial activity, it is well recognized that GLSs and their breakdown products exhibit inhibitory effects against a wide spectrum of bacteria and fungi. Generally, GLSs are not themselves active; the hydrolysis products are the main antimicrobial agents. Moreover, chemical structure influences biological activities, particularly antimicrobial activity. For instance, aromatic ITCs are more effective than aliphatic ITCs (Aires et al. 2009).

4.3.2.1 Antibacterial Activity

Cruciferous compounds that possess inhibitory effects against spoilage and pathogenic bacteria as well as intestinal microbiota have been well studied in recent years. Table 4.5 summarizes the antibacterial activity of GLSs and their hydrolysis products, primarily ITCs. Previous studies showed that Gram-negative bacteria were

Table 4.5 Antibacterial activity of GLSs and their derivatives

Antibacterial organosulfur compound	Explanation	Bacteria	Antibacterial level (%) or (MIC)	Reference
MSITC	50% lethal dose concentration (μM) was measured	*E. coli*	282	Tierens et al. (2001)
		Xanthomonas campestris	136	
		Pseudomonas syringae	28	
		Sarcina lutea	294	
AITC	MIC value (ppm) was measured	*Ped. pentosaceus*	300–400	Kyung and Fleming (1997)
		Leu. mesenteroides	500	
		Lactobacillus plantarum	300–400	
		Lb. brevis	300	
		L. monocytogenes	200	
		S. aureus	100	
		E. coli	50	
		Enterobacter aerogenes	300	
		Bacillus subtilis	50	
		S. Typhimurium	100	
AITC	The percentage of relative inhibition zone (%) at 3.0 μM dose was calculated[a]	*Citrobacter freundii*	48.5	Aires et al. (2009)
		E. coli	51.1	
		Enterobacter hormaechei	48.2	
		Hafnia alvei	46	
		K. pneumoniae	50	
		K. oxytoca	66.7	
		Morganella morganii	74.1	
		P. aeruginosa	66.1	
		Proteus mirabilis	54.9	
		S. Typhi	52.7	

AITC	The minimum inhibitory dose (MID) (μg/dish) was defined as the dose at which no growth was observed in petri dishes	*B. subtilis*	420	Isshiki et al. (1992)
		B. cereus	360	
		S. aureus	420	
		S. epidermidis	420	
		E. coli	110	
		S. Typhimurium	210	
		S. Enteritidis	420	
		Vibrio parahaemolyticus	210	
		P. aeruginosa	210	
AITC	MIC value (ppm) was measured	*E. coli*	100–200	Shofran et al. (1998)
		P. fluorescens	200	
		Aeromonas hydrophila	200	
		S. aureus	200	
		B. subtilis	200	
		Ped. pentosaceus	1000	
		Lc. mesenteroides	500	
		Lb. brevis	1000	
		Lb. plantarum	–	
SFN	The percentage of relative inhibition zone (%) at 3.0 μM dose was calculated[a]	*A. baumanii*	175	Aires et al. (2009)
		Citrobacter freundii	127.9	
		E. coli	155.2–183.2	
		Enterobacter asburiae	188.2	
		E. hormaechei	127.8	
		E. cloacae	144.4	
		Hafnia alvei	181.7	
		K. pneumoniae	116	
		K. oxytoca	183.3	
		Morganella morganii	123.5	
		P. aeruginosa	138.1	
		Proteus mirabilis	168.6	
		S. Typhi	50.2	

(continued)

Table 4.5 (continued)

Antibacterial organosulfur compound	Explanation	Bacteria	Antibacterial level (%) or (MIC)	Reference
ATC	MIC value (ppm) was measured	*E. coli*	200–400	Shofran et al. (1998)
		P. fluorescens	200	
		S. aureus	200	
BITC	The percentage of relative inhibition zone (%) at 3.0 μM dose was calculated[a]	*A. baumanii*	112.5	Aires et al. (2009)
		C. freundii	94.8	
		E. coli	198.2–206.4	
		Enterobacter asburiae	65.6	
		E. hormaechei	150	
		E. cloacae	55.6	
		Hafnia alvei	231.8	
		K. pneumoniae	79.7	
		K. oxytoca	83.3	
		M. morganii	No growth	
		P. aeruginosa	46.1	
		Proteus mirabilis	388.2	
		S. Typhi	153.3	
PEITC	The percentage of relative inhibition zone (%) at 3.0 μM dose was calculated[a]	*A. baumanii*	58.3	Aires et al. (2009)
		E. coli	56.8–61.7	
		Enterobacter asburiae	55	
		E. hormaechei	46.3	
		Hafnia alvei	54.6	
		K. pneumoniae	52.4	
		K. oxytoca	61.1	
		M. morganii	100	
		P. aeruginosa	44	
		Proteus mirabilis	62.8	
		S. Typhi	62.5	

PEITC	Inhibition zone was measured at 2.0 mg/disc concentration by paper disc agar diffusion method	*Clostridium difficile* *C. perfringens* *E. coli*	>30 >30 16–20	Kim and Lee (2009)
BITC	Inhibition zone was measured at 1.0 mg/disc concentration by paper disc agar diffusion method	*C. difficile* *C. perfringens* *E. coli*	21–30 >30 21–30	Kim and Lee (2009)
BOITC	Inhibition zone was measured at 5.0 mg/disc concentration by paper disc agar diffusion method	*C. difficile* *C. perfringens* *E. coli*	>30 >30 >30	Kim and Lee (2009)

MSITC 4-methylsulphinylpropyl isothiocyanate, *AITC* Allyl isothiocyanate, *ATC* Allyl thiocyanate, *SFN* Sulforaphane, *BITC* Benzyl isothiocyanate, *PEITC* Phenylethyl isothiocyanate, *BOITC* Benzoyl isothiocyanate, *MIC* Minimum inhibition concentration

[a]Percentage of relative inhibition zone diameter = (Inhibition zone diameter of sample – Inhibition zone of negative control)/Inhibition zone of antibiotic standard × 100

more susceptible than Gram-positive against ITCs (Isshiki et al. 1992; Lin et al. 2000). Several of the antibacterial actions of ITCs have been reported. AITC alters protein structures and thereby inhibits microbial growth (Kawakishi and Kaneko 1987). Lin et al. (2000) observed the antibacterial mechanism of AITC against *S.* Montevideo, *E. coli* O157:H7 and *L. monocytogenes* Scott A. They showed that AITC was effective in both the exponential and stationary phases of the bacterial growth. AITC affected cell membranes causing leakage of cellular metabolites and increased β-galactosidase activity of bacteria. Delaquis and Mazza (1995) reported that AITC might damage the essential intracellular enzymes of microorganisms through oxidative cleavage of disulfide bonds.

SFN was found to be effective in the inhibition of *Helicobacter pylori*, the cause of human gastritis and stomach cancer (Fahey et al. 2002; Haristoy et al. 2003, 2005; Yanaka et al. 2009). SFN was also reported as both bacteriostatic and bactericidal against all strains of bacteria. Broccoli sprouts are relatively rich sources of ITCs with a content of 20–50 times more precursor GLSs than mature broccoli. Broccoli sprouts were suggested as a potential digestive eradicator of *H. pylori* from the human gastrointestinal system (Galan et al. 2004).

Sinigrin, a thioglucoside of cruciferous plants and its hydrolysis products AITC, AC, ATC and 1-cyano-2,3-epithiopropane (CETP) were tested for their MIC in broth against 9 species of bacteria including the strains of *E. coli*, *Pseudomonas fluorescens*, *Aeromonas hydrophila, S. aureus*, *B. subtilis, Pediococcus pentosaceus*, *Leu. mesenteroides*, *Lb. brevis* and *Lb. plantarum* (Shofran et al. 1998). Sinigrin, AC and CETP were found to be ineffective in the inhibition of any of the bacteria. ATC showed an inhibitory effect against only *E. coli*, *Pseudomonas fluorescens* and *S. aureus*. *E. coli* was the most sensitive pathogen to AITC with the MIC value of 100 ppm. In this study, the effect of pH on the antimicrobial activity of AITC was also tested and the MIC values of AITC varied from 60 to 140 ppm against *E. coli* and 120 to 220 ppm for *S. aureus*, respectively. Kyung and Fleming (1997) tested the susceptibility of 15 different bacterial strains to sinigrin and some other sulfur compounds. As same in the previous study, they found sinigrin to be inactive against the bacteria while the MIC values of AITC varied from 50 to 500 ppm.

The balance of intestinal microflora is of importance because it helps to maintain human health, contributes to pathogen resistance and interacts with the host immune system (Lahtinen et al. 2009). Several studies have been conducted on the effect of GLSs and derivatives on intestinal microflora. Kim and Lee (2009) reported that PEITC isolated from *Sinapis alba* L. (white mustard) strongly inhibited the growth of *C. difficile* and *C. perfringens* at 1 mg/disc and moderately inhibited the growth of *E. coli* at a dose of 2 mg/disc *in vitro* while it did not exhibit any inhibitory effect on the growth of beneficial microbiota including bifidobacteria and lactobacilli. Aires et al. (2009) showed *in vitro* antibacterial activity of GLS hydrolysis products and specifically SFN and BITC against Gram-positive and Gram-negative pathogenic bacteria isolated from the human intestinal tract. On the other hand, several bacterial strains associated with GLS hydrolysis and belonging to *E. coli, Bifidobacterium longum, Enterobacter cloacae, Bacteroides thetaiotaomicron* and *Bacteroides vulgatus* species have been identified in the intestinal flora.

These strains degrade GLS during 24–48 h cultivation and cause a decline in medium pH from 7.1 to 5.2 (Traka and Mithen 2009).

Ogawa et al. (2000) investigated the combined effects of hydrostatic pressure, temperature and addition of AITC on the inactivation of different strains of *E. coli in vitro*. They found that the antibacterial effects of pressurization with the addition of AITC at 4°C and 40°C were greater than at 20°C and all bacteria tested were effectively killed at 200 or 250 MPa with 10–80 μg/mL of AITC.

The favorableness of GLSs and hydrolysis products for extending the shelf-life of foods, especially meat products, has been investigated. Muthukumarasamy et al. (2003) reported that AITC (about 1,300 ppm) completely eliminated *E. coli* O157:H7 in low inoculated ground beef and reduced the viability >4.5 log cfu/g at high inoculated beef sample by the end of the storage period for 25 days at 4°C. Nadarajah et al. (2005) also obtained similar results in ground beef patties. In another study, Chacon et al. (2006) used microencapsulated AITC to eliminate *E. coli* O157:H7 and total aerobic bacteria (TAB) in finely chopped beef samples. AITC levels lower than 1,000 ppm were ineffective in reducing *E. coli* O157:H7 numbers while both low and high levels of inoculated *E. coli* O157:H7 were completely eliminated by 4,980 ppm AITC addition after 15 and 18 days of storage, respectively. 4,980 ppm AITC kept APC levels <3 log cfu/g during 18 days of storage. Shin et al. (2010) investigated the efficiency of the AITC-MAP (modified atmosphere packaging) system to control the growth of *L. monocytogenes* and *S.* Typhimurium on raw chicken breast during refrigerated storage for up to 21 days. Both release rates of 0.6 and 1.2 μg/h plus MAP systems significantly reduced the population of *S.* Typhimurium whereas *L. monocytogenes* was weakly inhibited at the lower release rate of AITC.

Fresh fruit and vegetables are at the risk of outbreaks of foodborne illnesses resulting from the contamination of pathogens during pre- and post-harvest stages. ITCs have been investigated as natural antimicrobial agents to sanitize fresh produce. Obadiat and Frank (2009a) compared the effectiveness of AITC, carvacrol and cinnamaldehyde in the vapor phase to eliminate strain cocktails of *Salmonella* and *E. coli* O157:H7 from sliced and whole tomatoes. AITC exhibited the highest antimicrobial activity and lowest level of AITC (8.3 μL/liter of air) inactivated *Salmonella* on sliced tomatoes by 1.0 and 3.5 log at 4°C and 10°C, respectively, in 10 days and by 2.8 log at 25°C in 10 h. AITC also reduced the *E. coli* O157:H7 level on sliced tomatoes by 3.0 log at 4°C and 10°C in 10 days, but there was no inactivation at 25°C in 10 h. The same researchers also tested those antimicrobial compounds in vapor phase on the intact and damaged portions of lettuce and spinach leaves against *E. coli* O157:H7 (Obadiat and Frank 2009b). On intact lettuce surface, 4 μL/liter of air AITC inactivated >4 log of *E. coli* O157:H7 at 0°C and 4°C in 4 days and at 10°C in 2 days. Pathogen inactivation on spinach surface was lower than on lettuce by 1 log. Higher concentrations of AITC were required when the surfaces were damaged by cutting. Lin et al. (2000) showed that lettuce inoculated with *E. coli* O157:H7 and *S.* Montevideo (each 10^4 cfu/g) showed no detectable bacterial counts after exposure to 400 μL of AITC and MITC for 24 and 48 h, respectively. *L. monocytogenes*

was reduced to undetectable levels after the treatment with 400 μL of MITC for 2 days whereas 400 μL of MITC failed to achieve the same bactericidal effect.

4.3.2.2 Antifungal Activity

Although cruciferous plants produce inducible chemical defensive systems, they may be exposed to infection by a wide range of fungi. It was demonstrated that GLSs and their breakdown products, the major defensive compounds of crucifers, exhibit antimicrobial activity against fungal pathogens both *in vitro* and *in planta* (Sellam et al. 2007). The inhibitory effects of GLSs and their derivatives against various fungal species are shown in Table 4.6.

Postharvest fungal growth becomes a threat for many crops during their storage under unfavorable conditions and causes considerable economic losses worldwide. The antifungal effects of vapor-phase ITCs have been demonstrated in many studies. Mari et al. (2002) reported that the best control of blue mold (*P. expansum*) on pears was obtained by exposing fruits in a 5 mg/L AITC-enriched atmosphere for 24 h. Wu et al. (2011) tested the potential use of AITC and EITC as fumigants in *in vitro* and *in vivo* trials to determine their effects on *P. expansum* Link and *Botrytis cinerea* Persl. infection in apples. A 3:1 ratio of AITC:EITC showed the best inhibitory activity on *in vitro* spore germination of *P. expansum* and *B. cinerea*. In *in vivo* trials on artificially infected apples, AITC, EITC and their combination reduced the incidence by more than 85% after 3–4 days of apple incubation at 20°C. Kurt et al. (2011) investigated *in vitro* and *in vivo* antifungal activity of synthetic pure ITCs against *Sclerotinia sclerotiorum*, a necrotrophic pathogen causing *Sclerotinia* stem and root rot of tomato and other economically important crops. MITC, AITC and BITC completely inhibited mycelial growth of the mold in the volatile phase. Butyl ITC (BUITC) and BITC reduced the apothecial production of *S. sclerotiorum* at their highest concentrations. In *in vivo* assay, AITC and PEITC reduced disease incidence by 76.7% and 70%, respectively.

Goncalves et al. (2009) investigated the antifungal efficiency of AITC sachets in cottage cheese preservation. The sachets produced by the incorporation of AITC were attached to the inner surface of the lids of cups containing cheese and the cups were stored for 35 days at 5 ± 2°C and 10 ± 2°C. After 7 days, the AITC sachets were found to be effective in reducing yeast and mold counts.

4.4 Conclusion

Organosulfur compounds in intact vegetables of the *Allium* and *Brassica* spp. and particularly their hydrolysis products are considered as key compounds of these vegetables. They give bioactive properties to these vegetables such as antimicrobial, anticarcinogenic, antitumor etc. while also giving them their pungent flavor and odor. Garlic is the most studied *Allium* vegetable as regards the antimicrobial activity

Table 4.6 Antifungal activity of GLSs and their derivatives

Antifungal agent	Explanation	Fungi	Antifungal level	Reference
MSITC	50% lethal dose concentration (μM) was measured	*Alternaria brassicicola*	>1130	Tierens et al. (2001)
		Botrytis cinerea	>1130	
		Fusarium culmorum	124	
		F. oxysporum	325	
		Neurospora crassa	271	
		Nectria hematococca	260	
		Plectosphaerella cucumerina	147	
		Penicillium expansum	294	
		Verticillium dahlia	215	
AITC	MIC value (ppm) was measured	*Saccharomyces cerevisiae*	4	Kyung and Fleming (1997)
		Torulopsis etchellsii	1	
		Hansenula mrakii	4	Shofran et al. (1998)
		Pichia membranefaciens	2	
		Torulospora delbrueckii	4	
		Zygosaccharomyces rouxii	–	
AITC	The minimum inhibitory dose (MID) (μg/dish) was defined as the dose at which no growth was observed in petri dishes	*S. cerevisiae*	62	Isshiki et al. (1992)
		Hansenula anomala	124	
		T. delbrueckii	50	
		Z. rouxii	31	
		Candida tropicalis	62	
		C. albicans	62	
		Aspergillus niger	124	
		A. flavus	124	
		Penicillium islandicum	62	

(continued)

Table 4.6 (continued)

Antifungal agent	Explanation	Fungi	Antifungal level	Reference
		P. citrinum	62	
		P. chrysogenum	250	
		Fusarium oxysporum	62	
		F. graminearum	31	
		F. solani	110	
		Alternaria alternata	62	
		Mucor racemosus	250	
AITC (gas)	MIC value (μg/L) was measured	*A. flavus*	100	Delaquis and Sholberg (1997)
		Botrytis cinerea	100	
		P. expansum	100	
BITC	MIC value (mg/mL) was measured	*Alternaria alternata*	0.1	Troncoso-Rojas et al. (2005)
MITC	MIC values (μM/L) of ITCs in the volatile phase were measured	*Sclerotinia sclerotiorum*	732	Kurt et al. (2011)
AITC			714	
BUITC			840	
EITC			550	
PITC			836	
BITC			525	
PEITC			670	
MITC	MIC values (μM/L) of ITCs in the contact phase were measured	*S. sclerotiorum*	4387	Kurt et al. (2011)
AITC			3065	
BUITC			1688	
EITC			2358	
PITC			1672	
BITC			750	
PEITC			2011	

MSITC 4-methylsulphinylpropyl isothiocyanate, *AITC* Allyl isothiocyanate, *ATC* Allyl thiocyanate, *SFN* Sulforaphane, *BITC* Benzyl isothiocyanate, *PEITC* Phenylethyl isothiocyanate, *BOITC* Benzoyl isothiocyanate, *MIC* Minimum inhibition concentration

since it is the richest source of antimicrobial organosulfur compounds among the *Allium* ssp. whereas all species of crucifers incorporate organosulfur materials. Allicin and other thiosulfinates such as DAS, DADS, DATS and ajoene present in *Allium* plants are the principal organosulfur compounds to exhibit antimicrobial activity. In the *Brassica* family, GLSs are not generally active themselves; their hydrolysis products are the main antimicrobial agents. Volatile ITCs like AITC, ATC, MITC and SFN have been shown to be the most effective antimicrobial compounds formed by the hydrolysis of GLSs in cruciferous plants.

Overall, it can be concluded that organosulfur compounds have the importance not only for the plant but also in the food industry in virtue of their health benefits. These compounds as well as various extracts of edible parts of organosulfur-source plants might be used in food product formulations for health and/or antimicrobial purposes. Although specific flavors/odors of these materials adversely affect organoleptic properties of most foods used, they might be considered as convenient ingredients of especially some meat and dairy products. Furthermore, several studies should be focused on preventing the come into forefront of the negative flavor/odors of them in foods when they are consumed.

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Chapter 5
Antimicrobial Activities of Essential Oils

Danuta Kalemba, Martyna Matla, and Anna Smętek

Abstract Essential oils are one of the most important groups of plant constituents responsible for biological activity of herbs and spices, and especially for their medicinal and antimicrobial properties. Numerous *in vitro* studies have demonstrated activity of different essential oils against bacteria, moulds and yeast. The power of essential oils is connected with their main constituents. The oils containing phenols such as thymol, carvacrol, and eugenol, exhibit the most pronounced activity against all kinds of microorganisms. This chapter gives a literature review of recent *in vitro* investigations concerning antibacterial and antifungal activity of essential oils. The oils recognized as the most valuable antimicrobial agents and used as food ingredients will be presented, namely thyme oil, clove oil, different cinnamon, mint and citrus oils, and rosemary oil.

Keywords Essential oils • Antibacterial activity • Antifungal activity • Thyme oil • Clove oil • Cinnamon oils • Mint oils • Citrus oils • Rosemary oil

5.1 Introduction

Spices and herbs have been used as food additives since ancient times, both as flavoring agents and as natural food preservatives. Essential oils, odorous and volatile products derived from plants, have found a considerable range of applications. They are used mainly as flavors and fragrances in food and perfumery industries. However, due to their antimicrobial activity essentials oils are also important for food and cosmetic preservation and for the control of human, animal and plant diseases that are of microbial origin.

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With growing interest in the use of essential oils in the food, agricultural and pharmaceutical industries, examination of these natural products has become increasingly important. In recent years antibacterial and antifungal potential of essential oils have been extensively researched and reviewed (Kalemba and Kunicka 2003; Burt 2004; Edris 2007; Reichling et al. 2009; Tajkarimi et al. 2010). In this chapter *in vitro* antimicrobial potential of some essential oils will be presented in the light of the recent 15 years investigations. The focus will be directed to the essential oils commonly used as food ingredients which antibacterial and antifungal activity have been well documented.

5.2 Distribution of Essential Oils

Essential oils are complex mixtures of volatile compounds produced by plants as secondary metabolites. They are obtained of taxonomically defined plant material mainly by water or steam distillation with the exception of essential oils from citrus peels obtained by cold pressing. Fragrance- and flavor-producing substances can be isolated by many other methods. However, such products shall not be considered as essential oils (Franz and Novak 2010).

Essential oils are biosynthesized and accumulated in various plants including annual, biennial or perennial herbaceous plants, and deciduous or evergreen shrubs and trees. All parts of plants are used as sources of essential oils: leaves or leafy stems (e.g., mint, oregano, thyme, rosemary, sage); fruits (anise, coriander, fennel, cumin, citrus, peppers, star anise, juniper); seeds (cardamom, nutmeg); stems (citronella); roots (angelica); rhizomes (ginger, turmeric); flowers or blossoms (rose, orange); flower buds (clove); bark (cinnamon, cassia); wood (camphor); bulbs (onion, garlic). The majority of plants produce essential oils in different botanical parts. These oils can be similar in composition (e.g. oils of angelica root and seed, oils of clove buds and leaves) or entirely different (e.g. oils of cinnamon bark and leaves; bitter orange peels, flowers and leaves; coriander immature leaves and seeds).

Essential oils are stored in special organs: secretory cells, ducts and cavities located inside different plant tissues, or in glandular hairs situated in the outer cell layer, mainly of leaves or petals. Some plant species produce exudates such as resins and balsams that also can be used as sources of essential oils. The content of essential oil in resins achieves 30%, while the content in plant parts is lower and amounts to 0.02% for flowers, 1% for herbs and 3–5% for seeds and fruits. The exception is clove buds containing 15–20% of essential oil.

Essential oils are usually colorless or pale yellow liquids with strong odor resembling the source plant material. They are soluble in alcohol, plant oils and most organic solvents but they are immiscible with water. Their density is usually smaller than that of water, they are characterized by a high refractive index and most of them are optically active.

Essential oils, their fractions and isolates are utilized in food, perfumery, flavors and fragrances, cosmetics and toiletries, fine chemicals, pharmaceutical industries as well as in therapy and aromatherapy. They are also sources of aroma chemicals, particularly of enantiomers that are useful as chiral building blocks in syntheses. Essential oils derived from spices, aromatic or medicinal plants are used as food ingredients fulfilling two roles flavor and preservative. Majority of them is generally recognized as safe (GRAS). Twenty eight essential oils that are used in medicine have their monographs in the European Pharmacopoeia 5 (EP 5).

5.3 Antimicrobial Activity and Composition of Essential Oils

The antibacterial and antifungal properties of essential oils have been known and utilized for centuries. Each essential oil displays antimicrobial activity that is resultant of essential oil effectiveness and microorganism susceptibility. The power of any biological activity of essential oils is strictly connected with the oil composition and especially with the content of some highly active constituents. Essential oils are multicomponent mixtures containing usually more than 100 components. In some commercially important oils more than 300 constituents have been known so far. These are subdivided into two groups: hydrocarbons that are made up almost exclusively of terpenes (monoterpenes, sesquiterpenes and diterpenes) and oxygenated compounds that beside terpene have phenylpropanoid and aliphatic skeletons. Some compounds may also contain nitrogen or sulphur. Due to the wide variety of number of carbon atoms, constitutional isomers (acyclic, mono-, bi- and tricyclic) and stereoisomers combined with different functional groups (hydroxyl, carbonyl, carboxyl) a great diversity of structures can be found in each group of constituents. The compounds found the most frequently as essential oils constituents are monoterpenes.

The antimicrobial activity rank of essential oil components depends mainly on the functional group. On the basis of hundreds of previous investigations the order proposed by Kalemba and Kunicka (2003) is corrected to the following one:

phenols > cinnamic aldehyde > alcohols > aldehydes = ketones > ethers > hydrocarbons.

The highest activity was reported for phenols. Monoterpenes: thymol and carvacrol as well as phenylpropanoid – eugenol are the most frequent phenols found in essential oils. Essential oils with phenols as main compounds express the highest and broadest activity against both bacteria and fungi. These are thyme, oregano and savory oils containing thymol and carvacrol as well as clove and cinnamon leaf oils containing eugenol. The highest activity of phenols is explained by acidic character of the hydroxyl group forming hydrogen bound with an enzyme active center. Cinnamaldehyde – the main component of cinnamon bark oil, also falls into the group of essential oil constituents of the highest antimicrobial activity. Monoterpene aldehydes, e.g. citronellal, neral, geranial, that are major components of citronella

and lemon balm oils, show lower activity that is comparable to the activity of alcohols such as: linalol (coriander and lavender oils), menthol (peppermint oil), geraniol, nerol and citronellol (rose and geranium oils), α-terpineol and terpinen-4-ol (tea tree oil), borneol, farnesols. Other oxygenated terpenes include ketones: carvone (caraway oil), menthone (peppermint oil), pulegone, α- and β-thujones and camphor (sage oil); esters: mainly acetates of monoterpene alcohols, and oxides: 1,8-cineole (eucalyptus oil), anethole (anise oil, fennel oil), estragole, ascaridole, bisabolol oxides. Oct-1-en-3-ol, (*E*)- and (*Z*)-hex-3-enols belong to the most important aliphatic constituents. Monoterpene hydrocarbons that can be the most frequently found as essential oil constituents are: limonene, pinenes (α- and β-), phellandrenes (α- and β-), terpinenes (α- and γ-), sabinene, camphene and myrcene. β-Caryophyllene is the most common sesquiterpene hydrocarbon. Hydrocarbons are the main components of citrus and conifer oils. These general rules enable to predict to some extent the *in vitro* antimicrobial activity of essential oil with known chemical composition.

Chirality is an important aspect of essential oil compounds because enantiomers may possess different smell and taste, e.g. (*S*)-(+)-carvone which isomer is a main constituent of caraway oil has a caraway flavor while (*R*)-(−)-isomer being the major component of spearmint oil possesses a mint flavor. It is known that enantiomers of some compounds have such different biological activity that one of them is a drug and the other a poison, e.g. thalidomide. According to the recent research in case of antimicrobial activity of essential oil constituents chirality seems to be not significant point. Only a few research have been done on that score. Similar activity was shown for both linalool enantiomers against *Botrytis cinerea* (Özek et al. 2010), both carvone enantiomers and both limonene enantiomers against a wide spectrum of human pathogenic bacteria and fungi tested (Aggarwal et al. 2002; Jirovetz et al. 2004). On the other side, (*R*)-carvone and essential oils containing its high amount were more effective than those containing (*S*)-enantiomer against postharvest fungal pathogens of fruit (Combrinck et al. 2011). Similar observation were reported by Lis-Balchin et al. (1999) for α-pinene, 18 out of 25 different bacteria and 2 out of 3 filamentous fungi were found to be more affected by the (+)-α-pinene than by its (−)-enantiomer.

The biological activity of essential oils is strictly connected with their chemical composition. Since essential oils are natural products, their composition cannot be precisely quantified. Correct botanical description of the plant material is out of discussion. The genus *Mentha* L. comprises about 25 species and even 900 taxons, there are hundreds of eucalyptus species and varieties. Different thyme and oregano species or even genera are accepted universally as thyme or oregano, respectively. That is the main reason that oils marketed at the same name showed great variability between the antimicrobial action, e.g. eucalyptus or chamomile (Lis-Balchin et al. 1998).

Moreover, numerous species produce several subspecies, varieties or chemotypes with different dominant constituents. The most spectacular example is common thyme, *Thymus vulgaris* L. Thymol and carvacrol types are the most relevant but

other such as geraniol, cineol, or linalol types grow in different regions. Thyme oils derived from different chemotypes of *T. vulgaris* (Ferhout et al. 1999; Oussalah et al. 2006) and different *Thymus* species (Oussalah et al. 2007) varied not only in flavor but first of all in their antimicrobial properties. On the other side, several thyme species produce essential oil with thymol as the main constituent and according to EP 5 thyme oil is obtained from *T. vulgaris* L. and *T. zygis* Loefl.

This is also the case of two other very common pharmacopoeial essential oils that can be misleaded with others. Two varieties of *Foeniculum vulgare* Mill. are traded as spice: bitter fennel fruit and sweet fennel fruit. The later is usually used as flavoring and the former is listed in EP 5. Pharmacopoeial sweet orange oil is obtained by pressing from peels of *Citrus aurantium* L. var. *dulcis* (syn. *C. sinensis* (L.) Osbeck). Under the same name distilled orange oil, and terpeneless oils both distilled and pressed are traded. Moreover, bitter orange peel oil is also available. As far as fennel and orange peel oils concerned, the composition of different oils do not differ significantly. Entirely different is the case of two important essential oils produced from Ceylon cinnamon. Eugenol is the main constituent of cinnamon leaf oil while cinnamaldehyde of cinnamon bark oil. Strangely, some researchers – even in very recent reports – do not specify which of these two oils they tested. Other valid example is rosemary oil. Two pharmacopoeial types of this oil, Spanish as well as Marocco and Tunisian type differ significantly in chemical composition.

What is more, it should be stressed that even for the properly defined plant material and isolation method significant fluctuations in percentage composition of essential oil can be observed. They are mainly due to cultivation conditions (growing region, weather, climate, soil, etc.), harvesting time, methods of preparing material, distillation parameters. This is the reason that in pharmacopoeial or ISO requirements a rather broad range of the content of main oil constituent as well as minimal or maximal content of other important constituent is given. Chemical composition of essential oil under investigation has to be reported together with its antimicrobial activity. Although this rule is obvious it is not always comply, even in many of very recent investigations.

5.4 Methods for Antimicrobial Assessment of Essential Oils

For antimicrobial assessment of essential oils the conventional *in vitro* methods used for testing antibiotic effectiveness are usually applied. Since they have been critically reviewed (Kalemba and Kunicka 2003; Burt 2004), here only the most important aspects will be pointed. As the most suitable the serial dilution method in liquid broth or agar is widely accepted, with the microdilution on agar as the most common recently. Different ways of microorganism growth assessment are applied. Traditional counting of colonies has been replaced by turbidimetry, impedimetry or cytometry. The semi-quantitative agar diffusion method (disc or hole) is generally considered as inappropriate for essential oils that are volatile and likely to evaporate.

This method can be useful in screening of great number of essential oils against a broad range of microorganisms (Hili et al. 1997; Lis-Balchin et al. 1998). The mutation of agar diffusion method called microatmosphere method is used for the estimation of essential oil activity in vapor phase. It is especially suitable for defining the activity of essential oils which are to be employed as the atmospheric preservatives or drugs used by inhalation. It has been proved that activity of essential oils depends on the assay method used and method should correspond with the application mode (Tullio et al. 2007; Inouye et al. 2003; Suhr and Nielsen 2003).

Antimicrobial activity of essential oils have been assessed since the beginning of twentieth century and reviewed for at least 40 years. Although several authors have been stressed the need of standard, reproducible method for assessing essential oils, many methods are applied and developed. What is more, even for the same method used, the comparison of experimental results obtained by different authors still remains difficult. A number of factors influencing the results of antimicrobial activity can be standardized, e.g. culture conditions, solvents used to facilitate the oil dispersion. Some of factors are difficult to standardization such as susceptibility of microorganism strain even if they come from same collections. Only a few of researchers control the susceptibility of microorganisms toward approved antibiotics or antimycotics (positive control) and even fewer make a negative control. The next difficulty results from great diversity of ways of reporting the antimicrobial activity: various units of oil concentration (replaced sometimes by dilution), different definition of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values, usage of such factors as BA_{50}, IC_{50}, IC_{80} instead of MIC. And last but not least, not always the composition of the essential oil is given and often the denomination of oil is ambiguous, especially in the case of commercial names.

The majority of a huge number of available results on antimicrobial activity of essential oils was obtained by different *in vitro* methods. These should be treated only as the first step in efficacy assessment. It has been many times proven and reviewed by Burt (2004) and Tajkarimi et al. (2010) that there is no rational relationship between *in vitro* and *in vivo* (as far as therapy of infectious diseases concerns) or *in situ* testing (in the case when essential oil is food or cosmetic preservative or functional ingredient). Effective doses of essential oils in food and cosmetics are much higher than MIC values established by *in vitro* methods. *In vivo* and *in situ* research are expensive and difficult. However, they are indispensable for proper evaluation of essential oils as drugs and preservatives. Such research are described in the other chapters. In this chapter the *in vitro* antibacterial and antifungal activity of dietary essential oils will be reviewed on the basis of last 15 years research. The first criterion of essential oil selection was their importance in food industry as well as in medicine. The second criterion was the power of antibacterial and antifungal activity, the oils are listed according to their effectiveness. The description of appearance and requirements for the content of important oil constituents are referred to European Pharmacopoeia 5 (2005) (EP 5), while the yield, flavor and applications of essential oils to Wright (2004).

5.5 Thyme Oil

Thyme oil is obtained from the fresh flowering aerial parts of *Thymus vulgaris* L. and *T. zygis* Loefl. (shrubs, Lamiaceae) with the yield of 0.5–1.2%. Two kinds of oil are known: red (dark reddish-brown with crude strong, aromatic flavor) and white (redistilled, yellow of milder odor). The oil is used as an agent in seasoning blends and in traces in many flavors, e.g. of mandarin and orange.

The major components of the oil according to EP 5 are: thymol (36.0–55.0%), p-cymene (15.0–28.0%), γ-terpinene (5.0–10.0%), linalol (4.0–6.5%), carvacrol (1.0–4.0%), myrcene (1.0–3.0%), terpinen-4-ol (0.2–2.5%) (Fig. 5.1).

5.5.1 Antibacterial Activity

Thyme oil has always been among these most frequently investigated against the antimicrobial activity and it was always placed among the most effective against both bacteria and fungi. It has often been investigated parallel with oregano oil and exhibited similar antimicrobial activity.

Thyme oil exhibits very high antibacterial activity. According to Biavati et al. (1997) a majority of bacteria strains from the genera *Bacillus* (8 spp.), *Clostridium* (8 spp.), *Bifidobacterium* (7 spp.), *Lactobacillus* (7 spp. and ssp.), *Pseudomonas* (6 spp. and ssp.), *Enterococcus* (2 spp.), *Lactococcus lactis* (2 ssp.), as well as *Streptococcus salivarius*, *Agrobacterium vitis*, *Xanthomonas pruni*, and *Erwinia carotovora* was inhibited at 400–600 ppm of thyme oil. The most resistant were three *Pseudomonas* species and three *Bifidobacterium* species with MIC 1,200–>2,000 ppm. Twenty oils were assessed in this research and only oregano oil showed as high activity as thyme oil. Similar activity of thyme oil was established against 3 bifidobacterias from dental caries MIC 600–>2,000 ppm (Crociani et al. 1997). Thyme oil was among the most effective out of 52 essential oils against *Acinetobacter baumanii*, *Aeromonas sobria*, *Enterococcus faecalis*, *Klebsiella pneumoniae* and *Serratia marcescens* with MIC 0.12–0.5%. Likewise majority of oils tested in this report thyme oil was inactive even at 2% toward *Pseudomonas aeruginosa* and *Salmonella typhimurium* (Hammer et al. 1999). Thyme oil along

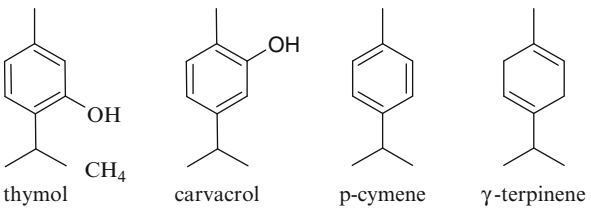


Fig. 5.1 Main components of thyme oil

with oregano and cinnamon bark oils were the most active out of 31 oils against *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella typhi*, *Streptococcus pyogenes* (625–1,250 mg/L), *Escherichia coli* (2,500 mg/l) and *P. aeruginosa* (5,000 mg/L) (Roengsumran et al. 1997). Significantly higher activity of thyme oil was observed against five food-borne bacteria: with MIC 0.02% for *S. aureus* and *Listeria monocytogenes*, 0.04–0.05% for *Salmonella enteritidis*, *Camphylobacter jejuni* and *E. coli*. Thyme oil was the most inhibitory from among 21 oils (Smith-Palmer et al. 1998). Similar BA_{50} values were established for *E. coli* and *Salmonella enterica* (0.05%), lower for *C. jejuni* (0.022%), and higher for two *L. monocytogenes* strains (0.091% and 0.22%, respectively). From 96 oils assessed in this research only three other (clove, cinnamon bark and leaf oils) showed equally high effectiveness (Friedman et al. 2002). The oil was effective against reference *Salmonella* Enteritidis (S. *enterica* subsp. *enterica* serotype Enteritidis) with MIC at 107 μg/ml as well as five clinical strains of this foodborne bacteria (MIC 67–320 μg/ml) (Rattanachaikunsopon and Phumkhachorn 2010). The same range of MIC values were reported for *S. aureus* (31.2 μg/ml) and *E. coli* (62.5 μg/ml) and for other bacteria, *Bacillus cereus* (15.6 μg/ml), *Proteus vulgaris* (31.2 μg/ml), *Proteus mirabilis* (62.5 μg/ml), *S. typhimurium* (125 μg/ml), *S. typhi* (250 μg/ml), *K. pneumoniae* and *P. aeruginosa* (both 500 μg/ml) (Al-Bayati 2008), as well as for antibiotic-resistant *Micrococcus luteus* (66.7 μg/ml) (Friedman 2006), and *Propionibacterium acnes* (0.016%) (Zu et al. 2010).

The antibacterial efficacy of thyme oil depended of the vegetation period of plant material and was highest for the oil of thyme in full flower that at 400 ppm caused total inactivation of five bacteria (e.g. *Sarcinia flava*, *Listeria innocua*) and at 800 ppm of all of other 15 strains of tested bacteria (e.g. *Pseudomonas fluorescens*, *Bacillus thuringiensis*) (Marino et al. 1999). The efficacy against seven food borne bacteria strains strongly depended on the origin of the oil. The biggest differences were observed toward *L. monocytogenes* with MIC 0.1–0.5 μg/ml for thyme oil from France and 2 μl/ml for oil from Spain (Rota et al. 2004).

Thyme oil was always one of the best in antimicrobial activity in comparison studies of some essential oils, irrespective of microorganism used. Thyme oil together with oregano oil showed the highest antibacterial activity against *Streptococcus pneumoniae* from among 73 essential oils (Horne et al. 2001), against *Pseudomonas putida* (MIC 0.05%) from among 60 oils tested (Oussalah et al. 2006), among 13 oils against 12 bacterial strains with *Lactobacillus sakei*, *Clostridium botulinum* and *Clostridium perfringens* as the most sensitive (Nevas et al. 2004), against *Lactobacillus* and *Staphylococcus* species commonly used in food industry and *Enterobacter* species related to food spoilage (Viuda-Martos et al. 2008). Thyme oil was the most active among 13 oils against *E. coli* O157:H7 (Burt and Reinders 2003), among 51 oils against 3 bacteria (*S. aureus*, *E. coli* and *P. aeruginosa*) (Hili et al. 1997), among 6 oils against 25 bacteria (Dorman and Deans 2000), among 14 oils against respiratory tract pathogens in gaseous contact with MID (Minimal Inhibitory Dose) 3.13–6.25 mg/l air (Inouye et al. 2001), and among five oils against two reference strains and nine isolated strains of *Vibrio alginolyticus* (MIC 0.078–0.31 mg/ml) (Hajlaoui et al. 2010).

It is worth to note that both bacteriostatic and bactericidal concentrations of white thyme oil against *E. coli* (625 and 1,250 μl/l, respectively) were two times lower than that of red thyme oil (Burt and Reinders 2003).

Essential oil of *T. zygis*, Spanish variety of *T. vulgaris*, was effective against poultry origin strains and pig origin strains from *Enterobacteriaceae* family showing MIC 0.5% against *Salmonella essen* and 2–4% against *Salmonella choleraesuis*, *S. enteridis*, *S. typhimurium*, *E. coli* (Penalver et al. 2005).

5.5.2 Antifungal Activity

Thyme oil at a concentration 1 μl/ml showed 92–100% inhibition of six pathogenic fungi, e.g. *Fusarium oxysporum*, *Penicillium brevicompactum*, and *Aspergillus fumigatus* (Zabka et al. 2009) and at 100 ppm 47–100% growth inhibition of 6 different fungi strains (Bourrel et al. 1995). The mycelial growth of *Aspergillus niger* and *Aspergillus flavus* was completely inhibited at the presence of thyme oil lower than 700 μg/ml (Paster et al. 1995; Viuda-Martos et al. 2007) and of A*spergillus parasiticus*, *A. ochraceus* and *Fusarium moniliforme* at 500 ppm (Soliman and Badeaa 2002). The oil applied against fungi colonising stored grain proved the possibility of using it as an alternative to chemicals in grain preservation (Paster et al. 1995; Soliman and Badeaa 2002). The oil effectively inhibited the growth of rye bread spoilage fungi both in agar test and in vapors at 250–270 μl/l (Suhr and Nielsen 2003). It controlled the growth of *Botrytis cinerea* and *Rhizopus stolonifer* and the decay of strawberries caused by these fungi (Reddy et al. 1998). Fungicidal effect of thyme oil against *B. cinerea* and *Mucor piriformis* in vapour phase was observed at significantly lower concentration (5 μl/l) than in liquid medium (600 μl/l) (Abdolahi et al. 2010).

Thyme oil exhibited high antifungal activity against some dermatophytes and *A. flavus* (MIC 156–625 ppm), as well as *Candida albicans* (MIC 1,250 ppm) (Amvam Zollo et al. 1998). Similar MIC toward *C. albicans* (0.12%) was established by Hammer et al. (1999) and lower one (0.31 μl/ml) by Donaldson et al. (2005). The fluconazole-resistant and fluconazole-susceptible strains of *Candida* species revealed similar susceptibility to thyme oil. *C. albicans, C. glabrata* and *C. crusei* were inhibited at MIC 0.32 mg/ml of thyme oil (Neves et al. 2009) while *C. albicans*, *C. dubliniensis*, *C. tropicali, C. krusei* and *C. glabrata* at MIC 400–3,200 μg/ml (Pozzatti et al. 2008). Thyme oil was the most active among 51 oils against and 4 yeast, e.g. *C. albicans* and *Torulopsis utilis* (Hili et al. 1997), as well as among 8 oils against plant pathogenic fungi with MIC 200–400 ppm (Giamperi et al. 2002).

Thyme red oil followed by clove oil showed the highest activity out of seven tested oils (e.g. fennel, sage, lavender) both by micro dilution method and in vapor contact against a total of 44 strains of environmental and clinically undesirable filamentous fungi, including *Microsporum canis*, *Epidermophyton floccosum*, *Aspergillus* sp. *Penicillium* sp. *Cladosporium cladosporioides* (Tullio et al. 2007).

5.6 Clove Oil and Cinnamon Leaf Oil

Clove oil and cinnamon leaf oil contain eugenol as the main constituent and are usually tested for their antimicrobial activity in the same investigations. Hence, they will be discussed together.

Clove oil is obtained from dried flower buds of *Syzygium aromaticum* (L.) Merill et LM Perry (*Eugenia caryophyllata* Thunb., Myrtaceae), yield between 15% and 20%. The oil is a clear yellow liquid which becomes brown when exposed to air, it has a characteristic clove-like aroma and burning, spicy flavor and is used in seasoning blends and in some natural flavors, especially banana, blackberry, cherry and smoke.

The major components of the oil are: eugenol (75.0–88.0%), eugenyl acetate (4.0–15.0%), β-caryophyllene (5.0–14.0%) (EP 5).

Cinnamon leaf oil is obtained from the leaves of Ceylon cinnamon tree *Cinnamomum zeylanicum* Blume (*C. verum* J. Presl, Lauraceae), yield of 1%. The oil is a reddish-brown to dark brown mobile liquid with spicy cinnamon, clove-like odor and taste. It is used as an alternative to clove oil in seasoning blends, and can be blended with cinnamaldehyde to approximate the character of cinnamon bark oil.

The major constituents of the oil are: eugenol (70.0–85.0%), β-caryophyllene (1.5–7.0%), linalol (1.5–3.5%), safrole (max. 3.0%), *trans*-cinnamic aldehyde (max. 3.0%), cinnamyl acetate (max. 2.0%), 1,8-cineole (max. 1.0%), coumarin (max. 1.0%) (EP 5) (Fig. 5.2).

5.6.1 Antibacterial Activity

Due to the similarity of essential oil composition, antimicrobial activities of clove oil and cinnamon leaf oil have been very often investigated parallel. Both oils acted strongly bacteriostatic at 0.03–0.05% and bactericidal at 0.04–0.1% concentrations toward *S. aureus*, *L. monocytogenes*, *S. enteridis*, *C. jejuni* and *E. coli* (Smith-Palmer et al. 1998), and with BA_{50} lower than 0.13% (Friedman et al. 2002). Although the same bacteria species were investigated in two research works with the exception of *S. aureus*, the susceptibility of *C. jejuni* to tested essential oils was the biggest according to Friedman et al. (2002) or the lowest one by Smith-Palmer et al. (1998). Higher MIC (0.25 ml/100 ml) and MBC (0.3 ml/100 ml) were established for clove oil against *E. coli* by Moreira et al. (2005). Clove and cinnamon oils in doses of 500 μg/ml caused 61–99% decrease in population growth of 3 bacteria (Hili et al. 1997). Cinnamon oil was slightly more effective (MIC 6.25–12.5 μg/ml) than clove oil (MIC 12.5 μg/ml for six strains and 50 μg/ml for *K. pneumoniae*) against seven major respiratory tract microorganism, e.g. *Haemophilus influenzae*, *Streptococcus agalactiae,* and *S. pyogenes* (Fabio et al. 2007). The effectiveness of both oils was similar in both solid diffusion and vapor diffusion test against four Gram-positive and four Gram-negative bacteria species. *Yersinia enterocolitica* was

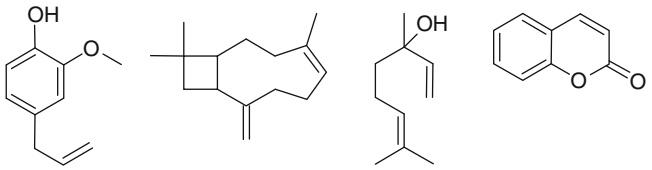


Fig. 5.2 Main components of clove oil and cinnamon leaf oil

the most susceptible and *P. aeruginosa* the least susceptible strain (Lopez et al. 2005; Goni et al. 2009).

Clove oil at the dilution 1/100 inhibited five of the six tested meat spoilage organisms (Ouattara et al. 1997), with MIC at 0.1% inhibited the growth of *P. putida* strain of meat (Oussalah et al. 2006), with MIC 500 μg/ml the growth of different *Aeromonas* isolates (Zaki et al. 2001), and with MIC 80–320 μg/ml the growth of a reference strain and five clinical strains of *Salmonella* Enteritidis (Rattanachaikunsopon and Phumkhachorn 2010). In disc diffusion test the oil was active against a large number of oral pathogenic bacteria and yeast (Kouidhi et al. 2010) and against 23 out of the 25 bacteria and all of 20 different isolates of *L. monocytogenes* in disc diffusion test (Deans et al. 1995).

5.6.2 Antifungal Activity

In comparative investigations with a number of essential oils, clove oil and cinnamon leaf oil always were on the top in activity, along with thyme oil, e.g.: among 51 oils against 3 bacteria and 4 yeast strains (Hili et al. 1997), among 20 oils against 45 bacteria and 8 yeast species (Biavati et al. 1997), among 45 oils against 7 bacteria and 3 fungi strains (Chao et al. 2000).

Clove oil and cinnamon leaf oil exhibited fungistatic and fungicidal activity against three postharvest pathogens of banana with inhibitory concentration 0.04–0.06% and lethal concentration (MLC) 0.06–0.11% (Ranasinghe et al. 2002) as well as were most active from among 49 essential oils tested against *B. cinerea* (Wilson et al. 1997). Clove and cinnamon oils in doses of 500 μg/ml caused significant decrease in population growth of 4 yeasts (59–100%) (Hili et al. 1997).

Clove oil exhibited fungistatic activity against four plant pathogens, e.g. *Aspergillus alternata* (MIC 0.05%), while above this concentration lysis of conidia and inhibition of mycelial growth were detected (Beg and Ahmad 2002). The oil is also effective in vapor phase against four fungal and four yeast species that are important food spoilage microorganisms (Matan et al. 2006). Mould and yeast strains isolated from ochnomycosis were inhibited by 2% of clove oil (Gayoso et al. 2005). The oil was active against 8 mould cellulolytic strains contaminated archive and museum reserves (Delespaul et al. 2000).

Cinnamon leaf oil showed antifungal activity (MIC 400 ppm) against *Stachybotrys chartarum* (Misra et al. 2000), and at 500 ppm completely inhibited spore production and germination as well as fungal colony development of five fungi such as *Cladosporium herbarum*, *R. stolonifer* and *B. cinerea* (Tzortzakis 2009). The oil is classified as the most effective against *Malassezia furfur* and *C. albicans* (Ferhout et al. 1999). The fluconazole-resistant and fluconazole-susceptible strains of *Candida* species (*C. albicans*, *C. dubliniensis*, *C. tropicali, C. krusei* and *C. glabrata*) were similarly susceptible to the oil with MIC 800–1,600 μg/ml (Pozzatti et al. 2008).

5.7 Cinnamon Bark Oil and Cassia Oil

Cinnamon bark oil and cassia oil have similar composition and will be presented together.

Cinnamon bark oil is obtained from the bark of Ceylon cinnamon tree *Cinnamomum zeylanicum* Blume (*C. verum* J.Presl, Lauraceae), yield of 0.5%. The oil is a light yellow liquid becoming reddish over time with spicy cinnamon odor and slightly bitter and pungent taste. It is used as an alternative to clove oil in seasoning blends, and can be blended with cinnamaldehyde to approximate the character of cinnamon bark oil. It is generally employed in cookery as a condiment and flavoring material.

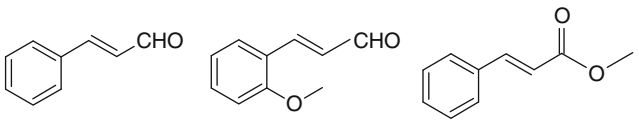
The main components of the oil are: *trans*-cinnamic aldehyde (55.0–75.0%), eugenol (max. 7.5%), linalol (1.0–6.0%), β-caryophyllene (1.0–4.0%), 1,8-cineole (max. 3.0%), benzyl benzoate (max. 1.0%), *trans*-2-methoxycinnamaldehyde (0.1–1.0%), coumarin (max. 0.5%), safrole (max. 0.5%) (EP 5). Coumarin and safrole are limited in food and beverages by EU.

Cassia oil is obtained from leaves, bark and young branches of *Cinnamomum cassia* Blume (Chinese cinnamon, a large tree, Lauraceae). The oil is a reddish-brown liquid and has odor reminiscent of cinnamon bark oil with the unique note of 2-methoxycinnamaldehyde which distinguishes cassia from cinnamon oil. Cassia oil is a major part of the traditional flavor of cola drinks. It is also used in confectionery and as ingredient in other natural flavors such as cherry, vanilla and some nut flavors.

The main components of the oil are: *trans*-cinnamaldehyde (70.0–90.0%), *trans*-2-methoxycinnamaldehyde (3.0–5.0%), cinnamyl acetate (1.0–6.0%), coumarin (1.5–4.0%), eugenol (max. 0.5%) (EP 5) (Fig. 5.3).

5.7.1 Antibacterial Activity

Antimicrobial activity of cinnamon bark oil was assessed many times while there are only a few reports on activity of cassia oil. The antibacterial activity of cinnamon bark oil was one of the highest among 45 essential oils (Chao et al. 2000) and higher



trans-cinnamaldehyde *trans*-2-methoxy-cinnamaldehyde cinnamyl acetate

Fig. 5.3 Main components of cinnamon bark oil and cassia oil

than that of 30 other oils investigated against *B. subtilis*, *S. typhi*, *S. pyogenes E. coli* and *P. aeruginosa* (Roengsumran et al. 1997). Low MIC values were established in this last research toward *P. aeruginosa* (1,250 mg/l) and higher toward *S. aureus* (2,500 mg/l) that were supported by activity of the main constituent of the oil – cinnamaldehyde, 625 mg/l for *P. aeruginosa* and 1,250 mg/l for *S. aureus*. The oil appeared to be strongly effective against 21 bacteria with *S. pneumoniae* and *Acinetobacter lwoffii* as the most sensitive at MIC <0.04 mg/ml and *S. aureus*, *S. pyogenes*, *Enterobacter aerogenes*, *B. cereus,* and five *Listeria* strains, as the most resistant at MIC 0.56 mg/ml (Unlu et al. 2010). In other research the oil showed similarly high activity with MIC at 20–25 μg/ml toward four Gram-positive (*B. subtilis*, *B. cereus*, *S. aureus*, *M. luteus*) and two Gram-negative bacteria (*K. pneumoniae* and *Serratia marcescens*) (El-Baroty et al. 2010). Cinnamon bark oil was the most active out of six oils against clinical isolates of six bacteria strains with MIC at 0.25 mg/ml (Sivamani and Sahul Hameed 2010). The oil was similarly active against six *S.* Enteritidis strains with MIC at 67–267 μg/ml (Rattanachaikunsopon and Phumkhachorn 2010) and less active against *E. coli* (MIC 4 μl/ml) and *P. aeruginosa* (11 μl/ml) (Pattnaik et al. 2010). High activity of the oil against five strains of respiratory tract pathogens (e.g. *S. pneumoniae*, *H. influenzae*) was also observed by gaseous contact at 1.56–6.25 mg/l air (Inouye et al. 2001).

Cassia oil and Ceylon cinnamon bark oil were equally effective in inhibiting the growth of various isolates of bacteria including Gram-positive and Gram-negative revealing MIC 75 μg/ml for *Vibrio parahaemolyticus*, MIC 150–300 μg/ml for *E. coli*, *P. vulgaris*, *P. aeruginosa*, *Vibrio cholerae*, and *S. typhymurium*, MIC 600 μg/ml for *S. aureus* and *E. aerogenes*, (Ooi et al. 2006) and MIC 0.03% for *C. jejuni* (Rossi et al. 2007).

Both cassia and cinnamon bark oil showed the highest activity out of 28 essential oils tested against four bacteria (*E. coli*, *S. typhimurium*, *L. monocytogenes* and *S. aureus*) being more effective than clove oil and cinnamon leaf oil with MIC 0.025–0.05% (Oussalah et al. 2007).

5.7.2 Antifungal Activity

Fungistatic and fungicidal activity of cinnamon bark oil against three postharvest pathogens of banana, e.g. *Fusarium proliferatum*, was higher than that of clove and cinnamon leaf oil, MIC 0.03–0.05%, MLC 0.04–0.8% (Ranasinghe et al. 2002).

Similar MIC values were established for three other plant pathogens, namely *Rhizopus nigricans* (0.64%), *A. niger* and *Penicillium expansum* (0.16%) (Xing et al. 2010). The oil inhibited the growth of *A. fumigatus* (MIC 25 μg/ml), *Trichophyton mentagrophytes* (MIC 12.5 μg/ml) and *C. albicans* (MIC 50 μg/ml). The effectiveness in gaseous contact was better than by solution contact (Inouye 2003; Inouye et al. 2003). The efficacy of cinnamon bark oil against two *C. albicans* strains was similar (MIC 0.07–0.12 mg/ml) and against *Candida parapsilosis* and *C. krusei* lower than 0.04 mg/ml (Unlu et al. 2010).

Both leaf and bark cinnamon oils were classified as the most effective against *Malassezia furfur* and *C. albicans* (Ferhout et al. 1999). These two oils were similarly efficient against six dermathophytes (e.g. *Trichophyton rubrum*, *T. mentagrophytes*, *Microsporum cannis* and *M. gypseum*) with MIC 0.08–0.16 μg/μl while bark oil revealed significantly higher effectiveness (MIC 0.08–0.16 μg/μl) than leaf oil (MIC 0.31–0.64 μg/μl) toward five strains of yeast (*C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata* and *Cryptococcus neoformans*) and *A. niger* (MIC 0.16 vs. 0.31 μg/μl) (Jantan et al. 2008). In the report comparing anti candidal and anti dermatophytic properties of essential oils form different parts of 11 cinnamon species Ceylon cinnamon leaf oil (MIC 0.55–5.91 μg/μl) and wood oil (MIC 1.51–6.04 μg/μl) were among the most active (Mastura et al. 1999). Both cinnamon oils were highly effective against 8 pathogenic fungus (4 *Aspergillus*, 2 *Fusarium* and 2 *Penicillium* spp.) isolated from food materials (Singh et al. 2007) and against *A. niger, F. oxysporum*, *Penicillium notatum* and *Mucora heimalis* (MIC 100 μg/ml) (El-Baroty et al. 2010).

Cassia oil effectively inhibited fungi including yeast (*C. albicans*, *C. tropicalis*, *C. glabrata* and *C. krusei*), MIC 100–450 μg/ml, filamentous molds (three *Aspergillus* spp. and one *Fusarium* sp.), MIC 75–150 μg/ml and three dermatophytes, MIC 18.8–37.5 μg/ml. (Ooi et al. 2006).

It is amazing that even in the latest reports about antimicrobial activity of cinnamon oil the authors were not clear as to whether the oil under investigation was leaf or bark oil. This is the case of strong activity of cinnamon oil against *E. coli*, MIC 300 ppm (Ceylan and Fung 2003), *Listeria monocytogenes* (Paparella et al. 2008) and three *Aspergillus* species (Carmo et al. 2008).

5.8 Peppermint Oil and Cornmint Oil

Peppermint oil is obtained from flowering tops and leaves of *Menta x piperita* L. (herbaceous perennial plant, Lamiaceae), yield of 0.3–0.7%. The oil is a colorless, pale yellow or pale greenish-yellow liquid. It has a characteristic mint odor and taste followed by the sensation of cold. It is used to give a peppermint flavor to a wide range of applications, at first as flavor additives in bubble gum and toothpaste. It is also used in mint and herbal blends and in liquor and sweets flavors.

Major components of peppermint oil are: menthol (30.0–55.0%), menthone (14.0–32.0%), methyl acetate (2.8–10.0%), isomenthone (1.5–10.0%), menthofuran

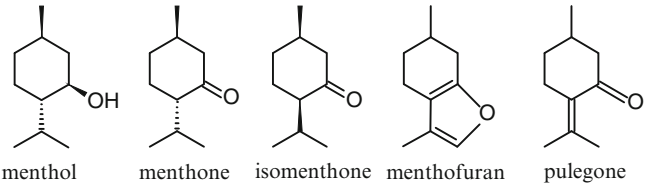


Fig. 5.4 Main components of peppermint oil and cornmint oil

(1.0–9.0%), pulegone (max. 4.0%), carvone (max. 1.0%), isopulegol (max. 0.2%) (EP 5).

Cornmint oil is obtained from flowering tops and leaves of *Mentha canadensis* L. (*M. arvensis* L., Japanese mint, Lamiaceae), yield of 0.5–2%. The content of menthol achieved 90% of the oil. Menthol is partly separated by crystallization and the remaining oil has appearance and odor resembling peppermint oil. The oil is used as a cheap alternative to peppermint oil, but easily recognized organoleptically because of its harsh flavor. Cornmint oil can be used in herbal blends and liquor flavors. Menthol obtained from the oil is mainly used in drug and cigarette production.

Major components of partly dementholized Cornmint oil are: menthol (30.0–50.0%), menthone (17.0–35.0%), isomenthone (5.0–13.0%), menthyl acetate (1.5–7.0%), limonene (1.5–7.0%), isopulegol (1.0–3.0%), pulegone (max. 2.5%), carvone (max. 2.0%), 1,8-cineole (max. 1.5%) (EP 5) (Fig. 5.4).

5.8.1 Antibacterial Activity

More than a half of mint oil production falls on peppermint oil and it is the most important because of its exceptional properties. Wide spectrum of therapeutic properties of this oil includes antibacterial and antifungal activities. Biological activity of mint oils is due to the content of their main constituent (1R, 3R, 4S)-(−)-menthol. Mint oils have shown high or middle activity against bacteria and fungi when compared with other essential oils. Peppermint oil was assessed more frequently than Japanese mint oil, that usually was tested as a raw but not dementholized oil.

Peppermint oil exhibited medium activity against *S. aureus*, *C. jejuni* and *L. monocytogenes* with MIC 0.03–0.1% being less active against *E. coli* and *S. enteridis* with MIC >1% (Smith-Palmer et al. 1998). Its BA_{50} was 0.3–0.7% against three tested bacteria and 0.07% against *C. jejuni* (Friedman et al. 2002). In research of activity of 20 essential oils action against 53 microbial strains peppermint oil was in the group of middle activity. From 45 bacteria the most susceptible to peppermint oil were seven *Clostridium* sp. and two *Lactococcus* sp. strains with MIC 400–600 ppm. The susceptibility of eight yeast species was similar. The activity of the oil against eight *Bacillus* sp. (MIC 400–1,800 ppm) and seven *Lactobacillus* sp. (MIC 1,400–2,000 ppm) was the lowest one (Biavati et al. 1997), being the same

against three bifidobacterias from dental caries (Crociani et al. 1997). Its MIC value against four *V. cholerae* strains varied from 0.27 to 0.80 μl/ml (Pattnaik et al. 1996) and against different *Aeromonas* isolates was of 1,250 μg/mL (Zaki et al. 2001). *E. coli* with MIC 5 μl/ml, and *S. aureus,* MIC 2.25 μl/ml appeared susceptible to peppermint oil while *P. aeruginosa* was resistant (MIC >20 μl/ml (Pattnaik et al. 2010). The same MIC value for *E. coli* as well as for *Staphylococcus epidermidis* (5.7 mg/ml) was reported by Schelz et al. (2006). The antibacterial study of four peppermint oils of different origin and composition has shown that for some out of 16 bacteria strains all oils were similarly active, e.g. against *K. pneumoniae* and *Yersinia enterocolitica* (both MIC 2.5 mg/ml) and for other strains quite different, e.g. *Pseudomonas syringae* (0.62–2.5 mg/ml) (Iscan et al. 2002). The oil inhibited also the growth of *S. aureus*, at the concentration <0.1% preventing from enterotoxin B formation (Tassou et al. 2000). Astonishingly low MIC values amounted to 1–3 μg/ml were determined for peppermint oil against 11 bacteria strains (e.g. *S. aureus*, *E. coli*, *B. subtilis*, *P. aeruginosa*) by Sokovic et al. (2010).

Parallel with the changes in peppermint oil composition depending on the planting time and mineral fertilisation, the oils shown different degree of inhibition: the oils from spring planted and fertilised crops were more active against some bacteria (Hussain et al. 2010). Essential oil of *M. piperita* exhibited a stronger antibacterial activity than other mint oils, in particularly against *E. coli* strains and the multiresistant strains of *Shigella sonnei* and *Micrococcus flavus* (Mimica-Dukic et al. 2003). Bacteriostatic effect of peppermint oil at 800 ppm was the most pronounced toward *E. coli* O157:H7 out of nine strains of Gram-negative and *Listeria innocua* out of six strains of Gram-positive food spoilage bacteria (Marino et al. 2001). In other research MIC of this oil against *E. coli* was established at 2.0 ml/100 ml (Moreira et al. 2005).

In parallel evaluation of both mint oils raw Japanese mint oil (80% of menthol) appeared to be significantly more effective than peppermint (28% of menthone and only 4% of menthol) against *S. aureus* (MIC 30 vs. 120 μg/ml) and *B. subtilis* (20 vs. 123 μg/ml) while their low effectiveness against *E. coli* was similar (ca. 300 μg/ml) (Hussain et al. 2010). However, in other investigation both oils revealed similar activity toward 13 bacteria species (Nevas et al. 2004). In the work comparing peppermint oil and dementholized cornmint oil (both ca. 40% of menthol) tiny differences were observed in antibacterial activity by disc diffusion method and pronounced ones by dilution method, especially in the case of *K. pneumoniae* with MIC 600 ppm for peppermint and 6 ppm for cornmint oil (Jirovetz et al. 2009). Both those essential oils inhibited the proliferation of *Helicobacter pylori*, *S. enteritidis*, *E. coli* and both methicillin-resistant and methicillin-sensitive *S. aureus* strains (Imai et al. 2001). Japanese mint oil showed microbicidal activity against seven bacteria strains (Thoppil et al. 2001).

5.8.2 Antifungal Activity

Peppermint oil was in the group of 13 oils with the highest activity from among 51 researched, at the concentration 500 μg/ml showing the growth reduction from 8%

for *Torulopsis utilis* to 94% for *Schizosaccharomyces pombe* (Hili et al. 1997). A pronounced activity against *A. niger* (Chao et al. 2000) and phytopathogenic fungi (Zambonelli et al. 1996) was observed. It inhibited also the growth of opportunistic *C. albicans* strain (Ezzat 2001). Mimica-Dukic et al. (2003) also reported good activity toward *C. albicans* (MIC 8 μl/ml) as well as toward *Trichophyton tonsurans* (MIC 4 μl/ml).

Japanese mint oil showed microbicidal activity against 8 fungi strains (Thoppil et al. 2001) and antifungal activity against toxic *A. flavus* (Varma and Dubey 2001) and two other moulds (Panday 2003). The oil at 1,000 ppm completely inhibited fungal growth of *A. ochraceus* and ochratoxin A production (Basilico and Basilico 1999) and was effective against 5 different human pathogens: MIC 1–3.90 μl/ml against four fungi and 62.5 μl/mL against *T. rubrum* (Rath et al. 2001).

5.9 Citrus Oils

The most valuable citrus peel oils are isolated by cold pressing of the fresh peel from the evergreen trees' fruit. However, citrus peel oils produced by hydrodistillation, as well as terpeneless citrus oils are also available on the market. These oils have less valuable quality but they are more stable and less sensitive to oxidation. The oils from leaves, twigs or flowers of different citrus trees were also produced. Three citrus peel oils have monographs in EP 5, lemon, mandarin and sweet orange oil.

Lemon (*Citrus limon* L.; Rutaceae). The essential oil is isolated by cold pressing of peels or peel pulp, yield of 0.4–4%. Lemon oil is a clear, pale yellow to greenish-yellow liquid that becomes cloudy at low temperatures. It has characteristic odor and taste of outer lemon peel part. Lemon oil is widely used in lemon and other natural flavors: pineapple, butterscotch and banana flavors, and can be mixed with other citrus oils like lime, orange and grapefruit.

Major components are: limonene (56.0–78.0%), β-pinene (7.0–17.0%), γ-terpinene (6.0–12.0%), sabinene (1.0–3.0%), geranial (0.5–2.3%), neral (0.3–1.5%), neryl acetate (0.2–0.9%), geranyl acetate (0.1–0.8%), α-terpineol (max. 0.6%), β-caryophyllene (0.5%) (EP 5).

Mandarin (*Citrus reticulata* Blanco; Rutaceae). The essential oil is isolated from rind of almost ripe fruits, yield of 0.5%. The oil is greenish or yellow to reddish orange liquid showing blue fluorescence. It is widely used alone or in conjunctions with orange oil in beverages, confectionery and in many natural flavors (mango, peach, apricot).

Major components are: limonene (65.0–75.0%), γ-terpinene (16.0–22.0%), α-pinene (1.6–3.0%), myrcene (1.5–2.0%), β-pinene (1.2–2.0%), p-cymene (max. 1.0%), methyl N-methylanthranilate (0.3–0.6%), sabinene (max. 0.3%) (EP 5).

Orange sweet (*Citrus sinensis* (L.) Osbeck, syn. *C. aurantium* var. *dulcis* L.; Rutaceae). The oil is pale yellow to orange, clear liquid that may become cloudy when chilled. It has a mild bitter, astringent flavor, yield of 0.3–0.5%. Orange oil is generally used in orange flavors and many other natural flavors.

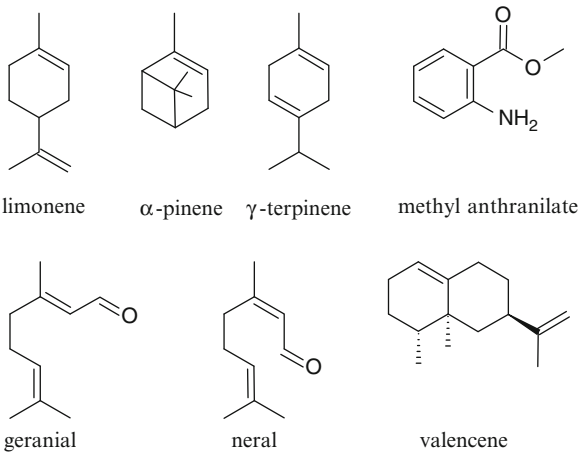


Fig. 5.5 Main components of citrus oils

Major components are: limonene (92.0–97.0%), myrcene (1.7–2.5%), sabinene (0.2–1.1%), linalol (0.2–0.7%), α-pinene (0.4–0.6%), valencene (0.02–0.5%), decanal (0.1–0.4%), octanal (0.1–0.4%), β-pinene (0.02–0.3%), geranial (0.03–0.2%), neral (0.02–0.1%) (EP 5) (Fig. 5.5).

5.9.1 Antibacterial Activity

The main constituent of all citrus peel oils is limonene – a monoterpene hydrocarbon. Antimicrobial activity of hydrocarbons is lower than that of oxygenated essential oil components. Despite of this fact, entirely good antibacterial and antifungal properties were observed for citrus oils mainly due to oxygenated monoterpenes. It is worth to mention that pressed citrus peel oils contain 1–15% non-volatile components.

The potential antimicrobial uses of citrus oils in food have been recently reviewed by Fisher and Philips (2008). In their previous work Fisher and Philips (2006) assessed antibacterial effect of lemon and sweet orange oil on the survival of five bacteria species. Both oils showed good activity against *L. monocytogenes* (MIC 0.25%) and weak activity against *C. jejuni*, *E. coli*, *S. aureus* and *B. cereus* (MIC 1–>4%). Lemon, mandarin and sweet orange oils exhibited high activity against *C. jejuni* (BA_{50} 0.009–0.044%), less against two *L. monocytogenes* strains (BA_{50} 0.0056–0.665%), and were merely active against *E. coli* and *S. aureus* (BA_{50} 0.41–0.67%) (Friedman et al. 2002). When tested against nine bacteria species (e.g. *E. faecalis*, *E. coli*, *K. pneumoniae*, *Serratia marcescens*) these three oils exhibited only weak antibacterial activity (MIC 2–>2%) with the exception of orange and lemon oil toward *Aeromonas sobria* (Hammer et al. 1999). Recently pronounced activity of mandarin oil was assessed against three Gram-positive bacterial strains (MIC 1–2 μl/ml) and lower one against Gram-negative bacteria such as

E. coli O157:H7, *S.* Enteritidis and *P. eruginosa* (MIC 5 µl/ml). Lemon oil and orange oil showed poor activity against Gram-negative bacteria while lemon oil revealed the highest activity against *Enterococcus faecium* and orange oil against *L. monocytogenes* (both MIC 0.5 µl/ml) (Espina et al. 2011).

Similar MIC 2.5 ml/100 ml for lemon oil against *E. coli* was reported by Moreira et al. (2005) and Ezzat (2001). According to Rossi et al. (2007) essential oils of *C. sinensis* and *Citrus reticulata* exhibited high activity against *C. jejuni* (MIC 0.125% and 0.25%, respectively), they were effective against *S. aureus* and showed weak activity against *E. coli*, *E. aerogenes* and *P. aeruginosa*. Orange oil at 300 ppm was partially sporicidal against *B. cereus* and *C. botulinum*, which was a little more resistant (Chaibi et al. 1997). It was only slightly active against 9 bacteria and 3 fungi (Chao et al. 2000). Lemon oil and sweet orange oil were among the most effective in the set of 21 essential oils against *P. vulgaris* (MIC 6.4 mg/ml) and 5 other bacteria (MIC 6.4–12.8 mg/ml) (Prabuseenivasan et al. 2006).

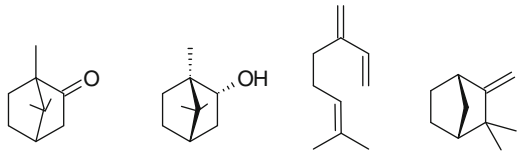
5.9.2 Antifungal Activity

The hydrodistilled fruit essential oils of lemon picked at three different period as well as 6 cultivars of sweet orange showed antifungal action on *Penicillium digitatum* and *Penicillium italicum*. Effective dose ED_{50} toward these two strains was 600–1,050 ppm and 1,400–2,500 ppm, respectively for lemon oil and 1,000–2,400 ppm and 3,200–5,400 ppm, respectively for orange oil (Caccioni et al. 1998). Orange oil was only slightly active against three fungi (Chao et al. 2000). Orange oil showed better activity than lemon and mandarin oils toward *C. albicans* (MIC 1%) (Hammer et al. 1999) and demonstrated fungistatic activity against *A. niger* at 1.5 µg/ml with 79% growth inhibition, at 750 ppm completely inhibited *A. parasiticus* and at 500 ppm aflatoxin B_1 production (Singh et al. 2010). However, orange oil had limited effect the growth of rye bread spoilage fungi, e.g. *Penicillium roqueforti*, *A. flavus* (Suhr and Nielsen 2003). Citrus oils at 1.6% reduce the level of *A. parasiticus* (Fisher and Philips 2008).

5.10 Rosemary Oil

Rosemary oil is obtained from flowering tops and leaves of *Rosmarinus officinalis* L. (evergreen shrub, Lamiaceae) with the yield of 0.5–2.5%. It is clear, mobile, colorless to pale yellow liquid with a characteristic odor. The main use of the oil is in seasoning blends. Two types of rosemary oil are available on the market, that differ mainly in percentages of main constituents, although several other chemotypes are known.

The main components of rosemary oil, Spanish type, are: α-pinene (18.0–26.0%), 1,8-cineole (16.0–25.0%), camphor (13.0–21.0%), camphene (8.0–12.0%), β-pinene



1,8-cineole camphor borneol myrcene camphene

Fig. 5.6 Main components of rosemary oil

(2.0–6.0%), limonene (2.5–5.0%), β-myrcene (1.5–5.0%), borneol (2.0–4.5%), α-terpineol (1.0–3.5%), verbenone (0.7–2.5%), bornyl acetate (0.5–2.5%), p-cymene (1.0–2.2%) (EP 5).

For rosemary oil, Moroccan and Tunisian type they are: 1,8-cineole (38.0–55.0%), camphor (5.0–15.0%), α-pinene (9.0–14.0%), β-pinene (4.0–9.0%), camphene (2.5–6.0%), borneol (1.5–5.0%), limonene (1.5–4.0%), α-terpineol (1.0–2.6%), p-cymene (0.8–2.5%), myrcene (1.0–2.0%), bornyl acetate (0.1–1.5%), verbenone (max. 0.4%) (EP 5).

It is very rarely indicated what type of rosemary oil was investigated for its antimicrobial activity and only sometimes the composition of the oil is given (Fig. 5.6).

5.10.1 Antibacterial Activity

Rosemary oil was among the most effective in the set of 21 essential oils against 6 bacteria, e.g. *B. subtilis*, *P. vulgaris*, *P. aeruginosa* and *K. pneumoniae* (MIC 6.4–12.8 mg/ml) (Prabuseenivasan et al. 2006) and in a set of other 21 oils against *S. aureus*, *L. monocytogenes* (MIC 0.04% and 0.02%, respectively) and *C. jejuni* (MIC 0.5%) (Smith-Palmer et al. 1998). According to Friedman et al. (2002) the oil showed higher activity toward *C. jejuni* (BA50 0.06%) and lower toward two *L. monocytogenes* strains (>0.6%).

The activity of rosemary oil toward four *E. coli* strains was quite good when compared with 10 oils tested, MIC 0.6 ml/100 ml (Moreira et al. 2005). According to other reports the oil was less effective against *E. coli* and *S. epidermidis* revealing MIC 11.3 mg/ml (Schelz et al. 2006) or 10–20 mg/ml (Celiktas et al. 2007) as well as against *P. putida* (MIC >0.8%) (Oussalah et al. 2007) and four other bacteria species (Celiktas et al. 2007). Rosemary oil had inhibitory effect on bacteria species used in food industry *Lactobacillus survatus*, *L. sakei*, *Staphylococcus xylosus*, *Staphylococcus carnosus*, and food spoilage bacteria *Enterobacter gergoviae* and *Enterobacter amnigenus* (Viuda-Martos et al. 2008).

Rosemary oil showed medium activity in the set of 13 oils against different strains of respiratory tract pathogens, e.g. *S. aureus*, *S. agalactiae*, *H. influenzae* (Fabio et al. 2007). However, its activity was lower (MID 50–100 mg/l air) than that of other 13 oils tested in vapor phase against respiratory tract pathogens (Inouye et al. 2001).

High activity of rosemary oil, Spanish type was established in diffusion test against 25 bacteria species (Baratta et al. 1998) and 29 bacteria strains (Mangena and Muyima 1999). Only tiny differences were found in activity of three oil samples of rosemary oil with different composition against five bacteria species (e.g. *E. coli*, *S. aureus*, *P. aeruginosa*) (Tommasi et al. 2009) and in activity of six oil samples (four with high 1,8-cineole content ca. 50% and two with similar 1,8-cineole and camphor content ca. 25%) toward three Gram-negative and five Gram-positive bacteria strains (Zaouali et al. 2010). In the latter research *P. aeruginosa* (MIC >10 µl/ml), *S. epidermidis* and *S. faecalis* (MIC 10 µl/ml) appeared to be resistant.

5.10.2 Antifungal Activity

Rosemary oil exhibited higher activity against yeast and moulds than bacteria (Luqman et al. 2007; Celiktas et al. 2007). It was in the group of 13 oils out of 51 tested demonstrating activity against four yeast species, e.g. *C. albicans*, *Saccharomyces cerevisiae* (Hili et al. 1997). MIC against two *S. cerevisiae* strains was 2.8–5.7 mg/ml (Schelz et al. 2006). Luqman et al. (2007) established MIC 2.75–5.5 mg/ml and MFC 5.5–11 mg/ml against ten drug-resistant mutants of *C. albicans* as well as MIC <2.75 mg/ml and MBC <5.5 mg/ml against dermatophytes. Hammer et al. (1999) reported similar MIC 1% while Angioni et al. (2004) considered rosemary oil as low active with MIC over 900 µg/ml against *C. albicans*.

From three rosemary oils the sample with composition corresponding to Moroccan and Tunisian type revealed significantly higher activity against *C. albicans* and *C. glabrata* then two others (Tommasi et al. 2009). High activity of rosemary oil, Spanish type was established in diffusion test against 12 yeast species (Mangena and Muyima 1999).

Rosemary oil was much more effective against rye bread spoilage fungi in the vapor than in agar medium. At 270 µl/l in air the oil totally inhibited the growth of *Endomyces fibuliger* and in 80% the growth of *Eurotium repens* being less active against other three fungi (Suhr and Nielsen 2003).

5.11 Conclusions

Essential oils are important natural products used for their flavor and fragrance in food, pharmaceutical and perfumery industries. The spectrum of biological and pharmacological activities of essential oils is exceptionally broad and has been extensively researched and reviewed. Their antimicrobial properties have been exploited intensively in recent years, mainly in respect to the extensive ban on antibiotics in the animal industries and antibiotic overuse in human medicine. These properties assessed by different *in vitro* methods are well documented and have been reported in this chapter for some essential oils selected for their importance as food additives.

However, it should be taken into account that essential oils are much more active in the *in vitro* conditions than in *in situ* (e.g. in food and cosmetic) or *in vivo* (in patients) model systems. The effective content of individual essential oil is usually too high to be acceptable for the application to food products because of the intensity of aroma. In the last decade, the assessments of antibacterial and antifungal activity of essential oils in product model systems have been more and more numerous. Such research revealed synergistic or at least additive effects in the mixtures of essential oils or essential oil with other food additives (Bassole et al. 2010; Tajkarimi et al. 2010). This suggests that such mixtures could be used in order to diminish the odor of each individual component and improve the preservative properties. Essential oils therefore will continue to be indispensable natural ingredients and they may provide alternatives to conventional antimicrobial additives in food.

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Chapter 6
Phytochemicals Against Drug-Resistant Microbes

Manuel Simões, Madalena Lemos, and Lúcia C. Simões

Abstract Bacteria are able to adapt to undesirable changes in nutrient availability, environmental conditions and presence of antimicrobial products, as well as to immunological defenses. Antibiotic resistant bacteria are increasingly prevalent and consequently new antimicrobials are needed to control these pathogens. Serious infections caused by bacteria that have become resistant to commonly used antibiotics have become a major global healthcare problem in the twenty-first century. Development of resistance, including multidrug resistance (MDR), is unavoidable because it represents a particular aspect of the general microbial evolution. Many bacterial diseases, which were thought to have been eradicated from developing countries, might once again become a serious health problem. There is thus an urgent need for products that act on novel molecular targets that circumvent resistance mechanisms. In this context, plant secondary metabolites (phytochemicals) have already demonstrated their potential as antibacterials when used alone, and as synergists/potentiators of less effective products. Moreover, phytochemicals can be used where bacterial resistance mechanisms, such as MDR, make conventional treatments ineffective and also in the control of biofilms. The aim of this chapter is to cover the recent advances on phytochemical antibacterial activities against drug-resistant bacteria.

Keywords Antimicrobial resistance • Antimicrobial mode of action • Biofilms • Phytochemicals • Structure-activity relationship

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6.1 Introduction

Since the 1970s, resistance to antimicrobials has become an escalating problem. There is a continuing effort in the pharmaceutical industry to develop new antimicrobial products for the treatment of resistant infections (Aksoy and Unal 2008). The challenge of developing effective antimicrobial strategies derives from the fact that bacteria are uniquely suited for survival in toxic environments. Bacteria express resistance mechanisms that are, in some cases, not specific to the antibacterial product to which they are exposed but are general mechanisms for minimizing the impact of adverse conditions. Moreover, the ability of bacteria to subsist on antibiotics and the potential to acquire resistance genes is a growing concern (Dantas et al. 2008). Natural products, mainly those from microbial origins, have provided the pharmaceutical industry with some of its most important sources of lead products in the search for new antimicrobials (Clardy and Walsh 2004). However, plants can also be effective sources of antimicrobials and have been used for centuries traditionally to inhibit microbial growth. Details of structures and sources of many antimicrobial phytochemicals have been widely compiled, and evidence for the functions of these products has also been reviewed (Simões et al. 2009a). In this chapter our focus will be on recent findings on the application of phytochemicals against antimicrobial resistant bacteria.

6.2 Conventional Antibiotics and the Problem of Microbial Resistance

Antimicrobial resistance is a complex process in which clinical, pharmacodynamic, pharmacokinetic and microbiological factors all play a part (Rodríguez et al. 2007). The use/misuse of antibiotics has led to an increasing prevalence of multidrug resistant (MDR) strains, and there is now an urgent need to develop new effective antibiotic agents (Cantrell et al. 2001). Dantas et al. (2008) demonstrated the resistance of diverse soil bacteria, including some closely related to clinically relevant pathogens. Those bacteria subsisted on antibiotics as their sole carbon source. The tested antibiotics included natural, semisynthetic, and synthetic products of different ages and from all major bacterial target classes (amikacin, carbenicillin, ciprofloxacin, chloramphenicol, dicloxacillin, d-cycloserine, gentamicin, kanamycin, levofloxacin, mafenide, nalidixic acid, penicillin G, sisomicin, sulfamethizole, sulfisoxazole, thiamphenicol, trimethoprim and vancomycin).

Some of the most clinical significant bacteria involved in drug-resistant infections include (Table 6.1): *Acinetobacter baumannii*, *P. aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* resistant to β-lactamases, along with methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Staphylococcus aureus* (VRSA), and *Mycobacterium tuberculosis*. (Alekshun and Levy 2007; Lee et al. 2008; Ojha et al. 2008; Weigel et al. 2007). Resistance mechanisms allow bacteria

Table 6.1 Antibiotics commonly used to treat problematic multidrug-resistant bacteria

Bacteria	Infection	Antibiotics
Acinetobacter baumannii	Lung, wound, bone, blood, indwelling devices, implants	Colistin, tigecycline
Extended-spectrum β-lactamase–producing *E. coli*	Blood, urinary tract, biliary, gastrointestinal, indwelling devices, implants	Colistin, tigecycline
Extended-spectrum β-lactamase–producing *Klebsiella pneumonia*	Lung, blood, indwelling devices, implants	Tigecycline
MRSA and VRSA	Skin and soft tissues, toxic shock syndrome, respiratory tract and blood, indwelling devices, implants	Trimethoprim, sulfamethoxazole, minocycline, quinupristin-dalfopristin, daptomycin, linezolid, tigecycline, vancomycin
Mycobacterium tuberculosis (extensively drug-resistant)	Lung	Drug combinations (streptomycin/isonicotinyl/hydrazine/rifampin/ethambutol/pyrazinamide/moxifloxacine/cycloserine/imipenem/co-amoxiclav/clofazimine/prochlorperazine/metronidazole), PA-824 and R207910
Vancomycin-resistant enterococci	Blood, cardiovascular, intra-abdominal, indwelling devices, implants	Quinupristin-dalfopristin, daptomycin, linezolid
MDR *S. pneumoniae*	Blood, ear, lung, cerebrospinal fluid	Fluoroquinolones, tigecyclines
P. aeruginosa	Lung, urinary tract, wound, indwelling devices, implants	Colistin, tobramycin

to survive in the presence of toxic conditions that can result from acquired or intrinsic cell changes. Bacteria may be intrinsically resistant to antimicrobial products, or may acquire resistance by *de novo* mutation or via the acquisition of resistance genes from other microorganisms (Fajardo et al. 2008). Acquisition of new genetic material by antimicrobial susceptible bacteria from those resistant counterparts may occur through gene transfer, by conjugation (via plasmids and conjugative transposons), transformation (via bacteriophages), or transduction (via incorporation into the chromosome of chromosomal DNA or plasmids) (Alekshun and Levy 2007; Hurdle et al. 2005; Tenover 2006). Once acquired, resistance genes are not easily lost. Instead, they become a relatively stable part of a genome. Additional resistance determinants may join those already prevailing, broadening the multidrug resistance phenotype. Acquired resistance genes may enable a bacterium to produce enzymes that inactivate the antibacterial product, to modify the target site, to produce an alternative metabolic pathway that bypasses the action of the antibacterial product, or to express efflux mechanisms that prevent the antibacterial from reaching its

intracellular target (Spratt 1994; Webber and Piddock 2003; Woodford and Ellington 2007). Efflux mechanisms, both drug-specific and multidrug, are important determinants of intrinsic and/or acquired resistance to these antimicrobials in important human pathogens (Lomovskaya et al. 2001). Efflux pumps are recognised as common membrane components in all cell types, from prokaryotes to complex eukaryotes, conferring a common and basic mechanism of resistance by extruding toxic molecules (van Bambeke et al. 2003). The MDR concept is used to describe a situation where insusceptibility to an antimicrobial is associated with insusceptibility to other chemically unrelated products through an efflux mechanism. Efflux pumps are widely involved in antibiotic resistance. Different pumps can efflux specifically an antimicrobial or class of antimicrobials, such as the NorA system that transports quinolones (Poole 2000), or TetA that transports tetracyclines (Levy 2002), or they can efflux a large variety of molecules, such as certain efflux pumps of *P. aeruginosa*, or MsrA efflux pumps specific for macrolides in *Staph. aureus* (Neyfakh et al. 1993).

Intrinsic resistance to antimicrobials is a natural property of bacteria. This is frequently associated with cellular impermeability imparted by the outer layers, limiting the uptake of antimicrobial products (Fajardo et al. 2008). The presence of efflux systems coupled with the narrow porin channels in the outer membrane which restricts diffusion of antimicrobials into the cells is responsible for the very high intrinsic resistance of Gram-negative bacteria (McDonnell and Russell 1999). In addition to the impaired uptake, some bacteria demonstrated intrinsic resistance through the inactivation and biodegradation of antimicrobial products by natural evolutionary mutations leading to modifications in proteins configuration (Dantas et al. 2008; Nishihara et al. 2000; Süssmuth et al. 1979).

Physiological adaptation of microorganisms induces the development of intrinsic resistance (Russell 2003). It is a natural tendency of microorganisms to attach to biotic or abiotic surfaces, to multiply and to embed themselves in a slimy matrix, resulting in biofilms. Biofilms are the leading example of physiological adaptation and are one of the most important sources of bacterial resistance to antimicrobials. It is now well recognised that bacteria embedded in biofilms behave quite differently from their planktonic counterparts (Fig. 6.1). In particular, microorganisms within biofilms are far more resistant to antimicrobial products (Davies 2003; Simões et al. 2009a). Nevertheless, there is no definitive answer to why and how bacteria, growing within a biofilm, develop increased resistance to antibacterials. In addition to the resistance mechanisms found in planktonic cells (gene transfer from resistant counterparts, efflux pumps, cellular impermeability imparted by the outer layers, enzymes that confer resistance and natural evolutionary mutations), there are six interesting hypothesized mechanisms (Spratt 1994; Alekshun and Levy 2007; Fajardo et al. 2008; Simões et al. 2009a):

1. Direct interactions between the biofilm extracellular polymeric matrix constituents and antimicrobials, affecting diffusion and availability. The extracellular polymeric substances (EPS) consist of various organic substances such as polysaccharides, proteins, nucleic acids and lipids (Sutherland 2001). The EPS matrix delays or prevents antimicrobial products from reaching target microorganisms

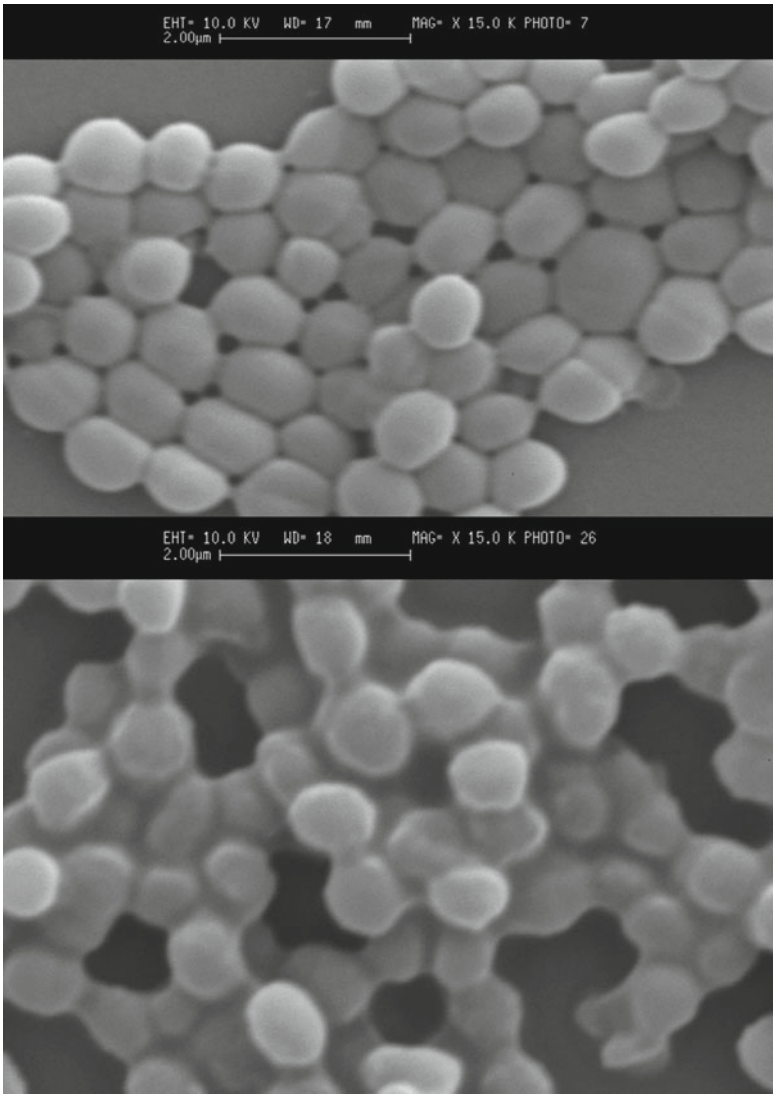

Fig. 6.1 Scanning electron microscopy photomicrographs of planktonic (**a**) and biofilm (**b**) cells of *Staphylococcus* spp. evidencing the presence of an extracellular polymeric matrix in biofilms (×15,000 magnification; bar = 2 μm)

within the biofilm by introducing diffusion limitation and/or chemical interaction with the EPS molecules (Davies 2003). Additionally, the cells present in the outer layers of the biofilm consume part or all of the reactant before it reaches the inner layers. As a consequence, the concentration of antimicrobial available for biofilm cells inactivation is reduced, particularly in the deeper zones, and thus,

the antimicrobial action is lower than in the planktonic tests. Moreover, the presence of sub-inhibitory concentrations will allow the appearance of (cross)-resistance scenarios within the biofilm population (Gilbert and McBain 2003). Another important EPS function is supposed to be their role as fundamental structural matrix elements determining the biofilm mechanical stability (Simões et al. 2009b). In addition to the potential of the biofilm matrix components to react directly and chemically quench reactive moieties, retention of enzymes with the capability to inactivate antimicrobial products within the biofilm matrix will amplify resistance (Heinzel 1998; Gilbert et al. 2002).

2. An altered chemical microenvironment within the biofilm, leading to areas of reduced or no growth (dormant cells) (Gilbert et al. 1990). When a bacterial cell culture becomes starved for a particular nutrient, it slows down its growth. Transition from exponential to slow or no growth is generally accompanied by an increase in resistance to antimicrobial products (Wentland et al. 1996; Lewis 2001). Because cells growing in biofilms are expected to experience nutrient limitation, it has been suggested that this physiological change can account for the resistance of biofilms (Mah and O'Toole 2001; Stewart and Franklin 2008). Oxygen gradients within the biofilm may also directly influence the activity of some antimicrobial products (Gilbert et al. 2002). Another phenomenon associated with biofilms is the existence of physiological gradients across biofilms. The peripheral cells will have growth rates and nutrient profiles that are similar to those of planktonic cells, allowing for the existence of physiological heterogeneity within the biofilm (Fux et al. 2005).
3. The development of biofilm/attachment-specific phenotypes. The physiological changes begin when cells attach to a surface, by expressing a biofilm phenotype that can confer resistance to stress conditions (Gilbert et al. 2002). This resistant phenotype might be induced by environmental stress, high cell density, efflux of the antimicrobials or a combination of these phenomena (Mah and O'Toole 2001). Bacteria can sense the proximity of a surface, up-regulate production of EPS and rapidly alter their susceptibility to antimicrobials after binding to a surface. In some instances, three to five fold decreases in susceptibility occurred immediately on attachment in the presence of antimicrobial products that exceeded the minimum inhibitory concentration (MIC) for planktonic cells (Fux et al. 2005). The magnitude of the decreases in susceptibility observed immediately after bacterial attachment is generally far less than that observed in mature biofilms and is insufficient to account for the reported levels of resistance in biofilm communities (Gilbert et al. 2002).
4. Some microorganisms in biofilms have been shown to express biofilm-specific antimicrobial resistance genes (Patel 2005).
5. Possibility of damaged bacterial cells undergoing apoptosis or programmed cell death. Following the absence of an adverse condition, the damaged cells would grow rapidly in the presence of nutrients released from their lysed community partners and the community would become restored. These cells would survive treatment phases and proliferate in the post-treatment phase, thereby stimulating considerable resistance upon the biofilm community (Lewis 2000).

6. Persister cells. It has been known for many years that small fractions of persistent bacteria resist killing when exposed to antimicrobials (Lewis 2001; Sufya et al. 2003). These persistent bacteria are not believed to be mutants. Rather it has been hypothesized that they are phenotypic variants and can exist in both planktonic and sessile populations. However, planktonic persisters are antimicrobial susceptible, while the biofilm persister cells are protected by the extracellular polymeric matrix (Davies 2003; Lewis 2007). The persistent cellular state is the newest explanation for biofilm insusceptibility to antimicrobial products (Lewis 2007).

Antimicrobial products have been the main weapons used to control unwanted biofilms. Although this strategy is widespread in biofilm control, there are no standardized antimicrobials with reliable efficacy. Strategies to remove unwanted biofilms must take into account the system characteristics, such as the biofilm colonizer species and the EPS composition (Simões et al. 2009b). It is expected that an effective and wide spectrum biofilm control strategy will overcome the resistance and cross-resistance problems (Gilbert and McBain 2003).

6.3 Plant Derived Antimicrobials

Natural products are typically secondary metabolites, produced by organisms in response to external stimuli (Strohl 2000). Natural products produced by plants, fungi, bacteria, insects and animals have been isolated as biologically active pharmacophores. Approximately one-third of the top-selling drugs in the world are natural products or their derivatives often with ethnopharmacological background. According to World Health Organization (2011), 70–95% of the world's population relies on traditional medicines for primary health care needs. Moreover, natural products are widely recognised in the pharmaceutical industry for their broad structural diversity as well as their wide range of pharmacological activities. The medicinal value of natural products lies in some chemical substances that produce a definite physiologic action on the human body. The interest in using plant secondary metabolites (phytochemicals) for treatment of microbial infections has increased in the late 1990s as conventional antibiotics become ineffective (Cowan 1999). However, only a small fraction of the known plant species of the whole world have been evaluated for the presence of antimicrobial compounds, and thus it is necessary to increase the efforts in collecting and screening plants for the development of novel and environmentally safe antimicrobials (Stein et al. 2005). Traditional plant phytomedicines, include crude vegetable drugs (herbs) as well as galenical preparations (extracts, fluids, tinctures, infusions) prepared from them. It has been estimated that less than 1–10% of the large diversity of plant species on Earth have been studied chemically and pharmacologically for their medicinal properties (Verpoorte 2000).

Plants produce an enormous array of phytochemicals and it is commonly accepted that a significant part of this chemical diversity is related to defence/stress mechanisms

including *in vitro* antimicrobial activity (Dixon 2001). This rich diversity of phytochemicals has partly arisen because of evolutionary selection for improved defence mechanisms against a broad array of microorganisms, insects, nematodes and even other plants (Dangl and Jones 2001). Plant "immune systems" effectively prevent infections caused by the majority of phytopathogens (Tierens et al. 2001).

The defence chemicals produced by plants are commonly classified either as phytoanticipins, which are molecules that are present constitutively in an inactive form (e.g. glucosides), or as phytoalexins, whose levels increase strongly in response to microbial invasion or are generated by *de novo* synthesis in response to a specific infection (Tegos et al. 2002). Phytoanticipins are low molecular weight products which are present in plants before the challenge by microorganisms or are produced from pre-existing constituents after microbial attack (VanEtten et al. 1994). These phytochemicals, such as glucosinolates, cyanogenic glucosides, and saponin glycosides, are normally stored as less toxic glycosides in the vacuoles or cell walls of plant cells. If the integrity of the cell is broken when penetrated by a microorganism or due to other damage, the glycoside comes into contact with hydrolyzing enzymes present in other compartments of the cell, releasing a toxic aglycone (Osbourn 1996).

Phytoalexins are low molecular weight products which are produced in response to elicitors such as microbial, herbivorous or environmental stimuli (Poulev et al. 2003). Once plants detect a pathogen signal, a complex mixture of secondary metabolites is produced to control the invader. These molecules are synthesized *de novo*, and thus involve the activation of certain genes and enzymes required for their synthesis (Kuć 1995). Phytoalexins are chemically diverse and may include many chemical classes such as simple phenylpropanoid derivatives, alkaloids, glycosteroids, flavonoids, isoflavonoids, various sulphur products, terpenes and polyketides (Hammerschmidt 1999). There is no boundary between phytoalexins and phytoanticipins, and in one plant species a certain chemical can function as a phytoalexin, whereas it has the function of a phytoanticipin in another species (Junghanns et al. 1998). It is important to point out that the distinction between phytoanticipins and phytoalexins is not based on their chemical structure but rather on how they are produced. Thus, the same chemical may serve as both phytoalexin and phytoanticipin, even in the same plant (VanEtten et al. 1994).

Phytochemicals with recognized antibacterial activity belong mainly to the following chemical structural classes: phenolics, terpenoids and other essential oils constituents, alkaloids, lectins and polypeptides, and polyacetylenes. The major subclasses are: simple phenols and phenolic acids, quinones, flavones, flavonoids and flavonols, tannins, coumarins, terpenoids and essential oils, alkaloids, lectins and polyketides, polyamines, isothiocyanates, sulfides, thiosulfinates, glycosides, phenanthrenes and stilbenes, among much others (Cowan 1999; Dorman and Deans 2000; Gibbons et al. 2004; Newman et al. 2000; Stavri et al. 2007). Each chemical class/subclass, besides their potential function against pathogen invaders, is believed to play other functions in plant physiology and functionality e.g. attraction pigments in flowers for pollinating insects, protection mechanisms against UV damage (flavonoids, anthocyanins, etc.) and oxidative stress (various simple and complex phenolics).

Phytochemicals have not been used as systemic antibiotics so far (Gibbons 2004; Lewis and Ausubel 2006). Although there are a significant number of phytochemical classes with antibacterial potential, they are not recognized by the medical community as therapeutic agents. In fact, the vast majority of phytochemicals have weak or narrow spectrum of activities (Tegos et al. 2002). Comparatively, molecules derived from microbial sources are often effective and have broad spectra of activity (Clardy and Walsh 2004; Clardy et al. 2006). Phytochemicals are routinely classified as antimicrobials on the basis of susceptibility tests that produce the MIC in the range of 100–1,000 μg/mL. Comparatively, typical antibiotics produced by bacteria and fungi produce MIC's of 0.01–10 μg/mL (Tegos et al. 2002). Moreover, there is missing the detailed structure-activity relationship (SAR) data for the majority of antimicrobial phytochemicals as has been done for many classes of microbial antibiotics. A major problem for the identification of new antibacterial products from plants is the variability in the extraction methods and antibacterial tests used. Cowan (1999) already proposed the advantage of standardizing extraction methods and *in vitro* tests to provide more systemic tests and, therefore, facilitate the interpretation of results and the development of reliable therapeutic antimicrobials. Moreover, and as was suggested by Gibbons (2004), there is the economical strategy. Pharmaceutical companies prefer to pursue antibacterials of microbial origin, of which there are many examples of highly effective products which can be readily generated leading to rapid economic rewards. The clear disadvantage is the rapid development of bacterial (cross)-resistance to many of these classes of microbial antibiotics.

6.3.1 Phytochemicals Antibacterial Mode of Action

A vast majority of phytochemicals are molecules with weak or narrow-spectrum activities, but can act on multiple biochemical targets. When current antibiotics aimed only at one target are used, the required high dosages for efficacy often produce bioavailability problems and unwanted side effects, and resistance problems may also emerge. Antibiotics act by: (i) inhibiting the synthesis of the bacterial cell wall; (ii) inhibition of protein synthesis; (iii) inhibition of DNA synthesis; (iv) inhibition of RNA synthesis; (v) competitive inhibition of folic acid biosynthesis; (vi) disorganizing membranes and other mechanisms (Madigan et al. 2000). Comparatively to the mode of action of antibiotics, phytochemicals can act on multiple biochemical targets of the bacterial cell. However, the exact mode of action and the reasons for phytochemical antibacterial specificity are not totally understood.

Essential oils and their constituents, such as terpenoids, carvacrol, thymol, occur widely in nature contributing to the characteristic plants flavours and aromas. Their mechanism of action against bacteria is not yet fully understood, but it is speculated to involve membrane disruption through lipophilic products (Griffin et al. 1999; Mendoza et al. 1997). This antibacterial action can result in membrane expansion, increase of membrane fluidity and permeability, disturbance of membrane embedded proteins, inhibition of respiration, and alteration of ion transport processes in

both Gram-positive and Gram-negative bacteria (Brehm-Stecher and Johnson 2003; Carson et al. 2002; Cox et al. 2000; Trombetta et al. 2005). Plant alkaloids, including berberine, found in *Berberis* species, and piperine, found in *Piper* species, can interact with the bacterial cytoplasmic membrane, intercalate with DNA, and inhibit efflux pumps in *Staph. aureus* (Jennings and Ridler 1983; Khan et al. 2006). Phenols and phenolic acids can cause the disruption of energy production due to enzyme inhibition by the oxidized products, through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Mason and Wasserman 1987). Phenolic extracts from *Origanum vulgare* and *Vaccinum macrocarpon* caused urease inhibition and the disruption of energy production by the inhibition of proline dehydrogenase at the plasma membrane of the Gram-negative human gastric pathogen *Helicobacter pylori* (Lin et al. 2005) Other polyphenols, such as flavonoids (robinetin, myricetin and epigallocatechin gallate) from *Elaegnus glabra*, can inhibit the synthesis of nucleic acids of both Gram-negative and Gram-positive bacteria (Cushnie and Lamb 2005; Mori et al. 1987). The authors suggested that the B ring of the flavonoids may play a role in intercalation or hydrogen bonding with the stacking of nucleic acid bases which may explain the inhibitory action on DNA and RNA synthesis. Quercetin, a component of propolis, binds to GyrB subunit of *E. coli* DNA gyrase and inhibits enzyme's ATPase activity (Plaper et al. 2003). This flavonoid was also reported to cause an increase in permeability of the inner bacterial membrane and a dissipation of the membrane potential (Mirzoeva et al. 1997). Epicatechin gallate and epigallocatechin gallate, two constituents of the major flavonoids found in green tea, inhibited antibiotic efflux pumps in MRSA (Gibbons et al. 2004). Epigallocatechin gallate is also a potent inhibitor of both the β-ketoacyl-ACP reductase (FabG) and the *trans*-2-enoyl-ACP reductase (FabI) components in the bacterial type II fatty-acid synthase system, a property that is common to a broad range of plant polyphenols (Zhang and Rock 2004). Glycoside saponins might induce pore-like structures which change the membrane permeability associated with alterations in the ionic homeostasis between intracellular and extracellular compartments (Melzig et al. 2001). They can also interfere with the energy metabolism through interaction with catabolic enzymes and the electron transport chain (Mandal et al. 2005; Sinha Babu et al. 1997). The diallyl thiosulfinate allicin, a phytochemical commonly obtained from *Allium sativum* (garlic), has potent antimicrobial activity and can interact with intracellular thiols and thiol containing proteins, inhibiting essential enzymes (for example, alcohol dehydrogenase, thioredoxin reductase and RNA polymerase) (Ankri and Mirelman 1999). Other authors also proposed that inhibition of RNA synthesis is the primary target of allicin action against *Salmonella* serovar Typhimurium (Feldberg et al. 1988). Plant peptides can act on bacterial cells by forming ion channels in the membrane and inhibiting adhesion of microbial proteins to host polysaccharide receptors (Suarez et al. 2005; Zhang and Lewis 1997). Peptides from *Moringa oleifera* caused membrane permeabilization and disruption of pathogenic Gram-negative and -positive bacteria including MRSA (Suarez et al. 2005).

Whilst the predictive site of action and some aspects of the mode of action of several phytochemicals have been studied, other factors such as the SAR are not

well understood (Griffin et al. 1999; Guz et al. 2001; Iwasa et al. 1998). Further research into the mechanisms by which phytochemicals cause inhibition of important cell functions and in some causes lysis, is required in order to understand and hence exploit any mechanisms and apply them efficiently in new therapeutic or biocontrol strategies. The more structurally complex libraries inspired by natural products including phytochemicals might be tremendous sources of new antibacterials. Phytochemicals often occur as a part of a family of related molecules so that it is possible to isolate a number of homologues and obtain SAR information. Lead compounds found from screening of natural products can be optimised by traditional medicinal chemistry or by application of combinatorial approaches. In a recent report, Kumar et al. (2008) evaluated a library of piperine-derived compounds and identified a class of compounds which were more potent than the parent molecule in potentiating the activity of ciprofloxacin through the inhibition of the NorA efflux pump in *Staph. aureus*.

6.3.2 Control of Resistant Bacteria with Phytochemicals

A significant example of phytochemicals antibacterial action against resistant bacteria is the essential oil-containing formulation, Polytoxinol™, which has been shown to be strongly bactericidal against a broad range of aerobic bacteria, including antibiotic resistant. Polytoxinol™ has been formulated to contain, in addition to constituents from *Eucalyptus* and *Melaleuca* species, components long recognised in traditional herbal medicine (Sherry et al. 2001). Berberine commonly found in *Hydrastis canadensis*, *Echinacea* species, and *Berberis* species is known to have antibacterial activity (Iwasa et al. 1998) and has shown good MDR inhibitor potential (Ball et al. 2006; Stermitz et al. 2000). Berberine from *Coptidis chinensis* rhizomes and from the *Phellodendri amurense* cortex was an efficient antibacterial against MRSA (Yu et al. 2005). Isoflavonoids isolated from several *Erythrina* species had antibacterial effects against MRSA (Sato et al. 2006; Tanaka et al. 2002) and vancomycin-resistant enterococci (VRE) (Sato et al. 2004). Cinnamaldehyde and eugenol have also been found to inhibit several different MDR Gram-negative and Gram-positive bacteria (Ali et al. 2005; Suresh et al. 1992). Allicin has a variety of antimicrobial activities. In its pure form, allicin was found to exhibit antibacterial activity against a wide range of Gram-negative and Gram-positive bacteria, including MDR enterotoxicogenic strains of *E. coli* (Ankri and Mirelman 1999). Chalcomoracin, a 2-arylbenzofurans isolated from *Morus* species, exhibited considerable antibacterial activity against MRSA (Fukai et al. 2005). The diterpene isopimaric acid, extracted from immature cones of *Pinus nigra*, was antimicrobial against MDR *Staph. aureus* and MRSA (Smith et al. 2005). Two abietane diterpenoids (11-hydroxy-12-oxo-7,9(11),13-abietatriene and 7α,11-dihydroxy-12-methoxy-8,11,13-abietatriene), isolated from the aerial material of *Plectranthus elegans*, inhibited the growth of Gram-positive bacteria (Dellar et al. 1996).

There is also a significant interest in the search for phytochemicals with the potential to inhibit bacterial efflux pumps. An effective efflux pump inhibitor could have significant benefits, including the restoration of antibiotic sensitivity in a resistant strain and the reduction in the effective dose of antibiotic, reducing adverse toxic effects (Kaatz 2005). The inhibition of efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN) decreased the level of intrinsic resistance significantly, reversed acquired resistance, and decreased the frequency of emergence of *P. aeruginosa* strains highly resistant to fluoroquinolones (Lomovskaya et al. 2001). Stavri et al. (2007) also proposed the use of an efflux pump inhibitor in combination with an antibiotic to delay the emergence of resistance to that antibiotic. An example is the plant alkaloid reserpine, which inhibits both TetK and NorA multidrug resistance mechanisms, involved in tetracycline and norfloxacin resistance in *Staph. aureus* (Gibbons and Udo 2000) but, unfortunately, it is cytotoxic at the concentrations required for this activity (Markham et al. 1999). Piperine, a major plant alkaloid present in *Piper nigum* and *P. longum*, in combination with the fluoroquinolone ciprofloxacin markedly reduced the MIC's and mutation concentration of ciprofloxacin for several *Staph. aureus* strains, including MRSA, by efflux pump inhibition (Khan et al. 2006). The phenolic diterpene totarol isolated from immature cones of *Chamaecyparis nootkatensis* demonstrated potential to inhibit the *Staph. aureus* NorA efflux pump (Smith et al. 2007). The activity of rhein, the principal antimicrobial from rhubarb, was potentiated 100- to 2,000-fold, depending on the bacterial species, by disabling multidrug resistance pumps. Comparable results were observed with the naphthoquinone plumbagin, the stillbene resveratrol, the polyphenolic aldehyde gossypol, the coumestan coumestrol and the alkaloid berberine (Tegos et al. 2002). *Dalea spinosa* (smoke tree) extracts potentiated antibiotic activity against MRSA related to the NorA pump of *Staph. aureus* (Belofsky et al. 2006). Tegos et al. (2008) demonstrated the NorA inhibition by the indole alkaloid reserpine, the flavonolignan 5′-methoxyhydnocarpin, and the polyacylated flavonol glycoside neohesperidoside. There is also a significant interest in the search for phytochemicals to restore antibiotic sensitivity in a resistant strain and the reduction in the effective dose of antibiotic, reducing adverse cytotoxic effects (Kaatz 2005; Markham et al. 1999; Sudano Roccaro et al. 2004).

The synergistic/potentiating effects of phytochemicals and antibiotics have been demonstrated for several Gram-negative and -positive pathogens. Yu et al. (2005) found an interesting potentiating effect between berberine and ampicillin, and a synergistic effect of berberine and oxacillin against MRSA, suggesting that this phytochemical may have potential to restore the effectiveness of β-lactam antibiotics against MRSA. Similar findings were reported with *Curcuma longa* ethyl acetate extracts (Kim et al. 2005). Epicatechin gallate and epigallocatechin gallate potentiated the antibacterial activity of β-lactam antibiotics against MDR strains of *Staph. aureus* (Hu et al. 2002; Zhao et al. 2001, 2002). Isoflavonoids from several *Erythrina* species were reported to act either synergistically or additively with vancomycin against MRSA and VRE (Sato et al. 2004, 2006; Tanaka et al. 2002, 2004). Isoflavones isolated from *Lupinus argenteus* were found to potentiate the antibacterial activity of α-linolenic acid, a phytochemical found in the same plant (Morel

et al. 2003). These isoflavones also enhanced the antibacterial activity of berberine and the synthetic fluoroquinolone antibiotic norfloxacin; they also increased the uptake of berberine into *Staph. aureus* (Morel et al. 2003). The additive effect of two phytochemicals was also reported by Stermitz et al. (2000). The presence of 5′-methoxyhydnocarpin-D or pheophorbide A, two phytochemicals from *Berberis* species, potentiated the antibacterial action of berberine against resistant *Staph. aureus*. Cinnamaldehyde from *Cinnamomum zeylanicum* bark, essential oil reduced clindamycin resistance of the Gram-positive *Clostridium difficile* (Shahverdi et al. 2007). Kubo et al. (1996) reported the potentiating effects of polymyxins by indole and (E)-2-hexenal, two plant metabolites found in cashew apple and green tea flavour respectively, against the Gram-negative pathogens *P. aeruginosa* and *E. coli*.

It is interesting to note that phytochemicals that have different antibacterial modes of action can potentiate the activity of the same antibiotic class. For instance, berberine (interact with the cytoplasmic membrane and with DNA) (Jennings and Ridler 1983) and epicatechin and epigallocatechin gallates (inhibit efflux activity and bacterial type II fatty acid synthesis) (Gibbons et al. 2004; Zhang and Rock 2004) have distinct antibacterial mode of action, however, they potentiate the antibacterial action of β-lactam antibiotics (Zhao et al. 2001, 2002; Hu et al. 2002; Yu et al. 2005). Other phytochemicals (piperine, reserpine, and triterpenoid saponins) sensitize bacteria through different mechanisms (Aeschlimann et al. 1999; Khan et al. 2006; Melzig et al. 2001; Schmitz et al. 1998; Trombetta et al. 2002) and potentiate the action of other antibiotic classes (quinolones and polymyxins) (Gibbons and Udo 2000; Kubo et al. 1996; Lomovskaya et al. 2001). Moreover, it is conceivable that phytochemicals with other mechanisms of action, such as those with membrane permeability effects, may potentiate the antibacterial activity of antibiotics that target intracellular sites (aminoglycosides, macrolides, quinolones, tetracyclines). In fact, this is an interesting chemotherapeutic strategy, where phytochemicals can sensitize bacteria and modulate their susceptibility to antibiotics at reduced concentrations.

Plants can support populations of surface-attached bacteria and produce phytochemicals that attenuate biofilm development through specific mechanisms (Morris and Monier 2003). Many plant species produce molecules that mimic AHL signals and affect quorum-sensing (cell-cell signalling events) in bacteria (Adonizio et al. 2006, 2008; Vattem et al. 2007). Successful quorum-sensing (QS) inhibition was found with the use of QS quenching molecules from *Medicago truncatula*, a plant widely adopted as a system for molecular analysis of plant-microbe interactions (Gao et al. 2003). Hamamelitannin from *Hamamelis virginiana* inhibited QS of methicilin-resistant *Staphylococcus* species (Kiran et al. 2008). Other plants from traditional medicinal use and from the human diet, including garlic (*Allium sativum*) extracts, also demonstrated the potential to inhibit QS events (Adonizio et al. 2008; Bjarnsholt et al. 2005; Girennavar et al. 2008; Vattem et al. 2007). Halogenated furanones produced by the macroalga *Delisea pulchra* inhibit AHL-dependent gene expression (Manefield et al. 2002). Those furanone compounds demonstrated the potential to specifically interfere with several AHL-regulated bacterial processes without any effect on bacterial growth or general protein synthesis capability

(Givskov et al. 1996; Manefield et al. 2000). The secondary lichen metabolite (+)-usnic acid (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzofurandione) is able to inhibit *P. aeruginosa* and *S. aureus* biofilm formation on polymer surfaces (Francolini et al. 2004). The mechanism of action expressed by (+)-usnic acid is still unknown. However, it is known that it inhibits RNA transcription and may influence QS in *P. aeruginosa* (Campanella et al. 2002; Francolini et al. 2004).

QS inhibition is only one of the possible mode of action of phytochemicals against bacterial biofilms. There are other studies indicating that phytochemicals can inhibit interspecies coaggregation (Weiss et al. 1998), prevent bacterial adhesion (Kuźma et al. 2007; Rukayadi and Hwang 2006), and inactivate mature single and multi-species biofilms (Knowles et al. 2005; Lebert et al. 2007; Niu and Gilbert 2004). The surface coating with the sesquiterpenoid xanthorrhizol prevented biofilm formation by *Streptococcus mutans* (Rukayadi and Hwang 2006). The diterpenoid salvipisone also demonstrated a potential anti-biofilm activity against antibiotic resistant *Staphylococcus* species (Kuźma et al. 2007). The monoterpene carvacrol demonstrated the ability to inactivate dual species biofilms formed by *S. aureus* and *Salmonella enterica* serovar Thyphimurium (Knowles et al. 2005). Epigallocatechin gallate, the main polyphenol component of green tea, has several antibacterial properties, including the ability to decrease polysaccharide production by *Staphylococcus* spp (Blanco et al. 2005). The monoterpene phenol thymol and the monoterpenoid phenol carvacrol, two components of the essential oils, demonstrated the ability to inactivate staphylococcal biofilms (Nostro et al. 2007, 2009).

6.4 Conclusions and Future Perspectives

The emergence of antibacterial resistance has motivated the exploration of new antibacterial products that target nonessential cell processes, reducing the possibilities of bacteria to develop resistance. Phytochemicals may act through different mechanisms from that of conventional antibiotics, and could, therefore, be of clinical value in the treatment of resistant bacteria. Preliminary investigations suggest that the use of antibacterial phytochemicals is a highly attractive practice, particularly with respect to the emergence of MDR bacteria in both planktonic and biofilm states. The perspective of their potentials in combination with other antibacterial products provides another attractive application of phytochemicals and should form a subject of further extensive study (Kumar et al. 2008). Moreover, besides the phytochemicals potential practical utility as antimicrobials and resistance modifying agents, this knowledge of phytochemicals chemical diversity and functionality provides new concepts with application to combinatorial synthesis and computational design of new drugs. There are some examples on the successful application of these concepts on the development of antibacterial strategies with potential therapeutic application (Pemberton et al. 2007; Cegelski et al. 2009). The synthesis of structurally analogous products, based on the novel scaffolds from phytochemical molecules, with increased efficiency and decreased cytotoxicity is a current practical application of phytochemicals discovery.

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Chapter 7
Phytochemicals as Anti-microbial Food Preservatives

Mehrdad Tajkarimi and Salam A. Ibrahim

Abstract Phytochemicals containing essential oils (EOs) in the range of 0.05–0.1% have demonstrated inhibitory activity against pathogens, such as *Salmonella* Typhimurium, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*, in food systems. Three major limitations for the broad application of phytochemicals in food are: limited data about their effects in food, strong odor, and high cost. New techniques and synergistic effect of compounds have been successfully applied in several food and *in-vitro* experiments. Several in-food and *in-vitro* applications of essential oils, phenolic and other components are discussed in this chapter.

Keywords Phytochemicals • Food preservatives • Food spoilage bacteria • Essential oils • Phenolic compounds

7.1 Introduction

Increasing occurrence of food-borne disease outbreaks and growing interest in consumer demand for safe, fresh, ready to eat and high-quality foods, raises considerable challenges (Chana-Thaworn et al. 2011; Tajkarimi et al. 2010). Application of chemical preservatives, synthetic antimicrobials and several processing techniques are commonly used to inactivate or inhibit the growth of spoilage and pathogenic microorganisms. However, they have not been considered as a comprehensive control method (Tajkarimi et al. 2010; Chana-Thaworn et al. 2011; Xing et al. 2010). As a consequence, naturally derived compounds such as plant extracts in food are receiving a good deal of attention as control agents for microorganisms.

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The potential use of plant extract as a natural antimicrobial agent in food preservation forms the basis for many applications (von Staszewski et al. 2011; Chana-Thaworn et al. 2011; Zouari et al. 2010; Thembo et al. 2010). Antimicrobials of plant origin are generally secondary metabolites in plants, that could act separately or jointly against food borne pathogens, in addition to contributing to the taste and flavor (Sotelo et al. 2010; Park et al. 2010). Strong odor and flavor of EOs such as thiosulfinates is a major issue, however, there are promising reports about the application of some EO constituents such as eugenol (*Eugenia caryophylata*), a major component of clove oil, against pathogens in fresh-cut apple without flavor change (Teng et al. 2010). Antimicrobials control and prevent natural spoilage processes (food preservation) and growth of microorganisms, including pathogens (food safety). Phytochemicals and plant origin materials such as spices and herbs have antimicrobial effects on plant and human pathogens in addition to their flavoring effects (Tajkarimi et al. 2010; Romeo et al. 2010).

There are new techniques such as pulsed light, high pressure pulsed electric, magnetic fields, incorporation of natural antimicrobials into packaging materials, micro emulsion, micro- and nanoencapsulation that could provide protection conditions to deliver bioactive compounds into food systems for food preservation and controlling pathogens and spoilage microorganisms in food (Nori et al. 2011; Tajkarimi et al. 2010; Zhang et al. 2010a; Khanzadi et al. 2010). For example, *Gelidium corneum* (GC), a type of agarose-containing red algae as an edible film packaging material, has successfully demonstrated antimicrobial activity by the addition of grape seed extract or thymol against *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Lim et al. 2010a, 2010b; Bisha et al. 2010). Grape skin extracts of Riesling *Vitis vinifera* L. grapes showed strong preservative effects against Gram-positive foodborne pathogens such as *Staphylococcus aureus*, *Enterococcus faecalis* and *Enterococcus faecium* (Corrales et al. 2010). Table 7.1 illustrates recent reports on the application of phytochemicals against Gram positive bacteria. However, some of the new technologies are not sufficiently effective for eliminating pathogens or delaying microbial spoilage. A growing body of data indicates considerable potential for the utilization of EOs derived from spices and herbs against *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* O157:H7, *Shigella dysenteria*, *Bacillus cereus* and *Staphylococcus aureus* at levels ranging between 0.2 and 10 μl ml^{-1}. There are a number of examples indicating more than 5 log10 CFU reduction in pathogenic microorganisms, using a combination of phytochemicals with common processing techniques (Tajkarimi et al. 2010). Phytochemicals could also improve the storage stability by means of active components including phenols, alcohols, thiosulfinates, aldehydes, ketones, ethers and hydrocarbons, especially in spices such as cinnamon, clove, garlic, mustard, and onion (Santas et al. 2010).

The purpose of this chapter is to provide an overview of some phytochemicals that have been reported to be effective against spoilage or pathogenic microorganisms, and practical methods used for screening these compounds.

Table 7.1 Inhibitory activities of plant-origin antimicrobials against gram positive pathogenic bacteria, listed alphabetically representative studies conducted within the last 10 years

Organism	Adverse effects	Inhibitors	References
Bacillus cereus	Food poisoning; emesis	Tea, eugenol, leaf essential oil, bark essential oil; bark oleoresin, E-cinnamaldehyde, clove, mustard, cinnamon, guarana extract, EOs of *Syzygium gardneri* leaves, supercritical fluid extract of the shiitake mushroom Lentinula edodes, hydro-distilled fresh leaves of *Pittosporum neelgherrense* Wight et Arn, Melianthaceae Bersama engleriana, leaves, bark and fruits of Neolitsea fischeri , EOs of the flower heads and leaves of *Santolina rosmarinifolia* L. thymol and carvacrol thyme, cinnamic aldehyde and eugenol extracted from cinnamon and clove, aerial parts of Ammoides atlantica, leaves, bark and fruits of Neolitsea fischeri, hydro distillation of Syzygium gardneri leaves and dried and oil samples of Thymus caramanicus EOs from Anzer teas and wild-grown leaves of Acorus calamus, aerial parts of fresh Plectranthus cylindraceus oil, EOs of Actinidia macrosperma, fruits of Eucalyptus globules, oil-macerated garlic, Petiveria alliacea L. root extract, Aristolochia indica L., tannins, dried and commercial garlic products, garlic oil, marjoram and basil essential oils, citral, linalool and bergamot vapor, methanol and acetone extracts of 14 plants belonging to different families, ethanolic extract from Rhodomyrtus tomentosa (Ait.) Hassk. Leaves, Salvia pisidica Boiss. and Heldr. ex Bentham (Lamiaceae) essential oil; relative inhibitory effects on Bacillus cereus: clove>mustard>cinnamon>garlic>ginger>mint. Hydro distilled extract of *Bunium persicum, Cuminum cyminum and Carum copticum*. EOs and methanol extracts of sweet basil *Ocimum basilicum* L. (Lamiaceae)	Tajkarimi et al. (2010), Ozkan et al. (2010), Oroojalian et al. (2010), and Hossain et al. (2010)

(continued)

Table 7.1 (continued)

Organism	Adverse effects	Inhibitors	References
Bacillus subtilis	Food poisoning	Teas, leaf essential oil, leaf oleoresin, eugenol, bark essential oil; bark oleoresin, E-cinnamaldehyde, oil-macerated garlic extract, tannins, polymers of flavanols, cassia bark-derived substances, crude extracts of bulbs (Lycoris chinensis), stems and leaves of (Nandina domestica), (Mahonia fortunei), (Mahonia bealei), stems of Berberis thunbergii and stems, leaves and fruits of Camptotheca acuminata, methanol and acetone extracts of 14 plants belonging to different families, *Eruca sativa* (aerial and root). EOs and methanol extracts of sweet basil *Ocimum basilicum* L. (Lamiaceae). Ponkan (*Citrus reticulata* Blanco). Seeds of *Zizyphus jujube.*	Tajkarimi et al. (2010), Khoobchandani et al. (2010), Hossain et al. (2010), Gao et al. (2010), and Al-Reza et al. (2010)
Clostridium spp.	Food poisoning; diarrhea	Clove EOs, *Zataria multiflora*	Tajkarimi et al. (2010), and Khanzadi et al. (2010)
Listeria monocyto-genes	Food poisoning; listeriosis	Tea, pure essential oils, oregano in whey protein isolate (WPI) films containing garlic essential oil, cabbage juice, cinnamon bark, cinnamon leaf, and clove, Brassica oleracea juice, lemon balm and sage essential oils, cassia bark-derived substances, citral, linalool and bergamot vapor, *Rosmarinus officinalis* oil, Thymol and carvacrol EOs, borneol extracted from sage and rosemary. Hydro distilled extract of *Bunium persicum, Cuminum cyminum and Carum copticum.* Asari Radix, the roots of *Asarum heterotropoides* F. Maekawa var. manshuricum F. Maekawa. *Alpinia galanga* (Linn.) Swartz. EOs and methanol extracts of sweet basil *Ocimum basilicum* L. (Lamiaceae). Raw state of brown Irish edible seaweeds, *Himanthalia elongata, Laminaria sachharina* and *Laminaria digitata*(100% inhibition). ethanol extracts of *Artemisia Herba Alba, Lavandula officinalis* L., *Matricaria Chamomilla, Eugenia caryophylata, Cistus salvifolius, Mentha suaveolens subsp. Timija, Thymus serpyllum* L., *Lippia citriodora, Cinnamomum Zeylanicum, Rosa centifolia, Thymus vulgaris* L, *Rosmarinus officinalis and Pelargonium graveolens.* Seeds of *Zizyphus jujube.*	Tajkarimi et al. (2010), Oroojalian et al. (2010), Oh et al. (2010), Hsu et al. (2010), Hossain et al. (2010), Gupta et al. (2010), Castano et al. (2010), Bayoub et al. (2010), and Al-Reza et al. (2010)

Mycobac-terium tuberculosis	Tuberculosis	Catechins, Bersama engleriana (Melianthaceae),	Tajkarimi et al. (2010)
Spore forming bacteria	Food poisoning	Catechins	Tajkarimi et al. (2010)
Staphylo-coccus aureus	Food poisoning; infection	Theasinensin, tea, cinnamon, oregano (Origanum vulgare), pure essential oils, leaf essential oil, leaf oleoresin, eugenol, bark essential oil, bark oleoresin, E-cinnamaldehyde, [oregano in whey protein isolate (WPI) films containing at 2% level, garlic essential oil at 3% and 4%], dried garlic powder, commercial garlic products, clove, mustard, rosemary (Rosmarinus officinalis), lemon balm (Melissa officinalis), sage (Salvia officinalis), chocolate mint (Mentha piperata), and oregano (Origanum vulgare), Bersama engleriana (Melianthaceae), chrysanthemum extracts, oil-macerated garlic extract, Petiveria alliacea L. root extract, Aristolochia indica L., tannins, polymers of flavanols, lemongrass and bay; certain combinations of carvacrol- thymol, citral, linalool and vapour, methanol and acetone extracts of 14 plants belonging to different families. Hydro distilled extract of *Bunium persicum, Cuminum cyminum and Carum copticum.* Methanol extract of *Salvia leriifolia* leaf. Steam distilled Herba Moslae. Hydro distilled extract of *Bunium persicum, Cuminum cyminum and Carum copticum.* Lantana camara Linn. (Verbenaceae), Ageratum houstonianum Mill. (Asteraceae) and Eupatorium adenophorum Spreng. (Asteraceae), *Eruca sativa* (aerial and root). *Alpinia galanga* (Linn.) Swartz. EOs from peel of Ponkan (*Citrus reticulata* Blanco). Seeds of *Zizyphus jujube. Ziziphus mauritiana* L. and *Ziziphus spinachristi* L.	Tajkarimi et al. (2010), Oroojalian et al. (2010), Mehr et al. (2010), Li et al. (2010), Kurade et al. (2010), Khoobchandani et al. (2010), Hsu et al. (2010), Gao et al. (2010), Castano et al. (2010), Al-Reza et al. (2010), and Abalaka et al. (2010)

7.2 Historical Overview of Plant Antimicrobials as a Food Preservative

Natural antimicrobial agents derived from plant oils have been recognized and used for centuries by the early Egyptians and Asian countries such as China and India. Spices such as clove, cinnamon, mustard, garlic, ginger and mint are still applied as an alternative health remedy in India. Processing and production of EO's can be traced back over 2,000 years to the Far East, with the beginnings of more modern technology occurring in Arabia in the ninth century. Spices have eastern origin and some of them such as chili peppers, sweet peppers, allspice, annatto, chocolate, epazote, sassafras, and vanilla have been recognized after discovery of the New World. In 1880s, the first scientific report about the potential of spices as preservatives was published. In the 1910s, the preservative effect of cinnamon and mustard in applesauce was reported. Other spices, such as allspice, bay leaf, caraway, coriander, cumin, oregano, rosemary, sage and thyme, have been reported by many researchers to have significant bacteriostatic properties since then (Tajkarimi et al. 2010). Hemp (Cannabis sativa L.), a therapeutic phytochemical, has been used in Asia 5,000 years ago (Nissen et al. 2010)

7.3 Major Plant Antimicrobials

Phytochemicals are used as natural components for extending the shelf life of foods, reducing or eliminating pathogenic bacteria, and increasing the overall quality of food products. Herbs, spices and fruits can be divided into subgroups based on their chemical structures. The most important groups include phyto-phenolics in herbs and spices, flavonoids and acids in fruits and berries, and glucosinolates in cruciferous vegetables, mustard, cabbage, and horseradish (Schirmer and Langsrud 2010). There are various parts of the plant are used to obtain phytochemicals including flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots. Phytochemicals, generally are mixtures of several components; some phytochemicals such as components in oregano, clove, cinnamon, citral, garlic, coriander, rosemary, parsley, lemongrass, sage, vanillin and lichens exhibit antimicrobial activity (Shukla et al. 2010; Tajkarimi et al. 2010; Schirmer and Langsrud 2010). The essential oils extracted from some of the above-stated spices contain chemical compounds such as carvacrol, cinnamaldehyde, eugenol and camphor, that play a major role as antimicrobial compounds (Xing et al. 2010; Tajkarimi et al. 2010; Weerakkody et al. 2010; Qiu et al. 2010; Mihajilov-Krstev et al. 2010). Other spices, such as ginger, black pepper, red pepper, chili powder, cumin oil and curry powder, also have antimicrobial properties (Hajlaoui et al. 2010; Holley and Patel 2005). Zingiberaceous plants, galangal (*Alpinia galanga*), turmeric (*Curcuma longa*), and finger root (*Boesenbergia pandurata*) extracts have been found to be effective against Gram-positive and Gram-negative pathogenic bacteria at 0.2–0.4% (v/v) for

finger root and 8–10% (v/v) for all of the other spices (Chen et al. 2008; Tajkarimi et al. 2010). Table 7.2 shows the use of phytochemicals against gram negative bacteria.

It has been well demonstrated that more than 1,340 plants with defined antimicrobial compounds have been isolated and used in the food industry. However, there are only few commercial EOs available with useful characterizations of preservative properties. Different methods including steam distillation (SD) and hydro distillation (HD) methods, cold, dry and vacuum distillation, solvent free microwave extraction (SFME) , supercritical carbon dioxide extraction and supercritical fluid extraction (SFE) are available in the production of commercial EOs (Okoh et al. 2010; Tajkarimi et al. 2010; Zhang et al. 2010b). Some of these methods enable better extraction properties (Okoh et al. 2010). Manipulating the parameters such as temperature, pressure and bioengineering contributes the number of commercially available products. Varieties of edible medicinal and herbal plants, and spices, have been successfully used alone or in combination with other preservation methods to extend the shelf life of foodstuffs or as antimicrobial agents against a variety of Gram-positive and Gram-negative bacteria. The efficacy of the components depends on the pH, storage temperature, the amount of oxygen, the EO concentration and active components presented (Tajkarimi et al. 2010; Rao et al. 2010).

7.3.1 Chemical Components Present in Plant-Origin Antimicrobials

There are various chemical components present in plant-origin antimicrobials including, saponin and flavonoids, thiosulfinates and glucosinulates. EOs may contain different components including terpenoids, sesquiterpenes and possibly diterpenes with different groups of aliphatic hydrocarbons, acids, alcohols, aldehydes, acyclic esters or lactones.

Saponin and *flavonoids* are found in fruits, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey; they commonly form a soapy lather after shaking in water or could be extracted from roots, stem bark, leaves and wood of the selected plants. *Thiosulfinates* are hydrolysis products of garlic and onion, formed when the bulb damages. They have a strong antimicrobial activity against pathogenic microorganisms such as *Listeria monocytogenes* (Santas et al. 2010). *Glucosinulates* are present in broccoli, brussels sprouts, cabbage, and mustard powder and cause the pungent flavor of mustard and horseradish; they exhibit a wide range of antibacterial and antifungal activity with direct or synergistic effect in combination with other compounds. Generally, phenolic compounds extracted from lemon, olive oil (oleuropein) and tea-tree oil (terpenoids), orange and bergamot have broader antimicrobial effects. Phenolic compounds present in eugenol oil (*Eugenia caryophylata*) and selected clonal herb species of the Lamiaceae family show inhibitory effect against a variety of microorganisms. Meanwhile, there are increasing reports of non-phenolic

Table 7.2 Inhibitory activities of plant-origin antimicrobials against gram negative pathogenic bacteria, listed alphabetically representative studies conducted within the last 10 years

Organism	Adverse effects	Inhibitors	References
Aeromonas hydrophila	Gastrointestinal diseases	Linalool and methyl chavicol vanillin extracted from sweet basil vanilla	Tajkarimi et al. (2010)
Campylobacter jejuni	Food poisoning; diarrhea	Black and green tea, cinnamaldehyde and carvacrol, marigold, ginger root, jasmine, patchouli, gardenia, cedar wood, carrot seed, celeray seed, mugwort, spikenard, orange bittle oils and coriander(*Coriandrum sativum*) oil, Linalool vapor of bergamot and linalool oils, cinnamic aldehyde and eugenol extracted from cinnamon and clove, linalool and methyl chavicol vanillin extracted from sweet basil vanilla , EO of *Origanum minutiflorum*	Tajkarimi et al. (2010), and Rattanachaikunsopon and Phumkhachorn (2010b)
Escherichia coli	Food poisoning; diarrhea	Cinnamon, oregano oil (Oreganum vulgare), pure essential oils, leaf essential oil, eugenol, bark essential oil, bark oleoresin, E-cinnamaldehyde, carvacrol, oregano oil, citra, lemongrass oil, cinnamaldehyde, cinnamon oil, Oregano in whey protein isolate (WPI) films containing garlic essential oil at, lemongrass, thyme, carvacrol, cinnamaldehyde, citral, and thymol, clove (Eugenia caryophyllata), glucosinolates naturally present in mustard powder, mustard, Bersama engleriana (Melianthaceae), chrysanthemum extracts, cabbage juice, Petiveria alliacea L. roots, Aristolochia indica L., catechin, chlorogenic acid and phloridzin, ground yellow mustard, Brassica oleracea juice, dried garlic powder, commercial garlic products, and garlic oil, onion, marjoram and basil essential oils, Scutellaria, Forsythia suspensa (Thunb), and rosemary and clove oil with 75% ethanol, cassia bark-derived substances, thyme (Thymus vulgaris), bay (Pimenta racemosa), ion extracts, lemongrass, crude extracts of Lycoris chinensis (bulbs), Nandina domestica/Mahoniafortunei/Mahonia bealei (stems and leaves), Berberis thunbergii (stems) and Camptotheca acuminata (stems, leaves and fruits), methanol and acetone extracts of 14 plants belonging to different families, caffeine, 1,3,7-trimethylxanthine, Guarana extract, chloroform extract of the plant of *Abrus precatorious* L. roots, EOs from leaves, stems and flowers of *Salvia reuterana* (Lamiaceae), linalool vapor of bergamot and linalool oils, water-distilled EO from leaves and flowers of *Micromeria nubigena* H.B.K. (Lamiaceae), hydro distillation leaf oil of *Cinnamomum chemungianum and Cinnamomum zeylanicum* Blume, *Rosmarinus officinalis* oil, Anzer tea EOs, EOs of *Actinidia macrosperma,* oleuropein extracted from olive oil, clove EOs, thymol and carvacrol EOs, aerial parts of *Ammoides atlantica,* mango	Tajkarimi et al. (2010), Trajano et al. (2010), Ozkan et al. (2010), Oroojalian et al. (2010), Kurade et al. (2010), Gao et al. (2010), Castano et al. (2010), Al-Reza et al. (2010), and Abalaka et al. (2010)

		seed kernel, EOs of the flower heads and leaves of *Santolina rosmarinifolia* L. (Compositae), *Salvia pisidica* Boiss. and Heldr. ex Bentham (Lamiaceae) extract, sorghum extracts and fractions, ydro-distilled fresh leaves of *Pittosporum neelgherrense* Wight et Arn, relative inhibitory effects on *Escherichia coli* as follows: mustard > clove > cinnamon > garlic > ginger > mint. Hydro distilled extract of *Bunium persicum, Cuminum cyminum and Carum copticum.* Lantana camara Linn. (Verbenaceae), Ageratum houstonianum Mill. EOs from peel of Ponkan (Citrus reticulata Blanco) (Asteraceae) and Eupatorium adenophorum Spreng. (Asteraceae). Seeds of *Zizyphus jujube. Ziziphus mauritiana* L. and *Ziziphus spinachristi* L.	
Pseudomonas aeruginosa	Food spoilage	Tea extract, [Milk protein-based edible films containing oregano, 1.0% (w/v), pimento, or 1.0% oregano pimento (1:1)], tannins, polymers of flavanols, lemongrass, oregano and bay, crude extracts of Lycoris chinensis (bulbs), Nandina domestica/Mahonia fortunei /Mahonia bealei (stems and leaves), Berberis thunbergii (stems) and Camptotheca acuminata (stems, leaves and fruits), certain combinations of carvacrol-thymol, oregano essential oil; methanol and acetone extracts of 14 plants belonging to different families, *Eruca sativa* (aerial and root). Raw state of brown Irish edible seaweeds, *Himanthalia elongate*(98% inhibition), *Laminaria sachharina (93%)*	Tajkarimi et al. (2010), Khoobchandani et al. (2010), and Gupta et al. (2010)
Pseodomonas fluorescens	Food spoilage	Teas, [Milk protein-based edible films containing oregano, 1.0% (w/v) pimento, or 1.0% oregano pimento(1:1)], tannins, polymers of flavanols, *Salvia pisidica* Boiss. and Heldr. ex Bentham (Lamiaceae) essential oil	Tajkarimi et al. (2010), and Ozkan et al. (2010)
Shigella spp. Tea	Diarrhea	Tea, Bersam engleriana(Melianthaceae), tannins, polymers of flavanols, crude extracts of Lycoris chinensis (bulbs), Nandina domestica/Mahonia fortunei/ Mahonia bealei (stems and leaves), Berberis thunbergii (stems) and Camptotheca acuminata (stems, leaves and fruits), *Eruca sativa* (aerial and root). EOs and methanol extracts of sweet basil *Ocimum basilicum* L. (Lamiaceae)	Tajkarimi et al. (2010) Khoobchandani et al. (2010), and Hossain et al. (2010)

(continued)

Table 7.2 (continued)

Organism	Adverse effects	Inhibitors	References
Salmonella spp.	Food poisoning; Salmonellosis	Teas, leaf essential oil; leaf oleoresin; eugenol; bark essential oil; bark oleoresin, E-cinnamaldehyde, [oregano in whey protein isolate (WPI) films containing at 2% level, garlic essential oil at 3% and 4%], oregano (Origanum vulgare), and cinnamon (Cinnamomum zeylanicum), lemongrass, thyme (Thymus vulgaris), (carvacrol, cinnamaldehyde, citral, and thymol), methanol extract of Aspilia mussambicensis (Compositae), Bersama engleriana (Melianthaceae), Aristolochia indica L., Brassica oleracea juice, dried garlic powder, commercial garlic products, and garlic oil, marjoram and basil essential oils, cassia bark-derived substances, lemongrass, bay, methanol and acetone extracts of 14 plants belonging to different families, thymol, EOs extracted from the aerial parts of cultivated *Salvia officinalis* L. Hydro distilled extract of *Bunium persicum, Cuminum cyminum and Carum copticum. Alpinia galanga* (Linn.) Swartz. EOs and methanol extracts of sweet basil *Ocimum basilicum* L. (Lamiaceae). lemon balm (*Melissa officinalis*), aqueous garlic extract (*Allium sativum*). Seeds of *Zizyphus jujube.*	Tajkarimi et al. (2010), Oroojalian et al. (2010), Hsu et al. (2010), Hossain et al. (2010), Dikbas et al. (2010), Belguith et al. (2010), and Al-Reza et al. (2010)
V. cholerae	Cholera	Tea extract	Tajkarimi et al. (2010)
V.parahaemolyticus	Mild gastroenteritis	Basil, clove, garlic, horseradish, marjoram, oregano, rosemary, thyme, cassia bark-derived substances. EOs and methanol extracts of sweet basil *Ocimum basilicum* L. (Lamiaceae). *Cuminum cyminum* L.	Tajkarimi et al. (2010), Hossain et al. (2010), and Hajlaoui et al. (2010)
Yersinia enterocolitica	Diarrhea	Teas, pure essential oils, *Salvia pisidica* Boiss. and Heldr. ex Bentham (Lamiaceae) essential oil	Tajkarimi et al. (2010), and Ozkan et al. (2010)

compounds such as allyl isothiocyanate, carvacrol, isoterpinolene, caryophyllene, camphene, pinene, and thymol, being effective against both Gram-positive and Gram-negative groups (Szabo et al. 2010; Schirmer and Langsrud 2010). EOs could be extracted from oregano, clove, cinnamon, citral, garlic, coriander, rosemary, parsley, lemongrass, purple (cultivar Ison) and bronze (cultivar Carlos) muscadine seeds, clary sage, juniper , lemon, marjoram and herb infusions such as *Ilex paraguaiensis* (Vaquero et al. 2010; Tajkarimi et al. 2010; Tserennadmid et al. 2010). Antimicrobial activity of Clove is higher than rosemary and lavender EOs (Gomez-Estaca et al. 2010). Terrenes, carvacrol, *p*-cymene, and thymol present in oregano, savory and thyme EOs, have demonstrated antifungal and antimicrobial activity that has attracted attention recently because of their potential in food safety applications (Mihajilov-Krstev et al. 2010).

7.4 Uses of Plant-Origin Antimicrobials

Food spoilage can occur through the whole production chain from raw food materials to process and distribution. Preserving food from spoilage and/or pathogenic microorganisms using plant origin compounds have dramatically increased since the 1990s. Plant origin antimicrobials have relatively low molecular weight, (Padovan et al. 2010) and strong antimicrobial activity as potential food preservatives (Dikbas et al. 2010). Water extracts of plants may have greater antimicrobial potential and less undesirable gastric disorders, for example water-phase and decoction extract of bamboo shavings and *Piliostigma reticulatum* (DC.) The bark of *Piliostigma reticulatum* showed antimicrobial activity against different pathogenic and spoilage microorganisms with minimum inhibitory concentrations (MICs) ranging from 0.28 to 32 mg/ml (Zhang et al. 2010b; Zerbo et al. 2010). The MIC values essential oil ranged between 0.039 and 10 mg/ml for *Hypericum scabrum*, *Myrtus communis*, *Pistachia atlantica*, *Arnebia euchroma*, *Salvia hydrangea*, *Satureja bachtiarica*, *Thymus daenensis* and *Kelussia odoratissima* against *Escherichia coli* O157:H7, *Bacillus cereus*, *Listeria monocytogenes* and *Candida albicans* (Pirbalouti et al. 2010). Terpinen-4-ol, linalool, nerol, geraniol, β-pinene, limonene, α-pinene, sabinene, γ-terpinene and myrcene, cineole, and geranyl acetate are antimicrobial compounds that have been effective against some food-borne pathogens and antibiotic-resistant *Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Campylobacter jejuni* (Tserennadmid et al. 2010; Tajkarimi et al. 2010; Park et al. 2010). Olive leaves (*Olea europaea*), rich in phenolic compounds, demonstrated strong antimicrobial effects and potential use in food processing including turkey breast packaging (Erbay and Icier 2010; Botsoglou et al. 2010). The crude extract of *Sorghum bicolor* Moench showed antimicrobial properties and showed variable antimicrobial properties (Tajkarimi et al. 2010; Lee and Lee 2010; Erbay and Icier 2010)

The influence of seasonal harvest of plants and geographical location still has to be investigated in detail in order to be able to draw the utmost benefit for industrial use. Seasonal variations showed some effects on EOs of the cerrado species;

however, they did not have a significant effect on *Myrcia myrtifolia* EOs. Different harvest locations of *Thymus pallescent* resulted in different antimicrobial activities; However, differences in antimicrobial/antioxidant activity of the Tunisian *Thymus capitatus* have not been attributed to location. Essential oil of the aerial parts of *Satureja hortensis* L. containing carvacrol showed strong antimicrobial effect against a variety of gram positive and gram negative pathogenic microorganisms and molds (Adiguzel et al. 2007; Razzaghi-Abyaneh et al. 2008).

Oregano and thyme, oregano with marjoram, and thyme with sage were the most effective EOs against pathogenic microorganisms. Phenolic compounds of spices and plants such as hydroquinone, thymol, carvacrol, BHA, as well as octyl gallate and tannic acid might be primarily responsible for bacteriocidal/bacteriostatic properties (Xia et al. 2011; Tajkarimi et al. 2010; Rua et al. 2010).

Tables 7.1, 7.2 and 7.3 presents the antimicrobial activities and components of various spices and herbs.

7.4.1 Mechanism of Action

The antimicrobial effect of EOs are basically demonstrated by causing structural and functional damages and altering the bacterial cell membrane, including the phospholipid bilayer as well as the toxicity caused by the optimum range of hydrophobicity. This antimicrobial function causes swelling and increases permeability, loosing cellular pH gradient (Mihajilov-Krstev et al. 2009; Serrano et al. 2011). Other mechanisms include disrupting enzyme systems, compromising the genetic material of bacteria, and forming fatty acid hydroperoxidase caused by oxygenation of unsaturated fatty acids. For example, tea tree oil changes the respiratory enzyme or metabolic event inhibition or leakage in potassium ions transfer. Mustard derived EOs showed multi-targeted mechanisms of action in metabolic pathways, membrane integrity, cellular structure and statistically significant higher release of the cell components of *Escherichia coli* O157:H7. Carvacrol extract increases the heat shock protein 60 HSP 60 (GroEL) and inhibits the synthesis of flagellin significantly in *E. coli* O157:H7. It has been suggested that essential oil constituents such as eugenol might inhibit the mechanism of virulence *agr* two-component system gene expression by interactive, hierarchical regulatory cascade among the *agr*, *sar*, and other regulatory gene products in *Staphylococcus aureus* (Qiu et al. 2010). Cytotoxic activity of cumin EOs might contribute to its antimicrobial effect (Allahghadri et al. 2010).

It has been well demonstrated that the antimicrobial efficacy of plant-origin antimicrobials depends on several factors including the EO extraction method, the inoculum volume, growth phase, culture medium used, and intrinsic or extrinsic factors of the food such as pH, fat, protein, water content, antioxidants, preservatives, incubation time/temperature, packaging procedure, and physical structure (Tajkarimi et al. 2010).For example, in galangal flowers, oven-dried samples extracted with ethanol was more effective compared to the freeze-dried samples extracted with

Table 7.3 Inhibitory activities of plant-origin antimicrobials against protein toxins and fungi, listed alphabetically representative studies conducted within the last 10 years

Organism	Adverse effects	Inhibitors	References
Botulinum	Neurotoxin botulism	Black tea	Tajkarimi et al. (2010)
Cholera	Cholera	Catechins, theaflavins	Tajkarimi et al. (2010)
Molds			
Aspergillus flavus, Aspergillus niger, Aspergillus parasiticus	Myco-toxicosis, Ochratoxins	Pure essential oils, leaf oleoresin, leaf essential oil, eugenol, bark essential oil, bark oleoresin, E-cinnamaldehyde, cassia bark-derived substances, naringin, hesperidin and neohesperidin, and enzymatically-modified derivatives of these compounds, higher levels of phenolic compounds in thyme, cinnamon and clove, Cinammon oil, Methanol extract of *Aspilia mussambicensis* (Compositae), Guarana extract , EO and methanol extract of *Satureja hortensis, Satureja hortensis* L. containing carvacrol and thymol, water-distilled EO from leaves and flowers of *Micromeria nubigena* H.B.K. (Lamiaceae), oleuropein extracted from olive oil, EOs from thymol, cinnamic aldehyde and eugenol extracted from cinnamon and clove, *Thymus vulgari* and *Citrus aurantifolia*, *Mentha spicata* L., *Foeniculum miller*, *Azadirachta indica* A. Juss, *Conium maculatum* and *Artemisia dracunculus*, *Carum carvi* L.; linalool and methyl chavicol vanillin extracted from sweet basil vanilla, hydro-distilled EOs of stems, leaves (at vegetative and flowering stages) and flowers of *Eugenia chlorophylla* O. Berg. (Myrtaceae), thyme, oleoresin extracted from cinnamon and cinnamic aldehyde and eugenol extracted from cinnamon and clove , allyl isothiocyanate and citralon in mustard and lemongrass, marjoram oil, essential oil of *Ageratum conyzoides.* Thyme and rosemary EOs isolated from hazelnut. EOs of *Ageratum conyzoides* L. Ocimum sanctum essential oil. *Eruca sativa* (aerial and root). EOs of oregano (*Origanum vulgare* L.)=citrus (*Citrus sinensis* L. Osbeck)>savory (*Satureja thymbra* L.)>laurel (*Latin's nob ills* L.)>myrtle (*Myrtus communis* L.). EOs from peel of Ponkan (Citrus reticulata Blanco). Mexican oregano (Lippia berlandieri Schauer) essential oil added to amaranth, chitosan, or starch edible films. *Ziziphus mauritiana* L. and *Ziziphus spinachristi* L.	Tajkarimi et al. (2010), Salas et al. (2011), Xia et al. (2011), Xing et al. (2010), Patil et al. (2010), Ozcakmak et al. (2010), Nogueira et al. (2010), Kumar et al. (2010), Khoobchandani et al. (2010), Gunduz et al. (2010), Gao et al. (2010), Avila-Sosa et al. (2010), and Abalaka et al. (2010)

(continued)

Table 7.3 (continued)

Organism	Adverse effects	Inhibitors	References
Fusarium semitectum		Naringin, hesperidin and neohesperidin, and enzymatically-modified derivatives of these compounds, higher levels of phenolic compounds in thyme, cinnamon and clove, methanol and hexane extracts of weedy plant species Vigna unguiculata and Amaranthus spinosus, allyl isothiocyanate and citralon in mustard and lemongrass, marjoram oil	Salas et al. (2011), Xia et al. (2011), and Thembo et al. (2010)
Penicillium expansum		Naringin, hesperidin and neohesperidin, and enzymatically-modified derivatives of these compounds , higher levels of phenolic compounds in thyme, cinnamon and clove, Cinammon oil, linalool and methyl chavicol vanillin extracted from sweet basil vanilla, hydro-distilled EOs of stems, leaves (at vegetative and flowering stages) and flowers of *Eugenia chlorophylla* O. Berg. (Myrtaceae), thyme, oleoresin extracted from cinnamon and cinnamic aldehyde and eugenol extracted from cinnamon and clove, allyl isothiocyanate and citralon in mustard and lemongrass, marjoram oil	Salas et al. (2011), Xia et al. (2011), Xing et al. (2010), and Avila-Sosa et al. (2010)
		Mexican oregano (*Lippia berlandieri* Schauer) EOs added to amaranth, chitosan, or starch edible films	

ethanol (Hsu et al. 2010). Other parameters such as lower pH level inside the bacterial cell such as *E. coli* and *Salmonella* are important. Gram negative bacteria are less sensitive to the plant origin antimicrobials because of the lipopolysaccharide outer membrane of this group, which restricts diffusion of the hydrophobic compounds (Tajkarimi et al. 2010). Gram positive bacteria are more sensitive to plant essential oils compared to gram negative bacteria (Wanner et al. 2010a, b).

Some spices have stronger antimicrobial activity than others. Generally, spices, herbs and their essential oils are used in food systems within the range of 0.05–0.1% (500–1,000 ppm). Application of higher amount of the spices of herbs raises concerns as it releases its aroma and taste into the food product. For example, there are concerns regarding the application of 1% oregano in food systems. Some studies suggest application of certain combinations of EOs in order to reduce the adverse sensory impact in food (Bassole et al. 2010). Different exposure methods of EOs also have effect on the mechanism of action. For example, application of mustard and clove EOs in vapor phase compared to the direct contact method, showed considerable difference in results. The slight modifications in stereochemistry, lipophilicity and other factors affected the biological activity of these compounds positively or negatively.

7.4.2 Synergistic and Antagonistic Effects of Components

Synergy is defined when the combined effect of substances is higher than the sum of the individual effects. When a combination shows less effect compared to the individual application, it means antagonism. Synergistic effects might happen by combination of different compounds and/or combination of compounds and techniques. The synergistic effect of different components and methods could offer a way to prevent possible off flavor caused by different plant origin materials, spices and herbs. Combination of a variety of plant extracts with nitrite ($NaNO_2$) shows stronger antimicrobial effect against *Clostridium botulinum* in meat without compromising the organoleptic properties (Cui et al. 2010). The synergic effect of some compounds such as organic acids and nicin, in addition to the major components in EOs, have been shown in some studies (Tyagi and Malik 2010; Tajkarimi et al. 2010; Tserennadmid et al. 2010). Combination of carvacrol- thymol and carvacrol-*p*-cymene can improve the efficacy of EOs against pathogenic microorganisms. Antimicrobial activity of a combination of cinnamon and clove EOs in vapor phase showed promising antimicrobial effect with less active concentration in the vapor phase compared to the liquid phase (Tyagi and Malik 2010; Tajkarimi et al. 2010). Oregano EOs showed higher antimicrobial activity in combination with low levels of sodium nitrite against *Clostridium botulinum* spores compared to sodium nitrite alone, depending on the number of inoculated spores. Combinations of EOs, oregano with thyme, oregano with marjoram and thyme with sage had the strongest effect against different pathogenic microorganisms (Gutierrez et al. 2008a). Synergistic

and antagonistic effects of thymol and carvacrol have been observed in different combinations of cilantro, coriander, dill and eucalyptus EOs and mixtures of cinnamaldehyde and eugenol, against different pathogenic microorganisms (Tajkarimi et al. 2010). Chinese cinnamon and winter savory EOs were used successfully used to increase the radio sensitivity in ground beef. Combinations of EOs and nisin showed enhanced antimicrobial activity against *Listeria monocytogenes* (Tajkarimi et al. 2010). Combination of EDTA–lysozyme–rosemary oil and EDTA–lysozyme–oregano oil were most effective against the growth of Gram-negative, Gram-positive and to a lesser extent on yeasts on the shelf-life of semi cooked coated chicken fillets stored under controlled condition (Ntzimani et al. 2010). Synergic effect of compounds in combination with processing techniques such as vacuum packing, negative air ions, high-hydrostatic pressure and modified atmosphere packaging (MAP), increasing temperature from 8ºC to 30ºC and increasing light exposure to plants, showed interesting results against different pathogenic microorganisms with a variety of plant compounds such as thymol, carvacrol, Oregano, hexane, ethyl acetate, n-butanol. Sensitivity of antimicrobials increases by lowering the exposure of microorganisms to the available oxygen (Tajkarimi et al. 2010). Application of both extracts and EOs of plant-origin such as floral parts of *Nandina domestica* Thunb could be used as potential alternative for synthetic preservatives (Tajkarimi et al. 2010; Joung et al. 2010). Table 7.3 summarizes the results of various experiments regarding the application of phytochemicals against molds and yeasts.

7.5 Review of Some Findings of *In-Vitro* Experiments

In-vitro experiments of plant-origin antimicrobials are well described in the literature; however, the antimicrobial activity of herbs and spices might vary based on the tested organism.

Significant antibacterial activity was shown on ethanolic, methanolic and acetonic extracts by the *Calligonum Comosum*, a Medicinal Plant from Tunisia by the agar-well diffusion method against *Listeria ivanovii*. (Riadh et al. 2011). Cardiac glycosides, polyphenols, saponins and tannins presented in ethanolic extracts of leaves of two species of genus Ziziphus showed strong antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* at MIC 1 mg and 5 mg/l respectively (Abalaka et al. 2010). Phenolics and flavonoids present in pomegranate fruit peels demonstrated strong antimicrobial activity against *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Yersinia enterocolitica*. The minimum inhibitory concentration (MIC) against Salmonella enteritidis was 4 mg/ml (Al-Zoreky 2009). Phenolic, flavonoid, carotenoid, terpenoid and triterpene present in Psidium guajava demonstrated strong antimicrobial activity (Gutierrez et al. 2008b). Antimicrobial activity of non-volatile ethanol extract of *Satureja montana* L. might be attributed to the presence of phenolic compounds such as carvacrol, carvacrol methyl ether, and thymol and terpenes (γ-terpinene) molecules (Serrano et al. 2011). Antimicrobial activity of

phenolic compounds have also been demonstrated in several studies including edible seeds extract of Chinese Mei (Prunus mume Sieb. et Zucc) (Xia et al. 2011), different Argentinean green tea varieties (von Staszewski et al. 2011), *Piliostigma reticulatum* (DC.) Hochst extracts (Zerbo et al. 2010), soybean extracts enriched for phenolic content via dark-germination sprouting or solid-state bioprocessing by the dietary fungus Rhizopus oligosporus or Lentinus edodes (McCue et al. 2005), dark germinated fenugreek sprouts phenolic compounds (Randhir et al. 2004) and wood smoke (Niedziela et al. 1998).

Flavonoids, extracted from Citrus species, such as naringin, hesperidin and neohesperidin, and enzymatically-modified derivatives of these compounds, also demonstrated strong antifungal activity (Salas et al. 2011) Black raspberry and Chardonnay seed extracts showed antibacterial activity against *Escherichia coli* and growth inhibition against *Listeria monocytogenes,* under experimental conditions (Luther et al. 2007).

7.6 Some In-Food Experiments with Plant-Origin Antimicrobials

In-food studies depend on several additional factors, which have not been tested in similar *in-vitro* studies and might not have an impact on the bacterial growth in complex food systems (Schirmer and Langsrud 2010). The difference is mainly because only a small percentage of EOs is tolerable in food materials. Factors such as type, effects on organoleptic properties, composition/concentration and biological properties of the antimicrobial and the target microorganism, processing and storage conditions of the targeted food product are also important. *In vitro* experiments using microbiological medium are more common. Consequently, the effectiveness of EOs when applied in food has been less understood due to their unacceptable organoleptic properties (Romeo et al. 2010). Generally, higher concentrations of EOs are necessary in food, compared to *in-vitro* trials: for example, two-fold increase compare to laboratory medium in semi-skim milk, ten-fold in pork liver sausage, 50-fold in soup and 25- to 100-fold in soft cheese. Generally, effective EOs in decreasing order of antimicrobial activity is: oregano > clove > coriander > cinnamon > thyme > mint > rosemary > mustard > cilantro/sage. The antimicrobial potential has shown some inconsistencies in some experiments; for example in a study, mint showed lower antimicrobial effect compared to mustard (Tajkarimi et al. 2010).

Odors created mostly by high concentrations, and the cost of these materials are the two major issues restricting the application of plant-origin antimicrobials in food. However, there is promising news about the application of agricultural by-products such as the hulls of Antep pistachio (*Pistacia vera* L.) as an effective antimicrobial against *E. coli* O157:H7 and *Listeria monocytogenes* (Ozturk et al. 2010; Tajkarimi et al. 2010).

7.6.1 Meat and Poultry Products

Plant extracts are useful for reducing pathogenic microorganisms of meat origin however; some studies demonstrated low antimicrobial effects of plant origin antimicrobials against meat pathogens. The low antimicrobial effect might be caused by the interaction of meat fat and greater solubility of EOs in lipids compared to aqueous parts of food. For example, a combination of 1% clove and oregano in broth culture did not show similar inhibitory effect against *Listeria monocytogenes* in meat slurry. A 5–20 μl g^{-1} level of eugenol and coriander, clove, oregano and thyme oil inhibits the growth of *Listeria monocytogenes*, *Aeromonas hydrophila* and autochthonous spoilage flora in meat products (Tajkarimi et al. 2010). According to Tables 7.1 and 7.2, in recent studies, certain oils such as eugenol existing in clove, oregano and thyme oils showed high effect against *Listeria monocytogenes*, *Aeromonas hydrophila* and autochthonous spoilage flora in meat products; however, mustard, cilantro, mint and sage oils were less effective or ineffective (Tajkarimi et al. 2010). Combination of water soluble extracts of oregano and cranberry, at a ratio of 50:50 and a concentration of 750 ppm, with 2% sodium lactate showed the best inhibitory effect against *Listeria monocytogenesis* in fresh and frozen meat and poultry (Apostolidis et al. 2008). Antimicrobial effect of Roselle (*Hibiscus sabdaridda* L.) against both susceptible and antibiotic-resistant Campylobacter strains were similar on *in-vitro* and in contaminated ground beef. Better EO delivery systems such as encapsulation showed better antimicrobial effect against *Listeria monocytogenes* in pork liver sausage (Tajkarimi et al. 2010; Ruiz et al. 2009).

Basil oil showed 3 log CFU reductions on *Salmonella enteritidis* population after 3 days of storage (Rattanachaikunsopon and Phumkhachorn 2010a). Combination of different methods such as cold temperature, pulsed light, high pressure, pulsed electric and magnetic fields, irradiation, or modified atmosphere packaging with the application of Winter savory (*Satureja montana*) EOs demonstrated to be economically reasonable as a natural antibacterial substance to control the growth of food-borne bacteria and improve the quality of minced pork. Radiation at 25 kGy in a cobalt-60 irradiator did not show changes in antimicrobial effect of *Glycyrrhiza glabra* roots (Khattak and Simpson 2010). Aqueous extract of rosemary, sage and thyme inhibited the rancidity of heat-treated turkey-meat products. Milk protein-based, chitosan, or starch edible films containing oregano, pimento, or oregano and pimento was effective against *Escherichia coli* O157:H7 or *Pseudomonas* spp. (Tajkarimi et al. 2010; Avila-Sosa et al. 2010; Aider 2010). Preservation methods using a combination of different techniques and natural plant antimicrobials for fresh poultry meat have been successfully conducted (Ntzimani et al. 2010). Chlorophyll-containing films were successfully tested against *Staphylococcus aureus* and *Listeria monocytogenes*. Marjoram (*Origanum majorana* L.) EO in fresh sausage was effective against several species of bacteria. *Staphylococcus aureus* and *Listeria monocytogenes* in cooked frankfurter were successfully reduced by using Chlorophyllin-gelatin films and

coating applications. *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas fluorescens* and *Lactobacillus sake* in modified atmosphere-packaged fresh pork and vacuum-packaged ham slices stored at 4ºC were reduced using individual extracts of clove, rosemary, cassia bark and liquorice. Clove and tea-tree oils showed potential bio-preservative capabilities to control *Escherichia coli* O157:H7 on blanched spinach and minced cooked beef (Tajkarimi et al. 2010). Methanol leaf extract of *Salvia leriifolia* successfully reduced *Staphylococcus aureus* numbers in hamburger (Mehr et al. 2010).

7.6.2 Sea Food

The antimicrobial effect of EOs against various microorganisms in foods containing high fat, such as meat and some fish products is low; however, some of the EOs such as oregano oil showed effective antimicrobial effect against the spoilage organism *Photobacterium phosphoreum* on cod fillets, which is a fatty fish. Oregano also showed strong antioxidant activity. *Salmonella enteritidis*, *Listeria monocytogenes* and the natural spoilage flora were inhibited using EOs on the surface of whole fish or as coating for shrimps. Wild thyme (*Thymus serpyllum*) application in fresh water fish significantly increased the shelf-life by about 15–20 days. Combined application of carvacrol + thymol with some other additives extended the shelf-life of carp fillets. Thymol, carvacrol and cinnamaldehyde, isoeugenol, eugenol, garlic oil, and citral increased the shelf life of carp fillets. *Aloysia sellowii* EO in brine shrimp was successfully applied against a variety of Gram-positive and -negative bacteria and yeasts. 0.5% eugenol plus 0.5% linalool contributed to the freshness sensation of tuna slices (Tajkarimi et al. 2010). Addition of 0.05% (v/v) thyme oil to packed sea bass could significantly reduce bacterial growth during storage (Schirmer and Langsrud 2010). 1% and 2% clove oil caused a reduction between 1 and 4 $\log_{10}$ CFU/g in *Listeria monocytogenes* on Salmon fish (Miladi et al. 2010). Pre-treatment of mackerel fillets with diluted quince-polyphenolic extract might be used to inhibit spoilage bacteria (Fattouch et al. 2008). One percent cinnamaldehyde or 1% carvacrol showed a 5 $\log_{10}$ CFU/g reduction in microbial populations of tested oysters (Ravishankar et al. 2010). *Vibrio parahaemolyticus* contamination risk has been successfully minimized using allspice, basil, clove, garlic, horseradish, marjoram, oregano, rosemary, and thyme and hurdle technology. A synergistic effect of treatment with anodic electrolyzed NaCl solution, combined with eugenol and linalool, was found to enhance the shelf-life of coated semi-fried tuna (Tajkarimi et al. 2010). Complex gelatin-chitosan film incorporating clove EO in packaged fish developed effective antimicrobial effect below detection limits after 6 days, especially for *Enterobacteriaceae* (Gomez-Estaca et al. 2010). Chitosan is desired in liquid and solid foods due to its convenience as an antimicrobial and an antioxidative-preservative and its stability at pH 6 (Friedman and Juneja 2010; Fernandez-Saiz et al. 2010a, 2010b; Diaz-Visurraga et al. 2010).

7.6.3 Dairy Products

The application of EOs in milk is positively affected by higher water activity in milk. Remarkable antimicrobial activity against *Escherichia coli* and extended shelf-life of pasteurized cow's milk was shown using the extract of mango seed kernel.

The botanical composition of meadows on the sensory properties of terpenes in pressed cheeses was not or only marginally involved. Natural antioxidant and aroma properties were shown using *Satureja cilicica* EO in dairy products. Some EOs such as cinnamon, cardamom and clove oils inhibit the growth of yogurt starter cultures; however, mint oil was effective against *Salmonella enteritidis* in low-fat yogurt. *Salmonella enteritidis* was inhibited using clove oil in full-fat cheese (Tajkarimi et al. 2010). Allyl isothiocyanate isolates from plants showed 3.6 log CFU reduction in yeasts and molds counts in mozzarella packed with the antimicrobial sachet over 15-day storage time (Pires et al. 2009). Application of bay, clove, cinnamon and thyme showed 1 log CFU reduction on *Listeria monocytogenes* in low-fat cheese. However, 1% clove oil showed similar reduction effect on full-fat soft cheese against *Listeria monocytogenes* at cold storage temperatures over a 14-day period (Smith-Palmer et al. 2001). In another study , a mixture of plant essential oils at 2,500 ppm showed bacteriostatic effect against *Listeria monocytogenes* and ineffective against *E. coli* O157:H7 (MendozaYepes et al. 1997). In fermented products such as Ayran, similar responses were obtained using mint, thyme, garlic, salt and their mixture and non treated product against *E. coli* O157:H7 (Simsek et al. 2007). However, combined techniques of High pressure-processing with mint essential oil appeared to be a promising technique to preserve Ayran (Evrendilek and Balasubramaniam 2011).

7.6.4 Fruits and Vegetables

Low fat content, accompanied by lower pH and/or temperature of these products enable more successful application potential for EOs as antimicrobials. For example, oregano oil was effective against *Escherichia coli* O157:H7 in eggplant salad and Cinnamaldehyde and thymol were effective against six *Salmonella* serotypes on alfalfa seeds (Tajkarimi et al. 2010). Carvacrol and cinnamaldehyde effectively inhibited the natural microflora of kiwi fruits (Tajkarimi et al. 2010). Post-harvest fungal disease caused by *Botrytis cinerea* in stored grape was reduced effectively by using natural fungicidal plant EOs. Alginate-based edible coating of fresh-cut Fuji apples using EOs showed more than 4-log $_{10}$CFU/ml reduction in the population of *E. coli* O157:H7 and a total inhibition of native microflora for 30 days at 5°C (Khwaldia et al. 2010). Promising antimicrobial and quality effects on fresh-cut melon was shown using Cinnamon, clove, and lemongrass EOs and their active compounds. 40 ppm cinnamaldehyde with 40 ppm of eugenol or 80 ppm eugenol preserved apple juice for 7 days. *Alicyclobacillus acidoterrestris* was inhibited with

more acceptable results in the test panels using a combination of cinnamaldehyde and eugenol in apple juice. The key antimicrobial and antioxidant component for fresh-cut apple and salads were polyphenols which are present in green, white and commercial tea (Tajkarimi et al. 2010; Chiu and Lai 2010). Sumac (*Rhus coriaria* L.) water extract and oregano oil suspension on tomato surfaces significantly reduced *Salmonella* Typhimurium populations without affecting the sensory properties of tomatoes (Gunduz et al. 2010).

7.6.5 Cereals

Alkaloids, tannins and cardiac glycosides found in lemon grass powder and essential oil is believed to have preservative and antimicrobial effects in maize and cowpea samples against moulds like *Aspergillus flavus, A. fumigatus, Microphomina phaseoli and Penicillium chrysogenum* and bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Ps. fluorescens, and Bacillus subtilis* (Adegoke and Odesola 1996). Antifungal and antiaflatoxigenic activity of *Pimpinella anisum* L. (anise), *Peumus boldus* Mol (boldus), *Hedeoma multiflora* Benth (mountain thyme), *Syzygium aromaticum* L. (clove), and *Lippia turbinate* var. integrifolia (griseb) (poleo) essential oils (EOs) at 2,000 and 3,000 μg/g has been demonstrated (Bluma and Etcheverry 2008). Three stored-rice pests (*Sitophilus oryzae*, *Rhyzopertha dominica* and *Cryptolestes pusillus*) were reduced using leaves of five different varieties of *Ocimum basilicum*. *Bacillus cereus* in rice was reduced using sage oil and carvacrol. Two major seed-borne fungi of rice was reduced using *Ocimum gratissimum* and *Thymus vulgaris* (Tajkarimi et al. 2010). It has been suggested that cinnamon and oregano oils could be effective in controlling the growth of Fumonisin B1 production in preharvest conditions (Velluti et al. 2003). However, edible bamboo shoots in Korea were able to inhibit bacterial growth (Park and Jhon 2010).

7.6.6 Animal Feed

Considering restrictions on the application of antibiotics and growth promoters especially in European countries, significant increase in EO use were reported in farm animal feeds. Carvacrol-rich and some other EOs have shown inhibitory effect against *Clostridium perfringens* and necrotic enteritis in poultry. Digestion improvement in ruminants, and improving the stability and palatability of animal feed, were some positive impacts of using EOs for animal health (Franz et al. 2010). Bacterial communities in animal feed were affected by the presence of thymol. There is limited effect on nutrient utilization using EO compounds in alfalfa and corn silage. However, cinnamon leaf oil showed inhibition effects on the total volatile fatty acid (VFA) concentration and prevented adverse effects on metabolism and increased the

productivity of ruminants (Tajkarimi et al. 2010). Oregano oil administration at 0.5% ZnO level in feed did not prevent diarrhea in weanling pigs (Henn et al. 2010).

7.7 Conclusions

Plant-origin antimicrobials are present at various levels and at different degrees of effectiveness in a variety of plants, spices and herbs. These compounds can naturally improve shelf life of food products.

Parameters such as low pH or modifying physical conditions such as vapor phase, may improve the inhibitory effects of EOs that might be a because of direct result of acidity or interaction of EOs with the lipid phase of the affected bacterial membrane (Tyagi and Malik 2010; Tajkarimi et al. 2010). Application of EOs in vapor phase, in combination with other techniques showed successful result for antimicrobial packaging development. Direct application of plant essential oils onto food packaging and developing active form of packaging are approaches (Ojagh et al. 2010). Phytochemicals such as eugenol could be used as a structural model for developing antimicrobial agents aimed at the bacterial virulence factors (Qiu et al. 2010). Majority of plant origin antimicrobials need to be addressed by regulatory authorities for most parts of these compounds. The US regulatory agencies have recognized EOs of cinnamon, clove, lemon grass and their respective active compounds (cinnamaldehyde, eugenol and citral, respectively) as generally recognized as safe (GRAS). However, some materials, such as thymol, have not been recognized as food-grade additives by European legislators. Carvacrol, carvone, cinnamaldehyde, citral, *p*-cymene, eugenol, limonene, menthol and thymol have been registered and recognized as safe-to-use materials in the EU countries (Tajkarimi et al. 2010; Qiu et al. 2010). Risk assessment of the effect of high doses of some EOs on intestinal cells should be considered seriously. Application of these compounds in the food industry needs further investigation from a synergism and antagonism point of view. Determination of EOs for target and effective range including MIC and safety data (toxicity, allergenicity) in food materials is a necessary requirement. Several successful application of EOs in conjunction with hurdle technology and modified atmosphere packaging created pleasant odor with longer shelf life. Other possible ways to reduce the organoleptic impact include: Minimizing perception of the presence of plant origin compounds in food by optimizing food formulation; Application of combined methods; Enhancing a calibrated vapor pressure capacity in order to increase the interaction between EO and the bacterial cell membrane.

Several studies showed higher antimicrobial activity of whole EOs present in plants compared to selective components and information on the effects of crude extracts of plant origin compounds against food-borne microorganisms is limited. Future studies are required to conduct a more in-depth review for individual, and in combination application of EOs with extracts from other parts of the plant, other effective EOs and other food-processing techniques.

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Chapter 8
Dietary Tannins on Microbial Ecology of the Gastrointestinal Tract in Ruminants

Amlan Kumar Patra, Byeng-Ryel Min, and Jyotisna Saxena

Abstract This review discusses the effects of tannins on nitrogen metabolism in the rumen and intestine, microbial populations (bacteria, protozoa, fungi and archaea), metabolism of tannins, microbial tolerance mechanisms to tannins, inhibition of methanogenesis, ruminal biohydrogenation processes and performance of animals. The discrepancies in responses of tannins among different studies are attributed to the different chemical structures (degree of polymerization, procyanidins to propdelphinidins, stereochemistry and C–C bonding), different concentrations of tannins, and type of diets. An establishment of structure-activity relationship would be required to explain differences among studies and obtain consistent beneficial tannin effects. This paper reviews progress with plant tannins occurring in both temperate and tropical forages for fulfilling the objective of mode of action of tannins, rumen microbial activity and rumen metabolisms.

Keywords Tannins • Microbial ecology • Gastrointestinal tract • Metabolism • Rumen fermentation • Resistance mechanisms • Ruminants

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8.1 Introduction

Tannins (hydrolysable and condensed tannins) are polyphenolic polymers of relatively high molecular weight with the capacity to form complexes mainly with proteins due to the presence of a large number of phenolic hydroxyl groups. They are ubiquitously distributed in nutritionally important forage trees, shrubs, legumes, cereals and grains. Intake of feeds and forages containing high concentration of tannins in animals may show adverse effects on feed intake, nutrient utilization and animal performance. However, tannins may modulate rumen fermentation favourably, such as decreased protein degradation in the rumen, prevention of bloat, inhibition of methanogenesis and increased concentrations of conjugated linoleic acid in ruminant-derived foods (Mangan 1988; Kumar and Vaithiyanathan 1990; Aerts et al. 1999; Barry and McNabb 1999; McSweeney et al. 2001a; Min et al. 2003; Mueller-Harvey 2006; Waghorn 2008; Patra and Saxena 2010). All these beneficial responses are mediated through the modulation of microbial populations in the rumen. Besides, tannins may exert antimicrobial effects in the intestine.

Tannins were primarily considered as anti-nutritional biochemicals due to their adverse effects on feed intake and nutrient utilization (Kumar and Vaithiyanathan 1990). Nevertheless in recent years, they have been recognized as useful phytochemicals for beneficially modulating the rumen microbial fermentation. The effects of tannins on ruminant production have been published in the past, which primarily focused on the adverse effects of tannins on animal system, with some discussion on their positive effects on protein metabolism and prevention of bloat (Mangan 1988; Kumar and Vaithiyanathan 1990; Aerts et al. 1999; Barry and McNabb 1999; McSweeney et al. 2001a; Min et al. 2003; Mueller-Harvey 2006; Waghorn 2008). This chapter focuses on the effects of tannins on ruminal microbial populations that affect N metabolism, methanogenesis and ruminal biohydrogenation process in the rumen.

8.2 Chemistry and Occurrence of Tannins

Tannins are water soluble polyphenols of relatively high molecular mass and have capacity to form complexes mainly with proteins, to a lesser extent with carbohydrates due to the presence of a large number of phenolic hydroxyl groups. They are usually classified into two major groups: hydrolysable tannins (HT) and condensed tannins (CT).

The HT is complex molecules with a polyol as a central core such as glucose, glucitol, quinic acids, quercitol and shikimic acid that is partially or totally esterified with a phenolic group, i.e. gallic acid (3,4,5-trihydroxy benzoic acid; gallotannins) or gallic acid dimer hexahydroxydiphenic acid (ellagitannins) (Haslam 1989; Fig. 8.1). The resulting phenolic groups may further be esterified or oxidatively crosslinked to yield more complex HT (Haslam 1989). Hydrolysable tannins are susceptible to

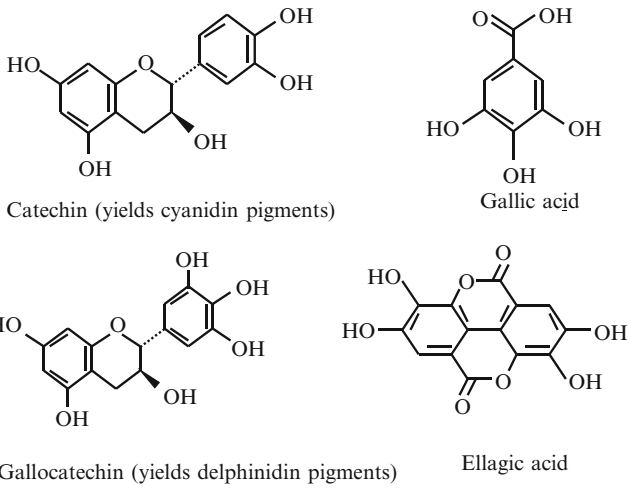

Fig. 8.1 Monomeric units of condensed (catechin and gallocatechin) and hydrolysable tannins (gallic and ellagic acid)

hydrolysis by acids, bases or esterases yielding polyol and the constituent phenolic acids (Haslam 1989).

The CT or proanthocyanidins are mainly polymers of the flavan-3-ol (epi) catechin and (epi) catechin units, which are linked by C4–C8 and C4–C6 interflavonoid linkages (Ferreira et al. 1999; Hagerman and Butler 1989). Many other monomers of CT, e.g. profisetinidins, probinetidins and proguibortinidins are also present (Haslam 1989). For example, quebracho tannins are of largely profisetinidin type (Hemingway 1989). In procyanidins and prodelphinidins, C4–C8 and C4–C6 linkages with a ratio of about 3:1 are more common and majority of tannins in these classes are of mixed stereochemistry with 2,3 *cis* to 2,3 *trans* ratios between 9:1 and 5:5 (Seigler 1998). The number of monomeric units can vary and this determines the degree of polymerisation from di-, tri- and tetraflavonoids to higher oligomers. These can then produce a large numbers of chemical structures, which in turn could produce different biological properties (Waghorn 2008). The CT is degraded to monomeric anthocyanidins (e.g. cyanidin and delphinidin) pigments upon treatment with acid butanol reaction (Porter et al. 1986; Haslam 1989). The CT can react by hydrogen bonding with plant protein to form stable and insoluble CT-protein complexes at pH 3.5–7.0, which dissociate and release protein at pH <3.5 (Jones and Mangan 1977).

Tannins are ubiquitously distributed in nutritionally important forage trees, shrubs and legumes, cereals and grains that often limit their utilization as feedstuffs. Generally, tannin concentrations are greater in vulnerable parts of the plants, i.e. new leaves and flowers (Terrill et al. 1992; Frutos et al. 2004) and various factors such as temperature, light intensities, water and nutrient stress, soil quality and topography influences the concentrations of tannins in plants (Frutos et al. 2004). Both HT and CT may occur in the same plant, but some plants may contain

predominantly HT whereas others contain CT (Haslam 1989). Again, although CT in plants represents a mixture of different monomeric units, a particular type of CT may be predominant in a particular plant, which may explain different physiological effects and animal performance (Waghorn 2008). For instance, CT from *Lotus corniculatus* (birdsfoot trefoil) predominantly has catechin subunits (67%), i.e. procyanidins type CT with average molecular mass of 1,900 (Foo et al. 1996), whereas CT from *Lotus pedunculatus* (big trefoil) has epigallocatechin subunits (64%), i.e. prodelphinidin type CT with average molecular mass of 2,200 (Foo et al. 1997). It has been noted that low molecular weight CT oligomers are more reactive and have higher protein precipitating capacities than high molecular weight polymeric tannins (Butler and Rogler 1992).

8.3 Effects of Tannins on Microbial Nitrogen Metabolism

The quantity and quality of protein flowing to the intestine is one of the major factors determining the productivity of ruminant livestock. The protein reaching the abomasum consists of a mixture of dietary and microbial protein. The flow of protein from the rumen to abomasum depends on proteolysis by rumen microorganisms and the efficiency of microbial protein synthesis in the rumen. When ruminants are fed on a high quality fresh forages containing high concentration of N (25–35 g/kg DM), most of the proteins become rapidly soluble in the rumen and are degraded by rumen micro-organisms resulting in surplus levels of ammonia (20–35%) which is absorbed from the rumen and excreted in urine (Ulyatt et al. 1975). Tannins may improve the protein metabolism in the rumen by reducing protein degradation via formation of insoluble tannin-protein complexes and decreasing the solubility of protein (Tanner et al. 1994; Min et al. 2000; Fig. 8.2). Preincubation of total leaf protein with the rumen bacteria *Streptococcus bovis,* isolated from sheep rumen fluid resulted in a rapid degradation of the large subunit protein (Rubisco) within 2 h of incubation (Fig. 8.3). This was followed by further gradual degradation of Rubisco over the remaining 24 h incubation. Preincubation of bacterial cells with CT extracted from *L. corniculatus* prior to the addition of total soluble leaf protein markedly reduced the rate proteolysis of large and small subunits of Rubisco. This study shows that CT binds to both Rubisco (plant protein) and bacterial cells, and both interactions lead to a decrease in the degradation rate of the large and small subunits of Rubisco.

An increase in dietary protein flow to the abomasum may also be due to the inhibitory effects of tannins on proteolytic bacteria and proteolytic enzyme activity. Besides, tannins might sometime also affect the efficiency of microbial protein synthesis. Tamarind (*Tamarindus indica*) seed husk (containing 14% tannins) improved the efficiency of microbial protein synthesis *in vitro* (Bhatta et al. 2001). Quebracho tannins also increased microbial protein synthesis in a lucerne diet fed to sheep at dosages of 2% and 3% of DM, but not at 3% of DM (Al-Dobaib 2009). However, some studies reported that the microbial protein outflow from the rumen was little affected due to feeding of tannin-containing forages (McNeill et al. 2000; Min et al. 2003).

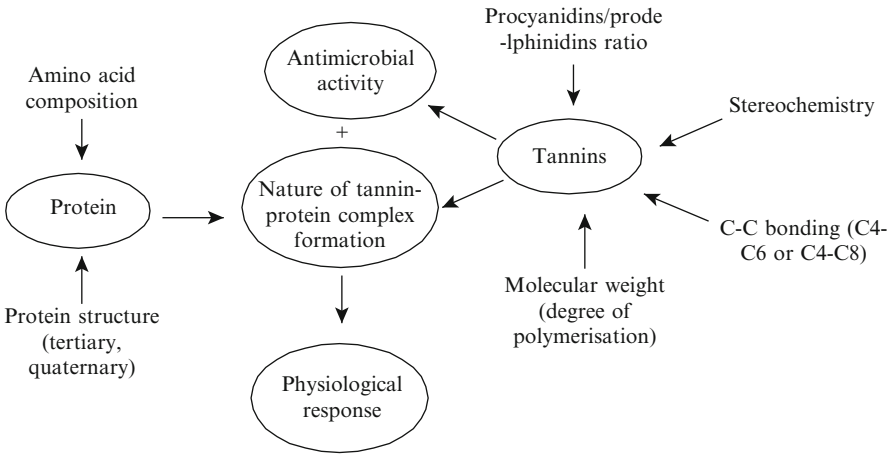

Fig. 8.2 Factors that may affect formation and dissociation of tannin-protein complex and antibacterial activities of tannins in the gastrointestinal tract (Adapted from Patra and Saxena 2011)

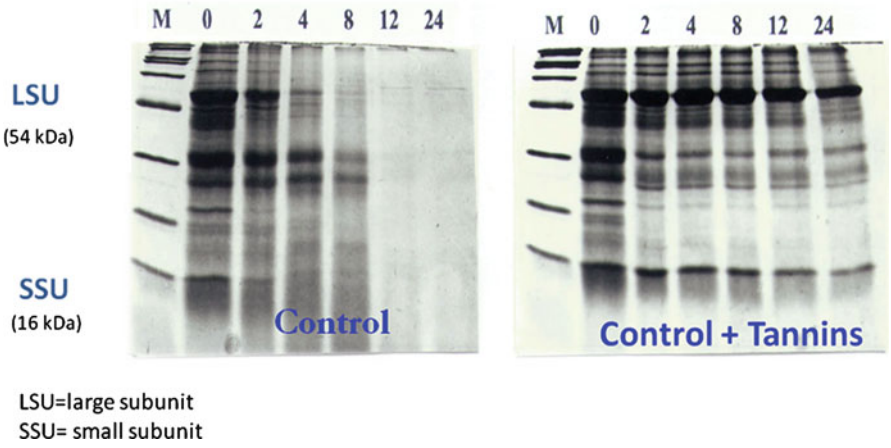

Fig. 8.3 The degradation of the large subunit (*LSU*) and small subunit (*SSU*) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) during *in vitro* incubation of total leaf protein extracted from white clover (*Trifolium repens*) with *Streptococcus bovis* rumen bacterial strains (105 cells innoculated) and condensed tannins extracted from *Lotus corniculatus* (birdsfoot trefoil). Incubations were performed with (**b**, CT-active) and without CT (**a**, CT-inactive). Samples were removed prior (0 h) and after 2, 4, 8, 12, and 24 h of incubation. The sample on the left side of each gel contains the broad range molecular weight (*MW*) markers (Bio-RAD, Hercules, CA; Min, 2012, unpublished data)

Proteolytic bacteria were present in relatively high numbers (10^8/g of digetsa) in sheep fed on *Calliandra* leaves (McSweeney et al. 1999). The changes in the activities of proteolytic bacteria and the protease enzyme by tannins could mediate nitrogen metabolism in the rumen (Tanner et al. 1994). A reduction in proteolysis might be attributed to the direct effects of CT on microbial proteolytic enzyme activity or

to the indirect effects on rumen metabolite concentration that can regulate proteolytic activity in some bacteria (Waghorn et al. (1994a, b). Rate of proteolysis by bacteria varies among bacterial species in the presence of CT. Min et al. (2005) reported that the proteolytic bacterial cultures without CT (from *L. corniculatus*) or with CT+ polyethylene glycol (PEG; which binds to tannins thus decreasing the effects of tannins) degraded large (LSU) and small (SSU) of Rubisco protein rapidly (6–8%/h). Cultures of *Prevotella ruminicola* C21a degraded both the LSU (11.3%/h) and SSU (9.8%/h) when grown in the presence of 1.5 g CT/L. Bacterial strains *S. bovis* B315 (11.5%/h) and strain NCFB 2476 (10.6%/h), *Eubacterium* sp. C12b (5.1%/h) and C124b (5.5%/h) degraded SSU more effectively than the LSU (2.6%, 2.3%, 3.8%, and 2.6%/h, respectively) in the presence of 1.5 g CT/L. *Fibrobacter succinogenes* S85, *Ruminococcus albus* 8, *P. ruminicola* 23, *Clostridium proteoclasticum* B316^T, *Butyrivibrio fibrisolvens* WV1 and C211a had low (0.3%/h) to moderate (3–4%/h) rates of proteolysis when CT was included in the medium (Min et al. 2005). The CT has been shown to inhibit endogenous enzyme activity (Oh and Hoff 1986; Horigome et al. 1988). It is suggested that at higher tannins/protein ratios, the inhibition of proteolysis is likely due to coating of the protein surface by the polyphenolic compounds (McManus et al. 1981), hence, leading to interference with the interaction of enzyme and substrate. Jones et al. (1994) studied the protease activity of four strains of ruminal bacteria as affected by CT from *Onobrychis viciifolia*. Total protease activity in cultures of *B. fibrisolvens* and *S. bovis* was reduced by 48% and 92%, respectively, at a concentration of 25 mg of CT/L; whereas, the total protease activity in cultures of *P. ruminicola* was 36% higher in the presence of 100 mg of CT/L than in the control. CT did not inhibit the protease activity of *Ruminobacter amylophilus* (formerly *Bacteroides amylophilus*) cultures at concentrations below 100 mg/L. The cell-associated protease activity in *S. bovis* was similar to that of the control up to 25 of mg CT/L), but the activity decreased considerably (32% of control) at 50 mg of CT/L. In contrast, there was no reduction in the cell-associated protease activity of *P. ruminicola* at any CT concentration up to 300 mg/L of CT.

Information on fungus on the degradation of tannin-protein complex is limited. A fungus, *Aspergillus niger* var Tieghem having tannin-protein complex-degrading activity was isolated from the faeces of such cattle fed largely on oak leaves (Bhat et al. 1996). In some animals, microbes responsible for degrading the tannin-protein complex have ecological advantages by cleaving the protein moiety from the complex. Tannin-protein complex degrading *S. bovis* biotype I and enterobacteria that digest tannin-protein complexes has been isolated from the faeces of koalas, *Phascolarctos cinereus* (Goldfuss), which almost solely fed on eucalyptus leaves with higher tannin content (Osawa 1992).

8.4 Effects of Tannins on Ruminal Methanogenesis

Methane produced during anaerobic fermentation in the rumen represents a 2–15% feed energy loss and contributes to greenhouse effects in the environment. Therefore, reducing methane emission has been a great interest for ensuring the sustainability

of ruminant production. Methane is produced normally during fermentation of feeds by methanogenic archaea. The removal of ruminal protozoa can also reduce methane production as some population of methanogens remains attached with protozoa (Hess et al. 2003). Tannin-containing forages and tannin extracts have been demonstrated to decrease methane production both *in vivo* and *in vitro* (Patra and Saxena 2010). The inhibitory effects of tannins on rumen methanogenesis have been ascribed due to direct effects on methanogenic archaea, protozoal associated methane production and indirectly through a depression of fiber digestion in the rumen. Animut et al. (2008a) also observed that feeding of different levels of Kobe lespedeza (*Lespedeza striata* K) decreased methane production linearly in goats and it has been attributed to the presence of CT (Animut et al. 2008b). Tavendale et al. (2005) reported that methane production (ml/g DM) at 12 h for *Medicago sativa* (alfalfa) containing negligible amount of CT (25 ml) was higher than *L. pedunculatus* (17.6 ml) that contains 0.10% CT and after addition of PEG increased methane production for *L. pedunculatus* (17%), but not for *M. sativa*. They also investigated the inhibitory effects of extractable CT fractions from *L. pedunculatus* on the common rumen methanogens *Methanobrevibacter ruminantium* strains YLM-1 and DSM1093. Oligomeric CT fractions did not have any influence on both strains, as determined by methane production measurements. The polymeric fraction completely inhibited methane production. Inhibitory effects in broth culture for strain YLM-1 were bacteriostatic, while strain DSM1093 did not recover growth, as indicated by methane production, even upon prolonged incubation. In a plate assay, the zone of inhibition with the polymeric fraction remained after a further week of incubation. Similarly, Huang et al. (2011) showed that the CT fraction with higher molecular weight (1,349 Da) isolated from *Leucaena* hybrid forage had pronounced effect on *in vitro* methane (62% inhibition) followed by lower molecular weight fractions. Besides, direct inhibitory to methanogens, tannins have been shown to lower protozoal numbers (Patra and Saxena 2009, 2011), which may also decrease protozoal associated methanogenesis (Finlay et al. 1994).

8.5 Effects of Tannins on Ruminal Biohydrogenation

Increasing the concentrations of conjugated linoleic acids (CLA) in foods derived from ruminants has been of recent interests in many researches due to its beneficial effects on health such as anti-cancer properties, reduced risks for cardiovascular diseases, reduction of body fat accretion and modulation of the immune system. The CLA represents *cis* and *trans* isomers of linoleic acid (C18:2) with conjugated double bonds, e.g. *cis*-9, *trans*-11 C18:2 (also called rumenic acid and a predominant isomer in meat and milk) and *trans*-10, *cis*-12 C18:2 (Bauman et al. 1999). The CLA concentrations in ruminant-derived foods can be increased by nutritional and management practices that facilitate higher fore-stomach output of CLA and vaccenic acid (trans-11 C18:1) for absorption and incorporated into animal tissues (Bauman et al. 1999). Many ruminal bacteria species of the genera *Butyrivibrio, Ruminococcus, Treponema-Borrelia, Micrococcus, Megasphaera, Eubacterium,*

Fusocillus and *Clostridium* are known to be associated in ruminal biohydrogenation of unsaturated fatty acids (Harfoot and Hazlewood 1997; Maia et al. 2007; Durmic et al. 2008). *Butyrivibrio* group are most active species among the group A bacteria, which form CLA from linoleic acid, while few species of bacteria such as *Fusocillus* spp. and *C. proteoclasticum* (group B) converts vaccenic acid to stearic acid (Maia et al. 2007; Paillard et al. 2007; Durmic et al. 2008). Therefore, it has been suggested that selective inhibition of group B bacteria without affecting group A bacteria may provide more vaccenic acids and CLA (Harfoot and Hazlewood 1997; Durmic et al. 2008).

The ability of plant extracts including tannins to modify the fatty acid composition of ruminant-derived food products (i.e. milk or meat) has received great attention recently. Sivakumaran et al. (2004) demonstrated that all three fractions (i.e. low, medium and high molecular weight) of proanthocyanidins from *Dorycnium rectum* forage (a perennial legume shrub) inhibited the growth of *C. proteoclasticum* at concentrations of 100, 200 and 300 mg/L of *in vitro* medium, whereas, its low and medium molecular weight fractions inhibited the growth of *B. fibrisolvens* at these concentrations, while the high molecular weight fraction stimulated the growth of *B. fibrisolvens* at the concentration of 100 mg/L. The effects of tannins on biohydrogenation by rumen microbial populations have been discussed in details in Chap. 9.

8.6 Effects of Tannins on Rumen Microbial Populations

8.6.1 Bacteria

Phenolic compounds are used as antimicrobial agents in an array of products, including food, paint, leather, metal working fluids, textiles and petroleum (Marouchoc 1979). The ability of an array of phenolic compounds, e.g. ferulic acid, CT (tea catechins), oleuropein, HT (ellagic acid) and p-coumaric acid, to inhibit the growth of bacteria (*Salmonella enteritidis*, *Staphylococcus aureus*, *Listeria monocytogenes*) and fungi in milk has been reported (Payne et al. 1989; Tassou and Nychas 1994, 1995; Rosenthal et al. 1997, 1999; Apostolidis et al. 2011). A number of studies showed that tannins also selectively inhibit the growth of microorganisms in the digestive tracts depending on the types of tannins. A long time ago, Tagari et al. (1965) reported that cellulolytic and proteolytic activities were inhibited in the presence of carob tannins (mainly CT types) in artificial rumen. The gallotannins strongly inhibited cellulolytic activity, but had slight effect on proteolysis (Tagari et al. 1965). Similarly, CT of *L. corniculatus* reduced the populations of *C. proteoclasticum*, *B. fibrisolvens, Eubacterium* spp. and *S. bovis* in sheep (Min et al. 2002). In a pure culture study, CT of *L. corniculatus* inhibited the growth of *F. succinogenes* at a concentration of 400 mg/L, but had no appreciable inhibitory effect on the growth at concentrations below 400 mg/L (Bae et al. 1993). The effects of tannins on ruminal

bacteria are reported to be species-dependent. Jones et al. (1994) studied the effects of CT of the legume sainfoin (*O. viciifolia*) on growth and proteolysis by four strains of ruminal bacteria. They observed that growth and protease activities of *B. fibrisolvens* and *S. bovis* were reduced by CT, but had little effect on a strain of *P. ruminicola* and *R. amylophilus*. Sivakumaran et al. (2004) reported that antibacterial activity of proanthocyanidin from leaves of the forage legume *D. rectum* ranged from the very sensitive to *R. albus* and *Peptostreptococcus anaerobius* to the less sensitive to *Clostridium aminophilum*, *C. proteoclasticum* and *B. fibrisolvens*. Addition of 200, 400 and 600 mg CT/L significantly reduced the growth rate of most bacterial strains (*S. bovis* NCFB 2476 and B315, *B. fibrisolvens* strains WV1 and C211, *P. ruminicola* 23, *Prevotella*-like strain C21a, *Eubacterium* spp. C12b and C124b and *F. succinogenes* S85) except *C. proteoclasticum* B316^{T}and *R. albus* 8, which showed transient increases in their growth rate at low (50–100 mg/L), but not at high (>200 mg/L) concentrations of CT. Morphological changes occurred in the bacteria that were inhibited by tannins, whereas, an extracellular material produced by *P. ruminicola* probably protected the bacteria from the direct action of tannins (Jones et al. 1994). Similarly, addition of phlorotannins from *Ascophyllum nodosum* (brown seaweed) at 0.5 mg/mL to rumen cultures inhibited the growth of *F. succinogenes*, but had minimal effect on *Ruminococcus flavefaciens* and *R. albus*, whereas, the growth of *Selenomonas ruminantium, S. bovis, R. amylophilus*, and *Prevotella bryantii* was stimulated (Wang et al. 2009).

The shifts of microbial populations in the digestive tracts generally occur in animals fed on tannin-containing diets because of selective inhibition of bacteria and tolerance of some bacteria to tannins. Plumb et al. (2000) demonstrated that members of the *Cytophaga-Flexibacter-Bacteroides* group predominated over members of the low G+C Gram-positive bacteria in both the sheep and the goats (78% versus 21%, and 82% and 11%, respectively) fed with *A. aneura* forage, while members of the low G+C Gram positive bacteria predominated (74% versus 25%) in the sheep fed grass. In a rat model, Smith et al. (2005) noted that the proportion of the low G+C Gram positive bacteria decreased, while the *Bacteroides-Prevotella-Porphyromonas* group and *Enterobacteriaceae* predominated in faeces of rats fed an *A. angustissima tannin*-containing diet. The levels of lactobacilli increased, whereas, the numbers of total bacteria and Bacteroidaceae decreased in the feces of 30-days-old pigs fed 0.2% tea polyphenols (Hara et al. 1995). Furthermore, Rosenthal et al. (1997, 1999) reported that tea CT (catechins) and ferulic acid inhibit the growth of pathogenic bacteria (coliforms and *Salmonella*) with little effect on lactic acid bacteria.

Tannins may also reduce the archaeal populations and/or activity directly or indirectly in the rumen depending upon the concentrations and type of tannins (Patra and Saxena 2010, 2011). There are also species and strain differences of methanogens showing sensitiveness to different molecular fractions of CT (Tavendale et al. 2005). The number of methanogens in the *Methanobacteriales* order decreased quadratically with increasing doses of CT from *Leucaena leucocephala* leaves (Tan et al. 2011a). The tannins modify the diversity of methanogens in the rumen probably due to greater sensitivity of some methanogens to tannins. For instance, Tan et al. (2011b) reported that the 16S rRNA gene library of the CT-fed animals had

lower proportions of archaea from the orders *Methanomicrobiales* (32% vs. 16.9%) and *Methanobacteriales* (8.5% vs. 1.7%) as compared to those found in the CT treatment clone library in both orders (32% and 8.5%, respectively).

8.6.2 Fungus

Muhammed et al. (1995) investigated the effects of tannic acid, ellagic acid, gallic acid and catechin on rumen fungus *Neocallimastix frontalis* strain RE1. All these compounds inhibited the cellulolysis and zoospore attachment to cellulose by *N. frontalis*. Gallic acid, ellagic acid and catechin showed more inhibitory effect to cellulolysis than tannic acid. However, ellagic acid was most inhibitory to zoospore attachment, perhaps indicating the involvement of different cell-surface receptors in this process. Paul et al. (2003) reported that fungus could grow at tannic acid concentration up to 20 g/L and the growth was not appreciably affected up to 10 g/L concentration acid. However, fibre-degrading ability of rumen fungi may be less sensitive to the inhibitory effects of CT compared to cellulolytic bacteria (McSweeney et al. 1998). Salawu et al. (1999) showed that quebracho tannins (5% of DM) reduced cellulase and xylanase activities of the rumen microbes and total rumen protozoa in sheep fed a grass-barley diet.

8.6.3 Protozoa

Effects of tannins on rumen protozoa are variable. Newbold et al. (1997) investigated that tannins were not responsible for the anti-protozoal activity of *Sesbania sesban.* Salem et al. (1997) observed a linear increase in protozoal numbers in rumen fluid of sheep fed on a lucern-hay based diets by addition of increased proportion of *Acacia cyanophylla* Lindl. foliage, which contained 4.5% CT. Similarly, CT present in *L. corniculatus* and *Hedysarum coronarium* (sulla) increased protozoal numbers in the rumen of sheep (Chiquette et al. 1989; Terrill et al. 1992). There are many reports indicating inhibitory effect of tannins on rumen protozoa. Makkar et al. (1995a) reported that quebracho CT (0.1–0. 4 mg/mL media) significantly reduced the numbers of total protozoa, entodiniomorphs and holotrichs, the effect being higher on holotrichs in Rusitec system. Monforte-Briceno et al. (2005) screened the defaunating properties of 15 multipurpose tree fodders, which generally contain tannins. Out of the 15 plants, inhibitory effect on protozoa was due to *Acacia farnesiana*, *Calliandra calothyrsus* and *Lysiloma latisiliquum* that contained CT higher than 1% of dry matter. Tannins extracted with ethanol and methanol from *Terminalia chebula* decreased total protozoa as well as large and small entodiniomorph (Patra et al. 2006). Tannins contained in kobe lespedeza also decreased protozoal number linearly in goats without affecting the total and cellulolytic

bacterial counts (Animut et al. 2008a). Benchaar et al. (2008) did not observe any effect on protozoal numbers in dairy cattle fed quebracho tannins (CT concentrations of 70%, 150 g/day) probably due to addition of a low dosage in the diet. Although rumen protozoa contribute to fiber digestion, there are studies demonstrating increased digestibility of fibrous feeds and the potential for improvement in ruminant productivity maintaining the defaunated state because protozoa increase microbial protein turnover in the rumen, which reduces the efficiency of protein utilization in ruminants (Wallace and McPherson 1987). Protozoal associated methanogenesis accounts for about 37% of rumen methanogenesis (Finlay et al. 1994). It has been suggested that many of the negative effects of defaunation may be transitory and disappear as the bacterial and the fungal populations increase and occupy the niches previously filled by the protozoa (Williams and Coleman 1997). Therefore, removal of protozoa has been a target of rumen microbiologists for rumen manipulation. The anti-protozoal effects of tannins might be beneficial for improving protein utilization and inhibiting methanogenesis in the rumen. However, the mechanisms of inhibition of rumen protozoa by tannins are not clearly known.

Feeding of tannin-containing forages may also show beneficial effects on shedding of pathogenic microbes in faeces depending upon dietary levels and sources of tannins. Min et al. (2007) demonstrated that chestnut tannins (15 g of tannins per day) infused intra-ruminally to steers fed on Bermuda grass hay diet decreased fecal shedding of *Escherichia coli*. Similarly, Berard et al. (2009) reported that tannin-containing sainfoin (*O. viciifolia*) forage (1.2% CT) reduced fecal *E. coli* numbers compared with alfalfa hay in cattle. However, feeding of tanniferous Sericea lespedeza (*Lespedeza cuneata*) forage to goats did not affect fecal shedding of *E. coli* and total coliform numbers in goats (Lee et al. 2009). There are contradictory reports on the effects of tannins on beneficial bacteria in the intestine. Hara et al. (1995) reported that a diet containing 0.2% tea tannins for 2 weeks resulted in significantly increased levels of lactobacilli and a decrease in the levels of total bacteria and *Bacteroidaceae* in the feces of pigs. In contrast, Salem et al. (2010) from *in vitro* study suggested that phenolic compounds present in *Acacia saligna* may be inhibitory to lactobacilli in the intestine.

8.6.4 Mechanisms of Antimicrobial Effects of Tannins

Several mechanisms have been proposed to explain the antimicrobial properties of tannins. The antimicrobial effects of phenolic compounds are probably related to the inhibition of bacterial enzymes, alterations in cell wall permeability, an increase in the hydrogen ion activity of the microbial environment, a reduction in the surface and/or interfacial tension and perhaps chelation of essential minerals, particularly iron with a concomitant impairment of the microbial oxidative metabolic system (Chung et al. 1998). The antimicrobial activities of tannins are ascribed to the interactions of goats tannins with the extracellular enzymes secreted and

the cell wall of bacteria causing morphological changes of the cell wall, destabilization of cytoplasmic and plasma membranes, direct action on microbial metabolism through inhibition of oxidative phosphorylation, deprivation of substrates for microbial growth and chelation of cations by tannins reducing its availability to microbes (Kumar and Vaithiyanathan 1990; Scalbert 1991; Jones et al. 1994; Smith et al. 2005). Catechins have been shown to cause leakage from liposomes disrupting membrane integrity (Ikigai et al. 1993). *F. succinogenes* grown on *L. corniculatus* CT (100–300 mg/L) had large amounts of surface materials, which were suggested to the formation of tannin-protein complex on the cell surface (Bae et al. 1993). Cell associated and extracellular endoglucanase activities of *F. succinogenes* were inhibited by CT (0.1–0.4 mg/mL) from *L. corniculatus* under *in vitro* conditions (Bae et al. 1993). It has been stated that tannins from carob pod extract change the morphology of bacteria to produce antimicrobial activity (Heins et al. 1964). Similarly, CT fractions from *O. viciifolia* leaf caused morphological changes in tannin-sensitive bacteria *S. bovis* and *B. fibrisolvens* indicating the action of CT on the cell wall (Jones et al. 1994). Chung et al. (1998) demonstrated that tannic acid inhibited the growth of some human intestinal bacteria, but not lactic acid bacteria. In their studies, addition of iron in the medium restored the growth of *E. coli*, which indicates that the iron-chelating properties of tannins contributed to the inhibitory activities. Although methylgallate and propylgallate inhibited the growth of the intestinal bacteria, iron was not responsible for the growth inhibition (Chung et al. 1998). However, cellulolytic activity of rumen inoculum was not found to be affected by chestnut tannins (Zelter et al. 1970). This indicated that different sources of tannins might have different anti-microbial effects. Flavonols with a trihydroxy B ring (gallocatechins) have generally more inhibitory effects on the non-rumen microbial species than catechin having a dihydroxy B ring (Sakanaka et al. 1989). It has been further suggested that the toxicity of tannins towards micro-organisms correlates with their molecular weight. Therefore, the toxicities of epicatechin gallate and epigallocatechin gallate towards *Clostridium botulinum* were greater than that of their ungallated counterparts – epicatechin and epigallacatechin (Okuda et al. 1985). McAllister et al. (1994) reported that CT of *L. corniculatus* caused a considerable detachment of *F. succinogenes* S85 from colonised filter paper after a 30 min exposure. Inclusion of 30% *Calliandra* leaves in the diet significantly reduced rumen cellulolytic bacteria including *F. succinogenes* and *Ruminococcus* spp. without affecting the total protozoal population, fungi and proteolytic bacteria (McSweeney et al. 1998, 2001b).

8.7 Tannins: Fiber Digestion by Micro-organisms

Tannins have profound inhibitory effects on the fibrolytic bacterial populations in the rumen at high doses depending upon the type of tannins, thus reducing the fiber digestion and decreases fiber digestibility. The presence of *Calliandra* tannins in the

diet (2–3% tannin) reduced the population of fibre degrading bacteria (McSweeney et al. 2001a). The major fiber degrading bacteria in the rumen such as *F. succinogenes*, *R. albus* and *Runinococcus flavefaciens* have been found to be inhibited by tannins although degree of inhibition varied among the studies depending upon the dose and type of tannins. For instance, Singh et al. (2011) noted that feeding of *Ficus infectiria* leaves containing 8–12% CT at 50% of the diet of goats did not decrease the number of *F. Succinogenes*, but reduced the number of *R. flavefaaciens*. Bae et al. (1993) studied the effects of CT extracted from *L. corniculatus* on the fiber degrading *F. succinogenes*. The proanthocyanidin from leaves of *D. rectum* was very sensitive to *R. albus* , but were less sensitive to *B. fibrisolvens* (Sivakumaran et al. 2004). Phlorotannins at 0.5 mg/mL inhibited the growth of *F. succinogenes*, but had minimal effect on *R. flavefaciens* and *R. albus* (Wang et al. 2009). Addition of 200, 400 and 600 mg CT/L significantly reduced the growth rate of *B. fibrisolvens* and *F. succinogenes* (S85); however the growth rate of *R. albus* transient increased at low (50–100 mg/L), but not at high (>200 mg/L) concentrations of CT (Min et al. 2005). The CT reduced the extracellular endoglucanase activity at concentrations as low as 25 mg/L; whereas, cell associated endoglucanase activity increased at CT concentration of up to 300 mg/L and then decreased at 400 mg/L. The exposure of *F. succinogenes* to CT appears to cause the formation of tannin-protein complex on the cell surface, which is suggested to interfere with the adhesion process of bacterial cells to the cellulose.

8.8 Resistance of Gut Micro-organisms to Tannins

8.8.1 Tannin Tolerant Microbes

Initially, many strains of *Streptococci* that degraded tannin-protein complexes were isolated from the feces of koalas and other animals (Osawa 1990; Osawa and Sly 1991). These strains, which ferment mannitol, were conventionally classified as *S. bovis* biotype I strains to distinguish them from biotype II strains, which do not ferment mannitol. Osawa and Walsh (1993) demonstrated that many *S. bovis* biotype I strains produce an enzyme, tannase, which hydrolyzes tannins to release gallic acid. Subsequently, it was noted that all strains of *S. bovis* that exhibited gallate decarboxylase activity belonged to a single DNA homology group described as *Streptococcus gallolyticus* (Osawa et al. 1995a). *S. gallolyticus* had been isolated from several environments, including the feces of koalas, kangaroos, possums, cows, horses, pigs, dogs, and guinea pigs, as well as animals with bovine mastitis, human clinical sources, and the sheep rumen (Sly et al. 1997).

Several species of tannin tolerant or degrading bacteria have been isolated from *Enterobacteriaceae* and genera *Streoptococcus* and *Selenomonas* from the gastrointestinal ecosystems of different animals including humans, laboratory and zoo animals (Table 8.1; Nelson et al. 1998; Odenyo and Osuji 1998; Skene and Brooker 1995; Odenyo et al. 2001), which enable animals to thrive in high tannin-rich diets.

Table 8.1 Tannin-tolerant bacteria isolated from different animals

Isolate	Animals	Morphology	Gram stain	Tolerance to tannins (g/L)	Tannase	References
Streptococcus bovis JB1	Cattle	Cocci	Positive	TA, 2; CT of *A. aneura*, 1.5	Negative	Odenyo et al. (2001)
Streptococcus ES1	Sheep	Cocci	Positive	TA, 30; CT of *A. aneura,* 4	Negative	Odenyo et al. (2001)
Butyrivibrio sp. EAT6	Antelope	Curved rods	Negative	TA, 60; CT of *A. aneura*, 8	Negative	Odenyo et al. (2001)
S. ruminantium D	Cattle	Curved rods	Negative	TA, 4; CT of *A. aneura*, 1.5	Positive	Odenyo et al. (2001)
S. ruminantium K2	Feral goat	Curved rods	Negative	TA, 20	Positive	Skene and Brooker (1995)
Selenomonas sp. EG4.2	Goat	Curved rods	Negative	TA, 70	Positive	Odenyo et al. (2001)
Streptococcus gallolyticus	Koalas	Cocci	Positive		Positive	Osawa et al. (1995a)
S. gallolyticus (*caprinus*)	Goats	Cocci	Positive	TA, 25	Positive	Brooker et al. (1994)
Selenomonas sp. ES3	Sheep	Curved rods	Negative	TA, 70; CT of *A. aneura*, 8	Positive	Odenyo and Osuji (1998)
Selenomonas sp. EG19	Goat	Curved rods	Negative	TA, 50–60; CT of *A. aneura*, 8	Positive	Odenyo and Osuji (1998)
Selenomonas sp. EAT2	Antelope	Curved rods	Negative	TA, 50–60; CT of *A. aneura*, 8	Positive	Odenyo and Osuji (1998)
Streptococcus sp. KN1	Goat	Cocci	Positive	TA, 30; *Desmodium* tannins, 0.8	–	Nelson et al. (1998)
Streptococcus sp. KN2	Sheep	Cocci, chain	Positive	TA, 2; *Desmodium* tannins, 0.7	–	Nelson et al. (1998)
Streptococcus sp. KN3	Goat	Cocci, single	Positive	TA, 2; *Desmodium* tannins, 0.6	–	Nelson et al. (1998)
Streptococcus sp. TW1	Elk	Cocci, chain	Positive	TA, 8; *Desmodium* tannins, 0.6	–	Nelson et al. (1998)
Eubacterium sp. TW2	Elk	Rod	Positive	TA, 2; *Desmodium* tannins, 0.5	–	Nelson et al. (1998)
Escherichia sp. KN4	Deer	Rods	Negative	TA, 2; *Desmodium* tannins 0.4	–	Nelson et al. (1998)

TA tannic acid, *CT* condensed tannins

The tannin-tolerant bacteria are present in the digestive tracts of animals irrespective of the geography, climate and host (Nelson et al. 1998). Tannin-tolerant bacteria could be both Gram positive and negative with cocci, straight or curved rods types. Brooker et al. (1994) isolated a HT and CT tannin-resistant *Streptococcus caprinus* (a subjective synonyms of *S. gallolyticus*; Sly et al. 1997) from feral goats browsing *Acacia aneura* (containing 11–14% tannins), which was absent in domestic goats and sheep. *S. caprinus* was able to grow in the medium containing 2.5% (w/v) tannic acid. *S. gallolyticus* and *S. caprinus* produces lactate, and small amount of acetate and ethanol and were unable to grow in the presence of ammonia only (Brooker et al. 1994; Osawa et al. 1995a). The trans-inoculation of these bacteria using rumen fluid from feral goats to sheep fed on *Acacia* improved DM intake and nitrogen digestibility in sheep and the population was maintained as long as the animal was fed the *Acacia* diet. Another anaerobic diplococcoid bacterium, isolated from the rumen of goat fed on *Desmodium ovalifolium* (contains 17% CT), was able to grow in the presence of up to 30 g of tannic acid and 4 g of *Desmodium ovalufolium* CT per litre of media and this bacterium degraded tannic acid to pyrogallol (Nelson et al. 1995). Nelson et al. (1998) isolated six tannin tolerant isolates from sheep, goat, mountain elk and white-tail deer. The 16S rRNA sequence analysis showed that four of the isolates (isolated from goat, sheep and elk) were members of the genus *Streptococcus,* and were most closely related to *S. bovis* and *S. gallolyticus*. One of the other isolates (elk), a Gram positive rod, clustered with the clostridia in the low G+C group of Gram positive bacteria. The sixth isolate (deer), a Gram negative rod, was a member of the family *Enterobacteriaceae* in the gamma subdivision of the class *Proteobacteria*. Similarly, Odenyo and Osuji (1998) isolated three strains of tannin-tolerant bacteria related to *Selenomonas* sp. from sheep, goat and antelope, which were able to grow in media containing 50 g/L of a tannin extract and 50–70 g/L of tannic acid. The presence of tannins tolerant bacteria in the digestive tracts may depend upon the previous exposure to tannins. Tannin tolerating bacteria were not isolated from sheep, goats, cows, which had no history of tannin consumption (Brooker et al. 1994; Nelson et al. 1998).

Inoculation of tannin tolerant bacteria to animals fed high tannin-containing diets have shown to improve utilization of nutrients and performance of animals. For example, feral goats in Australia tolerated diets containing *Acacia aneura* (mulga; contains 5–25% CT) better than sheep tolerated such diets. Rumen fluid (Miller et al. 1995) or an *in vitro* cultured rumen inoculum (Miller et al. 1997) from goats improved nitrogen digestion in sheep fed *A. aneura*. Similarly, rumen inoculation with tannin-resistant Gram positive rod to sheep fed peanut skin CT (7.1%) showed a positive effect on nitrogen balance compared with sheep that did not receive this inoculum (Molina et al. 1999). Wiryawan et al. (2000) also demonstrated that inoculation of a pure culture of an uncharacterized tannin-resistant bacterium from Indonesian goats increased body weight gain over 40 days in goats changed from a grass diet to a 100% *Calliandra calothyrsus* diet (containing 6–10% CT), compared to the body weight gain of uninoculated goats. However, inoculation of a pure culture of tannin-resistant *S. caprinus* was ineffective to improve the digestion of *A. aneura* forage in sheep (Miller et al. 1996).

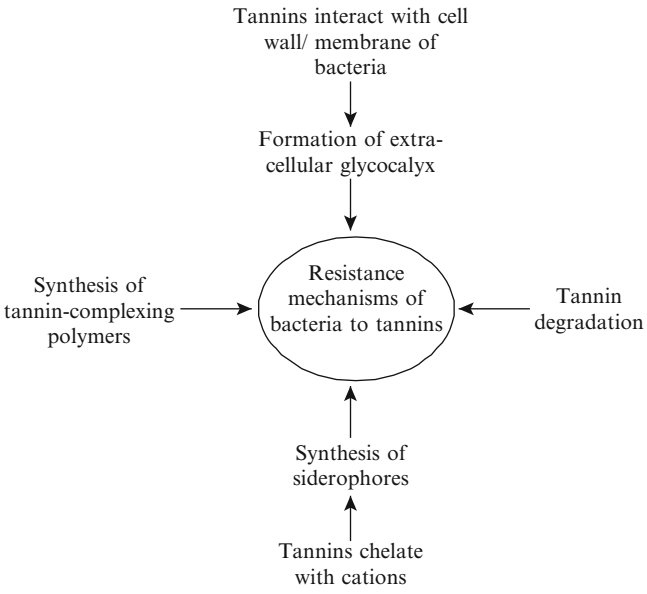

Fig. 8.4 Tolerance mechanisms of bacteria to dietary tannins (Adapted from Patra and Saxena 2011)

8.8.2 Resistance Mechanisms of Microbes to Tannins

Several mechanisms have been proposed for the resistance mechanisms of the bacteria to tannins (Fig. 8.4). Some bacteria grown in the presence of tannin secrete exo-polysaccharides (glycoproteins) that form a protective layer around the cells and thus protect the cells from the action of tannins (O'Donovan and Brooker 2001; Krause et al. 2005). Chiquette et al. (1988) demonstrated using transmission electron microscopy that ruminal bacteria produced a thick glycocalyx when grown in association with the high tannin containing *L. corniculatus* forage. The gene expression study in *E. coli* revealed that cytosolic and cell membrane damage caused by tannins may be overcome through an increase expression of cell envelop stress protein genes responsible for these protein synthesis (Zoetendal et al. 2008). Besides, the morphology of bacteria may be changed in the presence of tannins in media (O'Donovan and Brooker 2001). Goel et al. (2005) also observed that *Streptococcus* species isolated from non-adaptive cattle were converted from diplococi to an elongated chain of 40–50 cells with increasing concentrations of tannin acid in the media. Tolerance mechanism of bacteria to tannins may also involve degradation of tannins by secretion of tannase enzyme. The tannin-degrading microorganisms have been isolated from several species of ruminants and non-ruminants including sheep, goat, deer, horse, pigs and elk that have access to tannin rich forages (Sly et al. 1997; Odenyo and Osuji 1998). Odenyo et al. (2001) observed that some tannin-tolerant bacteria were not tannase-positive. Some intestinal bacteria such as

Lactobacillus and *Bifidobacterium* were inherently resistant to tannic acid because heme-containing enzymes are not present in these bacteria (Chung et al. 1998). Tannic acid has a strong iron binding capability, which depletes iron from media for the growth of bacteria having heme-containing enzymes (Chung et al. 1998). Besides, bacteria grown in the presence of tannins may synthesize siderophores (Scalbert 1991), which probably counteract the depletion of iron by tannins. Besides the development of tolerance mechanisms by the rumen bacteria, animals itself may develop tolerance to tannins by secreting increased amounts proline-rich proteins in the saliva (Mehansho et al. 1983). These proteins have strong affinity for tannins and may be constitutive or inducible depending upon the species of the animals (Mueller-Harvey 2006). The secretion of proline-rich proteins may diminish the responses of tannins to the N metabolism.

8.9 Metabolism of Tannins by Microorganisms in the Digestive Tract

The microorganisms of gastrointestinal ecosystems of animals are able to degrade HT and simple plant phenolic compounds to variable extent depending upon the types of phenolic compounds and species of microbes to counteract anti-nutritive effects of tannins (Fig. 8.5). Phenolic glycosides such as rutin and naringin, and flavonoid ring system of the common plant flavonoids (e.g. quercitrin, kaempferol and naringenin) are readily degraded by the rumen and intestinal micro-organisms by hydrolysis of the glycoside and cleavage of the heterocyclic ring to acetate, butyrate, di- and monohydroxyphenolics, and phloroglucinol (Simpson et al. 1969; Winter et al. 1989; Lowry and Kennedy 1996; Schoefer et al. 2003). The HT are primarily degraded by an enzymes, tannase (tannin acyl hydrolase, EC 3.1.1.20) by bacteria and fungi, which is active in galloyl residues of galloyl esters, hexahydroxydiphenoyl groups and other ellagitannins. It has both esterase and depsidase activities. The tannase-positive microbes have been reported in the cattle, sheep, goats, koalas, deer and humans, which consume tannin-containing diets (Skene and Brooker 1995; Osawa et al. 1995b, 2000). The ester bonds and depside bonds of HT are both cleaved in the rumen by *S. ruminantium* and *Streptococcus* spp. by tannase, (Skene and Brooker 1995; Nelson et al. 1998; Bhat et al. 1998; Goel et al. 2005) releasing glucose, gallic acid and ellagic acid. However, hydrolysis of tannic acid may sometime not involve the production of tannase (Odenyo et al. 2001). Gallic acid is then decarboxylated to pyrogallol and converted to resorcinol and phloroglucinol before cleavage of the phloroglucinol ring to acetate and butyrate by rumen micro-organisms (Murdiati et al. 1992; Bhat et al. 1998). Tsai and Jones (1975) isolated *Streptococcus* strains and three *Coprococcus* strains from the bovine rumen that were capable of degrading up to 80% of phloroglucinol within 2 days, where phloroglucinol was present as the only added carbon source. *Eubacterium oxidoreducens*, a strictly anaerobic rumen bacterium that degrades gallate, phloroglucinol, and pyrogallol to acetate and butyrate in the presence of hydrogen and formic

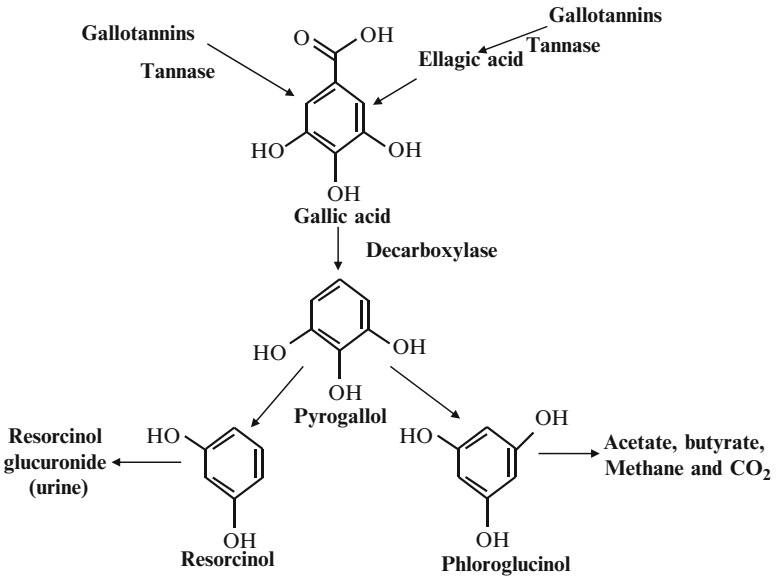

Fig. 8.5 Pathways for degradation of hydrolysable tannins in the rumen (Modified from Bhat et al. 1998)

acid (Krumholz and Bryant 1986). These simple monomeric compounds might be absorbed from the alimentary tracts and excreted through urine as conjugates. Sheep fed tannic acid and HT from leaves of *Terminalia oblongata* (containing ellagitannins) excreted glucuronides of resorcinol and 2-carboxy-2′,4′,4,6-tetrahydroxy diphenyl 2,2′-lactone as urinary metabolites (Murdiati et al. 1992). Compared to gallotannins, the ellagitannins are more difficult to be degraded by microbes than gallotannins due to their complex structures (Mingshu et al. 2006).

The colonic microbial populations also degrade HT to gallic acid, pyrogallol, phloroglucinol, and finally to acetic and butyric acid by sequential activities of different enzymes. *Enterococcus faecalis*, which inhibits gastrointestinal tracts of animals and humans has shown to produce tannase enzyme and degrade tannic acid to gallic acid, pyrogallol and resorcinol (Goel et al. 2011). The ellagitannins is degraded into ellagic acid and then transformed to urolithin B by colonic microbiota of humans and rats, which is detected as glucuronide form of urolithin B (Cerda et al. 2003). Catechin and gallocatechin can be degraded to velerolactone and phenylpropionic acid by intestinal micro-organisms, and glucuronic acid or sulphate conjugates of velerolactone have been observed to excrete through urine and faeces in rats and human (Hollman 2001; Mueller-Harvey 2006).

In contrast to HT, the metabolism of CT in the gastrointestinal tract is not clear. Some quantitative nutritional trials have shown apparent losses of 29.1% and 51.7% of CT from *Acacia barteri* and *Bauhania varigata*, respectively, up to 24 h of fermentation *in vitro* with no further increase in the degradability of CT after 24 h (Makkar et al. 1995b; Paul et al. 2006). With radioactively labeled CT,

Perez-Maldonado and Norton (1996) detected substantial losses of ^{14}C-labelled CT from *Desmodium intortum* in the gastrointestinal tract of sheep and goats. Wiryawan et al. (2000) noted that tannin degrading bacteria isolated from goats exposed to *Calliandra* tannins were able to decrease tannic acid and CT from the medium. It has been suggested that low molecular weight free CT or breakdown products of CT may be absorbed from the digestive tract (Perez-Maldonado and Norton 1996; Deprez et al. 2000; Serrano et al. 2009). For example, Deprez et al. (2001) observed that catechin, a proanthocyanidin dimer and trimer had similar permeability coefficients in intestinal cells *in vitro*, close to that of mannitol (a marker of paracellular transport); whereas, permeability of a oligomeric proanthocyanidin with an average polymerisation of six (molecular weight = 1,740) was approximately ten times lower. However, the absorption of low molecular weight proanthocyanidins from the digestive tract depends upon their properties. Jimenez-Ramsey et al. (1994) showed that proanthocyanidins soluble in water and ethanol were absorbed from the intestine, while proanthocyanidin fractions soluble in aqueous acetone but insoluble in water and ethanol were not absorbed in chicks. Further, it has also been postulated that apparent disappearance of CT from the digestive tract can be a result of minor conformational changes in the reactive CT ring structure and consequently these polyphenols are not quantified by the analytical techniques (Terrill et al. 1994; Perez-Maldonado and Norton 1996; Waghorn 2008). There may also be an underestimation of CT due to low recovery of CT in aqueous phase as CT may strongly bind with dietary macromolecules in the intestine (Deprez et al. 2000; Serrano et al. 2009). Although, the cleavage of carbon-carbon bonds of flavan-3-ols ring system (e.g. catechin and epicatechin) of CT by rumen and intestinal microorganisms has not been confirmed (McSweeney et al. 2001a; Serrano et al. 2009), there is evidence from the studies in simple stomach animals that a small amount of CT may be degraded into aromatic acid metabolites by the large intestinal microbiota (Gonthier et al. 2003; Deprez et al. 2000).

The tannins that reach the large intestine could be degraded by colonic microorganisms. The studies on metabolism of tannins by intestinal microbiota in ruminants are limited. Colonic microbiota of humans apparently metabolized the polymeric CT extensively after 48 h of incubation under *in vitro* anaerobic conditions (Deprez et al. 2000). Phenylacetic, phenylpropionic and phenylbutyric acids were the main metabolites detected, although, they represented only 2.7% of the initial radioactivity from the substrate (Deprez et al. 2000).

8.10 Conclusions

Tannins interact with proteins predominately via hydrogen bonds forming tannin-protein complexes thus preventing degradation of protein in the rumen, and these tannin-protein complexes are dissociated in the abomasum releasing protein. Tannins might also inhibit the growth of proteolytic bacteria, which can reduce proteolysis.

These increase non-ammonia N flow in the intestine for absorption. Moderate concentrations (depending upon the type of tannins) of tannins in diets improve body weight and wool growth, milk yields and reproductive performance. However, these effects have not been consistently observed. These discrepancies among studies are attributed to the mode of tannin-protein interactions, which do not always work in an optimal mode depending upon the chemical structures of tannins and protein. While tannins may shift N metabolism from rumen to intestine, the effects of tannins on large intestinal protein metabolism and microbial populations are not known. Recently, inclusion of tannins in diets has been shown to enrich CLA content in meat and milk. Tannins can exert beneficial effect environmentally by shifting N excretion from urine to faeces and decreasing methane output. Tannins lower methane production probably by directly inhibiting the activities of methanogenic archaea and/or reducing the fiber digestion in the rumen. Not all types of tannins produce beneficial nutritional and environmental responses. The elucidation of structure-activity relationships would be required to obtain consistent beneficial tannin effects in ruminants.

Alternative internal parasites management and food-borne pathogen control strategies using plant containing tannins and polyphenolic extracts have recently been suggested. It seems possible that consumption of plant tannins may reduce gastrointestinal parasites numbers, food-borne pathogens populations and improve animal performance through direct and indirect mechanisms.

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Chapter 9
Manipulating Ruminal Biohydrogenation by the Use of Plants Bioactive Compounds

Valentina Vasta and Rui J.B. Bessa

Abstract Ruminal microbial community is responsible for the biohydrogenation (BH) of the dietary unsaturated fatty acids ingested by ruminants. This process results in the production of saturated fatty acids (SFA) at the expenses of the unsaturated fatty acids (UFA). Animal scientists are attempting different possible strategies to manipulate ruminal BH process, in order to obtain meats and milk with a lower SFA content, which would be of great value for consumers' health. To avoid the use of synthetic molecules, such as some drugs or additives in livestock farming, animal scientists are focusing on the use of plant bioactive compounds (PBC) as modulators of ruminal BH. This manipulation is performed through a direct action of PBC on the bacterial and protozoa community involved in the BH process directly or indirectly. In this chapter, we report the effects of tannins, saponins and essential oils on ruminal BH with emphasis to their effects on the microbial ecosystem. A brief description of the impact of PBC on meat and milk fatty acid profile is given.

Keywords Ruminal biohydrogenation • Conjugated linoleic acid • Phytochemicals • Saponins • Tannins • Essential oils

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9.1 Introduction

The meat and milk from ruminant are often blamed for their high content of saturated fatty acids (SFA), intake of which is correlated to an increase in the risk of chronic diseases in humans (Burlingame et al. 1999) and for the relatively poor content of the health promoting polyunsaturated fatty acids (PUFA). At a first glimpse this would seem a paradox, as ruminants' diet is typically based on forages and grains which contain much greater proportions of PUFA than SFA (Morand-Fehr and Tran 2001).

The first explanation for this ruminant paradox was that animal tissues cause either a preferential oxidation or a direct saturation of dietary PUFA (Banks and Hilditch 1931). The evidence that dietary C18 PUFA are extensively hydrogenated in the rumen became available in the 1950s (Reiser 1951; Reiser and Reddy 1956). In late 1950s Shorland and co-workers demonstrated that the incubation of linoleic and linolenic acids (C18:2 *cis*-9 *cis*-12 [LA] and C18:3 *cis*-9 *cis*-12 *cis*-15 [LNA], respectively) with sheep rumen content led to the disappearance of LA and LNA and resulted in the production of stearic acid (C18:0, SA) and of a number of C18:1 isomers with *trans* double bond configuration (Shorland et al. 1957). These results have been attributed to the activity of ruminal bacteria and this pathway is currently named as ruminal BH of PUFA. In 1967, Kepler and Tove showed that the incubation of *Butyrivibrio fibrisolvens*, a bacterial species present in the rumen, with LA produced a conjugated dienoic fatty acid, identified as C18:2 *cis*-9 *trans*-11 and that the conversion of LA to the conjugated diene was carried out by LA isomerase enzyme (Kepler and Tove 1967). Although the presence of conjugated dienes in the ruminant edible fat was reported a long ago (Riel 1963; Parodi 1977), it was only in the late 1980s that conjugated linoleic acid (CLA) was identified as a potent anticarcinogenic agent (Ha et al. 1987). In 1996, the National Academy of Sciences (NRC 1996) concluded that ". . . conjugated linoleic acid is the only fatty acid shown unequivocally to inhibit carcinogenesis in experimental animals." Therefore, a further paradox of ruminants' meats and milk fatty acid profile is that, despite its high SFA content, it contains a nutraceutical component such as CLA.

The C18:2 *cis*-9 *trans*-11 is the major naturally occurring conjugated isomer of LA. The common name of this fatty acid, i.e., rumenic acid (RA) (Kramer et al. 1998) suggests that this is mainly produced in the rumen, however RA is also produced endogenously in the intestinal mucosa, in the muscle and in the mammary gland of mammals from the desaturation of C18:1 *trans*-11 (vaccenic acid, VA) (Corl et al. 2003), a fatty acid which arises from the BH of both LA and LNA.

While during the 1960s and 1970s the scientific community showed an interest in elucidating BH pathways, later on it was recognized that the best approach for improving the productivity of ruminants and increasing PUFA concentrations in meat and milk is minimizing the interactions between lipids and rumen microorganisms, thus avoiding any BH activity. This led to the development of protected fat sources which are extensively used in ruminant nutrition. A new surge of interest on BH and on its modulation started in the mid 1990s after the recognition of RA as a potent bioactive fatty acid, as shown by a large number of biomedical studies on its

mechanisms of actions. Recent advances on ruminal BH aim at unravelling the complexity of the microorganisms responsible of this pathway and identifying the BH intermediates.

The first animal production study aimed at identifying factors that affect RA concentrations in ruminant products (milk) was conducted by Jiang et al. (1996). Since then, the research on dietary factors that modulate the BH process and thus fatty acid composition of milk and meat has greatly increased. The exploration of plants secondary metabolites as dietary components capable to manipulate ruminal BH is very recent and will be reviewed here.

9.2 Fatty Acid Metabolism in Ruminants

9.2.1 The Fate of Dietary Fatty Acids in the Rumen: Lipolysis and Biohydrogenation

The BH process takes place in the rumen after the lipolysis of dietary triacylglycerols (present mostly in cereals) and of sulfo-, galacto- and phospholipids (predominating in green forages) (Dawson et al. 1977). In the rumen, the hydrolysis of ester linkages of dietary acyl lipids to non-esterified FA, or lipolysis, occurs rapidly (Garton et al. 1958; Dawson and Hemington 1974; Dawson et al. 1977). The presence of the free carboxylic group at the Δ-end of the fatty acids is an absolute requirement for the proceeding of the BH (Harfoot and Hazlewood 1997), so that the extent of lipolysis in the rumen is a major determinant of the extension of BH. Therefore, the partial inhibition of lipolysis in the rumen can be an effective approach to modulate both the amount of PUFA that escape BH and the pattern of biohydrogenation intermediates produced (Lourenço et al. 2010).

The lipases operating this process are both of plant (feed) and microbial origin, the contribution of each being still controversial (Lourenço et al. 2010). Fay et al. (1990) reported that 74 strains of ruminal bacteria showed lipolyzing capability, although the level of activity varied largely between the strains. The lipolytic activity in *Anaerovibrio lipolytica* (Harfoot 1978) and *Butyrivibrio fibrisolvens* (Hespell and O'Bryan-Shah 1988) was deeply investigated. Henderson (1971) reported that *Anaerovibrio lipolytica* did not hydrolyze phospholipids and galactolipids, while they hydrolyzed diglycerides more rapidly than triglycerides. These results suggest that *Anaerovibrio lipolytica* could play a minor role in lipolysis in forage-fed ruminants. Hespell and O'Bryan-Shah (1988) reported that among 30 strains of *B. fibrisolvens* tested the lipolytic activity largely varied and in some strains this activity increased with cell growth until the stationary growth phase was reached, after which the lipolytic activity remained constant.

Early studies also reported that protozoa might possess lipolytic activity (Wright 1961; Latham et al. 1972). When studying protozoa extracts, Wright (1961) observed that treating the cultures with penicillin reduced lipolysis and hypothesized that protozoa (mainly *Epidinium* spp.) could contribute up to 40% of ruminal lipolysis.

It has been also suggested that the protozoa lipolytic activity could be due to the feed lipases present in the chloroplasts engulfed by protozoa (Harfoot and Hazelwood 1988). Further studies supported by the recently available analytical techniques are needed to better understand the role of protozoa in ruminal lipolysis.

After lipolysis, the non-esterified unsaturated fatty acids liberated are mainly adsorbed into rumen particulate matter including bacterial surfaces (Keeney 1970), where PUFA can undergo through extensive BH. The reasons why rumen microbial ecosystem hydrogenate the unsaturated FA are not well understood. However, as pointed out by Jenkins et al. (2008), understanding why bacteria developed the enzymatic capacity to conduct BH may hold the key to find methods to manipulate BH in a predictable manner. Several hypotheses have been proposed so far. Lennarz (1966) hypothesized that the BH could serve as a hydrogen acceptor pathway to save the hydrogen to be rechanneled to other processes. Nevertheless, already at the beginning of the 1970, Czerkawski (1972) estimated that through the BH only small proportions of hydrogen could be saved. The hypothesis that BH is a detoxification mechanism was advanced by Kemp and Lander (1984), and it is based on the toxic effects of PUFA to most rumen bacteria. Recently, Maia et al. (2007, 2010) reported that the PUFA inhibited the growth of pure bacterial strains with BH activity, particularly the stearic acid producing bacteria.

Bessa et al. (2000) proposed that the generation of *trans* C18:1 isomers through incomplete BH could be an adaptive strategy of rumen ecosystem to deal with stressor stimuli including rumen lipid overload. This is because some BH intermediates, such as *trans* C18:1 isomers could have a protective role to rumen bacteria in certain environmental conditions as reported for other microbial systems (Keweloh and Heipieper 1996). In fact, *trans* C18:1 isomers are ubiquitous in the rumen ecosystem whatever the dietary conditions are.

At the end of the 1960s a scheme of the biohydrogenation pathway of LA was proposed by Kepler et al. (1966). From the very beginning of the studies of ruminal biohydrogenation it was clear that the scheme proposed was only one among the possible BH pathways, as a number of intermediary trienes and dienes (both conjugated and not conjugated) and monoenes isomers with 18 carbon chain were shown to arise from the BH of C18 PUFA (Kepler et al. 1966). In more recent studies some biohydrogenative pathways have been proposed and several intermediates of ruminal BH have been identified (Mosley et al. 2002; Buccioni et al. 2006; Bessa et al. 2007) and are listed in Table 9.1. Among all the possible BH pathways, it is noteworthy to describe in detail two mechanisms involving LA and LNA (Fig. 9.1).

1. the LA is first converted by LA-isomerase enzyme to RA, which is then hydrogenated to form VA by RA reductase enzyme. Then, VA is hydrogenated to form SA.
2. the LNA is isomerised to C18:3 *cis*9 *trans*11 *cis*15, which is then hydrogenated to C18:2 *trans*11 *cis*15. This fatty acid can be either directly hydrogenated to VA and C18:1 *cis*-15 or can be isomerised to form C18:2 *trans*11 *cis*13, which in a following step is hydrogenated to VA. The last step of the BH is the hydrogenation of VA to SA.

Table 9.1 Intermediates of ruminal biohydrogenation

	C18:2	C18:2	
C18:1	Non conjugated	Conjugated	C18:3
Trans-6	Trans-8, cis-13	Trans-6, trans-8	Cis-9, trans-11, cis-15
Trans-7	Cis-9, trans-12	Cis-7, trans-9	
Trans-9	Cis-9, trans-13	Trans-7, trans-9	
Trans-10	Trans-9, trans-12	Trans-7, cis-9	
Trans-11	Trans-9, trans-13	Trans-8, cis-10	
Trans-12	Cis-9, cis-15	Trans-8, trans-10	
Trans-13	Trans-12, trans-14	Cis-9, cis-11	
Trans-14	Cis-12, cis 15	Cis-9, trans-11	
Trans-15		Trans-9, trans-11	
Trans-16		Trans-10, trans-12	
Cis-11		Trans-10, cis-12	
Cis-12		Trans-11, trans-13	
Cis-13		Cis-11, trans-13	
Cis-14		Trans-11, cis-13	

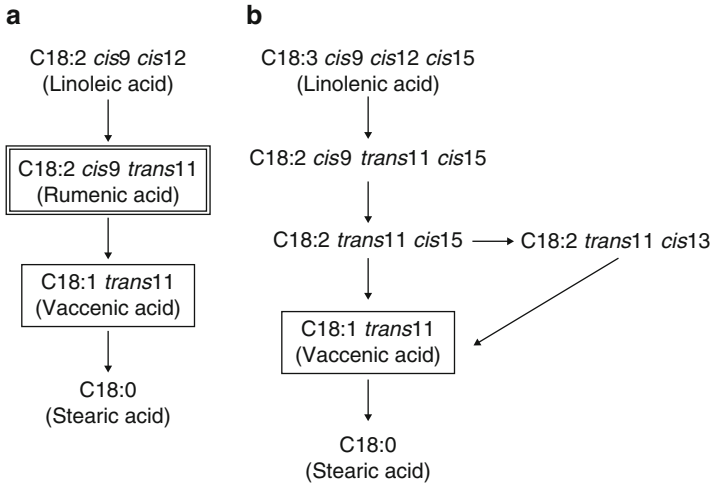

Fig. 9.1 The LA and LNA biohydrogenation pathways

While RA present in the rumen is generated only during the BH of LA, VA is synthesized during the hydrogenation of both LA and LNA. This process has major implications. In fact, Santora et al. (2000) and Piperova et al. (2002) shown that up to the 90% of RA detected in meat and milk is originated by the desaturation of VA operated in the muscle or in the mammary gland by Δ^9-desaturase enzyme; as a consequence, the RA synthesized during ruminal BH contributes a little to the overall RA content in meats and in dairy products. Therefore, it is now clear that, when attempting to increase RA content in meat and milk, the best strategy is enhancing VA formation in the rumen and its uptake in the duodenum, rather than increasing RA post-ruminal absorption.

9.2.2 Ruminal Microorganisms Participating to the Biohydrogenation

From the very beginning of the studies concerning the BH it was found that the bacteria that mainly carry out the BH are the cellulolytic bacteria belonging to the *Butyrivibrio* group (Kepler and Tove 1967) including the genera *Butyrivibrio* and *Pseudobutyrivibrio* (Paillard et al. 2007) and it is now largely agreed that the BH is undertaken by a consortium of bacteria (Lourenço et al. 2010). In 1984, Kemp and Lander (1984) proposed a division of the bacteria based on their participation to the different steps of the BH: group A bacteria -the *Butyrivibrio* group- that are able to hydrogenate linoleic and linolenic acid to VA, and group B: bacteria that are also able to saturate VA to form SA. The SA producing bacteria were identified as *Fusocillus* spp. (Kemp et al. 1975), which corresponds to *Clostridium proteoclasticum* isolated by Wallace et al. (2006), recently reclassified as *Butyrivibrio proteoclasticus* (Moon et al. 2008). Huws et al. (2011) have suggested that uncultured bacteria classified as *Prevotella*, *Anaerovoax* and unclassified Clostridiales and Ruminococcaceae may play a major role in the BH process.

The role of protozoa community in ruminal BH has been only little investigated, when compared to the large number of studies concerning bacteria, and so far there is an open debate on the role played by ruminal protozoa in BH. Devillard et al. (2006) reported that protozoa incorporate both RA and VA through the engulfment of ruminal bacteria. Yáñez-Ruiz et al. (2006) calculated that protozoa could contribute up to the 40% of RA and VA flow from the rumen to the duodenum. Controversial results are reported regarding an active role of protozoa in the BH pathway. A study conducted by Devillard et al. (2006) failed to detect the ability of protozoa from sheep to convert LA to RA or VA. On the contrary, Or-Rashid et al. (2008) reported that mixed rumen protozoa can convert LA to RA but cannot undertake the successive steps of the BH pathway. Boeckaert et al. (2009) evaluated *in vitro* the capability of *Isotricha prostoma* (a ciliate protozoon) to hydrogenate LA and its intermediates, and found that *I. prostoma* converted only little amount of LA to RA (less than 1%) and to C18:1 isomers. These authors concluded that *I. prostoma* cannot operate the BH of LA, while the bacteria associated (endo- and ectosymbionts) with this protozoan or engulfed by *I. prostoma* could be responsible for the observed results.

Little information is available with regards to the capacity of fungi in the ruminal BH process. Nam and Garnsworthy (2007) reported that rumen fungi can hydrogenate fatty acids *in vitro* until the formation of VA but the BH is slower compared to rumen bacteria.

The type of intermediate products and the completeness of the BH (leading to more saturated products) are strongly dependent on the diets offered to animals (Bessa et al. 2007). A large number of studies have investigated the effects of feeding green grass, silage or concentrates (French et al. 2000; Santos-Silva et al. 2002;

Aurousseau et al. 2004; for a review see Scollan et al. 2006) or the effects of lipid-enriched diets (Mele et al. 2007; Wąsowska et al. 2006; Noci et al. 2007) on ruminal BH and consequently on meat and milk fatty acid profile. However, this extensive topic is out of the scope of the present chapter and will not be further treated.

9.2.3 The Enzymatic Complex Operating Ruminal Biohydrogenation

The LA-isomerase (LA-I) enzyme catalyses the isomerization of LA to form RA. This enzyme was characterized in the late 1960s by Kepler and Tove (1969). So far, the activity of LA-I has been studied mostly with pure bacterial species, such as: *Butyrivibrio fibrisolvens* (Fukuda et al. 2006; Kepler and Tove 1967; Wąsowska et al. 2006), *Troponema* $B_2$5 strain (Yokoama and Davis 1971), *Clostridium sporogenes* (Peng et al. 2007), *Lacobacillus delbrueckii* (Lin 2006) and *Eubacterium lentum* (Verhulst et al. 1986). In an earlier study on the biohydrogenation activity in pure culture strains of *Butyrivibrio fibrisolvens*, Hunter et al. (1976) reported that the biohydrogenation *in vitro* of punicic acid (C18:3 *cis*9 *trans*11 *cis*13) to VA was higher with the incubation of both the cell free extract and the insoluble particulate fraction (which comprises bacterial cell walls) compared with the incubation of endocellular soluble fraction only. This happened because LA-I (Kepler and Tove 1967; Peng et al. 2007) and CLA reductase (Huges and Tove 1982) are membrane-bound enzymes. In another study (Polan et al. 1964), it had been found that the addition of boiled ruminal fluid to a reaction mixture of pure ruminal bacterial strains enhanced LA-I activity. As suggested by Polan et al. (1964), it is likely that some cofactor(s) present in the insoluble particulate fraction or in ruminal fluid might play a role in the reaction. However, a cell extract deriving from a pure culture (Kepler and Tove 1969; Verhulst et al. 1986), is a much simpler system compared with the whole ruminal environment in which the microorganisms responsible for the BH of fatty acids account only to a small proportion of the entire ruminal population. Vasta et al. (2009a) attempted to measure LA-I activity in cows ruminal fluid. The LA-I activity was observed only when the substrate was incubated in the presence of a lysate of ruminal microbial pellet (containing both the endocellular and the membrane fractions), while in the presence of the sole endocellular fraction it was not possible to measure LA-I activity.

9.2.4 Fatty Acids Metabolic Fate

The ruminal content comprising of bacteria and protozoa transits to the duodenum for the following digestive processes. In the lower tract of the small intestine fatty

acids, deriving either from feeds, or from free fatty acids or from digested protozoa and bacteria, are absorbed, re-arranged in the chylomicrons and transferred through lymph and blood to the liver and the tissues and become available for endogenous metabolism, including oxidation, transfer into milk and deposition in tissues. Some fatty acids generated in the rumen might undergo structural changes, particularly due to delta-9 desaturase enzyme action. Saturated fatty acids, like 18:0, are the major substrates for delta-9 desaturase enzyme, but some *trans* octadecenoates, namely those with double bonds from Δ-4 to Δ-13, except those with double bonds located at Δ-8, -9, and -10, served as substrates, originating 18:2 *trans*, *cis*-9 isomers, with the rate of desaturation being higher as the distance of the trans double bond from the Δ-9 position increased (Shingfield et al. 2008). Thus, accounting these predictable structural changes, the pattern of biohydrogenation intermediates (BI) observed in tissues is expected to reflect the BI rumen outflow pattern allowing the indirect monitoring of *in vivo* ruminal biohydrogenation. This is reinforced by the close relationship between duodenal flows and the biohydrogenation intermediates present in milk, as reviewed by Chilliard et al. (2007). This indirect evaluation of *in vivo* rumen biohydrogenation is particularly useful when no direct rumen fatty acid balance data are available, as in most of the studies on the role of PBC on rumen biohydrogenation.

9.3 The Impact of Plants Bioactive Compounds on Ruminal Biohydrogenation

The studies concerning the effects of plants bioactive compounds on ruminal biohydrogenation are quite recent. Most of the early studies focusing on plants (bushes, trees or forages) rich in secondary compounds aimed at evaluating the suitability of these feeds for livestock farming with respect to feed digestibility and animal growth performances, health and reproduction. The impact of dietary PBC on meat and milk fatty acid composition has been investigated only in the last few years (Vasta et al. 2008).

Most researches regarding PBC and ruminal BH have been conducted through *in vitro* studies, while the *in vivo* works available in literature are a few. In most of the studies here reviewed a detailed fatty acid profile of the ruminal content or of the fermentation medium is reported; nevertheless, so far the impact of PBC on the microorganisms responsible of the BH process has been seldom investigated. The following sub-sections will deal with the effect of tannins and phenolic compounds (Sect. 9.3.1), saponins (Sect. 9.3.2) and essential oils (Sect. 9.3.3) on ruminal biohydrogenation. Apart from the impact of PBC on ruminal microorganisms and BH, also a brief description of meat and milk fatty acid profile as affected by PBC will be given.

9.3.1 Tannins and Phenolic Compounds

9.3.1.1 Impact on Ruminal Microbial Community and Biohydrogenation

In vitro and *in vivo* studies conducted between 2003 and 2010 have shown that red clover (RC) (*Trifolium pratense*) reduced ruminal BH of PUFA and that the extent of lipolysis in RC was also hampered compared to other forages (for a review, see Van Ranst et al. 2011). These results were attributed to the interaction between the phenolic compounds from RC leaves and the polyphenol oxidase enzyme (PPO) (Loor et al. 2003), an enzyme which is highly active in RC. It was at first hypothesized that lipolysis could have been reduced by a stable binding between the quinones (produced by PPO through the oxidation of simple phenolics) and plant lipase enzyme (Lee et al. 2004, 2007; Van Ranst et al. 2009). Lourenço et al. (2008b) suggested that lipolytic enzymes from rumen bacteria could also be inhibited by quinones. However, this hypothesis was disproved: Van Ranst et al. (2009) reported no relation between measured lipase activity and lipolysis in red clover silages. Moreover, during ruminal fermentation the lipase of microbial origin seems to play a major role in lipolysis (Lee et al. 2007); therefore, the sole inhibition of plant lipase would not justify the reduced biohydrogenation observed *in vitro* and *in vivo*; Lee et al. (2010), by the use of immunogold labelling technique, concluded that the lipolysis of RC membrane lipids could be reduced through an entrapment of lipids within protein-phenol complexes. This means, in other words, that lipolysis is reduced because the substrate (membrane lipids) is unavailable to lipase enzyme (both of vegetal and of microbial origin). Considering that the lipolysis is a prerequisite for the biohydrogenation, it seems consequential that an impaired lipolysis also results in reduced BH of PUFA. In fact, Lee et al. (2007) and Cabiddu et al. (2010) found that the biohydrogenation of LA and LNA (calculated as the proportional loss of C18 PUFA during the incubation) was reduced by phenols, suggesting that the BH was inhibited. In addition, Cabiddu et al. (2010) also found that, compared to proteins-bound phenols, the tannic polyphenols had a stronger inhibitory effect on biohydrogenation probably because tannins reduced the BH depressing both lipolysis and biohydrogenation.

Khiaosa-Ard et al. (2009) and Vasta et al. (2009a) conducted *in vitro* studies aiming at elucidating the effect of tannins on ruminal BH. Incubating ruminal fluid with the CT extracted from *Acacia mearnsii* (Khiaosa-Ard et al. 2009) or *Schinopsis lorentii* (quebracho) (Vasta et al. 2009a) inhibited the last step of the BH, thus leading to the accumulation of VA at the cost of SA production. Nevertheless, in these studies the accumulation of RA was unaffected by the presence of tannins in the fermenter systems. Also Durmic et al. (2008) reported that when extracts from *Acacia iteaphylla* -which contains condensed tannins (Al-Soqeer 2008)- were incubated *in vitro* with sheep ruminal fluid the production of VA increased while SA decreased. This trend of fatty acids in the ruminal fluid was also observed in two *in vivo* studies (Vasta et al. 2009b, 2010) with lambs supplemented with quebracho tannins. An issue to be solved was whether tannins interfere with the BH through

depressing ruminal microorganisms proliferation or through a direct binding between tannins and the enzymatic system responsible for the BH of fatty acids. Khiaosa-Ard et al. (2009) and Vasta et al. (2009a) reported that the acetate: propionate ratio was reduced by the inclusion of tannins in the fermenters. Considering that the acetate is produced mostly by cellulolytic bacteria (which also operate the BH), the lower acetate: propionate ratio could indicate that the activity of cellulolytic bacteria was impaired by CT. Khiaosa-Ard et al. (2009) also reported that the CT extract increased the bacterial community, while reduced the total protozoa population compared to the control fermenters. Vasta et al. (2009a) investigated LA-isomerase (LA-I) activity in fermented ruminal fluid in the presence of CT. These authors observed that although the CLAs – measured by the unspecific absorbance of dienes bonds at 233 nm wavelength- produced in the LA-I assay were synthesized at lower extent in the presence of tannins, nevertheless LA-I activity (nmol CLAs/mg protein/min) was not affected by tannins because together with CLA the microbial proteins in the reaction mixture also decreased. This result suggested that tannins did not interfere with LA-I *per se*, but interfered with microbial activity, accordingly with the lower VFA production and microbial protein production observed in that study. This would be consistent with earlier observation of Jones et al. (1994), who reported that tannins form *Onobrichis viciifolia* induced morphological changes and reduced the growth in *Butyrivibrio fibrisolvens*.

Converse results were found *in vivo* in the rumen of lambs fed a basal diet (barley grains-based concentrate) with or without the supplementation of quebracho tannins (Vasta et al. 2010). In this study, the LA-I specific activity was lower for the tannins-receiving lambs while the concentration of total CLAs produced in the assay was not affected by tannins supplementation. In the same study, RA accumulated in the rumen of the tannins-fed lambs, while it was absent in the rumen content of the lambs not supplemented with tannins (Fig. 9.2), suggesting that tannins hampered the conversion of RA to VA.

The effect of tannins on ruminal microbial community is still controversial, and in addition, there is only scarce information regarding the interaction between tannins and the bacterial groups responsible for the BH of fatty acids. Vasta et al. (2010) found in an *in vivo* study that quebracho tannins strongly impacted the microbial community. The dendrogram in Fig. 9.3 obtained from clustering analysis of banding profile of rumen bacterial 16S rRNA showed that bacteria from the lambs fed the concentrate with (TY) or without (TN) tannins clustered separately. Concerning the bacteria involved in the BH, Vasta et al. (2010) reported that tannins increased the total protozoa and the relative abundance of *B. fibrisolvens* and decreased the relative abundance of *B. proteoclasticus* in the rumen. Previous studies have shown that tannins from *Lotus corniculatus* (Min et al. 2002) or from *Acacia* spp. (Durmic et al. 2008) reduce the proliferation of *C. proteoclasticum* B316^T and *C. proteoclasticum* P18, respectively. Therefore, it is likely that different bacterial strains are differently sensitive to tannins. Indeed Durmic et al. (2008) tested the inhibitory power upon biohydrogenating bacteria of large number of plants containing secondary compounds, most of which also contained tannins. They found that the minimum dose of plants needed to inhibit the proliferation of *B. fibrisolvens* JW11 (which forms RA)

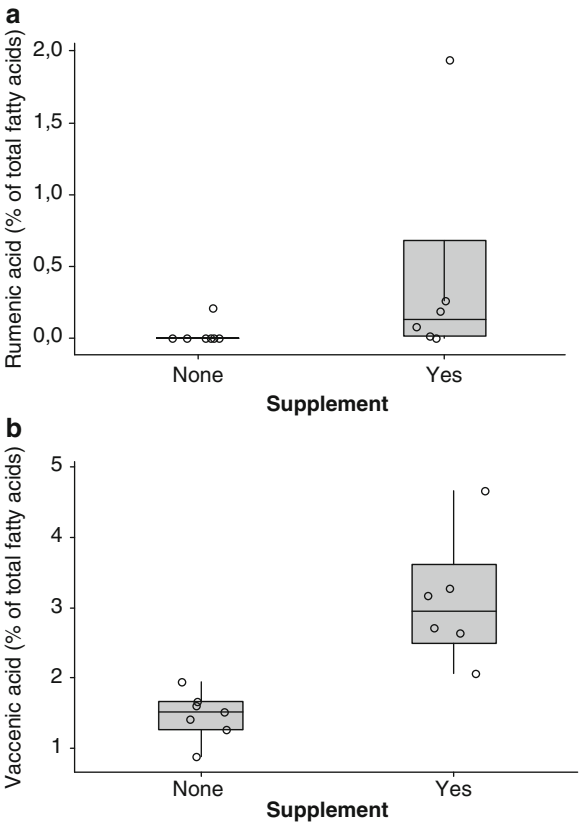

Fig. 9.2 Box plot of the distribution of RA (**a**) and VA (**b**) in the ruminal fluid of lambs fed concentrate with or without tannin supplementation. *Circles* represent individual animals

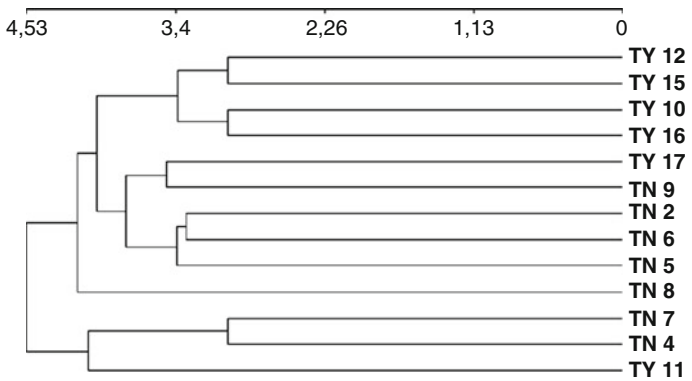

Fig. 9.3 Dendrogram derived from clustering analysis of denaturating gel electrophoresis banding profile of rumen bacterial 16S rRNA from lambs fed concentrate with (*TY*) or without (*TN*) tannins

was much greater than the dose needed to inhibit *Cl. proteoclasticum* P18, suggesting that *Cl. proteoclasticum* P18 is more sensitive to tannins than *B. fibrisolvens* JW11 (Durmic et al. 2008).

The effect of tannins on ruminal bacteria is also strongly dependent on the chemical structure of these compounds. In a study on the susceptibility of *B. fibrisolvens* and *C. proteoclasticum* to tannins, Sivakumaran et al. (2004) found that proanthocyanidins fractions from *Dorycnium rectum* with high molecular weight (HMW) inhibited the growth of *B. fibrisolvens* CF3, while low molecular weight (LMW) fractions did not interfere with bacterial growth. Conversely, *C. proteoclasticum* did not initiate the growth when incubated in the presence of LMW proanthocyanidins, while grew normally in the presence of HMW tannins. In the both cases, these results were also dose-dependent.

9.3.1.2 Tannins and Meat and Milk Fatty Acid Profile

The first study, in which an interaction between dietary condensed tannins (CT) and ruminal BH was hypothesized, was performed by Priolo et al. (2005). In that *in vivo* study, a group of lambs was fed sulla (*Hedysarum coronarium*; CT: 1.8% DM), while another group of lambs received sulla plus polyethylene glycol (PEG; a tannins binding agent). The supplementation of PEG did not affect the fatty acid profile of *longissimus dorsi* muscle: probably the low concentration of tannins in sulla was not enough to inhibit bacterial proliferation. Conversely, Vasta et al. (2007) found that the intramuscular fat of lambs receiving carob (CT: 2.7% feed DM) contained lower RA and VA, compared to lambs receiving the same diet but supplemented with PEG. In a study conducted by Vasta et al. (2009b) ruminal and muscle fatty acid profile of lambs supplemented with tannins were studied. The inclusion of quebracho tannins into concentrate diets resulted in greater amounts of VA, RA and total *trans*-C18:1 and in a reduction of C18:0 in meat (Vasta et al. 2009b). When quebracho was included in herbage, lamb meat contained greater amounts of PUFA compared to lambs fed the same herbage without added quebracho. These results observed in muscle reflected the fatty acid profile of rumen content. Jeronimo et al. (2010) reported that the supplementation of grape seed tannins in the diet of lambs did not affect abomasum or meat fatty acid profile. Feeding lambs a diet containing *Cistus ladanifer* (a tanniniferous bush) and linseed oil enriched meat with VA and RA, compared to the meat of lambs receiving the oil but not the *Cistus* (Jeronimo et al. 2010). These results quite encourage adopting the use of tannins-containing feed for improving meat fatty acid profile.

Cabiddu et al. (2009) analysed the fatty acid profile of milk from ewes grazing sulla (CT: 2.6% DM, average of three sampling dates) with or without supplementation of PEG. They noted that the RA and VA were lower, while C18:2 n-6 and C18:3 n-3 were higher, in the milk from the sulla-fed ewes compared to the milk from ewes receiving sulla + PEG. The authors concluded that tannins had impaired ruminal BH (Cabiddu et al. 2009). Benchaar and Chouinard (2009) reported that supplementing tannins from *Schinopsis balansae* did not affect the overall fatty acid profile

in cow milk. The ineffectiveness of tannins as modifiers of the fatty acid profile could be due to the low level of quebracho inclusion in the diet (0.45% of DMI) chosen for this study. Feeding cows *Lotus corniculatus* increased RA, LA and LNA and reduced VA and C18:0 in milk compared to *Lotus* + PEG (Turner et al. 2005).

It is sometimes doubted about the usefulness of dietary tannins for manipulating meat and milk fatty acid profile because of the generally reported detrimental effects of tannins on animal performances. However, the results reported in literature show converse effect of tannins on animal performance. In the studies from Priolo et al. (2000) and Vasta et al. (2007) lambs fed a diet containing carob pods (condensed tannins = 2.5% and 2.7% of feed DM, respectively) showed dramatically lower growth performance and carcass yield compared to lambs receiving the same diet, but with the addition of PEG. Also the inclusion of quebracho tannins (4.0% DM) in lamb's diets reduced animal growth performances compared to lambs not receiving the supplement (Vasta et al. 2009b). However, in the study of Vasta et al. (2009b), although the performances of the quebracho-supplemented lambs were reduced, animal health and carcass traits were still acceptable for Comisana breed lambs, while ruminal BH and meat fatty acid profile where strongly affected by tannins. As reported in an *in vitro* study by Vasta et al. (2009a), quebracho tannins have a "milder" inhibitory effect on ruminal microflora compared to carob tannins; this could explain the more detrimental impact of carob (2.7% DM) than quebracho (4.0% DM) on animal performance observed in the two studies form Vasta et al. (2007, 2009b). Jeronimo et al. (2010) found that tannins from *Cistus ladanifer* or from grape seed (2.5% DM) modulated fatty acid metabolism in the digestive tract, but they did not affect the average daily gain (ADG) and final live weight (LW) in lambs compared to the lambs fed a control diet. The effects of sulla on productivity were tested in dairy ewes (Molle et al. 2009; Cabiddu et al. 2009) and lamb (Priolo et al. 2005), and in both cases animal performances (milk yield and body condition score in ewes, and ADG and final LW in lambs) were similar to that of animals supplemented with PEG. Therefore, the use of tannins-containing feed seems to be promising. Further studies are needed to assess the opportune level of inclusion of each type of tannin in the diet, in order to manipulate ruminal biohydrogenation avoiding detrimental effects on animal productivity.

9.3.2 Saponins

9.3.2.1 Impact on Ruminal Microbial Community and Biohydrogenation

The impact of saponins upon ruminal BH has been studied only very recently. Similar to tannins, saponins have been shown to possess antimicrobial effects (Patra and Saxena 2009; Wallace et al. 2004). Makkar and Becker (1997) found that until 6 h incubation of *Quillaja s*aponins with ruminal fluid, saponins were not degraded, while saponins content in the fermenters decreased after 9 h incubation, suggesting that ruminal mixed cultures after an adaptation period are capable of degrading

Quillaja saponins. Wina et al. (2005) found that different bacterial groups within the fibrolytic bacteria responded differently to saponins exposition. With regards to the microorganisms mainly involved in ruminal BH, some early studies reported that *B. fibrisolvens* strains isolated from cattle rumen were able to degrade alfalfa saponins (Gutierrez et al. 1959). Conversely, Wallace et al. (2004) found that saponins from *Yucca schidigera* suppressed the growth of pure strains of *Butyrivibrio fibrisolvens* SH13, while prolonged the lag phase of *Streptococcus bovis* and enhanced the proliferation of *Prevotella ruminicola*. These results suggest that *Butyrivibrio* spp. might be more sensitive to saponins compared to other bacterial strains. Lourenço et al. (2008a) reported that incubating cow rumen content in continuous fermenters with added *Quillaja* saponins (up to a concentration level of 1 ppm) did not affect the production of fatty acids arising from the BH as compared to the control fermenters. In that study, VFA profile and the branched chain fatty acids, which can be considered as indicators of microbial growth (Vlaeminck et al. 2004) were unchanged by saponins, suggesting that the microbial activity was unaffected by saponins. However, it should be considered that changes in rumen microbial community caused by PBC do not necessarily imply changes in the biochemical pathways and *vice versa* (Goel et al. 2008). Khiaosa-Ard et al. (2009) found no effect of *Yucca schidigera* on ruminal BH *in vitro*. Moreover, in that study total rumen bacteria were not affected by saponins, while the protozoa community even increased. *In vivo* studies reported that feeding sheep 30 g/day *Yucca schidigera* saponins (Eryavuz and Dehority 2004) or cows 60 g/day (Benchaar et al. 2008) did not affect ruminal protozoa community. Nevertheless, *B. fibrisolvens* isolates and protozoa have been shown to be sensitive to saponins *in vitro* (Wallace et al. 2004). It is likely that saponins are less toxic to these microorganisms when added to mixed rumen bacteria or when fed to animals because in the consortium of the ruminal content some bacterial strains can degrade the saponins (Makkar and Becker 1997). Moreover, if some bacteria strains are inhibited by saponins, some other strains could take advantage, thus explaining the results from Khiaosa-Ard et al. (2009) and Benchaar et al. (2008). Nevertheless, it is mentioned that the response of ruminal microbial community to saponins seems to be dose-dependent (Patra and Saxena 2009).

9.3.2.2 Dietary Saponins and Meat and Milk Fatty Acid Profile

The studies aiming at investigating the effects of saponins-containing feed on meat or milk fatty acid profile are still very few. Ben Salem et al. (submitted) noted that lambs fed on diets containing up to 45 g (equal to 1.35 g diosgenin) of fenugreek seed (*Trigonella foenum-graecum* L.) and found no effect of saponin on longissimus muscle fatty acid profile. Similarly, Brogna et al. (2011) reported that the inclusion of *Quillaja saponaria* (level of inclusion in the diet: 30, 60 or 90 ppm) in the diets of lambs had no effects on the concentration in meat of those fatty acids arising during ruminal biohydrogenation (C18:1 *trans/cis* and C18:2 *trans/cis* isomers). Similarly, saponins did not modify cow milk fatty acid composition (Benchaar and Chouinard 2009).

9.3.3 Essential Oils

9.3.3.1 Impact on Ruminal Microbial Community and Biohydrogenation

The use of essential oils (EO) as modifiers of rumen fermentation and their impact on ruminal ecosystem has been largely investigated (Busquet et al. 2005; Duval et al. 2007; Calsamiglia et al. 2007). The term "essential oils" refers to a vast number of chemical compounds that can be classified as terpenoids (mono- and sesquiterpenes) and phenylpropanoids. These compounds possess potent anti-microbial properties. They adsorb on bacteria cell membranes causing conformational changes, membrane fluidification and leakage of ions across the membrane; and they also bind with enzymes, thus, hampering bacterial growth and activity (Calsamiglia et al. 2007). The sensitivity of bacteria to EO can vary depending on the: (i) chemical structure of the compound: molecules with carbonyl and hydroxyl groups are more toxic (Griffin et al. 1999), (ii) the type of bacteria: gram-positive bacteria seem to be more sensitive than gram-negative (Cox et al. 2001), and (iii) the concentration in the medium. Some mono- and sesquiterpenes can be degraded by rumen microorganisms. This has been shown *in vitro* by Broudiscou et al. (2007) who found that α-copaene, myrcene, β-ocimene, α-pinene and sabinene were degraded by caprine rumen micro-organisms, while camphene and thymol were not degraded. According to Busquet et al. (2005), ruminal microbial populations can adapt to the presence of EO. This implies that results on ruminal fermentation obtained in a short-term exposure of ruminal bacteria to EO might be only a partial view of the real impact of EO on ruminal microorganisms.

McIntosh et al. (2003) screened *in vitro* the sensitivity of some ruminal bacterial strains to a blend of EO (containing thymol, eugenol, vanillin and limonene) and they found that *B. fibrisolvens* SH13 was more sensitive to the exposure to EO among the 21 bacterial strains studied. Durmic et al. (2008) tested *in vitro* the resistance of *B. fibrisolvens* JW11 and *Cl. proteoclasticum* P18 to plant EO. The results of this study showed that the EO extracted from *Lavandula intermedia*, *Agonis fragrans*, *Malaleuca capreolata*, *Santalum spicatum*, *Eucalyptus plenissima*, *Eucalyptus staigeriana*, *Leptospermum petersonii* and *Malaleuca ericifolia* inhibited the growth of *B. fibrisolvens* JW11 and *Cl. proteoclasticum* P18 and that the minimal inhibitory concentration of these EO was different between the two bacterial strains. Lourenço et al. (2008a) found that the inclusion of eugenol (250 mg/l) in fermenter systems induced some minor inhibition of the BH process, while the inclusion of cinnamaldehyde (500 mg/l) had dramatically hampered the BH and bacterial activity, leading to a great accumulation of those fatty acids that are intermediate products of the BH process. From this study it is not easy to conclude whether the type of molecule (eugenol vs. cinnamaldehyde) or their concentrations in the fermenter (250 vs. 500 mg/l) were responsible for the different results. Supplementing dairy cows with 1 g/day of cinnamaldehyde did not modify ruminal volatile fatty acid concentrations (which is an indicator of microbial activity) and protozoa count (Benchaar et al. 2008).

9.3.3.2 Dietary Essential Oils and Milk Fatty Acid Profile

The above stated dramatic effect of cinnamaldehyde on the biohydrogenation observed *in vitro* by Lourenço et al. (2008a) was not confirmed *in vivo*: the inclusion of 1 g/day of cinnamaldehyde in the diet did not change milk fatty acid composition (Benchaar and Chouinard 2009). With regards to other terpenoids, the administration of 750 mg/day of EO to dairy cows did not alter the total counts of cellulolytic bacteria and protozoa and milk fatty acid profile compared to milk from cows not receiving the EO (Benchaar et al. 2007). In this study, the animals were subjected to the experimental treatment for 28 days, which probably allowed ruminal population to adapt to the presence of the EO blend. Similar results were reported by Malecky et al. (2009) in a study conducted with dairy goats receiving a monoterpene blend (linalool, p-cymene, α-pinene and β pinene; 0.43 g/kg DMI) through rumen cannula. The *in vivo* results do not encourage using the EO as modifiers of ruminal biohydrogenation.

9.4 Implications

Modulating ruminal biohydrogenation is a key point to improve meat and milk fatty acid profile. Increasing RA and PUFA and reducing SFA in ruminants' products would have a major impact on consumers' health. Among the possible strategies to manipulate ruminal BH, the use of some plant bioactive compounds seem to be effective in reducing the BH. Condensed tannins have been shown to impair the last step of the biohydrogenation. Saponins and essential oils seem to be less effective than tannins in modifying the BH pattern. The research should be extended also to other PBC such as simple phenols, alkaloids and oxalates. Nevertheless, more research is needed to unravel the complexity of the interaction between PBC and the BH. It is noteworthy to encourage the collaboration between nutritionists, ruminal ecologists and meat and dairy scientists. Research should be conducted at two different and subsequent levels: (i) assessing *in vitro* the effects of purified PBC and of feedstuffs containing PSC on the BH pattern and the microorganisms involved in the BH; and (ii) testing the PBC, both present in feed and purified in *in vivo* trials, in which ruminal ecosystem, functionality, BH process and meat/milk fatty acid profile are contextually analysed. In addition to ruminal fatty acid metabolism, it is worthy to study also abomasal and duodenal fatty acid composition and net flow, which would help to predict the type and amount of fatty acids that will be absorbed and transferred in animal tissue. It is also of paramount importance to evaluate the dose-response effect of PBC not only on ruminal BH and products fatty acid composition, but also on animal performances.

Under the economical and social sustainability, the use of PBC as modulators of ruminal BH is more desirable than other supplements such as oils or oil-rich grains. Through the modification of the BH pattern we do not claim to get "health-promoter meat/milk" but to obtain products with a better fatty acid profile compared to those products deriving from animals raised under intensive production systems.

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Chapter 10
Essentials Oils and Rumen Microbial Populations

Malgorzata Szumacher-Strabel and Adam Cieślak

Abstract In recent years there has been observed an increased interest in the potential use of plant essential oils rich in secondary metabolites to modify rumen fermentation. The goal of such modifications is to increase the efficiency of the symbiosis between ruminant and rumen microorganisms in order to improve profits in animal feeding without negative impact on environment. Essential oils belong to the group of natural components and may affect rumen fermentation processes, among others microbial populations. The action of essential oils can be considered in the aspect of synergism and antagonism of components that are present in essential oils but also that are present in the animal diets. The research are carried out to determine the effect of essential oils and their active substances on rumen fermentation parameters and to find out whether they can be used as feed antibiotics alternatives in ruminant feeding. On the basis of the performed research and the available literature, it can be stated that it is possible to use essential oils as alternatives for growth-promoting antibiotics, but before their commercial application in animal feeding, several issues must be evaluated, among other things interactions of (i) essential oils and the type of feed, (ii) essential oils and geographical region, which is connected with species of microflora inhabiting the rumen.

Keywords Essentials oils • Rumen microorganisms • Methanogenesis • Volatile fatty acids • Biohydrogenation

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10.1 Introduction

The specificity of the structure of the ruminant digestive tract, and above all the structure of the stomach consisting of four chambers, including three forestomachs, enables the symbiosis of a ruminant and microorganisms inhabiting the main chamber, i.e. the rumen. The microorganisms inhabiting the rumen include bacteria, protozoa, methanogens and fungi. The average capacity of the rumen of ruminants is 40–240 l, whereas the microbiological mass of bacteria and protozoa, the most numerous microorganism groups, amounts to about 1.463 g of bacterium cells and 455 g of protozoan cells per a cow.

Digestion in the rumen is a combination of microbiological fermentation and physical decomposition during chewing (Hart et al. 2008). Ruminants are characterized by the ability to utilize low-quality feed and to process substrate of poor quality into high-quality product, i.e. milk or meat (Greathead 2003). The ability results from symbiotic relationships between ruminants and the population of microorganisms inhabiting the rumen. Owing to the presence of microorganisms an animal uses nutrients, unavailable to the mammalian enzymatic system, and obtains energy from decomposition of structural carbohydrates (about 80% of the total demand) and protein from transformations of nitrogen compounds (from 60% to over 80% of the total demand). Volatile fatty acids, produced as a result of microbiological decomposition of structural carbohydrates, are absorbed mainly in the rumen, while amino acids resulting from decomposition of feed protein and microbiological protein, fatty acids and glycerol from decomposition of fat and glucose from decomposition of both structural carbohydrates (cellulose and hemicellulose) and non-structural carbohydrates (starch) are absorbed in the small intestine. This symbiotic relation animal – microorganisms has very limited utilization of feed ration energy and protein, which is reflected in the production and emission of respectively methane and ammonia (Van Nevel and Demeyer 1988; Calsamiglia et al. 2007; Cieślak et al. 2009a). Ineffectiveness of the utilization of protein and energy of feed ration leads to the economic loss in the form of reduction in the actual nutritional value of the provided feeds, reduction in animal productivity and above all to increased pollution of the natural environment (Patra 2010; Szumacher-Strabel and Cieślak 2010).

At present an increasing interest in the use of various compounds of plant origin in animal nutrition, including the nutrition of ruminants, can be observed, and the main emphasis is placed on a change in the direction of the rumen metabolism. However, the complexity of structure and diversity of plant phytofactors is a factor limiting the progress in research on the subject. Phytofactors are non-nutritional bioactive compounds present in plants. They differ from so-called primary compounds, e.g. carbohydrates, proteins, fats, in limited distribution, and generally are products of specific plants or plant groups. Even in particular plant species or single specimens there is a considerable diversity in their quality and quantity. Originally it was believed that they do not perform any function in plants, and at present we know that they are indispensable: they protect against pathogens, abiotic stress, e.g. UV radiation (Briskin 2000), and facilitate plant pollination by insects. They are

often synthesized as a response to a stress factor, e.g. frequent alfalfa (*Medicago sativa*) cutting increases the concentration of saponins (Marriott 2000).

The issue of animal nutrition is inextricably linked with other fields of science, for example with the quality of the obtained products and protection of the natural environment. In the last years the interest of nutritionists in bioactive plant factors as natural feed supplements has been rapidly growing because bioactive plant factors can: (i) modify the processes of fermentation in the rumen (defaunation), (ii) inhibit methane production and emission to the atmosphere, (iii) improve protein metabolism, and thereby reduce ammonia production and emission, and also (iv) increase the concentration of biologically active components in animal products, e.g. conjugated linoleic acid in milk or meat. Great diversity of bioactive phytofactors, such as saponins, tannins or essential oils, contained in many plant species, was identified as a factor potentially affecting the above-mentioned processes. Nevertheless, since their effectiveness depends on many factors, it has not been fully confirmed. The most literature data concerns essential oils and their effect on rumen metabolism. The mode of action of essential oils include plant and animal factors: factor type and place of location in the plant, its structure and activity, synergistic or antagonistic relationships with other feed ration components, animal species and production direction and the resulting concentration of structural carbohydrates in a feed ration, type of the used feeds, latitude of an animal's occurrence.

10.2 Historical Determinants of the Use of Phytofactors

The background of the present interest in the use of plants and their biologically active components as modulators of changes in the digestive tract of animals is not a new issue. So-called 'ethnoveterinary' and 'ethnomedicine' for ages have been using different plant forms (Greathead 2003). One of the first information on medicine based on plants come from 2600 BC from Mesopotamia (Newman et al. 2000; Greathead 2003) and China. The Chinese used plants in treatment already 5,000 years ago, while the Egyptians used plants and active compounds contained therein as food preservatives and mummification compounds in 1550 BC (Davidson and Naidu 2000 in Calsamiglia et al. 2007). Natural medicine was widely used (especially in the so-called West) until the mid-twentieth century, when a turnabout towards synthetic and effective drugs, patentable and highly profitable, occurred (Tyler 1999; Greathead 2003). Between 1950 and 1980 scientific research was focused mainly on substitutes of plant compounds. Since 1980 until the late 1990s, a turnabout towards the use of plants as a potential source of new chemotherapeutic compounds was observed. However, because of the difficulties (time and cost) related to the isolation and characterization of particular active substances from often very complex extracts, and then their chemical synthesis, the interest in these compounds was moderate. The development of modern techniques, and above all a change in society's attitude, once again direct the attention towards natural compounds of plant origin.

The use of antimicrobial properties of phytofactors including essential oils in many spheres of human life inclined scientists to apply these compounds also in animal nutrition. The attempts at changing the direction of the processes occurring with the participation of microorganisms and at increasing their effectiveness by modulation of the composition of microbial population in the rumen have been described in the world literature for many years. The main direction of action is supplementation of animal feeds with the compounds affecting the composition and number of microorganisms.

The first scientific study on antimicrobial properties of active compounds present in plants was published in the early 1920s (Hoffman and Evans 1911). The results of the first research on the use of biologically active compounds in ruminant nutrition come from the 1960s. Oh et al. (1967, 1968) and Nagy and Tengerdy (1964, 1968) described the influence of essential oils on fermentation processes in the rumen under *in vitro* conditions, including the production of postfermentation gases and activity of ruminal bacteria.

Nagy and Tengerdy (1968) concluded that the effects of action depends on the type of plant (*Artemisia nova* seems to be most effective) and as the consequence on volatile oil content. Borchers (1965) among other investigated the influence of secondary plant metabolite – thymol on the processes of protein transformations in the rumen. Thymol was used as the prevention factor against deamination of amino acids that were liberated by proteolysis. The obtained results indicated the possibility of modulation of ruminal processes in the desired direction. However, the legalization of the use of ionophore antibiotics as growth promoters, introduced in the 1970s, inhibited the development of research on the use of natural plant compounds in animal nutrition and for about 30 years few studies have been published on that subject (Broderick and Balthrop 1979). Another intensive development of research on the possibility of use of phytofactors, including essential oils rich in plant secondary metabolites, in animal nutrition is related to the announcement of the European Commission of the intended ban on the utilization of antibiotic growth stimulators so far used. Among other things it resulted from the pressure exerted by consumers to eliminate the use of all non-plant xenobiotic factors from animal feeds, and hence a turnabout (especially in Europe) towards the use of natural supplements. These actions (since 2000) have resulted in a very intensive development of scientific research and a dramatically rising number of the publications on that subject. Until 2006, mainly chemical supplements were used, such as antibiotics, ionophores, inhibitors of methane production and rumen-defaunating factors (Patra and Saxena 2009). The ban on their use (Directive 1831/2003/CEE, European Commission 2003), implemented in 2003 and effective since 1st January 2006, has resulted in an intensive development of research on searching and using effective natural compounds displaying similar properties.

The reasons for the ban on the use of chemical compounds in the modulation of ruminal processes can be found in: (i) their toxicity to an animal and microorganisms, manifesting itself among other things in lack of selectivity of their effect, (ii) lack of the ability of microorganisms to adapt to the used agent, (iii) passing into products of animal origin, (iv) increasing resistance to antibiotics, (v) little availability in the

practical use (e.g. defaunating factors), and, above all, (vi) decreasing social acceptance of the used substances. Other reasons for the ongoing search for substitutes of the above-mentioned chemical compounds are also: (i) increasing the animal genetic potential resulting in the necessity of using feeds with high concentration of effectively utilized nutrients, and, altogether, (ii) growing environmental pollution resulting from ineffective utilization of feed rations.

The above-described paradox caused a turnabout towards using substances naturally occurring in plants in animal nutrition, which are the basis of ruminant feed ration. The 'natural' compounds include probiotics, prebiotics, enzymes, organic acids and for instance essential oils rich in plant secondary metabolites or other chemical compounds of the same structure. Plant secondary metabolites, numbered among so-called phytocompounds, are bioactive factors which potentially can modulate fermentation processes occurring in the rumen (Wallace 2004). These are compounds that are not involved in the basic biochemical processes taking place in plants, such as growth, development and reproduction, yet they make the so-called line of defense ensuring the survival of plant structures and reproductive functions, thereby protecting a plant from predator attack (e.g. insects; Patra and Saxena 2009). For years the compounds have been considered as waste products of the primary metabolism. They are responsible for plant smell and color and are chemical transmitters between a plant and its environment, and also show wide antimicrobial activity against bacteria, yeasts and molds (Gershenzon and Croteau 1991 in Calsamiglia et al. 2007).

Assuming that the global market of antibiotic growth stimulators was worth 237 million US dollars in its peak in 1996 (Greathead 2003), it is not difficult to predict the implications related to the increasing interest in alternative fermentation modulators, including the whole plants, plant oils, plant extracts, essential oils or, finally, the very biologically active factors.

The research on compounds occurring in plants that can affect the rumen metabolism, and thereby animal productivity, should be not only the strategic but also the fundamental aim enabling to expand the knowledge of correlations between an animal and a 'cocktail' of phytochemical compounds, taken up by it every day in feed. Among all known groups of phytochemicals essential oils seems to be one of the most promising in their action.

10.3 Essential Oil

10.3.1 Origin and Classification

Essential oils are a mixture of plant secondary metabolites. They occur in plants called 'herbal', 'seasoning' or 'aromatic' and are responsible for the characteristic smell of these plants. They are mainly volatile compounds obtained by steam or solvent extraction from many plant parts, e.g. from seeds, leaves, flowers, stems, and their composition and activity are different depending on plant (including variety) or plant part from which they come (Dorman and Deans 2000). The chemical differences depend also on

genetic material, plant age and environmental conditions (Cosentino et al. 1999). Many essential oils seem to have antibacterial and bacteriostatic activity. They affect both bacteria and fungi, viruses and protozoa (Greathead 2003). The active compounds of essential oils in chemical terms can be divided into two groups: terpenoids and phenylpropanoids, deriving from two precursors of the primary metabolism, synthesized by different metabolic pathways (Calsamiglia et al. 2007).

Terpenoids are the most numerous and diverse group of plant secondary metabolites. About 15,000 terpenoids have been described in the literature (Gershenzon and Croteau 1991 in Calsamiglia et al. 2007). Essential oils are a mixture of mainly monoterpenoids (C_{10}) and sesquiterpenoids (C_{15}). They may also include diterpenoids (C_{20}) and various low-molecular-weight aliphatic hydrocarbons, acids, alcohols, aldehydes, acyl esters, lactones, compounds containing N and S, coumarins and phenylpropanoid homologues (Dorman and Deans 2000; Benchaar et al. 2008). Phenylpropanoids are synthesized in the shikimate pathway (from phenylalanine), the process occurring only in microorganisms and plants (Sangwan et al. 2001).

10.3.2 Biological Properties and Mechanisms of Action

Essential oils exhibit a range of biological properties that enable to use them as compounds preventing the development of many diseases, however, the most important ones used in animal nutrition are antimicrobial properties. Some research data indicate that mode of action of essential oils may be different than some ionophores (e.g. monensin) they are used to substitute (Meyer et al. 2009). Terpenoids and phenylpropanoids developed their antimicrobial properties above all against bacteria by affecting their cell membranes (Dorman and Deans 2000). This activity reflects the hydrophobic nature of cyclic hydrocarbons, which enables not only mutual influence but also accumulation of these compounds in the bacterial lipid membrane by their location between the chains of fatty acids (Ultee et al. 1999). It results in changes in the conformation of the cell membrane, which manifests itself in its expansion and fluidization, thereby causing permeation of ions and a decrease in concentration gradient. The effect of terpenoids and phenylpropanoids does not lead to cell death because the bacterial defense mechanism starts ion pumps. However, the process causes considerable energy losses, which limits bacterial growth by changing population size and proportions and hence the profile of ruminal fermentation (Griffin et al. 1999; Ultee et al. 1999). The antimicrobial activity is higher in the case of saturated cyclic hydrocarbons, and especially of those with phenolic structure, where the hydroxyl group and migrating electrons, owing to the hydrogen bridges, react with water, which makes them particularly active against microorganisms (Griffin et al. 1999; Dorman and Deans 2000; Calsamiglia et al. 2007).

Ultee et al. (1999) described an alternative mechanism of action working mainly towards the hydroxyl groups of aromatic compounds, where phenolic hydroxyl groups act as transmembrane carriers of monovalent cations and protons, similarly as ionophore antibiotics. The above mechanism does not operate in the case of non-aromatic compounds due to the presence of the electron transport system and

high acidity of phenols, and also because of the ability of hydroxyl groups to release its proton.

The described mechanisms are more effective against Gram-positive bacteria, whose cell membrane interacts directly with the hydrophobic nature of essential oils (Chao and Young 2000). In the case of Gram-negative bacteria the external cell wall surrounding the cell membrane is hydrophilic and does not let through lipophilic compounds. Most active compounds present in essential oils are lipophilic and do not have the ability to permeate the cell membranes of Gram-negative bacteria (Cox et al. 2001). The above-described process does not always work e.g. in the case of low-molecular-weight molecules that react with water (by hydrogen bridges), permeate through the cell wall, the lipopolysaccharide layer or protein membrane by diffusion and at that time interact with the bacterial lipid membrane (Griffin et al. 1999; Dorman and Deans 2000; Calsamiglia et al. 2007). On the other hand, Helander et al. (1998) notes the ability of some aromatic hydrocarbons present in essential oils to destruct the external membrane of Gram-negative bacteria, which results in a release of lipopolysaccharides and an increase in the permeability of the cytoplasmic membrane.

The above deliberations enable us to state that plant secondary metabolites of low molecular weight are effective both against Gram-positive and Gram-negative bacteria, which has its negative implications in the practical animal nutrition because it limits their selective effect.

Another possible effect of the above-discussed compounds on microorganisms is the potential of essential oils to coagulate some components of the cell membrane (presumably by protein denaturation), described by Gustafson and Bowen (1997). Phenolic compounds react with proteins by hydrogen and ion bridges or by hydrophobic interactions, while non-phenolic compounds react with other groups – e.g. the carbonyl group (Outtara et al. 1997, Prescott et al. 2004 in Calsamiglia et al. 2007). The compounds contained in the essential oils of garlic (*Allium sativum*) are a particular case – they are active against Gram-negative and Gram-positive bacteria, fungi, viruses and parasites, and the main mechanism of action is related to their ability to react with –SH groups (O'Gara et al. 2000).

To sum up, the antimicrobial effect of essential oils is related to their influence on bacterial cell membranes by influencing (i) electron transport, (ii) ion gradient, (iii) protein translocations, (iv) phosphorylation, and (v) other enzyme-dependent reactions (Ultee et al. 1999; Dorman and Deans 2000; Benchaar et al. 2008).

10.4 Essential Oils in Ruminant Nutrition

10.4.1 General Information

The effect of essential oils and other compounds modulating fermentation processes in the rumen is determined by their influence on the inhabiting microflora. Qualitative (e.g. PCR-DGGE) or qualitative and quantitative techniques (e.g. Real-time PCR or FISH) seems to be very useful to assess changes of rumen microflora as the effect

of different dietary factors (Pers-Kamczyc et al. 2011). Development of molecular techniques allow for better understanding of processes occurring within the rumen. Only a small percentage of all microorganisms inhabiting rumen may be described using traditional microbiological methods. During analysis of the effect of various compounds, including essential oils, on ruminal bacteria, one should be aware that in some ecosystems – and especially in those dominated by slowly growing, specialized microorganisms, only their small fraction (and often less than 1% of the overall population) can be registered with the available culture methods (Stewart et al. 1997). The data available in the literature indicate an ambiguous response of particular bacterial, protozoal and fungal groups to essential oils. Not all of ruminal microorganisms are sensitive to essential oils, despite the fact that antibiotic growth stimulators, e.g. monensin, previously used for the same purpose, were effective in the reduction of their number and activity and *vice versa*. McIntosh et al. (2003) analyzed the influence of a mixture of essential oils on different groups of microorganisms.

The authors observed a decrease in the activity of fungi inhabiting the rumen from the anaerobic species *Neocallimastix frontalis* with multiple flagella (Heath et al. 1983) with hydrogen production almost totally inhibited at the concentration of 40 ppm. Wanapat et al. (2008a) reported that essential oils from garlic powder supplemented to steer diets (80 and 100 g per day) tended to decrease protozoa and bacterial population, while fungal zoospore population was not affected.

The sensitivity of particular bacteria to the experimental factor depended on its concentration. Evans and Martin (2000) in their research on the influence of particular plant secondary metabolites on the ruminal bacteria *Selenomonas ruminantium* and *Selenomonas bovis* found that 90 mg/l of thymol showed a selective effect and inhibited the growth of only *Selenomonas ruminantium*, while 400 mg/l of thymol inhibited the growth of the both microorganism groups. McIntosh et al. (2003) confirmed that mixed essential oil (Crina® Ruminants) has an effects on most of the pure cultures of ruminal bacteria at concentration of less than 100 ppm. *Streptococus bovis* ES1, *Mitsuokella multicacidas* 46/5, *Megasphaera elsdenii* J1 and *Lachnospira multipara* D15d were identified as the most resistant species whereas *Prevotella ruminicola* 23, *Clostridium sticklandii* 12,662, *Peptostreptococcus anaerobius* 27,337 and *Ruminobacter amylophilus* WP109 were the most sensitive ones.

However, the analysis of the results is full of inconsistencies because of our lack of knowledge of effective concentration of essential oils or plant secondary metabolites in the rumen environment (Hart et al. 2008).

Essential oils should be also considered as active agents against pathogens inhabiting the animals' digestive tract. In some cases essential oils may increase the sensitivity of bacteria to pathogens (Rafii and Shahverdi 2007). A study by Ouwehand et al. (2010) confirms the sensitivity of bacteria of the genus *Clostridium perfringens* to 500 mg/l of biologically active compounds from essential oils (plant secondary metabolites), including among other carvacrol, cinnamaldehyde, limonene, thymol. Bacteria *Escherichia coli* were also sensitive to the above-mentioned compounds provided in the concentration of 5 and 50 mg/l. The quoted research included also the analysis of bacteria from the group *Bifidobacterium*, which showed their

significantly lower sensitivity to the used phytofactors. The studies confirm the specificity of effect of biologically active compounds on pathogenic factors.

Vidal et al. (2007) found activity of essential oils from peppermint (*Mentha piperita*) against *Giardia duodenalis*, a protozoan parasitizing cattle intestines. Benchaar et al. (2008) suggest that essential oils and their active factors may influence a range of other parasitic protozoa, e.g. *Cryptosporidium*, coccidia or nematodes. According to these authors, the effect of essential oils will depend on their activity in the particular parts of the digestive tract.

Varel and Miller (2001) indicated that decrease in pathogens digestive tract contamination as well as carcass contamination can be also achieved by treatment of feces with secondary plant metabolites present in essential oils e.g. carvacrol or thymol.

In the preliminary stage of research the interest in the use of essential oils in ruminant nutrition mainly resulted from their role in decreasing feed palatability of some plant species (Benchaar et al. 2008). As it was previously mentioned, the first reports on the subject of influence of essential oils on fermentation processes in the rumen come from studies by Oh et al. (1967, 1968) and Nagy and Tengerdy (1964, 1968). Nagy and Tengerdy (1968) found the influence of essential oils extracted from big sagebrush (*Artemisia tridentata*) on reduction in *in vitro* bacterial activity, while Oh et al. (1967) observed similar relationships as a result of using the extract from Douglas-fir needles (*Pseudotsuga menziessi*). The extent of effect each time depended on the chemical structure of a given active compound. It was proved that oxygenated monoterpens, especially alcohols and aldehydes, strongly inhibit the growth and metabolism of ruminal microorganisms, monoterpens containing hydrocarbon units in their structure slightly inhibit them, and under some conditions they may also stimulate the microorganism activity. The information contributed to further discussions and research on the influence of essential oils and their biologically active compounds on rumen metabolism, including mainly modulation of nitrogen transformations and utilization of energy.

Research on the possibility of use of essential oils in the nutrition of ruminants has been conducted for many years, however, due to the legislation changes, an increase in the intensity of research dates back to the turn of the twentieth and twenty-first centuries. The majority of the studies published so far is a result of the experiments conducted under *in vitro* conditions with the use of the techniques of batch culture or continuous culture (e.g. Rumen Simulation System – RUSITEC), describing changes of fermentation processes in the rumen resulting from the supplemented factor. The results obtained as an effect of short-term fermentation, especially in the case of the batch culture system, should be verified in a longer period of time both under *in vitro* and *in vivo* conditions (Castillejos et al. 2007). According to Chizzola et al. (2004), the reason is the ability of rumen populations to chemical reduction of the essential oils components to neutral alcohols. Such reactions may occur until the microorganisms are adapted to the applied factor, that is in the later stages of a given experiment.

Only in the last few years a growing number of publications on the possibility of use of essential oils in the direct nutrition of ruminants and describing the influence of essential oils on production performance has been observed.

The conducted and published studies used a range of essential oils and active compounds contained therein, and various levels of supplementation of feed rations, consisting of different components, were used. Therefore the results described in the world literature are sometimes contradictory and the described responses obtained as a result of research conducted under *in vitro* conditions are not always confirmed by experiments under production conditions (*in vivo*).

The expected directions of essential oils action are mainly connected with the modulation of rumen fermentation processes that are conducted by rumen microorganisms.

Favorable effects of the use of essential oils in ruminant nutrition can be expected, except of methane and ammonia emissions reduction, in the following aspects: (i) a change in the concentration of volatile fatty acids towards increasing the concentration of propionic acid without a change in the concentration of total volatile fatty acids, (ii) influence on the process of biohydrogenation of unsaturated fatty acids by inhibiting biohydrogenation on the level of *c*9, *t*11 C18:2 or *t*11 C18:1, which results in an increased concentration of the mentioned biologically active compounds in milk or meat, (iii) improvement of rearing and production performances. All above mentioned processes are the effects of rumen microbial actions.

10.4.2 Influence of Essential Oils on the Process of Methanogenesis in the Rumen

Announced legislation changes include the intended introduction of so-called cow tax by the European Commission – tax on farm ruminants. Its aim is a reduction in the emission of greenhouse gases to the atmosphere. The changes are a result of the Kyoto Protocol (1997; entered into force in 2005), supplementing the United Nations Framework Convention on Climate Change, that at the same time is an international agreement on prevention of global warming. It is actually due to the contribution of produced methane to the Earth climate warming and to causing measurable losses in animal production, that an increasing interest in the process of methanogenesis in the rumen of ruminants has been observed. Methane accounts for 18% of the total amount of greenhouse gases (Wuebbles and Hayhoe 2002), and its concentration has increased by 151% in the last few decades – the process is still going on. The amount of methane generated as a result of ruminant breeding accounts for 95–97% of the total animal production (Johnson and Ward 1996). The data compiled by Jensen (1996) indicates that the daily methane production by ruminants ranges from 30 l (a sheep with a weight of 40 kg) to 230 l (a cow with a weight of 500 kg). In the total amount of gases produced by ruminants, methane accounts for from 30% to 40%. The volume of methane production depends on several factors, including the intensity of changes in an animal organism (mostly rumen), production direction and the type of provided feed (Islam and Begum 1997). The loss of methane from fermentation processes in the rumen, emitted to the atmosphere, deteriorates feed utilization and thereby decreases the economic result of animal keeping. Johnson et al. (1993)

and Moss et al. (1994) assessed the energy losses occurring during the fermentation process at 2–12% of gross energy taken up in feed. The process of methanogenesis can occur only in anaerobic conditions with the participation of ruminal microflora as a result of interspecific symbiosis between methanogens and bacteria, protozoa or fungi. The symbiosis consists in, among other things, the transfer of H_2 generated in microorganisms' cells to methanogens. These microorganisms use inter alia CO_2 for H_2 reduction, and the energy obtained in the process is used for the generation of ATP. Owing to the process of methane production in the rumen, low concentration of H_2 is maintained in the rumen atmosphere, which favorably affects the carbohydrate transformations in this environment (Wolin and Miller 1988; Ushida and Jouany 1996). According to the available data, a cow uses up to 800 l of H_2 daily for methane production in the rumen (Hungate 1967). Skillman et al. (2004) state that the population of methanogens in the rumen may reach the maximum size of 10^8–10^9 per gram of rumen contents already in 3-week-old lambs. The methanogens dominating in the rumen of farm animals are microorganisms of the genus *Methanobrevibacter* and *Methanosarcina*, and especially the species: *Methanobacterium formicicum, Methanobrevibacter ruminantium, Methanosarcina barkeri, Methanosarcina mazei* and *Methanomicrobium mobile* (Stewart et al. 1997). However, the development of modern molecular techniques indicates that ruminant species determines the methanogen that dominates in the rumen (Janssen and Kirs 2008). Many scientific units in the whole world have been conducting intensive research on the reduction in the adverse effect of methane from animals on the natural environment and the associated economic effect of animal production. The research is largely focused on the possibility of control of the processes occurring in the rumen especially taking into account the microorganisms involved in these processes. According to Patra and Saxena (2009), plant secondary metabolites rich components, i.e. essential oils, can modulate the process of methanogenesis.

However, information on the influence of essential oils on the process of methanogenesis are ambiguous (Patra and Saxena 2009). Early research and literature reports indicate that 40 ppm of essential oils did not adversely affect the population of *Methanobrevibacter smithii* (Hobson 1969). However, subsequent studies demonstrated that an increasing content of essential oils is a direct cause of reduction in the population of *Methanobrevibacter smithii* PS (ATCC 35061; Newbold et al. 1988). It has been proved also by a study by McIntosh et al. (2003), who found that a reduction in the number of *Methanobrevibacter smithii* was observed only after using the supplementation with 1,000 ppm of a commercial mixture of essential oils. An increasing amount of limonene (40 and 400 mg/l), the main component of fir oil (*Abies alba*), reduced the total population of methanogens (on average by 25%) (Cieślak et al. 2009b), while using 4 mg/l of limonene had no influence on the discussed microorganism population. The authors state that not only the highest, but also medium amounts of the used experimental factor can modulate the populations of methanogens in the rumen environment. Similar conclusions were drawn by Agarwal et al. (2009), who analyzed peppermint oil (*Mentha piperita*; 0.33 μl/l) in their study. The substance caused a two-fold increase in the number of methanogens with 20% reduction in methane production. On the other hand, the supplementation

with both 1 and 2 μl/L of peppermint oil (*Mentha piperita*) reduced the process of methanogenesis by decreasing the number of methanogens on average by 82%. On the basis of the results one can conclude that lower doses of essential oils can increase the number of the methanogen groups that are less active in the process of methane production. Similar relationships were found in a study by Ohene-Adjei et al. (2008), where adverse effect of essential oils depended not only on their source and amount but also on the type of methanogen strains and their associations with protozoa. The authors supported this hypothesis by the conclusion that the use of essential oils in ruminant nutrition in the aspect of limitation of the process of methanogenesis may be ambiguous (Ohene-Adjei et al. 2008). Additionally, Busquet et al. (2006) reported that active components like garlic oil, diallyl sulfide and allicin, coming from the same herb plant (*Allium sativum*) and supplemented at the same concentration (300 mg/L) may have different effect on the decrease in methane production. Authors suggested that mechanism of action of these active components could be related to an individual potential of rumen methanogens population limitation. For example garlic oil might have inhibited these microorganism through an direct inhibition of the 3-hydroxy-3-methyl-glutaral coenzyme A which is necessary for normal cell function. However Busquet et al. (2006) stated that further study is needed to confirm this hypothesis.

10.4.3 Influence of Essential Oils on Protein Metabolism in the Rumen

Reduction in the amount of emitted greenhouse gases involves also the issue of limitation of ammonia production and emission by ruminants, which includes both two scientific and practical aspects that are worthy of further consideration: (1) increasing the utilization of dietary protein, and (2) protection of the natural environment.

The symbiotic correlation between a ruminant and microorganisms inhabiting its rumen enables an animal to use almost every source of nitrogen that is utilized by microorganism for creating protein of the highest biological value. However, the relationship does not meet the amino acids requirements of highly-productive animals. Although feed rations provided to ruminants are supplemented with protein components, whose aim is to rectify the deficiencies of this element, in the practical analysis this solution increases the costs of feed and does not always reach expected results. What is more, too high concentration of protein in a feed ration leads to its ineffective utilization, which results in nitrogen losses that pollute the natural environment. According to Lapierre et al. (2005), about 0.3 nitrogen unit is excreted in urine per each unit of nitrogen taken up by dairy cows. This value is averaged and largely depends on many factors, including e.g. (i) nitrogen level in the provided feed ration, (ii) type of nitrogen source in the feed ration and its susceptibility to degradation to ammonia in the rumen (non protein nitrogen; NPN), (iii) ratio of the amount of ammonia produced in the rumen to the amount available for synthesis of

bacterial protein in the rumen, (iv) type and enzymatic activity of bacterial flora inhabiting the rumen, and (v) outflow rate of rumen contents to the further parts of the digestive tract. Hence the attempt at influencing and modulating the protein transformations occurring in the rumen is one of the fundamental challenges of modern animal nutrition.

Supplementation with essential oils that have antimicrobial activity enables to consider them as natural feed supplements fulfilling these expectations, whose mechanism of action will be directly associated with the influence on bacteria specialized in ammonia production and indirectly with the influence on amino acid decomposition.

Early experiments by Borchers (1965) indicated that the supplementation with 1 g of thymol per 1 L of ruminal fluid in the presence of casein causes accumulation of amino acids and a decrease in the concentration of ammonia nitrogen, which suggests that amino acid deamination is hindered by ruminal bacteria. Broderick and Balthrop (1979) in their research confirmed the inhibition of amino acid deamination resulting from the thymol supplementation.

The above-described experiments show the influence of particular single substances on nitrogen transformations in the rumen. The world literature includes also publications of the results of research on supplementation with mixtures of essential oils and their chemical compounds, using the synergy phenomenon between them. An example of the described relationship are the results of research published by Busquet et al. (2005a), where the addition of 2.2 mg of essential oils from clove (*Syzygium aromaticum*) per a liter of ruminal fluid fermented in the continuous culture system lowered the concentration of large peptides by 80% but had no influence on the level of ammonia nitrogen, which suggests strong peptidolytic activity of the used supplement. Nevertheless, the active substance from clove oil – eugenol, used in the same amount, showed no effect, including no influence on the peptidolytic activity. These results suggest that the peptidolytic properties of essential oils from clove are a combined effect of the substances contained therein.

However, in many cases the activity is observed both in a single component and in a mixture of active substances. Busquet et al. (2006) showed that both oregano essential oil (*Origanum vulgare*) and its main biologically active component – carvacrol, lower the concentration of ammonia nitrogen analyzed in the batch culture system. The obtained results suggest that carvacrol determines the activity of the essential oil obtained from oregano. Essential oils can be provided as an extract directly from a given plant as well as a commercial mixture.

In 2003 McIntosh et al. found a 9% reduction in the rate of amino acid deamination resulting from 48-h *in vitro* fermentation of casein hydrolysate in the batch culture system. Ruminal fluid for incubation was collected from a cow fed a feed ration supplemented with 1 g per day of commercial mixture of essential oils Crina® ruminants (Akzo Nobel Surface Chemistry Ltd., Herefordshire, United Kingdom). Supplement Crina® ruminants contains 100–300 g/kg of phenolic components, including among other things: resorcinol, thymol, guaiacol, eugenol (Rossi 1994 in Benchaar et al. 2008). Similarly, Newbold et al. (2004) observed a decrease in amino acid deamination rate (by 24%) as a result of adding 110 mg of a mixture of

essential oils to ruminal fluid of sheep incubated together with casein hydrolysate in the *in vitro* system for 24 h.

In none of the quoted studies influence of the used supplements on peptidolytic and proteolytic activity was found, and excessive protein decomposition in the rumen, significant from the nutritional point of view, often exceeds the possibility of utilization of the final degradation products, that is e.g. free amino acids by microorganisms. The most proteolytic ruminal microorganisms include bacteria, of which some are strictly specialized in ammonia production – hyper-ammonia-producing bacteria (HAP). The bacteria are present in the rumen in a small amount (less than 0.01% of the bacterial population in the rumen) but they exhibit very high metabolic activity (Russell et al. 1988). According to the literature data, they are responsible for protein decomposition in the rumen in 50% and thereby for ammonia production (Hart et al. 2008). So far, 14 morphologically different species were identified, however, the greatest activity is observed in *Clostridium sticklandii, Clostridium aminophilum, Bacteroides ruminicola* and *Peptostreptococcus anaerobius*. They are Gram-positive bacteria, sensitive e.g. to ionophore antibiotics. For the first time hyper-ammonia-producing bacteria were isolated in New Zealand and Australia in sheep, cows and deer receiving green forage (Attwood and Reilly 1995). The presence of these bacteria in the ruminal fluid depends on the type of a feed ration and also on the latitude of an animal's occurrence.

Many authors have shown an effect of a mixture of essential oils on the activity and number of hyper-ammonia-producing bacteria, potentially favorable from an animal breeder's point of view. McIntosh et al. (2003) demonstrated that the mixture of essential oils in the form of Crina® ruminants preparation inhibits the growth of some bacteria (*Clostridium sticklandii, Peptostreptococcus anaerobius)*. Additionally, the research revealed different effect of essential oils on particular bacteria. The mixture of essential oils in the form of Crina® ruminants preparation less effectively influenced *Clostridium aminophilum*. According to Wallace (2004), the intensity of essential oil influence on HAP depends on protein concentration in a feed ration. In his research he found that the number of HAP was reduced by 77% in sheep fed low-protein feed supplemented with 100 mg of the mixture of essential oils per day, while in the group of sheep receiving high-protein feed supplemented with essential oils HAP was not affected. Wallace (2004) suggests that the effect of essential oils in the rumen may be regulated by their influence on *Ruminobactor amylophilus* – an amylolytic and proteolytic organism sensitive to essential oils. The author suggests that *Ruminobactor amylophilus* may play a significant role in the process of colonization of substances rich in protein and starch in the rumen.

The effectiveness of phytofactors influencing bacteria specialized in ammonia production depends also on their chemical structure, which was proved by Flythe and Kagan (39). They investigated red clover (*Triforium pratense*), rich in soluble phenols, and for direct experiments they used extracted pure phenolic compounds, such as biochanin A, and also the extract from the whole plant. The anti-effect of *Clostridium sticklandii*, one of the bacteria from the HAP group, was observed in the case of the extract from the whole plant and biochanin A, while no effect was found for other phenolic compounds. The results of research indicate that some

phenolic compounds from red clove may play a role in prevention of amino acid decomposition in the rumen.

Another factor which influences the effect of feed ration supplementation with essential oils on protein metabolism in the rumen is time of exposure to an experimental factor. Benchaar et al. (2008) suggest that short-term research under *in vitro* conditions (24, 48 h), unlike long-term research, does not reflect the actual influence of the used supplements on microorganism populations involved in protein transformations in the rumen. As mentioned by Benchaar et al. (2008) the results of research by Cardozo et al. (2004) and Busquet et al. (2005a) suggest that after 6–7 days of fermentation, microorganisms can adapt to altered conditions, including to the presence of a mixture of essential oils.

Also the chemical structure of an active compound of the used supplement has a decisive role in influencing protein metabolism in the rumen. The data published by Castillejos et al. (2006) on the influence of increasing doses of different active compounds from essential oils on the concentration of ammonia nitrogen under the conditions of 24 h *in vitro* fermentation indicate that vanillic aldehyde was ineffective in the concentration of 5, 50 and 500 mg/l of fermented ruminal fluid; 500 mg/l of limonene with monoterpene structure reduced the concentration of ammonia nitrogen; eugenol with phenolic structure reduced the concentration of ammonia nitrogen in the amount of 5, 50 and 500 mg/l of fermented ruminal fluid, while guaiacol (phenolic compound) was effective in all the used concentrations (5, 50, 500 or 5,000 mg/l) (Benchaar et al. 2008).

The above studies indicate that the effect depends also on the amount of the used supplement. Castillejos et al. (2005) in their experiments using the continuous culture system found no influence of 1.5 mg of a mixture of essential oils on ammonia concentration, flow of bacterial and feed nitrogen, decomposition of crude protein and effectiveness of microbial synthesis. Also the subsequent studies by Castillejos et al. (2005) with the use of the same essential oils in the amount of 5, 50 and 500 mg/l of ruminal fluid demonstrated no influence of the essential oils on nitrogen metabolism during 9-day incubation. According to McIntosh et al. (2003), the concentration of essential oils that ensures effective influence on the processes of protein transformations in the rumen amounts to over 35 mg per a liter of ruminal fluid under *in vitro* conditions. Benchaar et al. (2006a, 2007a) in their research found no changes in the parameters of nitrogen transformations, including among other things concentration of ammonia nitrogen and nitrogen retention, resulting from the supplementation of dairy cow feed rations with a mixture of essential oils in the amount of 0.75 and 2 g per day. The results of research by Busquet et al. (2006) showed that both some essential oils and their biologically active compounds significantly decrease the concentration of ammonia nitrogen when they are used in the doses as high as 3,000 mg/l, they are moderately active in the concentration of 300 mg/l and show no effect in the concentration of 3 mg/l.

The above-described effects concerning nitrogen transformations in the rumen are associated mainly with favorable influence of bacteria. On the other hand, protozoa play an adverse role in protein utilization by ruminants (Benchaar et al. 2008) because they consume and digest a considerable amount of ruminal bacteria thereby reducing

the outflow of net bacterial protein from the rumen to the duodenum (Ivan et al. 2000). Since protozoa have the ability to perform the processes of proteolysis and deamination, rumen defaunation (elimination of protozoa from the rumen ecosystem) results in an increase in the amount of nitrogen of microbial origin that reaches the duodenum. According to Ivan et al. (1992), the amount of bacterial protein that reaches the duodenum of defaunated sheep is greater by 35% than in non-defaunated ones. Since 2000 little information on the subject of effect of essential oils as factors defaunating the rumen has been published and obtained data are not unequivocal.

Ando et al. (2003) demonstrated that the supplementation of feed rations of heifers with 200 g of peppermint (*Mentha piperita*) per day reduced the total number of protozoa, including protozoa from the genera *Entodinium*, *Isotricha* and *Diplodinium*. According to some scientists, essential oils have low defaunating potential. McIntosh et al. (2003), Newbold et al. (2004) and Benchaar et al. (2008) in their studies did not confirm the adverse effect of essential oils on the population of protozoa however the study of Sallam and Abdelgaleil (2010) suggested that citrus essential oil as well as its secondary metabolite limonene reduced the protozoa count. Also ammonia concentration was decreased with high levels of supplements used (50 and 75 μL/75 mL buffered rumen fluid for citrus essential oils and 45 and 60 μL/75 mL buffered rumen fluid for limonene). Experiment by Cieślak et al. (2009b) also confirmed the potential of limonene to inhibit rumen protozoa population. The study by Wanapat et al. (2008b) evaluated the effect of lemongrass powder (*Cymbopogon citratus* Stapf.) on rumen ecology. Supplementation of diets for beef cattle with 100, 200 or 300 g/d of lemongrass powder with urea-treated rice straw improved bacteria and fungi population whereas decreased protozoa counts. The results of this experiment suggest that active components of lemongrass interact in different ways with particular groups of rumen microflora.

The interest in the influence of essential oils on the number and activity of particular protozoan groups has been investigated by several scientific groups. Newbold et al. (2004) analyzed the influence of 100 mg of a mixture of essential oils in a daily feed ration for sheep, whereas Benchaar et al. (2003) supplemented a feed ration for dairy cattle with 750 mg of a mixture of essential oils. In the both cases no influence of the used supplements on the number of protozoa was observed. Also an experiment by Santos et al. (2010) on the influence of a commercial mixture of essential oils on, among other things, the efficiency of utilization of feed ration nitrogen showed no effect of the used supplement consisting of eugenol, geranyl acetate and coriander oil (*Coriandrum sativum*).

10.4.4 Influence of Essential Oils on the Production of Volatile Fatty Acids (VFA) in the Rumen

Volatile fatty acids (VFA) are the basic source of energy for ruminants, and acetic acid is fundamental for synthesis of fatty acids, while other short-chain fatty acids (e.g. isobutyric, isovaleric and valeric acids) initiate the process of synthesis of

these acids (Wu and Huber 1994). The concentration of volatile fatty acids in the rumen also reflects the digestibility of nutrients of the used feed ration. The analysis of literature data indicates ambiguous influence of supplementation with essential oils on the concentration of volatile fatty acids in the rumen. Benchaar et al. (2007a) indicate that the influence of essential oils on the concentration of volatile fatty acids may depend e.g. on a feed ration. In the conducted research the supplementation of a feed ration with alfalfa silage with 750 mg of a mixture of essential oils per day caused a slight increase in total volatile fatty acids, while adding it to a feed ration based on corn silage reduced the amount of total volatile fatty acids in the rumen.

Another factor determining the influence of essential oils on the concentration and mutual proportions of volatile fatty acids in the rumen is the amount of the used supplement. In study by Busquet et al. (2006), the supplementation with a wide range of essential oils and particular secondary metabolites contained therein on *in vitro* fermentation processes was analyzed. In the described research increasing concentrations of the used supplements to as much as 3 g per a liter of incubated ruminal fluid were used. The analyzed supplements had no influence on the concentration of VFA, and only the highest supplement amount of 3 g/l resulted in a decrease in total volatile fatty acids, at the same time reducing the digestibility of the feed ration. A reduction in VFA production and as a result in their concentration in the rumen is an undesired phenomenon from the nutritional point of view because it decreases the utilization of dietary energy from structural carbohydrates that are one of the least expensive sources of nutrients for ruminants.

The expected results of the effect of essential oils include their influence on a change in molar proportions of VFA, increase in the concentration of propionic acid, reduction in the concentration of acetic acid without decreasing their total concentration. A research by Busquet et al. (2005b) revealed an increase in the concentration of propionic acid at the cost of acetic acid, however, the highest dose of the used experimental factor increased also the concentration of butyric acid. Changes in molar proportions may be dependable on the structure of particular secondary plant metabolites present in essential oils. In the study of Benchaar et al. (2007b) phenolic compounds: carvacrol, eugenol, thymol shifted volatile fatty acids fermentation pattern towards less propionate and more butyrate. The effect was also the result of evaluated concentration of particular phytofactor. The results of research by Castillejos et al. (2006) demonstrated that 500 mg of eugenol also decreased the concentration of propionic acid without a change in the amount of total volatile fatty acids. Concentration of total volatile fatty acids was not influenced by supplementation of three essential oils (cinnamon leaf, garlic and juniper berry) and two essential oils compounds (anethol and p-cymene) but cinnamon and garlic oil decreased the propionate concentration in *in vitro* experiments by Chaves et al. (2008).

Cardozo et al. (2005) suggest that changes in the concentration of volatile fatty acids in the rumen as a result of supplementation with essential oils depend on pH of ruminal fluid. At pH 7.0 the essential oil – a source of cinnamaldehyde, increased the ratio of acetic acid to propionic acid, while at pH 5.5 it decreased the ratio. The results of the study by Benchaar et al. (2007b) show that 200 mg/L of thymol

increased the final pH of rumen fluid *in vitro* and decreased the molar proportion of propionate.

Above mentioned data suggest that there is not always the substantiation for essential oils and secondary plant metabolites utilization in animal nutrition.

10.4.5 Influence of Essential Oils on Rumen Biohydrogenation, Production Performance and Animal Products Composition

So far little research was conducted on determination of the influence of essential oils on production performance of ruminants, including on the production and composition of milk and meat. Whereas, another reason for using essential oils in ruminant nutrition is the improvement in milk and meat quality among other things by changes in the process of biohydrogenation in the rumen and in the process of desaturation of monounsaturated fatty acids in the mammary gland. However, unsaturated fatty acids reaching the rumen more than once resulted in unintended changes in the composition of microflora (Kišidayová et al. 2006; Szumacher-Strabel et al. 2009; Cieślak et al. 2009c). The use of bioactive plant factors in ruminant nutrition is a chance of producing similar effects without the negative implications. In the case of favorable effects, the essential oils can be practically used in animal nutrition and protection of the natural environment.

The antimicrobial activity of essential oils and their biologically active compounds is aimed at Gram-negative and Gram-positive bacteria. The process of biohydrogenation of unsaturated fatty acids in the rumen involves several groups of Gram-positive bacteria (Harfoot and Hazlewood 1988), hence the suggestion that supplementation with essential oils may potentially modulate the process of biohydrogenation. The process occurs as a from of microorganism protection against unfavorable unsaturated fatty acids taken up by an animal together with dietary plants or other feed additives. The microorganisms for which fat is not a source of energy, transform unsaturated forms of fatty acids into less harmful saturated fatty acids. Biohydrogenation occurs in several stages, and conjugated isomers of unsaturated fatty acids, generated for example as a result of isomerisation, have beneficial health effect. The modulation of the process of biohydrogenation consists in inhibiting the activity of the involved bacteria and therefore increasing the concentration of generated unsaturated fatty acids. Unsaturated fatty acids, including conjugated isomers, produced as intermediate products of the process of biohydrogenation, pass into milk and thereby modify its composition in the direction desired by consumers (Cieślak et al. 2001, 2010; Potkański et al. 2009). The results of research published to date indicate a slight influence of essential oils on the process of biohydrogenation. An experiment conducted by Benchaar et al. (2007a) showed that the supplementation of feed rations for dairy cows with 750 mg of a mixture of essential oils had no effect on the composition of milk fatty acids, while the supplementation with 2 g of a similar mixture per day increased the concentration of conjugated linoleic acid.

Unpublished results of own experiments also did not confirmed the potential of essential oils to modulate rumen biohydrogenation. None of used essential oils originating from *Eugenia caryophyllata*, *Abies sibirica* or *Vanilla planifolia* improve concentration of conjugated linoleic acid isomers in the rumen and milk of dairy cows.

Research are also completed to evaluate the effect of essential oils on ruminants performance. Studies by Benchaar et al. (2006a, 2007a) revealed no changes in feed dry matter intake, milk production and composition as a result of using the supplementation with 750 mg or 2 g of a mixture of essential oils. Spanghero et al. (2009) analyzed the influence of increasing amounts of microencapsulated mixture of essential oils RumaXol Feed (Soda Feed Ingredients, MC 98000, Monaco) on the productivity of dairy cows in the first lactation. The supplemented essential oils had no influence on dietary dry matter intake, water intake and the content of dry matter in feces, and similarly the digestibility of nutrients was not changed by the used experimental factor. In addition, no influence on the productivity of milk and its components was found, however, the content of protein in milk increased as a result of using medium levels of phytofactors. The ambiguity of the results obtained so far has been confirmed by a study by Santos et al. (2010) on 310 dairy cows in early lactation, when the influence of commercial mixture of essential oils Agolin Ruminant (AGOLIN S.A., Biěre, Switzerland), containing mainly eugenol, geranyl acetate and coriander oil (*Coriandrum sativum*), on production performance was analyzed. The supplementation of feed rations with essential oils resulted in a decrease in dietary dry matter intake and a reduction in fat concentration in milk (expressed as g/d and g/kg) with unchanged milk production in groups with the analyzed factor. The supplementation with essential oils also caused a decrease in the cows' body condition score (BCS). The obtained results suggest an adverse effect of essential oils on the milk fat synthesis, presumably resulting from the decreased production of acetic acid and changed ratio of acetic to propionic acids in the rumen.

Several experiments on determination of the influence of essential oils on the production performance of young beef cattle has been conducted, however data on the influence of essential oils on the production performance of beef cattle is also ambiguous. Benchaar et al. (2006b) found that the supplementation with a commercial mixture of essential oils (Vertan®, IDENA, Sautron, France), which among other things consists of thymol, eugenol, vanillin and limonene, had no influence on feed intake and average weight gains. However, they observed a change in the ratio of weight gains to dietary dry matter intake, which as a result enables to increase the effectiveness of utilization of feed ration nutrients.

Meyer et al. (2009) found no significant influence of the examined factors (a mixture of thymol, eugenol, vanillin, guaiacol and limonene) on the analyzed performance, including daily average weight gains. However, Yang et al. (2010) investigated the effect of a pure component – cinnamaldehyde, on growth parameters of beef cattle and feed utilization. The obtained results indicate that the supplementation of feed for beef cattle with small doses of cinnamaldehyde improved feed intake in the first 30 days of the experiment (altogether 112 days), yet it had a minimal influence on daily weight gains, feed utilization and basic carcass parameters during the research.

So far little research was conducted on the influence of essential oils on rearing performance of young ruminants. Soltan (2009) determined the influence of a mixture, which included the following oils: eucalyptus, menthol and mint oil, on the rearing performance of two calf groups – before and after weaning. In the first group the mixture of oils was supplemented to milk replacer, and in the second group directly to water. In the group of calves before weaning the use of essential oils caused a decrease in dietary dry matter intake as a result of a reduction of concentrate intake, improvement of nutrient digestibility and also a decrease in the incidence of diarrhea. The group of calves after weaning, which received increasing doses of the oil mixture directly to water, showed better weight gains and reduced feed intake with greater feed utilization. The most favorable effects were obtained when medium doses of the supplemented essential oils were used, which suggests that the use of higher concentrations of essential oils is not justified by effectiveness and economic factors.

10.5 Summary

Summarizing the above-quoted results, several factors should be noted and developed in the near future, whose analysis seems to be necessary in order to use the described essential oils rich in plant secondary metabolites in practical animal nutrition on a large scale and modulate fermentation processes occurring in the rumen:

1. adaptive abilities of microorganisms and pathogens present in the digestive tract of ruminants to the used essential oils; extension of the time of experiments seems to be necessary;
2. mechanisms of action of particular essential oils on the particular groups of organisms inhabiting the digestive tract;
3. feed ration type and especially the level of protein and energy, including carbohydrate type (structural, non-structural);
4. reactions of synergism and antagonism among the essential oils mixtures and essential oils and dietary components
5. conducting experiments under production conditions, which will enable to take into account the environmental factors as well as the influence of animal species, breed, age, production type and physiological state.

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Chapter 11
Saponins: Effects on Rumen Microbial Ecosystem and Metabolism in the Rumen

Elizabeth Wina

Abstract Saponins are triterpene and steroidal glycoside compounds in which aglycone (sapogenins) are attached to one or more sugar moieties. They are polar compounds and have great diversity in their chemical structures. Different aqueous or polar solvents have been used to extract saponins. Saponins exhibit several physical and biological properties such as the formation of a stable foam, haemolysis, antimicrobial activity and defaunation of the rumen; however, there is little correlation among these properties. This paper describes the methods of saponin extraction, their chemical diversity and the effects on rumen microbial ecosystem along with metabolism of saponins in the rumen. Recent studies on methane emissions by ruminants provide evidence that saponins may have potential to be used as a antimethanogenic agent; however, the inclusion level of saponin from each source should be tested to get the optimum result. The practical utility of saponins or saponin containing plants as feed additives in sustainable and environmental friendly ruminant production warrants further investigation.

Keywords Saponins • Rumen • Microbial ecosystem • Metabolism of saponins • Extraction • Microbial diversity

Abbreviations

NMR	nuclear magnetic resonance
VFA	volatile fatty acid
DGGE	denaturing gradient gel electrophoresis

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PCR polymerase chain reaction
CLA conjugated linoleic acid
VA vaccenic acid
RNA ribonucleic acid
MPS microbial protein synthesis

11.1 Introduction

Saponins are a structurally diverse family of plant secondary metabolites. In general, saponins consist of an aglycone attached to one or more sugar moieties. They are classified as triterpene saponins and steroid saponins based on their aglycones which can be in the form of triterpene (30 C-atoms) or steroid (27 C-atoms), respectively. Recently, Vincken et al. (2007) proposed a new classification of saponins based on the biosynthesis of carbon skeletons of the aglycone. An enormous diversity found in saponin structures was the reason for this new classification. According to this classification, 11 main classes of saponins were distinguished, i.e. dammaranes, tirucallanes, lupanes, hopanes, oleananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes, and steroids. Out of these structures, oleanane is the most common aglycone occurring in plants with glucose, arabinose, rhamnose, xylose and glucuronic acid as the common sugars attached to it. Most saponins are defined as monodesmosides if they contain one saccharide chain attached to C3 atom of their aglycones or bidesmosides, if an additional saccharide chain is attached to C26 (furanstanol saponins) or C28 (triterpene saponins) (Güçlü-Üstündağ and Mazza 2007). Saponins can also be classified as neutral if the sugar attached to sapogenin is a common monosaccharide (glucose, xylose, arabinose etc.) or acidic if the sugar moiety contains uronic acid or one or more carboxylic groups (Lasztity et al. 1998). The aglycone component of the saponin is hydrophobic whereas the conjugated sugars are hydrophilic. Their unique structures allow saponins to give stable foams in water or aqueous solutions.

There are several recent reviews on the effect of secondary compounds including saponin or saponin containing plants on rumen function and animal production (Hart et al. 2008; Patra and Saxena 2009a). This review describes in more details saponin extraction methods, the structural diversity of saponins and their effect on rumen microbes and rumen fermentation. The information presented here could provide wider opportunities for the utilization of saponins and saponin-containing plants as feed additives in sustainable and environmental friendly ruminant production.

11.2 Biological Properties of Saponin

The most important property of saponins is their ability to form very stable foam as a consequence of their surfactant ability. The presence of polar groups in the sugar moiety together with the non polar character of the aglycone moiety enables saponins

to lower surface tension in aqueous systems. Traditionally, saponin-containing plants have been used as natural washing agents.

Other biological properties of saponins include their ability to haemolyse the red blood cells, to depress protozoal populations in the rumen, and to inhibit the growth of microbes, especially fungi. The same saponin extract can exhibit several of these biological properties.

The methanol extract of pericarp of *Sapindus rarak* fruit could make a stable foam, haemolyse the red cells (Wina, unpublished) and also depress the protozoa population both *in vitro* and *in vivo* (Wina et al. 2005a, 2006a). Saponins extracted from guar meal in 100% methanol had the ability to haemolyse the red cells and also showed antibacterial activities against the pathogenic bacteria, *Staphylococcus aureus, Salmonella typhimurium* and *Escherichia coli* (Hassan et al. 2010a, b). Gestetner et al. (2006) found that the lucerne saponin exhibited haemolytic property and also antifungal activity. Owing to these various phenomena, several authors have tried to link the biological activities of saponins as antifungal, antiprotozoal, antimicrobial or cytotoxic activity to haemolytic activity or foaming ability (Chwalek et al. 2006; Wang et al. 2007). The foaming test and haemolysis test for saponin are simple tests. Therefore, it would be faster and easier to screen many different saponins by either foaming test or haemolysis test and to relate the result to other biological activities of saponins, then, to choose the potential saponins for further use as feed additives or medicinal application.

The result of our study with 23 saponin-containing plants, however, did not find any correlation between height of foam or haemolytic activity to the antiprotozoal activity (Wina, unpublished). Wang et al. (2007) have disclosed from their study with 63 steroidal saponins that there was no correlation between haemolytic activity and cytotoxicity.

Lin and Wang (2010) studied the haemolytic mechanism of saponins using molecular dynamic simulation technique. Saponins first penetrate easily into the lipid membrane, and then accumulate in the lipid raft micro-domain. Saponins bind cholesterol in the lipid membrane and prevent the cholesterol from the interaction with sphingomyelin. Saponin-cholesterol micelles destabilize the structure of the lipid raft micro-domain and cause disruption of the lipid bilayer, which eventually lead to the haemolysis of red cells (Lin and Wang 2010; Baumann et al. 2000). The mechanism of protozoal lysis by saponins is similar to the mechanism of haemolysis of red blood cells by saponins. However, some aglycones still showed their ability to haemolyse the red cells but lost their activity toward protozoa. The presence of intact sugar moiety is very important for its activity against protozoa. Muetzel et al. (2005) reported that the aglycone of *Sapindus rarak* saponins (hederagenin) did not show its ability to depress protozoa population.

From several studies, it can be concluded that antiprotozoal, antimicrobial or cytotoxicity activity of saponins cannot be predicted from the height of foam or its haemolytic activity. Direct screening of the saponin extracts or saponin containing plants based on its specific biological property is recommended.

11.3 Biodiversity of Saponins in Plant Materials

Saponins are a complex group of compounds and different species or parts of plant synthesize different types of saponins (Lasztity et al. 1998; Mahato and Garai 1998). Diversity can also occur in one location of the plants. Several saponins in one plant usually have the same aglycone but different sugar moieties. Several species of *Sapindus* trees are found in different parts of the world such as *Sapindus rarak, Sapindus emarginatus, Sapindus mukorossi* and *Sapindus saponaria*. Their fruits contain foaming substances: it was reported that 20 different monodesmosidic saponins have been isolated and structural elucidated in *Sapindus rarak* fruit's pericarp (Hamburger et al. 1992; Asao et al. 2009).

Hederagenin is the aglycone found in these saponins. When arabinose is directly attached to hederagenin and rhamnose and arabinose attached to arabinose, this saponin is called sapindoside (Fig. 11.1). When arabinose attached to hederagenin and acetyl group attached to arabinose or rhamnose, this saponin is called rarasaponin. Asao et al. (2009) have isolated and identified six different structures of rarasaponin (I to VI) from *Sapindus rarak* fruit's pericarp. Other Sapindus, *Sapindus emarginatus* (Kanchanapoom et al. 2001), *Sapindus mukorossi* (Huang et al. 2008) and *Sapindus saponaria* (Tsuzuki et al. 2007) fruit's pericarp contained similar saponins to *Sapindus rarak* with hederagenin as the aglycone. Huang et al. (2008) named the isolated saponin as sapinmusaponin, which was almost identical with rarasaponin in *Sapindus rarak*. They also found damarane type of saponin in the gall of *Sapindus emarginatus*. Kanchanapoom et al. (2001) isolated saponins with acetyl hederagenin and oleanolic acid as the aglycone in *Sapindus emarginatus*. Lemos et al. (1992, 1994) found other saponins in *Sapindus saponaria* with glucose directly attached to hederagenin and rhamnose and arabinose linked to the glucose. The complexity of these saponins has not yet received any attention in relation to their individual activity on rumen microbes.

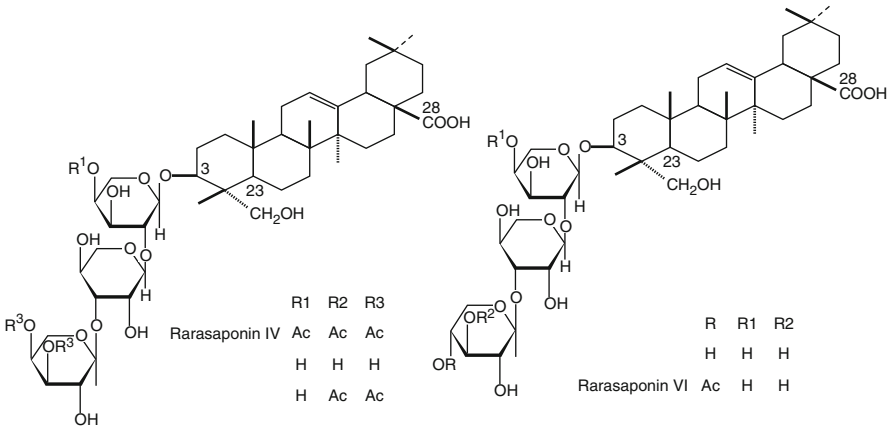

Fig. 11.1 Some saponin structures in the fruit's pericarp of *Sapindus rarak* (Asao et al. 2009)

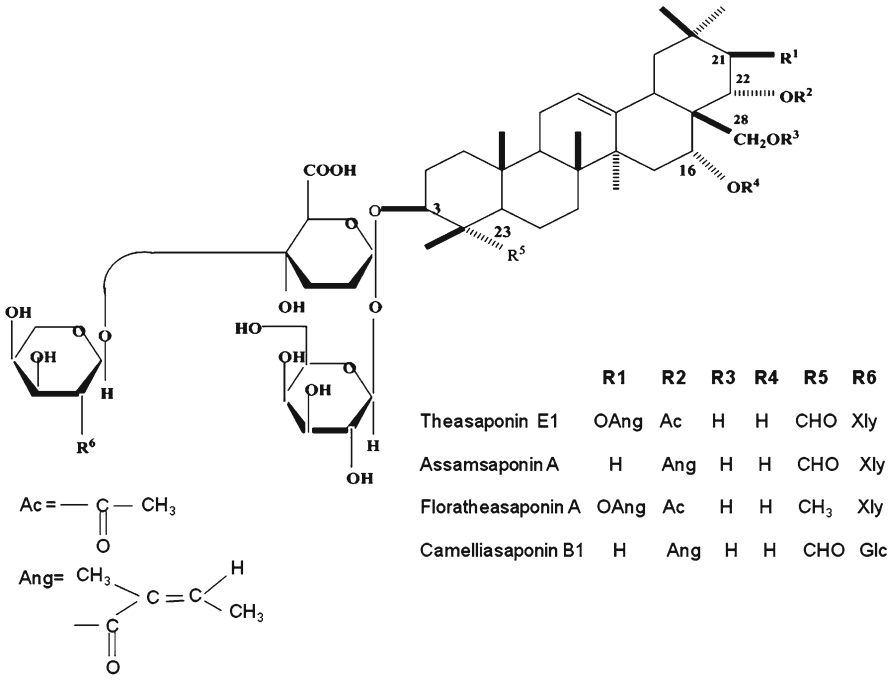

Fig. 11.2 Some saponin structures in tea (*Camellia sinesis*) seed (Yoshikawa et al. 2005)

Several structures of saponin have been identified in tea (*Camellia sinensis*) seeds, leaves and flowers. Saponin extract from tea seed is commercially available and used for killing small fish in shrimp ponds. Recently, it has been tested for a feed additive for ruminants and as a medicinal use. Yoshikawa et al. (2005) reported that 16 different structures of saponins have been identified in tea seeds. Further, they elucidated 12 new saponins in tea seeds (Yoshikawa et al. 2007). Therefore, in tea seeds, 28 different saponins have been isolated and identified as theasaponins, camelliasaponins, floratheasaponins, etc. (Fig. 11.2). Yoshikawa et al. (2005, 2007) showed that the position of acyl group that attached to the aglycone (theasapogenol) and different groups in the sugar moieties are important from a pharmacological point of view since different positions or groups attached to the aglycone or sugar moiety cause different activities.

Yucca schidigera stem contained as much as 10% of steroidal saponins which consisted of 28 different structures of spirostanol and furostanol glycosides. Yucca saponins have several aglycones, i.e. sarsapogenin, markogenin, smilagenin, samogenin, gitogenin and neogitogenin. They can be monodesmosides with one sugar chain attached at 3-O (Fig. 11.3, 1–4) and bidesmosides with two sugar chains attached at 3-O and 26-O positions (Fig. 11.3, 5–7). The predominant saponins in yucca are spirostanol saponins which primarily are glycosides of sarsapogenin (66%). Thus, the major property of Yucca saponins as described by Cheeke et al. (2006) is determined by spirostanol saponins (Oleszek et al. 2001).

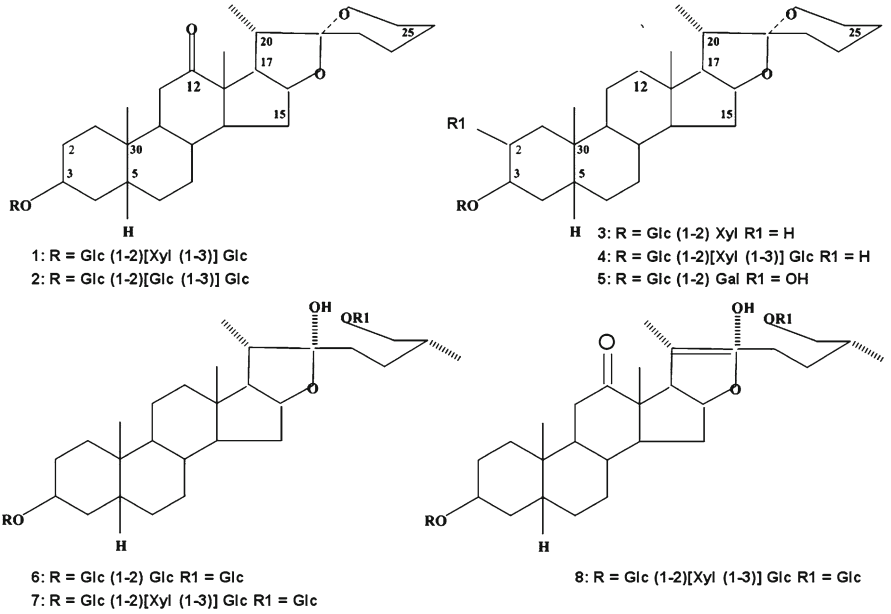

Fig. 11.3 Some saponins of *Yucca schidigera* bark (Cheeke et al. 2006; Oleszek et al. 2001)

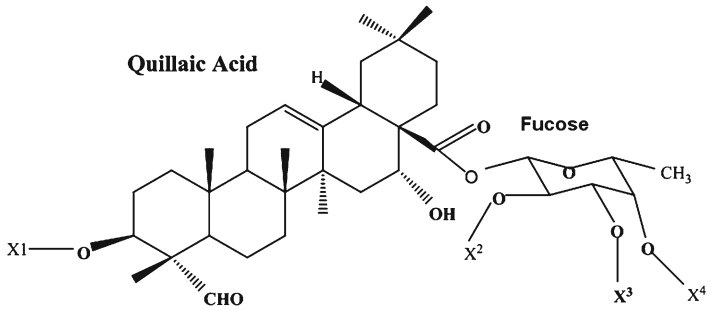

Fig. 11.4 The common basic saponin structure of *Quillaja saponaria* (Bankefors 2006)

Quillja saponaria Molina extract contains 10% total saponins, and more than 20 different structures of triterpenoid saponin have been structurally elucidated by NMR studies (Guo et al. 2000) Further, Bankefors (2006) did structural classification of 47 *Quillaja* saponins including minor compounds by electrospray ionisation ion trap multiple-stage mass spectrometry in combination with multivariate analysis from the chromatographic fractions. All studied structures are bidesmosides and consist of quillaic acid (as the aglycone) which is substituted with di or tri-saccharides at C-3 and a branched oligosaccharide at C-28. Bankefors (2006) found that saponin profiles in the young plants were different compared to older specimen; hence the biological and chemical activity differed between batches from different specimen (Fig. 11.4).

11.4 Extraction of Saponins

Saponins from plant materials can be extracted using different techniques and solvents. The conventional techniques for saponin extraction used soxhlet, liquid-liquid or solid–liquid extraction (Berhow et al. 2002; Hassan et al. 2010a, b). These methods consume a lot of solvent, time and may lead to potentially deleterious degradation of labile compounds (Kerem et al. 2005). Therefore, in recent years, new extraction techniques include accelerated solvent extraction, supercritical fluid extraction, solid-phase microextraction, sonication, extraction with supercritical or subcritical water, and microwave-assisted extraction have been developed and are considered to be more efficient than the conventional methods (Wu et al. 2001; Kerem et al. 2005; Ligor et al. 2005; Güçlü-Üstündağ and Mazza 2007). Ultrasonication-assisted extraction of ginseng saponins was about three times faster than the liquid-liquid extraction and can be carried out at lower temperature (Wu et al. 2001). Kerem et al. (2005) reported that methanol- microwave assisted method to extract saponin of chickpea proved to be faster and more efficient than soxhlet extraction.

Since saponins are polar compounds, many saponin extraction methods used water, aqueous methanol or ethanol, absolute methanol, ethanol or n-butanol (Kaur and Arora 2009; Xu et al. 2010; Tsuzuki et al. 2007; Zhang et al. 2005). The type of aglycone, type of sugar moiety and functional group attached to aglycone or sugar moiety, the concentration of saponin influence the saponin's ability to dissolve in different solvents, therefore different extracts exerted different activities.

Our study with *Sapindus rarak* fruit's pericarps showed that different extracts had different activity on suppressing protozoa population in the *in vitro* rumen (Wina, unpublished). The 50% methanol extract caused the highest suppression on protozoa population in the *in vitro* rumen fermentation, followed by 70% methanol and ethanol extract. While Kamra et al. (2006) and Agarwal et al. (2006) showed that the methanol extract of *S. mukorossi* was highly detrimental to protozoa in the *in vitro* rumen followed by ethanol and water extracts. Goel et al. (2008a) reported that either 50% methanol or 95% methanol extract of Fenugreek or *Sesbania sesban* gave similar effect in depressing protozoa, while water extract of *Sesbania sesban* showed lower activity to depress protozoa compared to 50% or 95% methanol extract. Patra et al. (2006) found that the ethanol extract of five plants (*Acacia concinna* pod, *Terminalia chebula*, *Terminalia belerica*, *Emblica officinalis* seed pulp and *Azadirachta indica* seed kernel) was more active than methanol or water extract in decreasing protozoa population and *in vitro* methane production. Sirohi et al. (2009) reported that the acetone extracts of several plants have more effective antimicrobial property than methanol or aqueous extracts, whereas aqueous extract of *Sapindus mukorossi* was the best inhibitor of methane production among other extracts. Hassan et al. (2010b) showed that saponins of guar meal that dissolved in 100% methanol had antibacterial activities against *Staphylococcus aureus*, *Salmonella typhimurium* and *Escherichia coli*, while only those dissolved in 20% and 60% methanol stimulated *Lactobacillus* spp. growth. These above reports

showed that different extracts of a specific plant had different activities against microorganism growth or population.

Unless it is purified, the saponins present in an extract may be in conjunction with other compounds. These compounds may or may not have an effect on the rumen fermentation. Soluble carbohydrates (simple sugars) are usually present in the methanol extract and these compounds may contribute to the increase of total production of short chain fatty acid during rumen fermentation (Wina et al. 2005b). Studies with a subfraction of methanol extract of *Sapindus rarak* showed that the ethyl acetate subfraction decreased protozoa numbers in the *in vitro* rumen fermentation much less than the aqueous subfraction (16% vs. 62%, respectively), and reduced total gas production 10% while no effect of aqueous subfraction was observed (Wina, unpublished). Beside saponins, a sesquiterpene glycoside, mukurozioside IIb has been identified in the fruit pericarp of related plant, *Sapindus mukurossi* and *S. emarginatus* (Kanchanapoom et al. 2001). This compound might dissolve in the ethyl acetate subfraction and not in the aqueous subfraction of *S. rarak* methanol extract

Different techniques or solvents used for saponin extraction may affect the purity of the extract. This was shown by different contents of sarsaponin in four commercial products of *Yucca schidigera* extract (Singer et al. 2008). *Y. schidigera* powder contained not only saponins but also phenolics (resveratrol, yuccaol) (Piacente et al. 2005), glycoprotein and stilbenes. These other compounds were present in non-butanol extractable fraction and exert several properties such as antiinflammatory, antioxidant (Cheeke et al. 2006), reducing air ammonia concentration and fecal odour (Piacente et al. 2005). Therefore, different results on the use of *Yucca* extract in the ruminant feed can be influenced by the presence of other compounds in the extract.

The use of pure saponin would allow the study of the effect of saponin on the rumen without any confounding effects from other impurities. The purification of the crude saponin extract usually requires a sequential approach from extraction, precipitation, adsorption, ultrafiltration and chromatography (Güçlü-Üstündağ and Mazza 2007)

11.5 Saponin Structure Activity Relationship

Studies on structure activity relationship mainly have been conducted to identify compounds for medical treatment or health. These findings would be useful in understanding the various results found in many animal experiments using saponins since no structure activity relationship of saponins to their activity toward ruminal microbes has been elucidated.

Saponin from *Sapindus mukorossi* and its monodesmosides, resulting from the partial degradation of saponin have exhibited an activity in reducing fungal growth (Saha et al. 2010). Further removal of the sugar moiety yielded a complete loss in activity. Esterification of the hydroxyl group has been found to influence the antifungal activity (Saha et al. 2010). Another study with steroidal saponins found

that antifungal activity against *Candida neoformans* was influenced by the aglycone moiety, number and structure of sugar moieties. The sugar moiety of four or five monosaccharide units displayed remarkable antifungal activity and when the sugar moiety contained less than 4 monosaccharides units, the activity was lost (Yang et al. 2006). The spirostanol type of steroidal saponin has more antifungal activity compared to the furanstanol type (Zhang et al. 2005). Conversely, Barile et al. (2007) found that furastanol saponin has higher activity than spirostanol.

Tomatidine, a steroidal alkaloid has inhibitory activity against *Saccharomyces cerevisiae* (Simons et al. 2006). The removal of a single sugar from the tetrasaccharide chain of α-tomatine (the aglycone) resulted in a substantial reduction in antimicrobial activity. But, the complete loss of sugars thus forming tomatine (the aglycone) led to enhanced antifungal activity. In contrast, the triterpene aglycones i.e. oleanic acid, β-amyrin, and hederagenin, did not exhibit any inhibitory activity (Simons et al. 2006).

Saponins from several plant sources have nematicidal activity. The partial hydrolysis of saponins showed more nematicidal activity than the related saponins at the same dose. The aglycones exerted nematicidal activity with hederagenin being the most active followed by medicagenic and bayogenin (Argentieri et al. 2008).

The haemolytic activity of saponins is dependent on the nature of aglycone and the glycoside chain (sugar moiety) including the configuration of the interglycosidic linkages, the substitution pattern and the type of sugar units involved (Chwalek et al. 2006).

The different plant source and structure of saponins, structure of algycone, length and position of sugar moiety may contribute various activities toward bacteria, fungi, protozoa and further studies are warranted.

11.6 Effect of Saponins on Ruminal Microorganisms

Wina et al. (2005a) compiled the data published from 1987 to 2004 on the saponin effect on rumen fermentation both *in vitro* and *in vivo* while Hart et al. (2008) modified the same data by adding the data of total production of volatile fatty acid and methane production. Recently, Patra and Saxena (2009a) presented the *in vivo* data published from 1987 to 2009 on saponin effects on rumen fermentation and the performance of animals. Tables 11.1 and 11.2 present the data published from 2005 to 2010 on the effect of saponin on rumen fermentation *in vitro* and *in vivo*, respectively.

11.6.1 Protozoa

Saponins depress the protozoa population and activity in the rumen (Wallace et al. 2002). The mechanism proposed is that saponin reduces the protozoa due to the interaction of saponins with cholesterol in the outer membrane of the protozoa

Table 11.1 Responses in the *in vitro* system due to the addition of different saponin extracts or saponin containing plants

No	References	Saponin	Concentration	*in vitro* system	Feed	Response
1	Wina et al. (2005a)	Methanol extract of *S. rarak* pericarps	0.25, 0.5, 1.0, 2.0–4.0 mg/ml	Gas test – holstein steers rumen liquor	Elephant grass: wheat bran (65:35 w/w)	Decreased: Protozoal numbers (11–49%); Ammonia production (8–16%) Increased: Gas production (2–7%), Total VFA production (2–8%), Molar proportion of propionate (1–45%), Microbial protein synthesis (4–42%)
2	Hu et al. (2005)	Tea (*Camellia sinensis*) saponin extract	0.2–0.4 mg/ml of buffered RL	Gas test- sheep rumen liquor	50% corn meal and 50% grass meal	Decreased: Gas production (4–10%), Protozoal numbers (13–16%); Ammonia production (19%); Methane production (12.7–14%) No effect on total VFA production Interactions between tea saponin and protozoa were mostly weak
3	Busquet et al. (2005)	*Trigonella foenum graecum* extract, 180 g/kg sarsaponin	2.2 mg/l of buffered RL = 7.1 mg/day	Continuous culture-dairy cow rumen liquor	Alfalfa hay 594, barley grain 201, corn grain 124, SBM 69 g/kg	No effect on total VFA, ammonia, propionate
4	Cardozo et al. (2004)	*Yucca schidigera* extract contained 80 g/kg saponins	0, 0.3, 3, 30, 300 mg/l buffered RL	Fermentor tubes-heifers fed forage: concentrate (1:9)	Forage: concentrate (1:9)	At pH 7: no effect acetate, propionate, butyrate Decreased ammonia (14–43%), Increased branched VFA (29–100%) At pH 5: decreased acetate (21.7–27.3%), Increased butyrate (12.7%)

5	Kamra et al. (2006)	Ethanol extract of *Sapindus mukorossi*	0.5 ml/30 ml buffered RL	Buffalo	Wheat flour: wheat straw (80:20)	Decreased: Protozoal numbers (76%); Methane production (96%), Ammonia production (2.7%), pH (1.7%), acetate (18.7%) Gas production (11%) Increased molar proportion of propionate (37%)
6	Pen et al. (2006)	*Yucca schidigera* extract contained 80–100 g/kg saponins	2, 4, and 6 ml/l buffered RL	Continuous-dairy cow rumen liquor	Oat and alfalfa hay (1:1,w/w)	Reduced: Protozoal numbers (29–56%), Ammonia production (26–48%), Methane production (17–42%) No effect on total VFA Increased: Molar proportion of propionate (16–54%) CO_2 production (0–28%)
7	Pen et al. (2006)	*Quillaja saponaria* extract contained 50–70 g/kg saponins	0, 2, 4, and 6 ml/l buffered RL	Continuous-dairy cow rumen liquor	Oat and alfalfa hay (1:1,w/w)	Decreased: Protozoal numbers (34–40%), Ammonia production (21–20%), Increased: Molar proportion of propionate (15–19%) CO2 production (1.5–28%) No effect on total VFA, methane production

(continued)

Table 11.1 (continued)

No	References	Saponin	Concentration	*in vitro* system	Feed	Response
8	Patra et al. (2006)	Water, methanol and ethanol extracts of *Sapindus mukorossi*	0.5 ml/30 ml buffered RL	Buffalo	Wheat flour: wheat straw (80:20)	All extracts decreased protozoal count and activity, ethanol extract gave the highest inhibition to: Methane production (22.7%) Total gas production (11.5%) and 0%, Acetate and butyrate reduced, DMD decreased Increased propionate by 26%, 57.6% and 37% with water, methanol and ethanol extracts, respectively
9	Goel et al. (2008a)	*Trigonella foenum-graecum* seed	0, 66 mg/40 ml buffered RL	Gas test -hay (concentrate) fed Holstein cattle	Hay (concentrate)	Depressed: Protozoal numbers (50%-hay, 56%-conc.) Methane production (5%-hay, 10%-conc.)
10	Goel et al. (2008a)	*Sesbania sesban* leaves	0, 66 mg/40 ml buffered RL	Gas test -hay (concentrate) fed Holstein cattle	Hay (concentrate)	Decreased: Protozoal numbers (48%-hay, 50%-conc.) Methane production (11%-hay, 12%-conc.)

11	Goel et al. (2008b)	80% methanol extract of *Sesbania sesban* leaves	0, 10.9 and 21.2 mg/40 ml buffered RL	Gas test- conc: hay fed holstein cattle	Concentrate: hay (1:1)	Decreased: Protozoal numbers (14–36%) Methanogens (78%), fungi (38%) Methane production (4.69–6.14%) Increased: Fibrobacter (45%) Ruminococcus (26%) No effect on total VFA, propionate
12	Goel et al. (2008b)	80% methanol extract of *Trigonella foenum-graecum* seed	0, 5.62 and 11.54 mg/40 ml buffered RL	Gas test-conc: hay fed holstein cattle	Concentrate: hay (1:1)	Depressed Protozoal numbers (15–39%) Methanogens (22%), Fungi (65%) Methane production (1.82–1.97%) Increased: Fibrobacter (42%) Ruminococcus (40%) No effect on total VFA, propionate
13	Goel et al. (2008b)	80% methanol extract of *Knautia arvensis* leaves	0, 3.88 and 7.76 mg/40 ml buffered RL	Gas test -conc: hay fed holstein cattle	Concentrate: hay (1:1)	Reduced protozoal numbers (14–25%), methane production (5.50–6.43%) Increased: Fibrobacter (24%), ruminococcus (18%) No effect on total VFA, propinoate. Methanogens (21%), fungi (30%)

(continued)

Table 11.1 (continued)

No	References	Saponin	Concentration	*in vitro* system	Feed	Response
14	Soliva et al. (2008)	*Sesbania sesban* leaves 10865, *Sesbania sesban* leaves 15019	300 mg/syringe	Gas test -conc (0.5 kg), hay ad lib. fed brown Swiss cow	No other substrate	*S. sesban* 10865: Methane 0.75 ml, methane/total gas 0.086 v/v; Protozoal count 3.01×10^4/ml, Bacteria 1.21×10^9/ml, ammonia 10.28 mmol/l, Total VFA 47.6; propionate 19.6 mmol/l; *S. sesban* 15019: Methane 6.56 ml; methane/total gas 0.144 v/v; Protozoal count 3.77 × 10^4/ml, Bacteria 1.29 × 10^9/ml; ammonia 16.13 mmol/l; Total VFA 78.0 mmol/l; propionate 20.1 mmol/l
15	Guo et al. (2008)	Tea saponin	0.4 mg/ml buffered rumen liquor	Bottle-gas test- rumen sheep fed alfalfa hay : concentrate (60:40)	Corn meal and grass meal (750 mg; 50:50, w/w)	Depressed: Protozoal relative abundance (51%), Fungal relative abundance (79%), Methane production (8%), Expression of mcrA gene (76%) Increased: Molar proportion of propionate (11%) *F. succinogenes* (41%) No effect on total VFA production, methanogens, *R. flavefaciens*

16	Soliva et al. (2008)	*Sapindus saponaria* fruit, *Enterolobium cyclocarpum* fruit	300 mg/syringe	Gas test -conc (0.5 kg), hay *ad lib.* fed brown Swiss cow	No other substrate	*S. saponaria*: Methane 5.14 ml, methane/total gas 0.12 v/v; Protozoal count 1.86×10^4/ml, Bacteria 1.03×10^9/ml, ammonia 18.17 mmol/l, Total VFA 92.5 mmol/l; propionate 21.7 mmol/l; *E. cyclocarpum*: Methane 12.71 ml; Protozoal count 0.175×10^4/ml, Bacteria 1.41×10^9/ml; ammonia 9.57 mmol/l; Total VFA 102.7 mmol/l; propionate 26.5 mmol/l
17	Holtshausen et al. (2009)	*Yucca schidigera* whole plant powder (6% saponin)	15, 30, 45 mg/g substrate	Batch culture -holstein cow rumen liquor	51:49 forage (barley silage):concentrate	Depressed: Total gas (2–4%), ammonia (81–100%) and Methane production (8–26%) Increased molar proportion of propionate (10–36%) No effect on total VFA production

Table 11.2 Responses in the *in vivo* system due to the addition of different saponin extracts or saponin containing plants

No	References	Saponin	Concentration	Animal	Feed	Response
1	Lila et al. (2005)	Sarsaponin (*Yucca schidigera*)	5–10 g/kg DM = 11.2–22.4 g/day	Holstein steers	Sudangrass hay: concentrate mixture (1.5:1)	Depressed: Protozoal numbers (67–71%), Methane production (8–12%), Total VFA production (2–2%), Acetate (2.5–3.8%) Increased: Propionate (4–7%), butyrate (7–10%), Ammonia production (5.7–10%)
2	Wina et al. (2006a)	Methanol extract *S. rarak* fruits pericarps	0.24, 0.48, 0.72 g/kg BM – daily for 100 days = 4, 8, 12 g/day	Thin tail sheep	Elephant grass: wheat bran (65:35 w/w)	Reduced: Protozoal numbers (34–80%), Ammonia production (12–18%) Increased propionate (14–19%) No effect on total VFA production, fungi, Methanogens, MCP
3	Wina et al. (2006b)	Methanol extract *S. rarak* pericarps	0.48–0.72 g/kg BM – daily feeding for 27 days	Thin tail sheep	Elephant grass: wheat bran (65:35 w/w)	Decreased: Protozoal numbers (76–78%), Ammonia production (21–29%) Increased propionate (13–14%) No effect on total VFA production, MCP
4	Wina et al. (2006b)	Methanol extract *S. rarak* pericarps	0.48–0.72 g/kg BM-interval feeding 3 days for 27 days	Thin tail sheep	Elephant grass: wheat bran (65:35 w/w)	Depressed: Protozoal numbers (69–64%), Ammonia production (7–21%) Increased propionate (6–7%) No effect on total VFA production, MCP

5	Lovett et al. (2006)	*Yucca schidigera*	0, 25, 50 g/head/day	Steer	Maize silage: grass silage: concentrate (61:23:15 DM)	Depressed: Protozoal numbers (19–34%), Propionate (23–19%), Total VFA (10–6%)
6	Pen et al. (2007)	*Yucca schidigera* extract (directly fed to rumen)	1.31–1.64 g saponin/day	Cheviot weather	Concentrate: Italian ryegrass hay (2:3 w/w)	Reduced total VFA (18%) Increased digestible energy (4%), ammonia (20%) No effect on methane emission, CO_2, MCP, Total energy loss
7	Pen et al. (2007)	*Quillaja saponaria* extract (directly fed to rumen)	0.8–1.3 g saponin/day	Cheviot weather	Concentrate: Italian ryegrass hay (2:3 w/w)	Reduced: Total VFA (12%), Ammonia (11%) Increased digestible energy (6%) No effect on protozoal numbers, methane emission, CO2, MCP, digestible energy
8	Santoso et al. (2007)	*Biophytum petersianum* Klotch aqueous extract	0, 13, 19.5 and 26 mg saponin/kg BW	Kacang goat	Elephant grass silage: grain-based concentrate (70:30 DM)	Reduced: Protozoal numbers (35–41%), Total VFA (9–20%), Ammonia (26–32%) Acetate, butyrate, iso acids reduced Increased propionate (16–33%) and MCP (2–38%)
9	Baah et al. (2007)	*Quillaja saponaria* extract (100 g saponins/kg)	8 g/kg diet DM	Jersey heifer	Rolled barley grain (885), barley silage (92), canola meal (11)	Reduced protozoal numbers (34%) No effect on ammonia production, total VFA production, Propionate, enzyme activities

(continued)

Table 11.2 (continued)

No	References	Saponin	Concentration	Animal	Feed	Response
10	Koenig et al. (2007)	*Enterolobium cyclocarpum leaves (saponin 0.8 mg/g leaves)*	16 g/100 g diet	Canadian arcott weathers	Barley silage (600 g/kg)and a barley grain and soyabean meal pelleted concentrate (400 g/kg)	Reduced: protozoal numbers (25%), Ammonia production (42%), OM digested in the rumen (7%) Higher pH, total VFA (28%) and isoacids Lower propionate (9%) No effect on acetate, butyric
11	Benchaar et al. (2008)	*Yucca schidigera* extract (saponin 100 mg/g)	60 g/day	Holstein lactating cow	Grass silage 39.6, Corn, 15.9, Beet pulp 12.0, CGM 9.9, Wheat bran 9.8, SBM 48% CP 8.8, NaHCO31.5, Limestone 1.1	No effect on protozoal numbers, ammonia production, Total VFA, propionate
12	Singer et al. (2008)	*Yucca schidigera* extract (sarsaponin 95.4 mg/g)	0, 50, 100, 150 g/cow/day	Holstein cow	Alfalfa hay 361, barley grain 111, corn grain 166, DDGS 34, SBM 20.7, cottonseed 95.6, beet pulp 55, almond hull 120, fat 8 g/kg	Increased molar proportion of propionate (P=0.08) A tendency decreased ammonia No effect on protozoal numbers and pH
13	Holtshausen et al. (2009)	*Yucca schidigera* whole plant powder (6% saponin)	10 g/kg DM	Holstein cow	51:49 forage (barley silage):concentrate	No effect on protozoal numbers, ammonia, methane production, total VFA, propionate
14	Holtshausen et al. (2009)	*Quillaja saponaria* whole plant powder (3% saponin)	10 g/kg DM	Holstein cow	51:49 forage (barley silage):concentrate	No effect on protozoal numbers, ammonia, methane production, total VFA, propionate

15	Poungchompu et al. (2009)	*Sapindus saponaria* fruit (15.7% saponin) + Mangosteen (*Garcinia mangostana*) peels (12.7% tannin) :44:51	40 g/kg feed	Holstein friesian heifers	Rice straw and concentrate (cassava chip 66%, rice bran 5%, brewer grains 9%, cottonseed meal 13%, urea 3%)	Decreased protozoal numbers and fungal numbers, acetate Increased VFA, propionate and methanogens No effect on ammonia, butyrate and total bacteria
16	Suharti (2010)	Methanol extract *S. rarak* whole fruit	100, 200 mg/kg BW	Crosbred ongole cattle	Native grasses: concentrate (70:30 DM)	Decreased: Protozoal numbers (not significant) MCP(not significant), Ammonia (43% at high level *S. rarak*), Ratio acetate:propionate (13–14%) Increased: Total VFA (46%, 31%) Molar proportion of propionate (11–13%) No effect on digestibility, acetate or butyrate
17	Mao et al. (2010)	Tea (*Camellia sinensis*) saponin extract	3 g/day	Huzhou lamb	Concentrate: Chinese rye (40:60)	Reduced: Protozoal numbers (41%), Ammonia production (15%), Methane production (28%), *R. flavefaciens* (51%) Enhanced: Total VFA (13%), MCP (22%) No effect on propionate, fungi, *F. succinogenes,* Methanogens

(continued)

Table 11.2 (continued)

No	References	Saponin	Concentration	Animal	Feed	Response
18	Zhou et al. (2010)	Tea (*Camellia sinensis*) saponin extract	3 g/day	Hu lamb	Concentrate: Chinese rye (40:60). Concentrate : a mixture of ground corn 626, wheat bran 114, soybean meal 104, and rape seed meal 156	*Refaunated:* Decreased: Protozoa (43%), *F. succinogenes* (79%) Ammonia production (13%), Methane production (11%), Increased: Propionate (11%), MCP (16%) No effect on total VFA, methanogen, fungi, Ruminococcus *Defaunated vs. control (refaunated):* Decreased: Protozoa (100%), *F. succinogenes* (100%) Ammonia (34%), Methane (18%) Increased: Propionate (34%), MCP (36%) No effect on total VFA, methanogens, fungi, Ruminococcus

making a pore and, hence, lysing the protozoa membrane (Wallace et al. 2002). Another mechanism proposed suggests that saponins with its ability to reduce surface tension has a similar effect to detergent causing toxicity to protozoa (Cheeke 2000; Francis et al. 2002). The sugar moiety has an important role in depressing protozoa; once, the saponin is completely hydrolysed, the inhibitory effect on protozoa was reduced or completely disappeared (Teferedegne et al. 1999; Wang et al. 2000; Muetzel et al. 2005). Partial acid hydrolysis of lucerne saponins decreased protozoa numbers in sheep rumen (Lu and Jorgensen 1987) suggesting that partial acid hydrolysed saponins might have some sugars attached to the aglycone , thus still retained its activity to inhibit protozoa.

Most of *in vitro* experiments in either batch or continuous systems showed that the reduction in protozoa population caused by saponins occurred very fast and was not specific. Koenig et al. (2007) showed that the protozoal numbers in sheep rumen markedly reduced 2 h after feeding *Enterolobium cyclocarpum*. *Entodinium* protozoa were present in the rumen as the dominant protozoal species, *Diplodinium*, *Isotricha* or *Dasytricha* were also present in the *in vitro* and *in vivo* experiments. In these experiments, addition of saponins did not influence the proportion of protozoa species (Baah et al. 2007; Koenig et al. 2007; Suharti et al. unpublished). However, when the level of saponin increased, all protozoa species decreased (Ivan et al. 2004; Wina et al. 2005a; Suharti et al. unpublished). Saponins reduced not only the numbers but also the diversity of protozoa. This was shown by a denaturing gradient gel electrophoresis (DGGE) study which revealed a lower diversity of protozoa after 21 days feeding of 3 g of tea saponin to sheep (Wang et al. 2010). *Eremoplastron dilobum* band on DGGE gel disappeared on the tea saponin treatment while *Entodinium furca monolobum* which was indicated as a faint band on the control treatment, appeared as a strong band in the saponin treatment (Wang et al. 2010; Zhou et al. 2010). It could be concluded that saponins may depress only specific protozoa species. Predation among protozoa may also explain the higher growth of one species of protozoa than the others (Dehority 1998; Ohene-Adjei et al. 2007). More studies should be conducted to confirm whether saponins affect specific protozoa species or if there is any predation among the protozoa or both. Table 11.1 shows that the reduction of protozoa numbers by saponin in most of *in vitro* experiments varied from 11% to 80% while in the *in vivo* experiments, the reduction varied from 0% (no reduction) to 71%. The variation among the experiments could be due to the different diet, saponin source, type of saponin, level of saponin and animal species used in the different studies.

Several studies showed that the presence of saponins in the rumen caused only partially defaunation. In the *in vitro* fermentation, *Enterolobium cyclocarpum* and *Sesbania sesban* which both contain saponins showed inhibiting effect on protozoa population (Leng et al. 1992; Teferedegne et al. 1999). When supplementing *Enterolobium cyclocarpum* to sheep, however, the antiprotozoal effect of *E. cyclocarpum* was only transient as the protozoa numbers started to increase after 12 days (Ivan et al. 2004; Koenig et al. 2007). When sheep received *Sesbania sesban* supplementation, protozoa numbers also increased after several days of supplementation (Newbold et al. 1997). Protozoa numbers in sheep rumen remained lower

compared to that in control sheep when fed *S. rarak* pericarp's extract at the level of 0.48–0.72 g/kg body mass for 100 days (Wina et al. 2006a). However, protozoal numbers were not significantly reduced when saponin extract of *S. rarak* whole fruit was fed to local cattle at the level 200 mg/kg BW/day for 90 days (Suharti 2010). The *S. rarak* saponins do not always depress protozoa numbers in the rumen perhaps due to the difference in composition of diet, the ratio of forage to concentrate, the animal breed, the level or type of saponin extract. The inconsistent defaunation effect of saponin between *in vitro* and *in vivo* experiments occurred when using other saponin sources such as in Yucca and Quillaja saponins (Pen et al. 2006, 2007; Lovett et al. 2006; Baah et al. 2007). Probably adaptation of the ruminal microbes, the nutrient flow or dilution effect might be responsible for the different results observed in the *in vitro* and *in vivo* studies (Benchaar et al. 2008).

11.6.2 Methanogens

Recently, methanogens and their diversity have been the subject of increasing interests due to the methane production by ruminants (Firkins et al. 2008). Much research is still in progress to study methanogens in order to mitigate methane emission from ruminants.

Beside living freely in the liquid mat or living associated with particles in the rumen, some methanogens remain associated with ruminal protozoa. It was estimated that 10–20% of total methanogens exist in close association with the protozoa either on the surface (ectosymbiosis) or inside the protozoal cell (endosymbiosis). Oligotrichs have endosymbiotic methanogens and entodiniomorph protozoa have ectosymbiotic methanogens (Ohene-Adjei et al. 2007). Using fluorescent-microscopic technique, Vogels et al. (1980) showed that nine genera of protozoa from the family of *Ophryoscolecidae* (order *Entodiniomorphida*) associated with methanogens. Physical structure (pellicle, surface structure and interior structure of cell cortex) of protozoa may affect this association. Study using molecular technique revealed that about 99% of protozoa-associated methanogens belong to the family of *Methanobacteriaceae* (Karnati et al. 2009a), and 20 novel sequences which differed from sequences previously known for protozoa-associated methanogens were obtained from rumen samples of goat, sheep and cow (Regensbogenova et al. 2004; Morgavi et al. 2006). DGGE profile also showed that inoculation of different species of protozoa to defaunated sheep resulted in different association of methanogens (Ohene-Adjei et al. 2007). The above findings indicate that various cultured and uncultured methanogens and their association with protozoa require further study.

Protozoa hydrolysed carbohydrate to produce hydrogen which then be used directly by associated methanogens to produce methane. Ushida et al. (1997) showed the occurrence of interspecies hydrogen transfer between the rumen ciliate *Polyplastron multivesiculatum* and the methanogenic archebacterium, *Methanosarcina barkeri*. Therefore, reducing protozoa will also reduce ruminal methanogens, thus reducing methane production. Tables 11.1 and 11.2 show that the studies mostly reported the

effects of saponins on ciliate protozoa, nitrogen metabolism, and methane production but only few studies reported the effect of saponins on methanogens. It was shown that saponins in the *S. rarak* extract at the level of 2 mg/ml in the *in vitro* fermentation did not affect methanogens RNA concentration (Wina et al. 2005a) The same result was observed with the feeding of *S. rarak* at a dose of 0.48 g/kg body to sheep (Wina et al. 2006a). In contrast, Goel et al. (2008a) found that the addition of saponin extracts from sesbania, fenugreek or knautia decreased methanogens by 78%, 22% and 21%, respectively in the *in vitro* rumen fermentation.

Tea saponins did not inhibit *M. ruminantium* as pure culture (Guo et al. 2008), but at the level of 0.4 mg/ml in the *in vitro* rumen fermentation, it inhibited significantly the activity of the methyl coenzyme-M reductase (*mcr*A) gene of *Methanobrevibacter ruminantium* by 76% (Guo et al. 2008). The *mcrA* is crucial to the final step of methanogenesis where it is involved in the reduction of the methyl group bound to coenzyme-M. Tea saponin has been reported to decrease methanogens diversity (Zhou et al. 2010), without having any effect on the relative abundance of methanogens in sheep (Mao et al. 2010). The study on the use of different saponin containing plants related to the expression or activity of *mcrA* gene in the rumen is very limited. Further studies are warranted to unravel the mechanism by which saponins reduce the ruminal methane production.

11.6.3 Bacteria

Beside the antiprotozoal activity, many published reports showed that saponins are antimicrobial or antifungal agents. These properties were observed when saponins were tested on pure cultures. Wang et al. (2000) showed that saponins isolated from *Yucca schidigera* reduced the growth of *Streptococcus bovis* and *Ruminobacter amylophilus*. Wallace et al. (1994) previously reported that yucca extract reduced the growth of *S. bovis* and *Butyrivibrio fibrosolvens*. Yucca saponins also reduced filter paper and endoglucanase activities of *Ruminococcus albus* and *R. flavefaciens* but not those of *Fibrobacter succinogenes* (Wang et al. 2000). *Selenomonas ruminantium* but not *Prevotella bryantii* growth, was enhanced by yucca saponins (Wang et al. 2000) while the reverse result was reported by Wallace et al. (1994). The inconsistency of these results may due to the isolation procedure and type of saponin.

The response of the mixed rumen microbes to saponins may be due to a direct effect of saponin on bacteria and an indirect effect of saponin reducing protozoal numbers. As the protozoal population decreased due to the use of saponins, the total bacteria numbers increased (Wina et al. 2005a; Pen et al. 2006; Goel et al. 2008a). However, the bacteria increase depended on the level of saponins added to the substrate. Wina et al. (2005a) found that total ruminal bacteria numbers increased significantly when *S. rarak* extract was included at the level of 1 mg/ml in the *in vitro* fermentation system, but the bacterial numbers decreased when the inclusion of saponin was increased to twofold to fourfold. It was further reported that saponins in the *in vitro* fermentation system caused a shift in bacterial population which was

shown by different intensity of bands on DGGE gel (Suharti et al. unpublished). Further analysis of three intensified bands show that the sequences of these bands have high similarity with sequences from *Prevotella ruminicola* (98–100%) *Pseudobutyrivibrio ruminis* or *Butyrivibrio fibrosolvens* (99%) and *Coprococcus eutactus* (99%) (Suharti et al. unpublished). In dual-flow continuous culture fermenters, removing protozoa increased population of *Prevotella*, *Eubacterium* spp., *Ruminococcus* spp., *Butyrivibrio fibrisolvens* which were shown as increased intensity bands on DGGE gel (Karnati et al. 2009b).

On pure culture, the growth of *Prevotella ruminicola* was stimulated by the addition of yucca extract (Wallace et al. 1994). In the *in vitro* fermentation using cattle rumen, a significant increase of *Prevotella ruminicola* due to the addition of *S. rarak* saponin extract was also reported (Suharti et al. 2010) and this result confirmed a previous qualitative analysis by DGGE (Suharti 2010).

A preference of protozoal predation toward *Butyrivibrio fibrisolvens* (Williams and Coleman 1992) explains the increased intensity of band matched with *B. fibrisolvens* during partial defaunation. Saponins also affected three major fibrolytic bacteria, *Ruminococcus albus, Ruminococcus flavefaciens* and *Fibrobacter succinogens* in the rumen. *S. rarak* saponins reduced RNA concentrations of *Ruminococcus albus* and *R. flavefaciens* both in the *in vitro* fermentation and *in vivo* during short term feeding (6 days) (Wina et al. 2005a, 2006a), but this effect did not occur during a long term feeding trial on sheep (100 days). RNA concentration of *Fibrobacter succinogenes* was unaffected in sheep rumen during short and long term feeding of saponin (Wina et al. 2006a). Quantitative analysis by real time PCR showed that *S. rarak* extract did not inhibit *R. flavefaciens* and *F. succinogenes* population in the *in vitro* fermentation (Suharti et al. 2010). However, sesbania saponin in the *in vitro* fermentation increased *F. succinogenes* (21–45%) and *Ruminococcus flavefaciens* (23–40%) populations measured by real time PCR (Goel et al. 2008a). Tea saponin did not affect the number of *R. flavefaciens* but increased that of *F. succinogenes* in the *in vitro* system (Guo et al. 2008). The effect of tea saponin was different in the *in vivo* trial using Hu lambs. Mao et al. (2010) found no effect of tea saponin on relative abundance of *R. flavefaciens* and *F. succinogenes* while Zhou et al. (2010) reported a decrease of *F. succinogenes* but no effect on *R. flavefaciens*, *R. albus*, *Butyrivibrio fibrisolvens*. These inconsistent results still can not be explained and warrant more studies to be conducted.

11.6.4 Fungi

Studies on the effects of saponins on fungi were mostly performed on fungi which caused disease problems in humans or plants (Zhang et al. 2005; Barile et al. 2007; Coleman et al. 2010). Steroidal saponins from *Tribulus terrestris*, saponins from *Allium minutiflorum*, plant saponins have shown potent activity against fluconazole-resistant fungi (Zhang et al. 2005), soil-borne and air-borne pathogenic fungi

(Barile et al. 2007) and *Candida albicans* (Coleman et al. 2010). Similar to what observed on protozoa, the mechanism of saponins inhibiting fungal growth could be the binding between saponins and sterol present in outer membrane of fungi causing its disruption (Armah et al. 1999). However, no correlation was found between antifungal activity and antiprotozoal or haemolytic activity of saponins as mentioned above. Studies on the effect of saponins on individual rumen fungi are of scarcely available. Pure culture study showed that saponins from *Yucca schidigera* completely inhibited the growth of ruminal fungi, *Neocallimastix frontalis* and *Piromyces rhizinflata* (Wang et al. 2000). *S. rarak* saponins reduced RNA concentration of ruminal fungi both in the *in vitro* fermentation (at the level of 2–4 mg/ml) and *in vivo* during short term feeding but this effect did not occur in a 100 days of feeding trial (Wina et al. 2005a, 2006a). Tea saponin showed activity to depress fungal growth in the *in vitro* system (Guo et al. 2008) and no such activity in the *in vivo* system (Mao et al. 2010; Zhou et al. 2010). Goel et al. (2008a) reported that knautia, sesbania and fenugreek extracts reduced the population of ruminal fungi in the *in vitro* fermentation by 30%, 38%, 65%, respectively. This fungal inhibition may or may not disappear when the extracts are given *in vivo* and thus further investigation is required

11.7 Adaptation and Resistance of Ruminal Microbes to Saponins

The transient effect of saponins on ruminal protozoa has been reported by several authors (Newbold et al. 1997; Ivan et al. 2004; Koenig et al. 2007). Beside transient effects, the varying effects of the same saponin on protozoa occurred. Feeding *Sapindus saponaria* fruits to sheep suppressed protozoal numbers as demonstrated by Diaz et al. (1993) and Navas-Camacho et al. (1993), but increased protozoal numbers in the study of Abreu et al. (2004). This variability of the anti protozoal effect exerted by saponins may be caused by an adaptation of mixed microbes to saponins although breed and exposure of the animals to different environment or diets may also contribute to the variability (Teferedegne 2000; Abreu et al. 2004). Adaptation mechanism of rumen microbes to saponins may occur. *F. succinogenes* was resistant to saponins. *F. succinogenes* is a Gram negative bacterium, with two membranes, and a thin surface carbohydrate coat of radiating fibers. The outer membrane contains of two polysaccharides, i.e. lower-molecular-mass fraction designated glycolipid and a high-molecular-mass capsular polysaccharide fraction. The presence of 2-aminoethylphosphonic acid on the surface of both polysaccharides which covalently linked to the membrane polymers, enhances membrane stability of *F. succinogenes* (Vinogradov et al. 2001). So, not only *F. succinogenes* but most of Gram negative bacteria may also exert resistance to saponins. Wang et al. (2000) reported that thickening the cell wall membrane as found in *Prevotella bryantii* is another adaptation mechanism of microbe to saponins.

11.8 Effect of Saponins on Rumen Fermentation

11.8.1 Methane Production

Recent reviews on the use of secondary metabolites including saponins to reduce methanogenesis (methane production) are available (Rochfort et al. 2008; Patra and Saxena 2009b).

The use of *Sapindus rarak* fruit's pericarp powder, the crude extract of the fruit's pericarp or that of the whole fruit suppressed *in vitro* methane production by 21%, 31% (Thalib 2004) and 10%, respectively (Suharti 2010); however there are no reports yet on the effect of *S. rarak* saponin on *in vivo* methane production. Similar effect of suppression was obtained by adding aqueous extract of *Sapindus mukorrosi* to the *in vitro* fermentation (Kamra et al. 2008; Sirohi et al. 2009). Goel et al. (2008b) found reduced methane production in the *in vitro* fermentation with the addition of sesbania, fenugreek or knautia leaves but not with their extracts. Saponins from *Yucca schidigera* or *Quillaja saponaria* reduced methane production in the *in vitro* fermentations (Pen et al. 2006; Holtshausen et al. 2009) but not in the *in vivo* experiments (Pen et al. 2007; Holtshausen et al. 2009). Depression on methane production occurred both in the *in vitro* and *in vivo* experiments was reported when using sarsaponin (Lila et al. 2003, 2005), and tea saponins (Hu et al. 2005; Mao et al. 2010; Zhou et al. 2010). But the depression was significant only in faunated rumen fluid, suggesting that this effect was mediated through associated effects on protozoa. In the partial defaunation by saponins, the population of methanogens that associated with protozoa would decline, hence the methane production reduced. However, saponin rich extracts of *S. sesban* leaves, *Knautia arvensis* leaves and fenugreek seeds did not decrease methane production *in vitro* although these extracts reduced protozoa number and methanogen population (Jayanegara et al. 2010) The lack of inhibition on methane production with decreased methanogens could be caused by changes in composition of the methanogen community and their increased efficiency of methane production (Jayanegara et al. 2010).

The formation of methane in the rumen requires the involvement of several microbes and enzymes. Reducing the activity of the methyl coenzyme-M reductase (*mcr*A) gene of *Methanobrevibacter ruminantium* by saponin (Guo et al. 2008), indicated that tea saponin inhibited the rate of methanogenesis and not the numbers of methanogens. It is likely that H_2 availability rather than the number of methanogens controlled the methanogenesis. High proportion of H_2 in the rumen is produced by protozoa when digesting starch, but when protozoa are inhibited, H_2 production in the rumen is limited and thus methane production is reduced. Saponin may suppress the H_2-producing bacteria (Wang et al. 2000) such as cellulolytic bacteria and bacteria that use pyruvate-ferredoxin oxidoreductase, hence reduce H_2 availability for methanogens.

Increasing H_2 utilization by organisms other than methanogens also reduce methane production. This process requires addition of an appropriate electron acceptor and an efficient type of rumen bacteria that can perfectly utilize such an acceptor

in the production of acetate or propionate, hence, reduced methane production (Abdl-Rahman et al. 2010).

Thalib and Widiawati (2008) suggested a combination of feeding *S. rarak* saponin extract with *Acetoanaerobium noterae*, an acetogenic bacteria that reduced *in vitro* methane production by 20%. Further, Thalib et al. (2010) showed that this combination also reduced methane production in sheep by 24%.

Caution should be taken in interpreting methane production data since methane production is expressed in different units. In the *in vitro* system, the units of methane production could be expressed as the total production per day or concentration relative to the total gas. Hess et al. (2004) showed that methane release could be expressed relative to metabolic weight or organic matter digested or energy intake or body nitrogen retained. Different units of methane release give various values which may lead to different conclusion. From several results, it can be suggested that saponins have potential to be used as a methane reducing agent, however, the inclusion level of saponin from each source should be tested to get the optimum result.

11.8.2 Biohydrogenation of Fatty Acid

Recently, there has been a renewed interest by several researchers in the study of biohydrogenation of fatty acid in the rumen after a growing demand for producing healthy animal products for human consumption. Conjugated linoleic acid (CLA) and vaccenic acid (VA) are fatty acid intermediates produced in the rumen. CLA is known as potentially health-promoting agent since many studies showed that it contributed to cancer prevention, decreased atherosclerosis and improved immune response (Pariza 2004; Palmquist et al. 2005). The most abundant *trans*-18:1 isomer, vaccenic acid (18:1 *trans*-11) was 6.6% of total fatty acids in protozoa and 2.0% of total fatty acids in bacteria. The *cis*-9, *trans*-11 CLA was 8.6-times greater in the protozoal fraction (1.32% of total fatty acids) than in the bacterial fraction (0.15%) (Or-Rashid et al. 2007). Proportion of CLA and VA in the rumen fluid of faunated 1.6–2.5 times higher than those of defaunated cattle (Sultana et al. 2011) The supply of CLA for post rumen absorption depend on the flow of protozoa from the rumen (Yanez-Ruiz et al. 2006). Only limited data of the effects of saponins on CLA and VA concentration in the rumen is available. Addition of quillaja saponins in the dual–flow continuous culture at the level of 500–1,000 mg/L did not affect the total or individual volatile fatty acid nor change the extent of biohydrogenation (Lourenço et al. 2008). No effect of saponin from *Yucca schidigera* on the rate of α-linolenic acid biohydrogenation was reported when added at the level of 1.12% of dry matter feed in a Rumen simulation technique system (Khiaosa-Ard et al. 2009). Defaunation by using a certain size filter only slightly reduced biohydrogenation in the rumen (Karnati et al. 2009a) and, hence, the presence of protozoa was not necessary for biohydrogenation to occur. More studies need to be done on the effect of different saponins on biohydrogenation of fatty acid in the rumen and the production of cis-9, trans-11-CLA and VA (18:1 trans-11).

Only bacteria in the same group as the *B. fibrisolvens* group formed cis-9, trans-11-CLA or trans-11-18:1 as intermediates in the process of biohydrogenation of linoleic acid (Jenkins et al. 2008). The DGGE result to study rumen bacterial diversity showed that the addition of *S. rarak* saponin extract caused *B. fibrisolvens* to appear as a more intense band (Suharti et al. unpublished). Defaunation by other compounds also increased some *Butyrivibrio* (Karnati et al. 2009a). Further study to quantify *B. fibrisolvens* following saponin addition may explain about its role in the CLA production in the rumen. The effects of various phytochemicals on microbial biohydrogenation in the rumen have been discussed in details in Chap. 9.

11.8.3 Nitrogen Metabolism

Protein consumed by ruminants is partly degraded in the rumen to peptides, amino acids and finally to ammonia. Therefore, there is a relationship between ammonia concentration in the rumen and percentage of total protein in the diet (Eugene et al. 2004). Another source of ammonia in the rumen comes from the proteolysis of bacterial protein when protozoa engulf ruminal bacteria as their principal source of protein amino acids. Reduced ammonia concentrations in the rumen typically occurred when total defaunation was applied (Eugene et al. 2004). The reduction of protozoal numbers resulted in less predation of bacterial and, hence, less lysis of bacterial protein to ammonia. Therefore, the observed ammonia level in the rumen is affected by the rate of degradation of feed protein to ammonia, the rate of bacterial lysis by protozoa, and the uptake of ammonia for microbial protein synthesis. Fewer protozoa also could influence the ammonia concentration since protozoa contributed to 10–40% of the total rumen nitrogen. The excess of ammonia is diffused from the rumen, converted to urea and excreted from the animal.

Saponins which partially reduced protozoal numbers, also reduced ammonia concentration in the rumen both in the *in vitro* (Table 11.1) and *in vivo* experiments (Table 11.2). Experiments using tea saponin or *S. rarak* extract to partially defaunate the rumen either *in vitro* or *in vivo* showed lower ammonia production. Tables 11.1 and 11.2 showed inconsistency in the effect of saponin on ruminal ammonia production between the *in vitro* and *in vivo* experiments using the same source of saponins. Saponin extract of *Yucca schidigera* (Lila et al. 2005; Cardozo et al. 2004; Pen et al. 2006, 2007; Singer et al. 2008; Holtshausen et al. 2009) or *Quillaja saponaria* (Cheeke 2000; Pen et al. 2006, 2007; Holtshausen et al. 2009) caused different effect on ammonia production. Comparison of all the results is rather complicated because of the different source of saponin, different level of saponin, process of extraction, dietary ingredient of forage and concentrate, breed and physiology of the animal.

There is limited information on the effect of saponins on peptide and amino acids metabolism in the rumen. Bacteria are responsible for most peptide degradation within the rumen and among them, Gram-negative bacteria *Prevotella ruminicola* appears to be the most important peptidolytic bacteria (Broderick et al. 1991).

Suharti et al. (2010) found that addition of *S. rarak* whole fruit extract to *in vitro* fermentation increased the relative abundance of *Prevotella ruminicola.* Further investigation is required to prove whether saponins increase the production of peptides in the rumen. However, the inhibition of rumen proteolytic activity by yucca saponins has been observed by Wallace et al. (1994). The reduction of ruminal N digestion was also observed when sheep was fed *E. cyclocarpum* leaves (Koenig et al. 2007). However, Muetzel et al. (2005) did not find any effect of *S. rarak* saponin on protein degradation *in vitro*. There is no effect of quillaja or yucca saponin on deaminative enzymes (Hristov et al. 1999; Baah et al. 2007).

Fenugreek extract in the continuous culture system showed no effect on large peptides, small peptides and amino acids productions in the rumen (Busquet et al. 2005) while Yucca extract increased the average peptide N concentration by 26.2% but no effect on amino acid concentration and ammonia was found (Cardozo et al. 2004).

Accumulation of peptides in the rumen fluid would be beneficial to ruminants since it would increase microbial protein synthesis and the flow of dietary amino acids to the lower gut (Griswold et al. 1996). Microbial protein synthesis (MPS) was enhanced by quillaja saponin in the *in vitro* fermentation (Makkar et al. 1998), *S. saponaria* (Abreu et al. 2004) and tea saponin in the *in vivo* trials (Mao et al. 2010; Zhou et al. 2010). However, MPS *in vivo* was not affected by applying *S. rarak* extract (Wina et al. 2006a, b), Quillaja or Yucca saponin (Pen et al. 2007). The application of yucca saponin at low level (15 μg/ml) increased microbial protein synthesis, in contrast, MPS reduced at high level of saponins (75 μg/ml) (Wang et al. 2000). Efficiency of MPS improved by 13% in sheep fed a diet supplemented with *E. cyclocarpum* (Koenig et al. 2007) and by 51% when supplemented with *Biophytum petersianum* Klotzsch (Santoso et al. 2007).

From the rumen, the bacteria and protozoa flow to duodenum could be observed by measuring the markers, ^{15}Nitrogen and phosphatidylcholine (PC) in the rumen and duodenal digesta (Ivan et al. 2006). Not only the microbial protein synthesis, but also the flow of microbial protein to duodenum was enhanced by *Sapindus saponaria* (Abreu et al. 2004) and tea saponin (Mao et al. 2010; Zhou et al. 2010). These positive effects on the microbial protein synthesis, efficiency microbial protein synthesis, the microbial N flow and Non Ammonia Nitrogen (NAN) may help to improve dietary N utilization by ruminants (Busquet et al. 2005).

11.8.4 Carbohydrate Metabolism

Interaction of several diverse species of bacteria, fungi and protozoa facilitates the breakdown of cellulose and other carbohydrate fractions in plant materials in the rumen. With the decrease of protozoal numbers, this interaction may be disturbed as some carbohydrate degrading enzymes have less activity. Wina et al. (2005b, 2006a) showed that the xylanase activity in the rumen decreased when *S. rarak* saponin extract was administered to *in vitro* fermentation or directly to sheep. The CMCase activity was not affected in the *in vitro*, however, it was depressed by

S. rarak extract entered the rumen of sheep. Protozoa also produced carbohydrate degrading enzyme including xylanase and CMCase (Williams and Withers 1991; Devillard et al. 1999) and a positive correlation was observed between protozoal numbers and both xylanase and CMCase activities in the sheep rumen (Wina et al. 2006a). Amylase activity in the *in vitro* rumen fermentation increased in the presence of saponin extract of *S. rarak* whole fruit (Suharti 2010). However, CMCase, xylanase, glucanase and amylase activities in heifer's rumen were not affected by Quillaja saponin extract (Baah et al. 2007). Eventhough the fibrolytic enzyme activity might be reduced in the rumen and, hence, reducing the fibre digestibility in the rumen, the total tract digestibility was not affected by saponin addition (Wina 2005).

The products of carbohydrate degradation in the rumen would be volatile fatty acids. The higher total VFA production was observed by *S. rarak* extract addition in the *in vitro* (Wina et al. 2005b; Suharti et al. unpublished) and in cattle rumen (Suharti 2010) indicating that saponin improved the fermentation while many experiments showed no effect or depressed effect of saponin on total VFA production. Consistent results were seen in the *in vitro* system and both sheep and cattle feeding trials in that the molar proportion of propionate increased in the presence of *S. rarak* saponin extract (Tables 11.1 and 11.2). *Y. schidigera* and *Q. saponaria* extracts increased propionate production *in vitro* (Pen et al. 2006) but not in sheep rumen (Pen et al. 2007). There are other reports in agreement and showing an increase in the molar proportion of propionate in the presence of saponin although some did not agree with this result (see Tables 11.1 and 11.2). The higher molar proportion of propionate could be due to higher activity or numbers of *Prevotella ruminicola* to form propionate. *Prevotella ruminicola* in pure culture increased in the presence of *Y. schidigera* saponin fraction (Wallace et al. 1994) and its relative abundance as a percentage to the total bacteria increased significantly in the presence of *S. rarak* extract in the *in vitro* fermentation (Suharti et al. 2010). Lower acetate and butyrate production which were the major fermentation end products in isolated protozoa caused a shift in molar proportion of individual VFAs to a higher proportion of propionate and a reduced ratio of acetate to propionate. A higher molar proportion of propionate means a higher glucogenesis and would be beneficial for animals fed high roughage diets that are commonly found in tropical countries where available sources for feed are mainly roughages of low quality. The potential usage of saponin extract as a natural substance to improve propionate production requires more study.

11.9 Metabolism of Saponins in the Rumen

Rumen microbes produce a variety of intracellular or extracellular enzymes. These enzymes hydrolyse all substances at different rates. Saponins are glycosylated compounds and are highly soluble in water or aqueous medium. Saponins can dissolve easily in the rumen and therefore be readily degraded by the various glycosidases

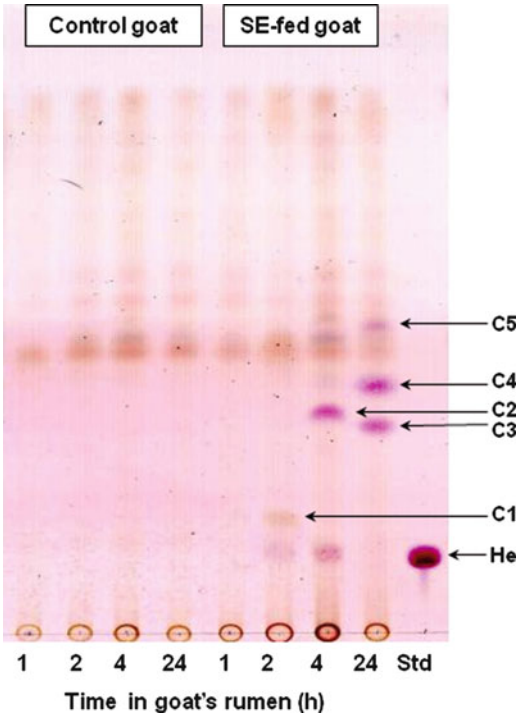

Fig. 11.5 Sapogenin fraction extracted from rumen of control and SE (saponin extract) fed goat taken at 1, 2, 4 and 24 h after feeding and separated on thin layer of Silica gel plate. *C1–C5* represented the metabolites of saponins (in the form of sapogenin), *He* hederagenin, the main aglycone of *Sapindus rarak* saponins

produced by the different microbes. In the *in vitro* study, Makkar and Becker (1997) reported that 81% of quillaja saponins still undegraded in buffered rumen liquor up to 9 h of incubation and were degraded rapidly after this time. Wina (2005) studied the degradation of *S. rarak* saponin in the *in vitro* rumen and showed that after 6 h of incubation only one saponin compound could be detected by thin layer chromatography. With further incubation for 12 h, this compound was detected with lower intensity. Using Rusitec system, Wang et al. (1998) found that after 2 h of incubation, most of yucca saponins (93.5%) remained intact in the rumen but with longer incubation, the degradation process occurred quite rapid and more than 50% of saponins disappeared at 8 h of incubation (Wang et al. 2000). Different rates of saponin degradation are shown in the *in vitro* systems (Makkar and Becker 1997; Wang et al. 1998, 2000; Wina 2005) and the degradation rate seems faster in the *in vivo* system especially if the saponin was directly introduced to the rumen. Saponins from an extract of *Costus speciosus* rhizomes was rapidly hydrolysed at 1 h after dosing to the rumen (Meagher et al. 2001). However, Wina (2005) did not find any degradation product of *S. rarak* saponins 1 h after feeding. The degradation products (aglycone structures) appeared after 2 h of feeding (Fig. 11.5). Hederagenin (He) as the aglycone of *Sapindus rarak* saponins was detected up to 4 h after feeding and then, it disappeared and perhaps structurally changed to several other degradation products after 24 h of the saponin extract feeding.

Flåøyen et al. (2002) reported that the saponin from *Yucca schidigera* was hydrolysed in the rumen to its main algycone, sarsapogenin, but then they assumed that this sarsapogenin was oxidized and reduced at C-3 position to become the corresponding epi-sarsapogenins. This result was in agreement with their previous study with saponins from *Narthecium ossifragum* which caused photosensitization to sheep (Flåøyen et al. 2001). Several free aglycones are formed in the rumen and the ring structure was not being degraded further in the rumen (Flåøyen et al. 2001, 2002). *Fibrobacter succinogenes* was reported to deglycosylate yucca saponin (Wang et al. 2000). There might be many other rumen microbes that have glycosidases activity and can hydrolyse saponin but have never been reported yet.

11.10 Summary of the Effects of Saponin on Rumen Fermentation

A scheme of effects of saponins on rumen microorganisms, activities and its products is presented in Fig. 11.6. Evidence obtained from numerous experiments suggests that the effects of saponins or saponin containing plants initially affected the microorganism in the rumen. Effect of saponin or its degradative products on microbial intestinal or caecum of ruminants, however, are limited known.

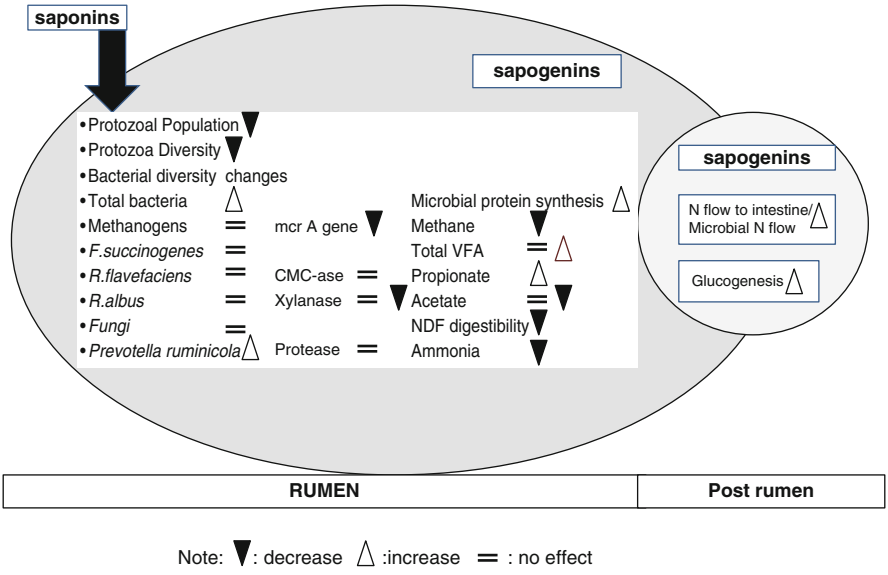

Fig. 11.6 Schematic diagram of possible effects of saponins or saponin containing plants on rumen microbes, activity and products

When saponins enter the rumen, the first effect is to reduce protozoal population (Fig. 11.6) Total bacterial numbers increase as less predation of bacteria by protozoa after saponins reduce protozoal population. The increase in total bacteria numbers means that the microbial protein synthesis enhances, hence, increases the microbial nitrogen flow to post rumen or intestine. Individual bacteria responses differently to saponins. Several studies show that the activity of methanogens but not methanogens population is reduced by saponins, resulting in a decrease in the methane produced in the rumen. Three major fibrolytic bacteria (*F. succinogenes, R. flavefaciens* and *R. albus*) are unaffected by saponin in the *in vivo* system Similar to fibrolytic bacteria, ruminal fungi are not affected by saponin. Protozoa and fungi possess carbohydrases (CMCase, xylanase), therefore partial defaunation by saponin causes a reduction in carbohydrate degrading enzymes. The reduced activity of these enzymes by saponin causes NDF digestibility in buffered rumen to decrease (Wina et al. 2005a). Saponin does not inhibit the activities of carbohydrate degrading enzymes in sheep or cattle rumen and, hence, does not impair total tract digestibility. Saponin increases VFA and propionate production in the rumen, therefore, it is expected that glucogenesis in post rumen is enhanced.

With reduced protozoal population, increase of total bacteria population, propionate production, microbial biomass production, microbial nitrogen flow and glucogenesis and without any effect of saponin on total tract digestibility, it is expected that the performance of ruminant is improved by saponin addition.

11.11 Conclusion

Various methods of extracting saponins from plant materials and the diversity of saponins influenced the activity of rumen microorganisms. Saponins could manipulate the rumen fermentation by depressing the protozoal population in the rumen. The consequences of reduced protozoal population leads to some changes in the rumen microbial composition and population, a shift in individual volatile fatty acids toward higher propionate and increased microbial nitrogen flow to duodenum. Saponins could also decrease methane production with or without decreasing the numbers of methanogenic archaea. Evaluation on the dose of specific saponins to be added into the diet and the interaction of saponins with different composition of diets require further studies so that saponins could be potentially used as a defaunating agent, a propionate enhancer and an inhibitor for methane production in the rumen.

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Chapter 12
Effect of Plant Secondary Metabolites on Rumen Methanogens and Methane Emissions by Ruminants

Devki Nandan Kamra, Mahesh Pawar, and Beddyuti Singh

Abstract Methanogenesis occurs in the rumen to take care of reducing power generated during fermentation of feed and accounts for a significant loss of energy offered to the ruminants as feed. Once carbon dioxide is reduced to methane, it cannot be oxidized to release energy under the anaerobic conditions prevailing in the rumen. To save this energy loss, several chemicals have been tested and some of them are very effective in selectively inhibiting methanogenesis, but these chemicals cannot be used in practical feeding of livestock due to their adverse effects on other rumen microbes, health of the animals and the quality of livestock products. Therefore, plants containing secondary metabolites might be superior feed additives to control methanogenesis without affecting other microbes of the rumen. *In vitro* screening experiments conducted in many laboratories have indicated that methanogenesis can be inhibited by inclusion of plants/plant extracts in the substrate. Some of the plants which showed *in vitro* methane inhibition are : *Allium sativum, Azadirachta indica, Emblica officinalis, Eugenia jambolana, Ficus benghalensis, Foeniculum vulgare, Lotus pedunculatus, Mangifera indica, Ocimum sanctum, Populus deltoides, Psidium guajava, Quercus incana, Sapindus mukorossi, Sapindus rarak, Sesbania sesban, Syzygium aromaticum, Trachyspermum ammi, Terminalia chebula* and *Yucca schidigera,* but some of them do have adverse effects on rumen fermentation and feed digestibility. Several of the above plants have been tested *in vivo* as feed additives in different ruminants either alone or in a combination and have shown significant decrease in *in vivo* methane emission and no adverse effect on feed utilization when used at the rate of 1–2% of dry matter intake. There is a need to screen larger number of plants containing secondary metabolites and to study the effect of feeding these compounds on the feed utilization and the quality of livestock products.

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Abbreviations

ADF	Acid detergent fibre
ADG	Average daily gain
CH_4	Methane
CO_2	Carbon dioxide
CT	Condensed tannins
D	Dalton
DDM	Digested dry matter
DGGE	Denaturing gradient gel electrophoresis
DM	Dry matter
DMI	Dry matter intake
EMP	Embden-Meyerhof pathway
EO	Essential oils
EOm	Essential oil mixture
HAP	Hyper ammonia producing
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NDF	Neutral detergent fibre
NH_3-N	Ammonia nitrogen
NO_3	Nitrate
OM	Organic matter
PCR	Polymerase chain reaction
PSM	Plant secondary metabolites
rDNA	Recombinant deoxyribonucleic acid
SO_4	Sulphate
TS	Tea saponins
VFA	Volatile fatty acids

12.1 Introduction

Ruminants have a unique capability of converting a non utilizable energy source of feed (lignocellulosic agro-industrial by-products) to an utilizable form of energy i.e. volatile fatty acids, which the ruminants can use as a source of energy. The other animals like monogastrics or non ruminant herbivores either are not able to digest lignocellulose or they do it only partially and inefficiently. The process is completed in three steps: cellulose and hemicellulose are released from the lignocellulosic

feeds by the activity of esterases; cellulose and hemicellulose are depolymersized to monomers (hexoses and pentoses) by the activity of glucanases and finally these monomers are partially oxidized to volatile fatty acids with a release of carbon dioxide and hydrogen. The capability of any animal to digest fibrous lignocellulosic feed depends upon the volume of fermentation sac (rumen, caecum or colon) where these fibre degrading microbes harbour and help in anaerobic oxidation of feed. In ruminants (cattle, buffalo, sheep, goat, deer etc.), such fermentation sac, the rumen is the first and the largest compartment of complex stomach, making up about 80% of total stomach volume and around 15–20% of the body weight of animals. Therefore, the ruminants are ideal animals to convert agricultural wastes like straws, stovers, oil cakes, brans and green fodders into edible meat and milk products and do not face any competition with the human beings for their feed.

When hexoses and pentoses are fermented anaerobically as in the rumen, reduced cofactors like NADH and NADPH are produced through EMP pathway. For re-use in the anaerobic ecosystem, these reduced cofactors have to be oxidized to NAD^+ and $NADP^+$ by electron transfer to acceptors other than oxygen like CO_2, SO_4, NO_3 etc., as a result these electron acceptors are reduced to methane, hydrogen sulphide and ammonia, respectively. If this reducing power concentrated in the reduced cofactors is not utilized for reduction of an oxidized compound, further fermentation of carbohydrates will be stopped and no release of energy from feed will be possible. Therefore, to continue release of energy for the animals, methane (by reduction of carbon dioxide by the reduced cofactors) has to be generated in the rumen. But in this process about 5–15% of gross energy intake by the animals is wasted in the form of methane (Johnson and Johnson 1995). Therefore, research efforts are needed to suppress methane emission for eco-friendly and economic livestock production.

There are many possibilities of mitigation of methane emission by the livestock e.g. use of chemicals, selective removal of ciliate protozoa, elimination of high methane producing or unproductive animals, microbiological and biotechnological interventions to inhibit growth of methanogens in the rumen, but each one of the methods listed above have one or the other limitation or have toxic effects on fermentation of feed in the rumen. Therefore, their practical application in the field is not visible in the near future. During the last one decade, the researchers have shown interest in using plants containing secondary metabolites (PSM) as feed additives to control methanogenesis. In this chapter efforts have been made to compile information on these PSM to assess the possibility of using them as feed additives to inhibit methanogenesis and their effect on productivity of the livestock.

12.2 Plant Secondary Metabolites

The plant secondary metabolites (PSM) are a vast variety of chemical compounds synthesized in plants that are not involved in the primary biochemical processes of growth and reproduction. These bioactive compounds, which have anti-microbial

activities, are meant for protection of the host plant against invasion by the foreign particles including pathogenic microbes. Therefore, these compounds have been used as medicine in traditional system of medicare in India, China, Srilanka, Japan and other Asian and African countries, for preservation of foods and as spices in kitchen in many parts of the world since time immemorial. More than 200,000 defined structures of plant secondary compounds have been identified. These PSM can generally be classified into three major groups: saponins, tannins and essential oils (EO).

Plant secondary metabolites might inhibit methane emission by the following modes:

- They might directly inhibit methanogens as these compounds have anti-microbial activities against different microbial groups.
- The plant secondary metabolites might have anti-protozoal activity, which might indirectly result in reduced number of methanogens. As the ciliate protozoa and methanogens have an ecto-symbiotic relationship, the latter might lose their symbiotic partners and hydrogen supply due to killing of ciliates by the plant secondary metabolites and therefore, might result in reduced production of methane.
- As the plant secondary metabolites have anti-microbial activity, which might reduce the numbers of bacteria and fungi and thus result into lower digestibility of feed and consequently cause a decrease in methanogenesis as the feed degradation and methane production are directly related to each other.

12.2.1 Tannins

The word "Tannin" was originally coined by Seguin to describe substances present in vegetable extracts, which are responsible for converting animal skin into leather. In plant extracts, these substances exist as polyphenols of varying molecular sizes and complexities. One of the most appropriate definitions of tannins was given by Horvath (1981), "Any phenolic compound of sufficiently high molecular weight containing sufficient hydroxyl and other suitable groups like carboxyl to form effective strong complexes with protein and other macromolecules under the particular environmental conditions being studied".

Bate–Smith (1972) defined tannins as water soluble phenolic compounds having a molecular weight between 500 and 3,000 D. These polyphenols contain a large number of hydroxyl or other functional groups (1–2 per 100 D) and therefore are capable of forming cross–linkages with protein and other macromolecules. Tannins are usually subdivided into two major groups: hydrolysable and condensed tannins and are considered to have both adverse and beneficial effects depending upon their concentration and nature besides other factors such as animal species, physiological state of animal and composition of diet (Makkar 2003).

Hydrolysable tannins split into sugars and phenolic carboxylic acids both in acid and alkaline conditions (White 1957). These are further classified according to the

products of hydrolysis, into gallo-tannins (gallic acid and glucose) and ellagi–tannins (ellagic acid and glucose) (McLeod 1974). Condensed tannins are often referred to as proanthocyanidins because they produce red anthocyanidins when heated in acid (Haslam 1982). Proanthocyanidins are phenylpropanoid polyphenols and are categorized by the type of monomers they contain–flavan-3-ols or flavan-3, 4-diols–into catechins or leucanthocyanidins (Horvath 1981). Besides hydrolysable tannins and proanthocyanidins, a group called beta–tannins also exists (Swain 1979; Horvath 1981), which are protein precipitating compounds and are insoluble in water. They form very stable bonds with protein and have lower molecular weight than other tannins.

The molecular weight (MW) and chemical structures of CT play a key role in their biological activity. The CT fractions with the highest MW had the highest inhibition (62% lower than the control) (Huang et al. 2011). Inclusion of 15 mg of CT/500 mg DM reduce CH_4 production by 47%, total methanogens and total protozoa number decreased with increasing levels of CT (Tan et al. 2011). However, higher CT inclusions, with further reduction in CH_4 emissions, have substantive negative effects on DM digestibility.

Methane production was completely inhibited in pure cultures of methanogens incubated with big trefoil (*Lotus pedunculatus*) compared with alfalfa, suggesting that these phenolics directly inhibit methanogen metabolism (Tavendale et al. 2005). In contrast, Beauchemin et al. (2007) reported that the diet of growing beef cattle supplemented with 18 g CT /kg DM from quebracho Colorado trees had no effect on enteric CH_4 emissions or DM digestibility. The effects of forage legumes containing condensed tannins on methane and ammonia production in continuous cultures of mixed ruminal microorganisms did not influence apparent digestibility of dry matter or neutral detergent fiber (Williams et al. 2011).

Terminalia belerica (bahera, 23.6–37.4% tannins) and *Terminalia chebula* (harad, 30–32% tannins in dry fruit pulp) contain tannins as secondary metabolites and the methanol extracts of both the plants showed antimethanogenic activity but harad (almost complete methane inhibition) was more effective as compared to bahera. The methane inhibition in this case was also associated with reduction in protozoa population and feed degradability (Patra et al. 2006b). The results indicated that tannins inhibited rumen methanogenesis through interaction with the rumen microbes. Adverse effect of tannins on feed digestion, microbial population and enzyme activity has been demonstrated in many studies (McSweeney et al. 2001; Hristov et al. 2003). As a mixture of both types of tannins (in extracts or ground samples of plant parts) has been used in most of the studies, it is difficult to ascertain which individual part is more effective against methanogenesis in the rumen. The condensed tannin rich leguminous fodders have anti-methanogenic activity, hence, it might be possible that condensed tannins are more detrimental for methanogens (Puchala et al. 2005). As the tannins have direct effect on methanogens, there might be some adverse effects on fiber degradation and lower hydrogen production.

Different sources of tannins have different effects on gas production and methane production. In an *in vitro* study with three condensed tannins (quebracho, grape seed, and green tea tannins) and four hydrolysable tannins (tara, valonea, myrabolan, and

chestnut tannins), Quebracho, valonea, myrabolan and grape seed decreased gas production and the maximum rate of CH_4 production, whereas addition of chestnut, green tea and tara tannins neither affected total gas nor CH_4 production (Pellikaan et al. 2011).

Findings from PCR-DGGE and RT-PCR analysis suggest that inclusion of CT altered the diversity of rumen methanogens without affecting total methanogen number (Mohammed et al. 2011). They also reported that *Methanobrevibacter smithii* B181, *Methanosphaera stadtmanae*, Methanogenic archaeon LGM-AFM09 and *Methanobrevibacter smithii* strain ALI-A increased while *Methanobrevibacter* sp. WBY1 and *Methanobrevibacter millerae* strain ZA-10 decreased with an increase in the proportion of CT in the diet.

12.2.2 Saponins

As the name indicates saponins are soapy in nature and are glycosides consisting of a steroid (C27) or a triterpenoid (C30) sapogenin nucleus with one or more carbohydrate branches. The degradability of saponins in the rumen depends upon many factors including its structure. Makkar and Becker (1997) reported that quillaja saponins are degraded by mixed microbial population of the rumen of cows and that of alfalfa (lucerne) were rapidly released into the rumen fluid and extensively degraded in the digestive tract of sheep, but the final products of degradation have not been identified (Mathison et al. 1999).

Methanogen population was decreased in the presence of *Sesbania sesban* saponins by 78%, Knautia saponins by 21% and fenugreek saponins by 22% in *in vitro* fermentation from cattle rumen liquor (Goel et al. 2008). The addition of 0.14 and 0.29 g/l of *Trigonella foenum-graecum* seed extract containing 34.5% saponins did not reduce methanogen numbers in an *in vitro* gas production test (Goel et al. 2008). It has been suggested that saponins may decrease methanogen populations through a reduction in the numbers of protozoa. Methanogens associated with protozoa may account for decreased methane production by 9–25% (Newbold et al. 1995) and as much as 37% (Finlay et al. 1994).

Saponins have anti-protozoal activity and affect the rumen fermentation significantly. Thalib et al. (1996) reported that methanol extract of *Sapindus rarak* fruit (0.07% of body weight, every 3 days) resulted in 57% reduced protozoal population, 69% increased bacterial numbers, significantly reduced ammonia nitrogen, greater daily body weight gain and improved feed conversion efficiency. It has been observed that ethanol extract of soapnut was superior to methanol and water extracts as far as the inhibition of protozoa and methane are concerned (Agarwal et al. 2006).

The saponins affect different bacteria of the rumen differently as evidenced by Wang et al. (2000) and confirmed by Hess et al. (2003) who reported that total bacteria and methanogens were not affected adversely by *Sapindus saponaria*, but rumen protozoa were significantly lowered. It was interesting to note that methanogenesis was reduced by 20% without affecting the degradation of fiber.

Y*ucca schidigera* and *Quillaja saponaria* containing 4.4% and 10% saponins (Wang et al. 1998) decreased polysaccharide degrading enzymes (carboxymethylcellulase and xylanase) considerably and inhibited protozoa to the extent of 42% and 54% respectively (Hristov et al. 1999, 2003). Wang et al. (1998) studied the effect of yucca extract (0.5 mg/ml buffer) on rumen fermentation in RUSITEC and reported no effect on dry matter digestibility, gas production and volatile fatty acid production, but the protozoa numbers were significantly reduced, while the number of bacteria was not affected.

Lila et al. (2003) studied the effects of different concentrations of sarsaponins of *Yucca schidigera* on ruminal microbial methane production using different substrates. Ammonia nitrogen and the numbers of protozoa were decreased with increasing dose of saponins. Total volatile fatty acids and gas production were increased. Molar proportion of acetate was decreased and propionate was increased with a corresponding decrease in acetate: propionate ratio. There was a decrease in methane production from 20% to 60% on different substrates.

The modified or partially degraded saponins, the sapogenins are degraded more slowly, but are not toxic for the ciliate protozoa. Interestingly in the experiment of Newbold et al. (1997) too the protozoa numbers reached the initial levels within 9 days. Thus the anti-protozoa effect is not consistent. This is not attributed to the fact that protozoa become resistant, but some other microbial group becomes active in degrading the anti-protozoal component of the extract. *Equisatum arvense* and *Salvia officinalis* inhibited methanogenesis and methane reduction was not associated with numbers of protozoa in the rumen liquor (Broudiscou et al. 2000).

Tea saponins (TS) and soybean oil had an inhibitory effect on methane production in growing lambs when they were added to the diets, but they showed different action against the protozoa, methanogens and other rumen microbes involved in feed degradation. Lambs fed diets with TS showed decreased daily methane production by 27.7%. The concentrations of total volatile fatty acids and microbial protein were increased with addition of TS with little effect on fungal population but protozoa populations relative to total bacterial 16S rDNA were decreased. Addition of TS with Soybean Oil had an inhibitory effect on the population of methanogens, fibrolytic microbes including *R. flavefaciens* and *F. succinogenes* (Mao et al. 2011). Tea saponins had similar effect on methane reduction as that of defaunation (2.1 L/day) with TS to (2.5 L/day) with defaunation (Zhou et al. 2011). A detailed discussions on thee effects of saponins on rumen microbial populations and fermentation characteristics have also been presented in Chap. 11.

12.2.3 Essential Oils

Essential oils (EOs) are steam-volatile or organic-solvent extracts of plants and are present in different parts of plants such as flowers, leaves, stems, bark, fruit pulps, roots and seeds. The concentrations of EOs might vary with the type of plant, stage of growth, plant health and environmental factors such as light, temperature, moisture and stress (Hart et al. 2008). Like other plant secondary metabolites, the

EOs too protect the plants against bacterial, fungal or insect invasion. Their use as food preservative and in traditional medicine is also very well known only because of their antimicrobial effects. In addition, they also possess biological activities as antioxidants, as hypocholesterolemics, as stimulant of digestive systems and as liver function enhancer (Craig 1999; Ramakrishna et al. 2003; Hernandez et al. 2004).

The most commonly occurring EOs are classified in two groups depending upon their chemical structure: terpenoids (monoterpenoids and sesquiterpenoids) and phenylpropanoids, which are synthesized through the mevalonate and shikimic acid metabolic pathways, respectively (Gershenzon and Croteau 1991; Calsamiglia et al. 2007). The terpenoids and phenylpropanoids act against bacteria through interaction with the cell membrane (Dorman and Deans 2000), which causes conformational changes in the membrane structure, resulting in its fluidification and expansion. The loss of membrane stability results in the leakage of ions across the cell membrane and causes a decrease in the trans-membrane ionic gradient. In most cases, bacteria can counter balance these effects by using ionic pumps and cell death does not occur, but large amount of energy is diverted to this function and bacterial growth is slowed down (Griffin et al. 1999; Ultee et al. 1999; Cox et al. 2001).

Essential oils have been examined for antimicrobial properties against rumen bacteria and many of them have been found to have strong activities. Therefore, research to exploit EOs as feed additive in animal nutrition has been accelerated in search for an acceptable feed additive, which can be used as a rumen modifier. McIntosh et al. (2003) reported that a mixture of EOs inhibited the growth of some hyper-ammonia producing (HAP) bacteria (*Clostridium sticklandii* and *Peptostreptococcus anaerobiosus*), but other HAP bacteria (*Clostridium aminophilus*) were less sensitive. This inhibition of HAP bacteria is diet dependent as reported in a study by Wallace (2004), where the number of HAP bacteria reduced by 77% in sheep receiving a low protein diet supplemented with EOs at 100 mg day^{-1}, but that EOs had no effect on HAP bacteria when sheep were fed a high-protein diet. At low doses, EOs could selectively inhibit the HAP bacteria, but all micro-organisms are adversely affected at higher concentrations (Wallace 2004). Similarly, Evans and Martin (2000) also reported that thymol selectively inhibited the growth of *Selenomonas ruminantium* at 90 mg L^{-1}, but not *S. bovis,* while at 400 mg L^{-1} all rumen microbes were inhibited. Individual EOs had different effects on mixed ruminal bacteria. Monoterpene hydrocarbons were less toxic and sometimes stimulatory to microbial activity compared with the corresponding oxygenated compounds, the monoterpene alcohols and aldehydes (Oh et al. 1967, 1968, Table 12.1).

There are several reports which indicate that essential oils do not have any adverse effect on ciliate protozoa in the rumen. McIntosh et al. (2003) observed that the bacteriolytic activity of rumen ciliate protozoa was unaffected in dairy cows supplemented with 1 g day^{-1} of mixed EOs and Newbold et al. (2004) and Benchaar et al. (2007a) reported that ruminal protozoa counts were not affected when sheep and dairy cows were fed 110 and 750 mg day^{-1} of a mixture of EOs, respectively. The extract of fennel (containing essential oils) had no effect on protozoa as reported by Patra et al. (2010).

Mentha piperita and *Eucalyptus globulus* oils, however, adversely affected ciliate protozoa *in vitro,* where the adverse effect increased with increasing concentration

Table 12.1 Essential oils, their active principles and effect on rumen fermentation and the animal

Plant part	Active principle	Effects	Reference
Trachyspermum copticum (ajwain) oil	Thymol [5-methyl-2-(1-methylethyl) phenol; $C_{10}H_{14}O$]	Active against a wide range of Gram-positive and negative bacteria, reduced methane production	Evans and Martin (2000), Pawar (2011)
Cinnamomum zeylanicum (cinnamon) oil	Cinnamaldehyde (3-fenil-2-propenal phenol; C_9H_8O)	Inhibits peptidolysis and methanogenesis, reduces acetate to propionate ratio	Cardozo et al. (2004), Busquet et al. (2005a), Pawar (2011)
Eugenia (Syzygium) aromaticum (clove) oil	Eugenol (4-allyl-2-methoxyphenol; $C_{10}H_{12}O_2$)	Inhibits methanogenesis, ammonia and VFA production	Davidson and Naidu (2000), Patra et al. (2009)
Allium sativum (garlic) oil	Allicin ($C_6H_{10}OS_2$), diallyl sulfide, diallyl disulfide and allyl mercaptan	Antiparasitic, insecticidal, anticancer, antioxidant, immunomodulatory, anti-inflammatory, hypoglycemic, anti-methanogenic	Reuter et al. (1996), Busquet et al. (2005b), Patra et al. (2009)
Eucalyptus globulus (eucalyptus) oil	Cineole, pinene and other terpenes, phellandrene	Effective against bacteria, methane inhibition	Kumar et al. (2009)
Mentha piperita (peppermint) oil	Menthol ($C_{10}H_{12}O$)	Methane inhibition, adverse effect on VFA production	Craig (1999), Agarwal et al. (2009)
Origanum vulgare (oregano) oil	Carvacrol	Increased butyrate, reduced propionate	Busquet et al. (2006), Evans and Martin (2000)
Pimpinella anisum (anise) oil	Anethol	Decreased VFA, acetate and propionate	Davidson and Naidu (2000), Busquet et al. (2006)

of essential oils in the medium (Agarwal et al. 2008; Kumar et al. 2009). It has also been observed that clove extract containing EOs decreased total numbers of protozoa, small entodiniomorphs and holotrichs, but did not affect large entodiniomorphs (Patra et al. 2010). In addition to *in vitro* studies, Ando et al. (2003) also reported *in vivo* that feeding 200 mg day^{-1} of peppermint oil (*Mentha piperita* L.) to Holstein steers decreased total number of protozoa and that of *Entodinium, Isotricha* and *Diplodinium.* However, Cardozo et al. (2006) observed that addition of a mixture of cinnamaldehyde (180 mg day^{-1}) and eugenol (90 mg day^{-1}) to the diets of beef heifers increased number of holotrichs and had no effect on entodiniomorphs, but there was no effect on numbers of these protozoa species when the mixture contained higher concentrations of cinnamaldehyde (600 mg day^{-1}) and eugenol (300 mg day^{-1}). Recently, Yang et al. (2010b) also observed that cinnamaldehyde supplemented at the rate of 0.4–1.6 g day^{-1} in steers did not affect total protozoa as well as *Isotricha, Dasytricha* and *Entodinium* sp.

The rate of fermentation of feed in the rumen is affected by several factors. Any prominent change in fermentation pattern is reflected first of all by variation in pH of the rumen liquor. Benchaar et al. (2006b) observed that ruminal pH was increased (6.50 *vs*. 6.39) by the addition of essential oils (Crina Ruminants; 2 g/day) and it tended to increase in lactating dairy cows fed essential oils. Beauchemin and McGinn (2006) observed that feeding essential oils (Crina Ruminants; 1 g/day) in cattle had no effect on ruminal pH.

The VFA concentration was not affected in lactating cows fed on alfalfa silage based diet, but were decreased when fed on the corn-silage based diet with the addition of 0.75 g day^{-1} of an EO mixture (Benchaar et al. 2007a). The acetate to propionate ratios were increased (Benchaar et al. 2007b; Macheboeuf et al. 2008; Agarwal et al. 2009) or some times were not affected (Wang et al. 2009; Kumar et al. 2009). Similarly, Yang et al. (2007) reported that the pH, concentration of ammonia nitrogen and total VFA were not affected by dietary supplementation of garlic oil (5 g/cow/day). In contrast, some studies showed an increase in concentrations of total VFA in the rumen liquor due to supplementation of cinnamaldehyde at the rate of 0.2 g kg^{-1} DM intake (Chaves et al. 2008a, b) and EO extract from oregano at 0.25 g kg^{-1} DM intake (Wang et al. 2009).

Thymol (0.4 g L^{-1}), the main component of EOs derived from Thymus and Origanum plants, a strong inhibitor of *in vitro* methane production (Evans and Martin 2000), caused a reduction in methane to the extent of 99% at 6 mM concentration (Macheboeuf et al. 2008). Anethole at 20 mg L^{-1} of medium caused an inhibition of methane *in vitro* (Chaves et al. 2008c). Other EOs like, Juniper berry EOs and cinnamon oil (Chaves et al. 2008c) and peppermint oil (Tatsouka et al. 2008; Agarwal et al. 2009) have been shown to have a strong inhibitory effect on methanogenesis. The active component of cinnamon oil i.e. cinnamaldehyde caused a depression in methane production to the extent of 94% at 5 mM (Macheboeuf et al. 2008). Methanol and ethanol extracts of fennel seeds and clove buds inhibited *in vitro* methane production (Patra et al. 2010). Eucalyptus oil inhibited methane production up to 58% at 1.66 mL L^{-1} (Kumar et al. 2009), 90.3% at 2 mL L^{-1} (Sallam et al. 2009) and 70% at a dose of 0.33 g of α-cyclodextrin-eucalyptus oil complex

(Tatsouka et al. 2008). The component of eucalyptus oil, p-cymene decreased methane by 29% at a concentration of 20 mg L^{-1} (Chaves et al. 2008c), however, α-cyclodextrin cineole did not influence methane up to a concentration of 0.33 g L^{-1} (Tatsouka et al. 2008).

Sallam et al. (2011) used four different EO *in vitro* isolated from *Achillea santolina, Artemisia judaica, Schinus terebinthifolius* and *Mentha microphylla*. The main components of the EO were piperitone (49.1%) and camphor (34.5%) in *A. judaica,* 16-dimethyl 15-cyclooactdaiene (60.5%) in *A. santolina,* piperitone oxide (46.7%) and cis-piperitone oxide (28%) in *M. microphylla*, and g-muurolene (45.3%) and α-thujene (16.0%) in *S. terebinthifolius*. The EO from *A. santolina* and *A. judaica* at all levels increased the gas production significantly, but *S. terebinthifolius*, *A. santolina* at different levels and all levels of *M. microphylla* decreased gas production significantly in comparison to that in control. The highest levels of *A. santolina, A. judaica* and *M. microphylla* inhibited the methane production along with a significant reduction in true degradation of dry matter, organic matter, protozoa count and NH_3-N concentration. It can be suggested that the EO has a promising methane mitigation effect. Whereas in another study with eucalyptus oil (*Eucalyptus citriodora*), methane emission (mL/g DM) was reduced by 53% and 57% with eucalyptus (Sallam et al. 2010).

The *in vivo* studies of Wang et al. (2009) showed that inclusion of 0.25 g day^{-1} of EO mixture from oregano plants in the diet of sheep for 15 days lowered methane emission. However, *in vivo* studies of Beauchemin and McGinn (2006) with EO mixture fed to beef cattle (1 g day^{-1}) for 21 days did not reveal any effect on methanogenesis. Many a times it has been observed that some essential oils have an inhibitory effect on methane production *in vitro*, but when tested *in vivo*, a similar effect might or might not be observed. There might be many reasons for that as *in vitro* tests usually have many limitations (Flachowsky 2009).

No effect on dry matter intake (DMI) was observed when lactating dairy cows were fed a mixture of essential oils (750 mg/day; Crina Ruminants) or on supplementation of a mixture of essential oils (1 g/head/day) (Beauchemin and McGinn 2006). Similarly, Yang et al. (2007) also reported no change in intakes of dry matter (DM), organic matter (OM), neutral detergent fibre (NDF) and acid detergent fibre (ADF) by dietary supplementation of garlic oil or juniper berry oil (2 g/cow/day) in cows. However, Kung et al. (2008) observed that dry matter intake was greater in cows fed a blend of essential oils (1.2 g/cow/day) compared with cows fed control diet (28.3 *vs.* 26.4 kg/day). Benchaar et al. (2006a) reported that feeding higher amounts (2 and 4 g/head/day) of a blend of essential oil compounds (Vertan; IDENA, Sautron, France) increased DMI of growing beef cattle fed silage based diets. But Cardozo et al. (2006) reported that supplementation of a mixture of cinnamaldehyde (0.6 g/day) and eugenol (0.3 g/day) oils decreased DMI, whereas, feeding capsicum oil (1 g/day of capsicum extract containing 15% capsaicin) increased DMI in Holstein heifers.

The retention of nitrogen was not affected in lactating dairy cows or in beef cattle (Benchaar et al. 2006a) fed different doses of a mixture of essential oil compounds. In contrast, Yang et al. (2007) observed that ruminal digestibility of nitrogen increased with the dietary supplementation of garlic oil (5 g/cow/day). There is not

much information available on effects of EOs or their compounds on performances of ruminants. Bampidis et al. (2005) observed no change in average daily gain (ADG) and feed conversion efficiency when growing lambs were fed diets supplemented with oregano leaves (*Origanum vulgare* L.) providing 144 or 288 mg of oregano oil (850 mg g^{-1} of carvacrol) per kilogram of dietary DM. Similarly, Beauchemin and McGinn (2006) observed no change in ADG of cattle supplemented with a mixture of essential oils (Crina Ruminants; 1 g/day). Benchaar et al. (2006b) reported no change in ADG of beef cattle fed a silage-based diet supplemented with 2 or 4 g day^{-1} of a mixture of EOs consisting of thymol, eugenol, vanillin and limonene. However, the EO mixture had a quadratic effect on feed conversion with a dose of 2 g day^{-1} improving feed conversion efficiency as compared to the dose of 4 g day^{-1}. Also, Yang et al. (2010) observed that dietary supplementation of cinnamaldehyde at 400, 800, or 1,600 mg/steer/day improved growth performance in steers. However, higher ADG (250 or 254 *vs.* 217 g day^{-1}) was observed when cinnamaldehyde or juniper berry EO was added to a barley-based diet at a similar concentration (0.2 g kg^{-1} of dietary DM). Soltan (2009) assigned 100 Holstein male calves to investigate the effect of essential oil mixture (eucalyptus oil, menthol crystal, mint oil; EOm) supplementation in milk replacer (0, 94, 187 and 281 mg/calf/day) during 8 weeks (pre-weaning period) and in drinking water (0, 15.6, 31.2 and 46.8 mg/l) for the next 16 weeks (post-weaning period). The results showed EOm supplementation at different levels in milk replacer had no effect on body weight gain when compared with the control which was attributed to lower concentrate intake by the treated calves during the whole period of experiment. However, during post-weaning period, calves administrated 15.6 mg of EOm/l of drinking water improved daily body weight gain, reduced feed intake and improved FCR as compared to control. Thus, it appears that the influence of EO on growth performance is diet dependant.

12.3 Selection of Plants Containing Secondary Metabolites

In the last one decade a large number of laboratories, throughout the globe have screened a lot of plants containing secondary metabolites with different objectives to:

- inhibit methanogenesis and ciliate protozoa,
- stimulate the activity of fiber degrading anaerobic fungi or bacteria to extract more energy from lignocellulosic feeds,
- inhibit ammonia production in the rumen for economic utilization of nitrogen by the animal,
- inhibit protein degradation in the rumen so that intact protein is available in the lower part of the gastro-intestinal tract or
- detoxify anti-nutritional factors if these are present in the feed offered to the animals.

The degree of success achieved in each of the experiments is variable. A large number of plant extracts have been screened for their potential to inhibit

methanogenesis and ciliate protozoa growth in the rumen of buffalo (Kamra et al. 2006, 2008, 2009). In addition to these two important parameters, fibre degrading enzyme profile, *in vitro* feed digestibility, ammonia production, volatile fatty acid production and sometimes microbial profile were also studied to get as much information as possible on each of the plant products so that possibility of their use as a rumen modifier could be explored. Out of 93 plant extracts tested, 11 inhibited *in vitro* methanogenesis to the extent of 25–50% and nine plant extracts inhibited more than 50%. Among these 20 extracts exhibiting antimethanogenic activity, nine were ethanol extracts, ten were methanol extracts and only one was water extract. Some of these plant extracts inhibited ciliate protozoa as tested by microscopic examination and ^{14}C-labelled radio-isotopic technique, but the protozoa inhibition was not correlated with methane inhibition, indicating that the methanogens sensitive to plant secondary metabolites might or might not be having any symbiotic relationship with ciliate protozoa. Methane inhibition was accompanied with a drastic fall in the number of methanogens as determined by real time PCR. Plants that appeared to have some potential as feed additives to control methanogenesis by the ruminants are: (i) seed pulp of *Sapindus mukorossi* (rich in saponins) and *Terminalia chebula* (rich in tannins), (ii) leaves of *Populus deltoides, Mangifera indica,* and *Psidium guajava* (rich in tannins and essential oils) and (iii) flower buds of *Syzygium aromaticum* and bulb of *Allium sativum* (rich in essential oils). The anti-methanogenic activity of most of these plants, especially the spices has been reported for the first time (Patra et al. 2006a, b). Some of these results have been summarized in Table 12.2. Most of this work was conducted in a multi-locational project funded by International Atomic Energy Agency, Vienna, Austria.

Similarly another large experiment on screening of plants containing secondary metabolites has been completed in the "RUMEN-UP" project in Europe. Primarily the foliage plants (450 in number) were screened *in vitro* for their potential to inhibit methanogenesis by the rumen microbes. The selection of plants was restricted to those which were growing or could be grown in European countries, therefore excluding most of the plants growing in the tropical regions of the world. Out of 450 plants examined in this project, 35 plants inhibited methane by more than 15% and only six plants (*Carduus pycnocephalus* L., *Populus tremula* L., *Prunus avium* L., *Quercus robur* L., *Rheum nobile* Hook. F. and Thoms., and *Salix caprea* L.) inhibited methane by more than 25% (Bodas et al. 2008). These plants did not have any adverse effect on any of the fermentation parameters tested, indicating that the secondary metabolites present in these plants were selective inhibitors of methanogenic archaea and did not affect any other rumen microbe at the level used in this experiment. These plants are reported to contain essential oils like hexadecanoic acid in *Carduus* sp. (Esmaeili et al. 2005), flavenoids in *Pycnocephalus* sp. (El Lakany et al. 1997), flavenoids and anthranoid derivatives in *Rheum nobile* (Iwashina 2003), some non-protein amino acids and cyanogenic glycosides in *Prunus avium* and phenolics and isoprenoids in *Populus* sp. and *Salix* sp. (Ikonen et al. 2002). These secondary metabolites have been proposed to be the major principles responsible for anti-methanogenic activities of these plants.

Table 12.2 Effect of seed pulp, tree leaves, spices and their extracts (in water, WE; methanol, ME and ethanol, EE) on inhibition of methane, *in vitro* true digestibility and ciliate protozoa in buffalo rumen liquor

Botanical name	Common name and part of the plant	CH_4 inhibition (%)[a]	Protozoa inhibition (%)	IVTD Reduction (%)	Reference
Cannabis indica	Bhang, leaves	34.42 (EE)	28.05	0.90	Kamra et al. (2008)
Eugenia jambolana	Jamun, leaves	24.27 (ME)	50.21	19.78	Kamra et al. (2008)
Ficus benghalensis	Banyan, leaves	20.17 (P)	7.14	8.24	Zadbuke (2009)
Mangifera indica	Mango, leaves	35.67 (ME)	55.74	−2.71	Kamra et al. (2008)
Ocimum sanctum	Tulsi, leaves	24.81 (ME)	11.26	4.66	Patra et al. (2008)
Populus deltoides	Poplar, leaves	85.86 (ME)	17.91	−2.63	
Psidium guajava	Guava, leaves	81.79 (EE)	52.76	5.30	Kamra et al. (2008)
Quercus incana	Oak banjh, leaves	37.12 (P)	5.81	8.12	Zadbuke (2009)
Quercus semicarpipholia	Oak khurson, leaves	30.00 (P)	5.48	3.17	
Allium sativum	Garlic, bulb	69.73 (ME)	−17.42	5.31	Kamra et al. (2008)
Curcuma longa	Turmeric, root	21.89 (ME)	0.90	5.04	Zadbuke (2009)
Foeniculum vulgare	Fennel, seeds	70.72 (ME)	23.86	9.77	Patra et al. (2006b)
Syzygium aromaticum	Clove, flower bud	85.61 (ME)	52.42	27.39	Patra et al. (2006b)
Trachyspermum ammi	Ajwain, seeds	42.28 (EE)	29.95	0.28	Pawar (2011)
Azadirachta indica	Neem, seeds	34.59 (EE)	14.56	7.71	Patra et al. (2006a)
Emblica officinalis	Amla, seed pulp	27.68 (ME)	−16.50	9.21	
Sapindus mukorossi	Soapnut, seed pulp	95.80 (EE)	52.29	47.64	Agarwal et al. (2006)
Terminalia belerica	Bahera, seed pulp	28.11 (ME)	24.27	3.00	Patra et al. (2006a)
Terminalia chebula	Harad, seed pulp	99.79 (ME)	37.86	14.13	

P powdered, *WE* water extract, *ME* methanol extract, *EE* ethanol extract

[a]Only those plants have been included which showed methane inhibition more than 20% in comparison to their respective controls. Methane inhibition has been calculated in terms of ml of methane produced/g DM

12.4 *In vivo* Feeding Trials

Most of the above studies have been conducted in *in vitro* conditions. There are only a few experiments conducted to report methane inhibition *in vivo*. The results indicate that there are many plants which contain secondary metabolites and are active against rumen methanogenesis. Many times methane inhibition by secondary metabolites in *in vitro* conditions might not be translated into similar effects in *in vivo* conditions. This might happen due to improper selection of the dose of these metabolites in the ration of animals.

In an *in vivo* experiment in sheep with tea saponins it has been reported that saponins inhibit protozoa, methane emission and improved rumen fermentation, where the reduction of methane emission was mediated through inhibitory effect on protozoa (Zhou et al. 2010).

Some experiments indicate positive results of including plants/plant extracts on inhibition of methanogenesis. In one experiment *Terminalia chebula, Allium sativum* and the mixture of two were fed to sheep at the rate of 1% of DMI, resulted in a decreased ($p = 0.09$) methane production by 24%, 11% and 23.5% in *T. chebula, A. sativum* and the mixture of the two, respectively, when expressed as L/kg digestible DM intake (Patra et al. 2010). *T. chebula* is a rich source of tannin (4.89% of DM), whereas, *A. satium* is rich in essential oils. The data indicated that *T. chebula* was more effective as compared to garlic. The reason for low *A. sativum* activity might be due to the instability of allicin, the main secondary metabolite responsible for antimicrobial activity of *A. sativum*.

Murrah buffaloes fed on a diet of wheat straw and concentrate mixture (50:50) and supplemented with a feed additive (a mixture of *Allium sativum,* 1% and *Mentha piperita* oil, 0.1% of DMI) (Mix 1) on every alternate day resulted in 7% reduction in methane emission (l/kg DMI), but this reduction in methane emission was attributed to reduction in dry matter intake (Verma et al. 2009). There was no adverse effect on rumen fermentation pattern, enzyme and microbial profiles.

In another experiment, a mixture of three plants (Mix 3) fed to buffalo calves at the rate of 1%, 2% and 3% of DMI, resulted in a dose dependent inhibition in methane emission (l/kg DDM) since per cent inhibition increased with an increase in dose of the feed additive (Chaudhary et al. 2009) without affecting dry matter digestibility at any of the levels of feed additives tested. The VFA and fibre degrading enzyme activities were not affected, whereas, there were a few changes in the rumen microbial profile as estimated by real time-PCR, but these were not responsible for any significant change in rumen fermentation.

As discussed above there are many plants which have a potential to inhibit methanogenesis in the rumen, but that is not the end. Therefore, screening of plants should be a continuous process to search for more useful ones, which can be used for rumen manipulation. In the secondary screening process, only selected plants should be tested in *in vivo* experiments to examine their potential for practical application.

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Chapter 13
Phytochemicals and Gut Microbial Populations in Non-ruminants

Artabandhu Sahoo and Nira Manik Soren

Abstract Phytochemicals are bioactive non-nutrient plant compounds, which have been great interests to the researchers because of their potential effects as antioxidants, antiestrogenics, anti-inflammatory, immunomodulatory, and anticarcinogenics. However, the bioavailability and effects of polyphenols greatly depend on their transformation by components of the gut microbiota. Phytochemicals and their metabolic products may also inhibit pathogenic bacteria while stimulate the growth of beneficial bacteria, exerting prebiotic-like effects. Gut microbiota influences the development and maturation of the digestive and immune systems and is a source of regulatory signals, some of which may be suitable for exploitation for therapeutic purposes. This chapter focuses on interaction between phyto-metabolites or plant secondary metabolites and gut microbial population in non-ruminants and harvesting nutritional, health and environmental benefits, consequently in the interest of human population.

Keywords Phytochemicals • Polyphenols • Essential oils • Saponins • Gut microbes • Non-ruminants

13.1 Introduction

The increased interest in phytochemicals in animal diets has been spurred on by the reduction in and general market resistance to the use of 'in feed' antibiotics, the removal of animal protein from diets, and thus the increased variety and inclusion levels of vegetable protein sources. Nevertheless, such effects may be the essential indicator of desirable properties, such as therapeutic potential, especially when the

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mechanism of bioactivity can be delineated. Careful observation of cause and effect, followed by a coordinated approach to identify the responsible entities, has proved extremely fruitful in discovering roles for phytochemical constituents.

Phytochemicals are described as plant secondary metabolites (PSM), and are produced in response to specific signals, and provide an important link between the plant, its potential predators and the environment in which they both live. The primary function of many PSM is probably for plant defence (Harborne 2001), e.g. defence against grazing herbivores and insects, defence against microorganisms including bacteria, fungi and viruses, defence against other plants competing for nutrients and light and protection against the damaging effects of UV light (Bennet and Wallsgrove 1998; Schäfer and Wink 2009). These bioactive phytochemicals often cause dramatic adverse consequences (anti-nutritional or toxic) in other organisms that may be exposed to them and have no direct nutritional value (Mazid et al. 2011). However, the greatest challenge would be to identify the specific compound(s) responsible for the observed adverse effect, defining their mode of action and finally delineating a useful function for the phytochemical or its derivatives as a 'therapeutic drug'. The ability of certain PSMs such as polyphenolic compounds to act as scavengers of free radicals (Michalak 2006), besides their antioxidant and antimicrobial properties, is raising the possibility of their food and pharmaceutical applications (Saikia and Upadhyaya 2011). Therefore, traditional medicines all over the world are nowadays being reevaluated by extensive research on different plant species with reference to their therapeutic principles and the plant kingdom might provide a useful source of new medicines and pharmaceutical entities or, alternatively, may be used as simple dietary adjuncts to existing therapies.

13.2 Phytochemicals

A number of phytochemicals, which are commonly found in foods, has been listed in Table 13.1. Besides, essential oils, non-starch polysaccharides/oligosaccharides, polyunsaturated fatty acids (PUFA) are other bioactive components of foods which are of special interest.

13.2.1 Phytochemicals-Herbivore Interaction

Plants possess a wide variety of compounds and growth forms that are termed PSM and they posses "anti-quality" factors to reduce forage value, yield toxic effects and deter grazing. Herbivores, on the other hand, possess several adaptive mechanisms (Karban and Agrawal 2002) to lessen the impacts of anti-quality factors, viz. (i) herbivores graze selectively to limit consumption of potentially harmful plant compounds (able to detect plant nutritional value or toxicity by relating the flavor of a plant to its positive or negative digestive consequences) (Foley and Moore 2005),

Table 13.1 Different classes of plant bioactive compounds found in foods

Alkaloids	*Monoterpenes*
Caffeine	Geraniol
Theobromine	Limonene
Theophylline	*Organosulfides*
Anthocyanins	Allicin
Cyanidin	Glutathione
Carotenes	Indole-3-Carbinol
Lycopene	Isothiocyanates
Coumestans	Sulforaphane
Flavan-3-ols	*Phenolic Acids*
Flavonoids	Capsaicin
Hesperidin	Ellagic acid
Kaempferol	Gallic acid
Naringin	Rosmarinic acid
Quercetin	Tannic acid
Resveratrol	*Phytosterols*
Rutin	Beta-Sitosterol
Hydroxycinnamic Acids	*Saponins*
Chicoric acid	*Triterpenoids*
Coumarin	Ursolic acid
Ferulic acid	*Xanthophylls*
Scopoletin	Astaxanthin
Isoflavones	Beta-Cryptoxanthin
Daidzein	*Other Phytochemicals*
Genistein	Damnacanthal
Lignans	Digoxin
Silymarin	Phytic acid
Monophenols	
Hydroxytyrosol	

(ii) enhance diet selection skills by adaptive intake patterns that limit the deleterious effects of plant allelochemicals that includes cautious sampling of sample new foods, consuming a varied diet, and eating plants in a cyclic, intermittent, or carefully regulated fashion (Dearing et al. 2005), (iii) grazing animals possess internal systems that detoxify or tolerate ingested phytotoxins, e.g. may eject toxic plant material quickly after ingestion, secrete substances in the mouth or gut to render allelochemicals inert, rely on rumen/intestinal microbes to detoxify allelochemicals, absorb phytochemicals from the gut and detoxified them in body tissues, or develop a tolerance to the toxic effects of plant allelochemicals (Launchbaugh et al. 2001).

Understanding the behavioral and metabolic abilities of herbivores suggests several livestock management practices to help animals contend with plant anti-quality characteristics, which include offering animals proper early life experiences, selecting the appropriate livestock species and individuals, breeding animals with desired attributes, and offering nutritional or pharmaceutical products to aid in digestion and detoxification (Launchbaugh et al. 2001). Further, PSM are non-nutritional components that occur in numerous feed materials and are able to exert toxic effects

in animals. Innate defense strategies developed by different animal species to avoid excessive exposure to PSM are (i) pre-systemic degradation of PSMs by rumen microbiota, (ii) the intestinal barrier including efflux transporters of monogastric species, (iii) pre-hepatic and intra-hepatic biotransformation processes (Gremmels 2010). These physiological barriers determine systemic exposure and ultimately the dose-dependent adverse effects in the target animal species. Considering the large number of potentially toxic PSM, mechanistic data related to tissue disposition and excretion pathways of PSM (e.g. milk, meat, egg, etc.) could substantially support the assessment of the risks for consumers of foods derived from PSM-exposed animals.

13.3 Non-ruminants, Gut Microbiota and Their Interaction

Non-ruminants are a wide group of animal species which can be grouped as non-ruminant herbivores and other simple stomach animals. Sloths, pandas, equids, kangaroos, hamsters, voles, colobine and langur, monkeys, hippopotamus, elephants, etc. belong to the former group. Animals of larger body size tend to feed less selectively and hence consume a diet of 'lesser quality', i.e. higher fibre content (Owen-Smith 1988). They make use of this ubiquitous dietary niche 'herbivory', not because of a 'more efficient digestion', but because of a shift of digestive priorities.

Most gut microbes exist as commensal flora providing a beneficial protective barrier to pathogenic bacteria and as a mechanism of nutrient metabolism and supply to the host, particularly in herbivorous animals (Sears 2005). There is wide diversity in gut microbial species in different non-ruminant hosts, although mostly influenced by diet (Prajwal et al. 2011). Until now, the number of microbial species in human and pig gut is estimated to be >1,000 and 400. These bacteria are natural symbionts of the gastrointestinal tract, and have adapted to their human and animal hosts over millions if not billions of years of evolution. The study of the interactions between hosts and their associated microorganisms is complicated by the fact that most associated microorganisms have not been cultured and that most of the interactions involve more than one microorganism with the host, e.g., the human gut microbiota (Ley et al. 2006). Recent analyses of ribosomal RNA sequence diversity have demonstrated the extent of bacterial diversity in the caecum or colon, and have provided new tools for monitoring changes in the composition of the gut microbial community (Zoetendal et al. 2004; Dethlefsen et al. 2007). Specific examples of harnessing microbe-microbe interactions, host-microbe interactions, and host-microbe-dietary interactions have immediate clinical implications. The future of drug discovery in gastroenterology is likely to reside in the lumen, which opens the possibility of a "bugs to drugs" program of discovery, in which the gut ecosystem is explored as a repository from which bioactives or novel drugs might be mined and translated to human health care (Shanahan 2010).

The microbial ecology in the gastro-intestinal tract of various animal species is characterized by high population density, wide diversity, anaerobic nature

and complexity of interactions. The major groups of microbes which inhabit the gastro-intestinal tract include bacteria, protozoa, fungi, yeasts and bacteriophages (Hobson and Stewart 1997). It would however appear that diet and geographic location have a larger influence on the composition of gut microbial population than the genetics of the host animal. As the commensal microbes are a dynamic ecosystem whose population and activities can be modulated by diet they represent an important nutritional target for maintaining gut health. Gut microbial activity in farm animals also influences the nutritional quality of animal food products and the properties of animal wastes. *Escherichia coli* are commensal bacteria that can account for up to 1% of the bacterial population of the gut. The pathogenic bacteria *E. coli* strain O157:H7 are being shed up to 30% in feedlot cattle, since the ration constitutes rapidly ruminally fermented grains (e.g., barley, distillers' grains) which may contaminate the carcass and enter in to the human food chain (Callaway et al. 2009; Berry and Wells 2010). Research has shown that diet does affect *E. coli* O157:H7 populations, but the effects have varied in magnitude and impact, e.g. 1,000-fold decline in total *E. coli* by switching from a high grain diet to an all hay diet (Callaway et al. 2009). The effects of forage feeding on *E. coli* O157:H7 populations may be due to concentrations of tannins and phenolic acids in forages (Min et al. 2007). Thus composition of diet and optimal nutrition in both animals and man is intrinsically linked with optimum gut health and consequently, may have major impacts on human health and on the environment.

Enhancing gut function through nutritional modulation of commensal microbes is a promising approach to disease prevention in both animals and man. A number of human medical disorders such as inflammatory bowel disease and bowel cancer show a significant correlation with diet and with the metabolic activity of the large intestinal microflora (Thomas and Versalovic 2010). Certain disorders may be linked to the inappropriate priming or "education" of the gut immune system during early life or to a breakdown in the normal relationships between commensal microbes and gut tissue function. Nutrient mediated modulation of gut function offers an attractive alternative therapy for such disorders (Cunningham-Rundles et al. 2005). There is a need to understand how type and number of microbes impact on the gut immune response and whether there are optimum "windows of opportunity" for programming the tolerant responses that can provide subsequent protection, e.g. commensal microbes could have more direct benefit in bowel cancer. Short chain fatty acids such as butyrate, generated from the microbial breakdown of polysaccharide, may protect against colon cancer and colitis while other microbial metabolites act as carcinogens (Topping and Clifton 2001; Andoh et al. 2003). Elucidating how diet can alter beneficial nutrient production by commensal bacteria provides a further important potential avenue towards improved health.

While the commensal microbes that inhabit the gut contribute to the priming of the mucosal-immune system and provide a barrier against pathogenic attack they also make a major contribution to the breakdown and synthesis of macronutrients (Sekirov et al. 2010). The microbial ecosystem that establishes in the gut is extremely complex, yet there is a dearth of information about how it is established or maintained, and in particular the functional contribution of specific bacteria.

Mechanistic understanding of any aspect of gut microbial metabolism requires that we identify the organisms involved and the genes and enzyme systems responsible (Kau et al. 2011). This applies to the breakdown of dietary substrates such as polysaccharides and proteins and to the formation and conversion of fatty acids. In particular, major unanswered questions pertain to the formation of short chain acids such as butyrate in the human colon and the hydrogenation of CLAs in the rumen. Another important activity of commensal microorganisms in the gut is the inhibition of pathogenic species through a variety of mechanisms including predation and the production of inhibitory fermentation products (Kelley et al. 2005). Research in these areas requires a combination of modern molecular genetic techniques in microbial ecology, gene identification and enzymology, together with established expertise in the culture of anaerobic microorganisms and in tracing metabolite fluxes. New research programmes in microbial genetics can use genome sequence information to identify genes involved in gut survival and host interactions. Work on gene transfer has shown that the gut microflora is a highly dynamic community where there is gene flow of antibiotic resistance genes and pathogenic determinants throughout the system (Sears 2005).

Although there is association between the colonic environments with inflammation, very few studies have addressed the involvement of gut bacteria and their ability to modulate inflammatory compounds derived from "non-nutritive" phytochemicals consumed in the diet (Ricciardiello et al. 2011). The human gut microbiota has been recognized as a metabolically versatile digester playing an essential role in the regulation of the host metabolome, energy utilization and storage (Wikoff et al. 2009; Backhed and Crawford 2010; Velagapudi et al. 2010). Changes in the gut microbial composition /function are implicated in the development of disease such as inflammatory bowel disease and colorectal cancer. It is demonstrated that prostanoid production in normal colon fibroblast cells was modulated to varying degrees by a group of structurally related phenolic compounds following cytokine-induced upregulation of prostaglandin synthesis (Russell et al. 2008). However, it is likely that these parent compounds will be transformed by the colonic microflora, requiring assessment of the structure of the metabolites and the overall effect of metabolism on inflammatory processes.

13.4 Intestinal Microbial Fermentation

The caecum/colon represents the main fermentation site in non-ruminants. Dietary components that escape digestion by endogenous enzymes in the upper gastrointestinal tract become available as substrates in the large intestine. Caecal/colonic microorganisms convert nutrients leaving the small intestine to volatile fatty acids (VFA), gases (CH_4, CO_2, and H_2), ammonia and compounds incorporated into microbial cells (Stevens and Hume 1998). On the other hand, the non-nutrient phytochemicals or their metabolites may influence caecal/colonic ecology due to interaction with host microbiota, colonocytes or the absorptive process of the GIT.

Methane production is almost absent from caecal fermentation before weaning and it is suggested that reductive acetogenesis is a major characteristic of this fermentation process in young rabbits (Piattoni et al. 1996), being replaced gradually and partially by methanogenesis as the intake of solid food increases, but reductive acetogenesis still appeared to be important (Belenguer et al. 2008). The metabolic outputs of the gut microbial community depend not only on available substrate, but also on the gut environment, with pH playing a major role. Alterations in diet composition result in both quantitative and qualitative changes in the supply of substrates to the large intestinal microbiota (Louis et al. 2007). The impact of dietary changes upon microbial metabolism is sequel to several inter-related mechanisms, (i) metabolism regulated within each individual species of gut bacterium, i.e. alternative substrates giving rise to different products as a result of fermentation via different metabolic routes depending on their rate of supply, or the physiology and environment of the bacterial cell (Louis et al. 2007); (ii) changes in the supply of non-digestible dietary carbohydrates that can lead to shifts in the species composition of the colonic bacterial community (prebiotic effect) (Walker et al. 2011); (iii) local conditions of pH, oxygen and hydrogen, metabolite concentration and gut transit time that strongly influence the gut environment including bacterial metabolism and bacterial competition (Louis et al. 2007); (iv) host secretions and secondary bacterial metabolites, such as antimicrobials and quorum-sensing molecules influencing the gut environment; (v) adaptations to varying substrates (including non-digestible phytometabolites), and environmental conditions may lead in to more prominent changes of activity rather than bacterial populations or both.

The amounts and types of fermentation products formed by colonic bacteria depend on the relative amounts of each substrate available, their chemical structures and compositions, as well as the fermentation strategies (biochemical characteristics and catabolite regulatory mechanisms) of bacteria participating in de-polymerization and fermentation of the substrates (Macfarlane and Macfarlane 1997). Protein breakdown and dissimilatory amino acid metabolism result in the formation of a number of putatively toxic metabolites, including phenols, indoles and amines. Production of these substances is inhibited or repressed in many intestinal microorganisms by a fermentable source of carbohydrate. Owing to the anatomy and physiology of the colon, putrefactive processes become quantitatively more important in the distal bowel, where carbohydrate is more limiting.

13.5 Functional Food Components

Prebiotics, polyunsaturated fatty acids (PUFAs) and phytochemicals are the most well characterized dietary bioactive compounds. The beneficial effects of prebiotics mainly relay on their influence on the gut microbiota composition and their ability to generate fermentation products (short-chain fatty acids) with diverse biological roles (Laparra and Sanz 2010). PUFAs include the ω3 and ω6 fatty acids, whose balance may influence diverse aspects of immunity and metabolism.

Moreover, interactions between PUFAs and components of the gut microbiota may also influence their biological roles. Therefore, the intestinal microbiota is both a target for nutritional intervention and a factor influencing the biological activity of other food compounds acquired orally (Cencic and Chingwaru 2010).

The use of phytochemicals or the natural plant products is one of the available options to achieve optimum nutrient utilization and efficient productivity (Wenk 2003; Kong et al. 2011). The addition of synthetic chemicals was the choice for the last many years. However, during the last couple of years there is a lot of consumer awareness to avoid any agricultural or animal products, which contain synthetic chemicals. As a sequel to these concerns, the use of chemicals in animal feeding systems has been stopped in the European Union (Demir et al. 2003). Other advanced countries are following suit. Therefore, it is imperative that we develop suitable feeding systems based on plant products (phytochemicals) for achieving the twin objectives of enhancing animal productivity and decreasing emissions of fermentation gases (methane and ammonia). Such research and development (R&D) is a lot imperative in the interest of competitive exports of animal products. The phytochemicals constitute plant secondary metabolites (PSMs) such as tannins, saponins, essential oils, and flavonoids which are the most attractive candidate compounds for this purpose and this approach is the focus of R&D attention in European Union and Australia. The forests are the ideal locale for finding novel plant resources to look for the promising plant products. PSMs have hitherto been considered essentially antinutritional. However, more strategic studies during the last couple of years have shown that the dose of the compounds ingested or included in the feeds plays a crucial part in the ultimate beneficial or harmful effects. Usually, the lower optimal doses of PSMs have a number of beneficial effects that help obviate the use of synthetic chemicals (Villalba and Provenza 2009).

13.6 Phytochemicals and Gut Microbiota

13.6.1 Tannins and Polyphenols

The antimicrobial activities of tannins are ascribed to the interactions of tannins with the extracellular enzymes secreted and the cell wall of bacteria causing morphological changes of the cell wall, tannin-induced membrane disruption, direct action on microbial metabolism, deprivation of substrates for microbial growth and chelation of cations by tannins reducing its availability to microbes (Scalbert 1991; Chung et al. 1998; Smith et al. 2005). These aspects have been have been discussed in details in Chaps. 1 and 8. Hydrolyzable tannins and phenolic monomers such as flavonols can be degraded in the anaerobic environment of the intestinal tract (Osawa et al. 1995; Schneider and Blaut 2000), but intestinal bacteria that can degrade condensed tannins and their monomeric units, flavan-3-ols. Monomeric polyphenols from green tea were shown to affect gastrointestinal bacteria in humans (Okubo et al. 1992), pigs (Hara et al. 1995), and chickens (Hara 1997) based on cultivation

of specific bacterial groups. Condensed tannins of *Acacia angustissima* has altered fecal bacterial populations in the rat gastrointestinal tract, resulting in a shift in the predominant bacteria towards tannin-resistant gram-negative *Enterobacteriaceae* and *Bacteroides* species (Smith and Mackie 2004). *Clostridium* and *Eubacterium* genera, which are phylogenetically associated, are other common elements involved in the metabolism of many phenolics. It is possible that previous exposure to dietary tannins may result in a shorter adaptation period on subsequent exposure. Thus, determining the mechanisms by which bacteria can resist the inhibitory effects of tannins is important to effectively implement a strategy of increasing the proportion of tannin-resistant bacteria in the gastrointestinal tract. More beneficial effects can be harvested by enhancing the resistance of certain strains or to produce tannin-resistant strains of essential organisms, such as fiber degraders. Antimicrobial activity of tannic acid against coliform is observed initially (day 14) in pig, which however nullified later (day 28) due to microbial degradation of tannic acid (Lee et al. 2010). Certain bacterial populations seemed to be better adapted than others to environmentally adverse conditions, such as less access time to nutrients due to higher motility and rate of passage of digesta caused by extreme temperatures, or antimicrobials such as tannins, possibly due to an influence of their biogeographical location within the gut (Romero-Pérez et al. 2011). Dietary phenolic compounds are often transformed before absorption, and these transformed phenolic compounds could also exert the different biological activity and antimicrobial properties in the intestine (more discussion has been provided in Chap. 8). Different studies have been carried out to understand gut microbiota transformations of particular polyphenol types and identify the responsible microorganisms. Although there are potentially thousands of different phenolic compounds in the diet, they are typically transformed to a much smaller number of metabolites. On the other hand, the health benefits from phenolic consumption should be attributed to their bioactive metabolites which modulate floral composition in the large intestine. The modulation of gut microbial population by phenolics and the two-way phenolic – microbiota interaction must thus be understood to explore GI health benefits of tannin and polyphenols.

It has been reported that some CT containing forage legumes exert direct and/or indirect inhibition of methanogens, may be through a reduction in hydrogen availability (Tavendale et al. 2005; Puchala et al. 2005). CH_4 production was completely inhibited in pure cultures of methanogens incubated with big trefoil (*Lotus pedunculatus*) compared with alfalfa (*Medicago sativa* L.), suggesting that these phenolics directly inhibit methanogen metabolism (Tavendale et al. 2005). Effects of various phytochemicals on methane emissions in ruminants have been discussed in Chaps. 8, 11, and 12. Although the hindgut has more acetogens compared to methanogens, the un-degraded/ undigested tannin polyphenols might have some antagonistic effect on rectal/colonic flora involved in fiber degradation and methanogenesis. Further, antimicrobial effect of tannins against pathogenic *E. coli* can be harvested to check the growing problems of zoonosis emanated from contamination of animal produce with *E. coli* O157:H7. The use of high-quality phytochemical/ tannin-containing forage may have the dual benefit of being good-quality forage, as well as reducing *E. coli* shedding (Berard et al. 2009). A perennial temperate forage

legume, sainfoin (*Onobrychis viciifolia*), is one such fodder that contains fairly good amount of protein with moderate condensed tannin levels (3–5% of dry matter) and also possesses antimicrobial properties against *E. coli* (Berard et al. 2009). Interestingly, there is no adaptation to sainfoin-tannin antimicrobiosis. A combined action of phenolic and flavonol glycosides which have altogether different chemical structure is proposed to be the mode of action against pathogenic *E. coli*. Additionally, inhibitory compounds are released from the degradation of sainfoin and as discussed above, availability of soluble sugars to *E. coli* declines, thus reducing its growth and multiplication.

Chung et al. (1998) demonstrated inhibition of the growth of some human intestinal bacteria, but not lactic acid bacteria by tannic acid. Tannins are inhibitory for structural carbohydrate-fermenting bacteria, consequently reduce the release of soluble sugars from the plant cell wall matrix, which would be a source of carbon for *E. coli* (Flint et al. 2008).The antimicrobial activities of tannins are ascribed to the interactions of tannins with the extracellular enzymes secreted and the cell wall of bacteria causing morphological changes of the cell wall, tannin-induced membrane disruption, direct action on microbial metabolism, deprivation of substrates for microbial growth and chelation of cations by tannins reducing its availability to microbes (Patra and Saxena 2011).

13.6.2 Saponins

Saponins, a structurally diverse family of secondary plant metabolites, contribute to cardiovascular and gastrointestinal health in addition to its anti-carcinogenic, hypolipemic, hypocholesterolemic, and immune-enhancing activities. It has been observed that some plant products lose their effects on continuous ingestion of the plants by animals and their effects are short-lived due to microbial adaptation (Makkar et al. 2007). This calls for development of strategies (for example: ingestion on alternate day or ingestion for 3–4 days followed by a break for 1–2 days) to beat the microbial adaptation. Katsunuma et al. (2000) observed that the total viable counts in the faeces were not different between the pigs given a diet supplemented with or without saponins. However, bifidobacteria, eubacteria, and staphylococci were more and veillonella was less abundant in the faeces of pigs given a diet supplemented with saponins, as compared with the pigs given the non-supplemented diet.

The forage alfalfa (*Medicago sativa*) is high quality forage that can support high levels of production and its content of saponin, phyto-estrogens can be utilized against gut pathogenic bacteria and the negative effect saponin against methanogenic population can further contribute to reducing GHG in the environment. Beneficial effect of addition of alfalfa extract in the culture media containing *Bacteroides ovatus* from guinea pigs has been demonstrated wherein the extract inhibited the organism completely. These organisms are responsible for increasing the concentration of faecal mutagen responsible for colon cancer in humans

(Johanning et al. 1984). The antimicrobial properties of saponins and their effects on gut microbiota have also been discussed in Chap. 1 and Chap. 11.

13.6.3 Essential Oils

Essential oils (EO) are a blend of secondary metabolites obtained from the plant volatile fraction by steam distillation (Gershenzon and Croteau 1991) and have attracted attention for their potential as alternatives to feed antibiotics and growth promoters in livestock (Burt 2004). The current trends regarding research on EO as allelochemicals mediating plant-plant, plant-animal, plant-microbe interactions envisage shifting of the bacterial population balance in favour of those bacteria that can tolerate them or even use them as a carbon and energy source. EO has antimicrobial activities that act in a similar way to monensin by inhibiting gram-positive bacteria. The principal site of action is the cell membrane, and their hydrophobic nature makes them more active against gram-positive bacteria. The hydrophobic nature of the cyclic hydrocarbons in EO interact with cell membranes and accumulate in the lipidic bilayer of bacteria, and causes conformational changes in the membrane structure, loss of membrane stability and cell death (see review, Calsamiglia et al. 2007). In general, the antimicrobial activity is highest in oxygenated cyclic hydrocarbons, and particularly in phenolic structures such as thymol and carvacrol, in which the hydroxyl group and the dislocated electrons allow for the interaction with water through hydrogen bridges as the main active site, making them particularly active against microorganisms (Table 13.2). Moreover, their low molecular weight molecules makes them also active against gram-negative bacteria as they can interact with water (through hydrogen bridges), cross the cell wall slowly by diffusion through the layer of lipopolysaccharides or through membrane proteins, and interact with the lipid bilayer of cells. Besides interacting with cell walls, EO has also the potential to coagulate some cell constituents, probably by denaturation of proteins. The antimicrobial properties of EO (Chap. 5) and the modulation of rumen fermentation and microorganisms (Chap. 10) have been discussed elsewhere in this book.

13.6.4 Other Phytochemicals-Rich Feedstuffs

Feedstuffs like legumes contain several compounds that have been traditionally considered antinutrients, such as protease inhibitors, phytate, saponins, plant sterols and isoflavones. However, recent information suggests that most of these compounds may actually benefit the consumer's/ animal's health. Legumes (pulses and soybeans) are excellent foods to increase dietary fibre consumption besides providing starch, vegetable protein, oligosaccharides, phytochemicals (especially the isoflavones in

Table 13.2 Essential oils with antimicrobial activity, their main active components, and susceptible microorganisms

Essential oil of	Name	Active components	Susceptible microorganisms	References
Allium sativum	Garlic	Allicin	*E. coli*, *Staphylococcus aureus*,	Rees et al. (1993)
		Ajoene	Gram-positive, gram negative and yeast	Naganawa et al. (1996)
		Allicin, diallyl sulfide	Enteropathogenic bacteria	Ross et al. (2001)
		Aqueous, chloroform and ethanol extract of garlic	*S. aureus*, *E. coli, S. pneumoniae* and *P. aeruginosa*	Abubakar (2009)
		Aqueous extract	*S. aureus*	Deresse (2010)
Anethum graveolens	Dill	Limonene, carvone	Gram-positive and gram-negative bacteria	Deans and Ritchie (1987)
		Carvone, limonene, *cis*-dihydrocarvone, diplaniol	*S. aureus*, *Candida albicans*	Yili et al. (2009)
		Flavanoids, tannins, alkaloids, saponins	G+ve: *Enterococcus faecalis, S. aureus,; G-ve: E. coli, P. aeruginosa, Salmonella typhi*	Kaur and Arora (2009)
Capsicum annum	Paprika	Capsaicin	Gram-positive and gram-negative bacteria	Deans and Ritchie (1987)
		Capsaicin	*S. aureus, E. coli , P. aeruginosa , B. subtilis, S. lutea, C. albicans*	Soetarno et al. (1997)
		Acetone extract	*P. aeruginosa* and *R. rubra*	Keskin and Toroglu (2011)
Cinnamomum cassia	Cassia	Cinnamaldehyde	*E. coli, S. aureus, Listeria monocytogenes, Salmonella enteritidis*	Ouattara et al. (1997), Mahmoud (1994)
		Cinnamaldehyde, eugenol, cinnamic acid, weitherhin, mucilage, diterpenes, proanthocyanidins	*Streptococcus oralis*, *Streptococcus anginosus*, *Streptococcus intermedius* and *Streptococcus sanguis*, *Enterobacter aerogenes*, *Micrococcus roseus.*	Smith-Palmer et al. (1998), Chaudhry and Tariq (2006)
		Cinnamaldehyde, 2-hydroxycinnamaldehyde, coumarin, cinnamyl acetate	*Clostridium perfringens, Bacteroides fragilis*	Choi et al. (2001)
Juniperus oxycedrus	Juniper	Cadinene, pinene	*Aeromonas sobria, E. fecalis, S. aureus*	Hammer et al. (1999)
		α-Pinene, Sandaracopimara-8(14)-15-diene, γ –Muurolene	*S. aureus, E. faecalis, E. coli, Pseudomonas aeruginosa, Candida albicans*	Medini et al. (2009)

Melaleuca alternifolia	Tea tree	Terpinen-4-ol, γ-Terpinene, α-Terpinene, 1,8-Cineole, Terpinolene	*S. aureus,*	Carson et al. (1995)
		Terpinen-4-ol	*S. aureus, E. coli, gram-positive* and gram-negative bacteria	Chao and Young (2000), Cox et al. (2001)
Origanum vulgare	Oregano	Carvacrol, thymol	Gram-positive and gram-negative bacteria	Sivropoulou et al. (1996), Dorman and Deans (2000)
		Limonene, γ-cariofilene, canfor, linalol. α-pinene, carvacol and thymol	*Citrobacter, Salmonella typhi, E. coli, Proteus vulgaris*, *Aeromonas hydrophila, Klebsiella pneumoniae* and *E. coli*	Chaudhry et al. (2007)
Pimpinella anisum	Anise	Anethol	*Aeromonas hydrophila, Brevibacterium linens, Brochothrics thermosphacta*	Deans and Ritchie (1987)
		trans-Anethole, Estragole, Anisaldehyde	*E. coli, S. aureus, Salmonella typhimurium, Candida albicans*	Hammer et al. (1999)
Rosmarinus officinalis	Rosemary	1,8-Cineole	*S. aureus, L. monocytogenes Campylobacter jejuni, L. monocytogenes*	Ouattara et al. (1997), Smith-Palmer et al. (1998), Rožman and Jeršek (2009)
		α-Pinene, bornyl acetate, camphor, 1,8-cineole	*S. aureus, Bacillus cereus, E. coli, Pseudomonas aeruginosa*	Genena et al. (2008)
Syzygium aromaticum	Clove	Eugenol	*E. coli, S. aureus, L. monocytogenes, S. enteritidis, C. Jejuni*	Ouattara et al. (1997), Smith-Palmer et al. (1998)
		Eugenol acetate, eugenol and caryo-phyllene	*Pseudomonas aeruginosa*	Saeed and Tariq (2008)
			Bacillus subtilis, B. megaterium, B. Sphaericus, B. polymyxa, S. aureus, Escherichia coli	Pundir et al. (2010)
Thymus vulgaris	Thyme	Thymol, carvacrol	*Salmonella typhimurium, S. aureus, Aspergillus flavus*	Juven et al. (1994), Ouattara et al. (1997), Mahmoud (1994)
		Thymol, carvacrol, linalool, eugenol	*Salmonella typhimurium, E. coli*	Rota et al. (2008), Ayachi et al. (2009)
Zingiber officinale	Ginger	Zingiberene, zingerone	Gram-positive and gram-negative bacteria	Chao and Young (2000)
		Zingiberene, β-sesquiphellandrene, bisabolene , farnesene	*Coliform bacillus, Straphylococcus epidermidis, Streptococcus viridians*	Malu et al. (2009)

soy) and minerals. The use of dietary fibre (both soluble and insoluble fibre) and oligosaccharides as prebiotics and their role in the modulation of gut microbiotica (promotion of bifidobacteria development thereby inhibiting pathogenic bacteria such as *Clostridium perfringens* and *Escherichia coli*, faecal bulking and production of SCFA) is widely recognised (Gibson and Roberfroid 1995).

Moringa (*Moringa oliefera*) seed protein (MSP) has bactericidal activity, e.g. reduction of viability Gram-positive and Gram-negative bacteria, pathogenic to humans (Suarez et al. 2005), while viability of *E. coli* was inhibited by four orders of magnitude (Suarez et al. 2003). Some of the compounds that have been isolated from moringa seeds are 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate (3%), 4-(4′-O-acetyl-α-L-rhamnopyranosyloxy) benzyl isothiocyanate, 4-(α-L-hamnopyranosyloxy) benzyl isothiocyanate (10%), niazimicin, and pterygospermin (see review, Makkar et al. 2007). *Helicobacter pylori*, a major cause of gastric and duodenal ulcers, and is a major risk factor for gastric cancer, is found to be highly susceptible to 4-(a-L-rhamnopyranosyloxy) benzyl isothiocyanate and various other isothiocyanates (Haristoy et al. 2005).

13.7 Conclusion

Advances in understanding the regulation of nutrient use in agricultural animals have led to the development of technologies referred to as *metabolic modifiers* that have the overall effect of improving productive efficiency (weight gain or milk yield per feed unit), improving carcass composition (lean: fat ratio) in growing animals, increasing milk yield in lactating animals, and decreasing animal waste per production unit. Several researchers are able to investigate the effect of phytocompounds on parameters of rumen metabolism, but would need to extend their expertise and facilities for studying post-ruminal effects, analysing different matrices for the presence of phytocompounds or metabolites thereof in order to explore the fate of phytocompounds. These would then be integrated into products to enhance the performance of animals to provide quality animal food products for the consumer.

Better understanding of the colonic microbial ecosystem will help to explain and predict the effects of dietary additives, including non-digestible carbohydrates and phytochemicals or their metabolites. Genomic analyses of the gut microbiota could revolutionize our understanding of these mechanisms and provide new biotechnological tools to unravel possible interrelationship amongst nutritive and non-nutritive (phytochemicals) substrates, host and host microbial ecology which can be modulated for the benefit of the host or the primary consumer, the human being.

Exploitation of the bioactive properties of plant secondary metabolites (PSM) by animals can provide a “treatment” against various challenges that perturb homeostasis in animals considering the fact that the probability of PSM exploitation is determined by the relative difference between the cost of a homeostatic challenge and the toxicity of the PSM. Animals can exploit the biological activity of PSM to

mitigate the costs of infection by parasites, enhance reproduction, moderate thermoregulation, avoid predation, and increase alertness. Thus, a better understanding of animal behaviour and considering a broader view of avoidance or selection of PSM relative to the homeostatic state can be exploited to advance practices of animal management which may lead to discovery of new drugs.

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