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

M. Simões (✉) • M. Lemos
LEPAE, Department of Chemical Engineering, Faculty of Engineering,
University of Porto, Rua Dr. Roberto Frias, s/n, 4200-465 Porto, Portugal
e-mail: mvs@fe.up.pt

L.C. Simões
IBB-Institute for Biotechnology and Bioengineering, Centre
of Biological Engineering, University of Minho,
Campus de Gualtar, 4710-057 Braga, Portugal

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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

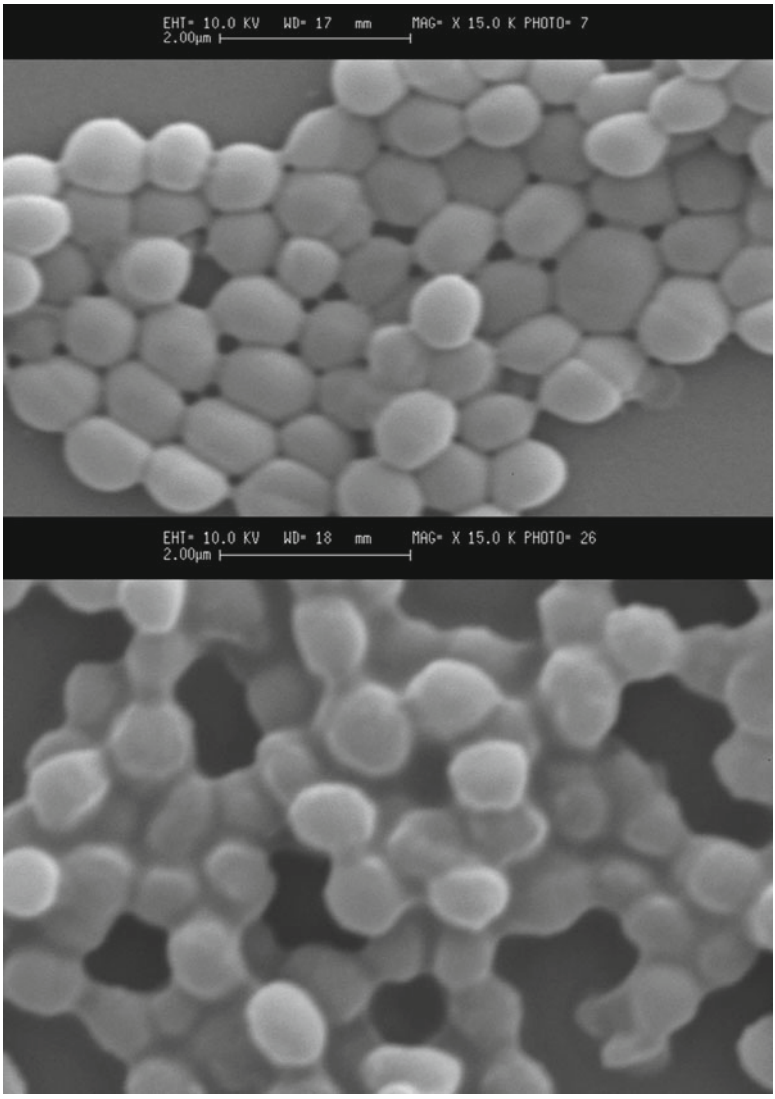

Fig. 6.1 Scanning electron microscopy photomicrographs of planktonic (**a**) and biofilm (**b**) cells of *Staphylococcus* spp. evidencing the presence of an extracellular polymeric matrix in biofilms (×15,000 magnification; bar = 2 μm)

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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