Branislav Ranković *Editor*

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Bioactive Properties and Pharmaceutical Potential

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ISBN 978-3-319-13373-7 ISBN 978-3-319-13374-4 (eBook)
DOI 10.1007/978-3-319-13374-4
Springer Cham Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014959829

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

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Chapter 1
Lichens as a Potential Source of Bioactive Secondary Metabolites

Branislav Ranković and Marijana Kosanić

Abstract Lichens are complex symbiotic associations between fungi and algae which are important constituents of many ecosystems. The production of various unique extracellular secondary metabolites known as lichen substances is the result of this symbiosis. These compounds exist within the thalli and typically form crystals on the surface of the fungal hyphae. Thus far, more than 800 secondary metabolites of lichens have been discovered, most of them being exclusively present in lichens. In recent date, lichens have been taken up for many researches concerning the phytochemical and pharmaceutical applications. Lichens and their secondary metabolites have many pharmaceutical roles, primarily including antimicrobial, antioxidant, antiviral, anticancer, antigenotoxic, anti-inflammatory, analgesic and antipyretic activities. Hence, the present study was undertaken to explain the lichens as the important potential sources of bioactive secondary metabolites.

1.1 The Lichens: Lichenised Fungi

Lichens are association between fungi (**mycobionts**) and photoautotrophic, algal partners (**photobionts**). Since the mycobiont is unique in the symbiotic association and usually dominates the association, lichens are traditionally classified as a life-form of fungi. About 18,500 different lichen species have been described all over the world. The fungal partners are mostly (98 %) *Ascomycota* (Honegger 1991; Gilbert 2000) and the others belong to the *Basidiomycota* and anamorphic fungi. Approximately 21 % of all fungi are able to act as a mycobiont (Honegger 1991); thus, lichens form the largest mutualistic group among fungi. Only 40 genera are involved as photosynthetic partners in lichen formation: 25 algae and 15 cyanobacteria (Kirk et al. 2008).

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B. Ranković (ed.), *Lichen Secondary Metabolites*,
DOI 10.1007/978-3-319-13374-4_1

The most common photobionts are the genera *Trebouxia*, *Trentepohlia* and *Nostoc*. The genera *Trebouxia* and *Trentepohlia* are of eucaryotic structure and belong to the green algae, while the genus *Nostoc* belongs to the prokaryotic cyanobacteria. Eucaryotic photobionts are sometimes called **phycobionts** (90 % of lichens), while cyanobacterial photobionts are termed **cyanobionts** (10 % of lichens).

In lichen associations, both partners have benefit. The mycobiont has two principal roles in the lichen symbiosis: to protect the photobiont from exposure to intense sunlight and desiccation and to absorb mineral nutrients from the underlying surface or from minute traces of atmospheric contaminants. The photobiont also has two roles: to synthesise organic nutrients from carbon dioxide and, in the case of cyanobacteria, to produce ammonium (and then organic nitrogen compounds) from N_2 gas, by nitrogen fixation. In some ecosystems such as desert soils, tundra heaths and Douglas-fir forests of the Pacific Northwest of the United States, lichens can provide the major input of nitrogen which supports other forms of life (Hale 1983; Nash 1996).

Thus, through the lichen partnership, the photobionts are protected and able to grow in conditions in which they could not grow alone; they also benefit from the highly efficient uptake of mineral nutrients by the lichen fungi. The fungi, in turn, obtain sugars and in some cases organic nitrogen from the photosynthetic partner, enabling them to grow in environments deficient in organic nutrients (Hale 1983; Nash 1996).

A lichen thallus usually consists of layers such as an upper and lower cortex, algal layer and medulla (Fig. 1.1). The layers differ in thickness and are better developed in some species than in others. Fungal hyphae make up most of the thallus; photobionts are cells of only a small percentage (about 7 %) of the total volume (Ahmadijan 1993). There are three main types of thalli: crustose, foliose and fruticose (Fig. 1.2). A crustose thallus lacks a lower cortex and is generally considered to be the most primitive type. Thalli of the *Lepraria* species do not have layers but consist only of powdery granules. Many crustose lichens stick tightly to the substratum and appear to be painted on it. Squamules are a specialised type of crustose thallus and are attached at only one and to the substratum. A foliose thallus

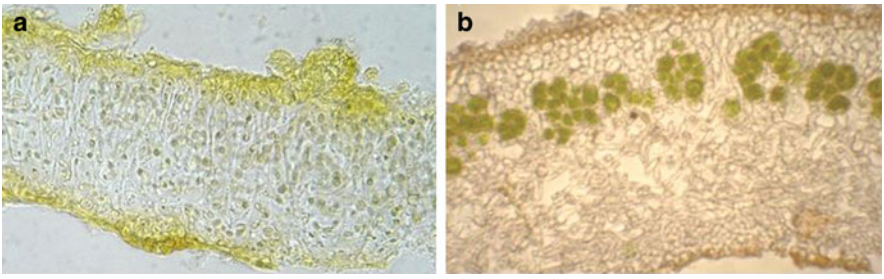

Fig. 1.1 A vertical section of lichen thallus showing anatomical features. (**a**) Homoiomerous thallus; (**b**) heteromerous thallus

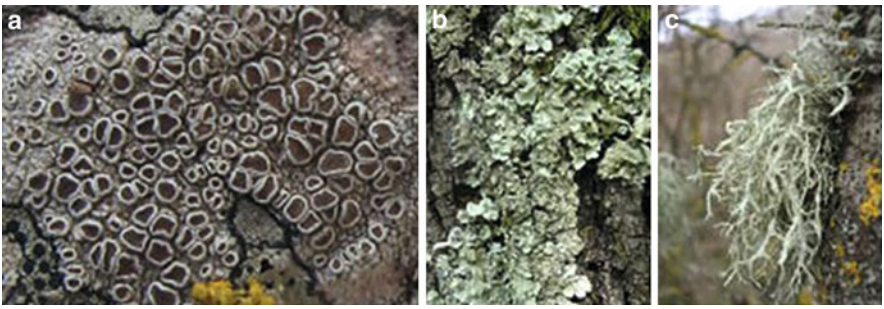

Fig. 1.2 Main morphological groups of lichen thallus. (**a**) Crustose; (**b**) foliose; (**c**) fruticose

has an upper and lower cortex, an algal layer and medulla and is usually loosely attached to the substrate by hair-like structures called rhizines. Some foliose lichens have thalli that are attached to the substrate by only one central point. Fruticose thalli are upright or hanging, round or flat and often highly branched. The layers of a fruticose thallus may surround a central thick cord, as in *Usnea*, or a hollow space as in some *Cladonia* species (Ahmadijan 1993; Nash 1996).

Some lichens, such as *Collema*, which have *Nostoc* as a photobiont, do not form a well-organised thallus (Fig. 1.1). In these cases, fungal hyphae grow inside the thick gelatinous sheaths of the photobiont, which make up much of the thallus (Paracer and Ahmadijan 2000).

In nature, lichens grow very slowly. Their radial growth is measured in millimetres per year. Hale (1973) made the following generalisation about growth rates of lichens: most foliose species grow 0.5–4 mm/year, fruticose species 1.5–5 mm/year and crustose species 0.5–2 mm/year.

Lichen grow practically everywhere—on and within rocks, on soil and tree bark, on almost any inanimate object. They grow in deserts and in tropical rainforests, where they occur on living leaves of plants and ferns. They have been found on the shells of tortoises in the Galapagos Islands and on large weevils in New Guinea. In the dry valleys of Antarctica, endolithic lichens, such as *Buellia* and *Lecidea*, grow inside sandstone crevices. *Dermatocarpon fluviatile* and *Hydrothyria venosa* grow in freshwater streams, and species of *Verrucaria* are common in the intertidal zones of rocky, ocean shores. *Verrucaria serpuloides* is a permanently submerged marine lichen that grows on stones and rocks 4–10 m below mean low tide off the coast of the Antarctic Peninsula. *Verrucaria tavaresiae* is another unusual marine lichen with brown algal photobiont. Douglas Larson has estimated that about 8 % of the earth's terrestrial surface is dominated by lichens. Lichens abound in areas with high annual humidity, such as the fog belt zones of Chile and Baja California. Extensive lichen populations also grow in the cool, northern forests of the world, where hundreds of miles of forest floor are covered with thick carpets of reindeer lichens (*Cladonia*). Trees along the coasts of the northwestern United States may be blanketed with beard lichens such as *Alectoria* and *Usnea* (Hale 1973; Moe 1997; Cox 2003).

Lichens with organised thalli do not grow well in areas that are continuously wet, such as tropical rainforests. Only poorly organised species of *Lepraria* and leaf-inhabiting lichens are found in these regions. *Lecanora conizaeoides* and *Lecanora dispersa* colonise trees and gravestones in industrial cities and towns, but most lichens cannot tolerate the polluted atmosphere and persistent dryness of urban areas. The sensitivity of lichens to atmospheric pollutants such as sulphur dioxide, ozone and fluorides has made them valuable indicators of pollution in cities and industrial regions (Richardson 1992).

Interactions between the symbiotic partners allow lichens to live in unusual environments (Bačkor and Fahselt 2008). Lichens are able to survive in extreme ecological conditions; they can adapt to extreme temperatures, drought, inundation, salinity, high concentrations of air pollutants and nutrient-poor, highly nitrified environments (Nash 2008). Despite this extreme range of ecological adaptations, most lichens are sensitive to changes of their preferred ecological conditions and can hardly grow in non-native habitats.

1.2 Lichen Metabolites

Specific conditions in which lichens live are the reason of production of many metabolites that they provide good protection against various negative physical and biological influences. Metabolites synthesised by lichens were divided into two groups: primary and secondary (Lawrey 1986).

The primary (intracellular) metabolites include proteins, amino acids, carotenoids, polysaccharides and vitamins. They are generally soluble in water and can be easily isolated from the lichens by boiling water. Some of the primary metabolites are produced by fungi and some by algae. Most of these metabolites are non-specific and also may occur in free-living fungi, algae and higher plants. Presence of free amino acids is similar to their presence in the plants. In general, the amount of nitrogen compounds is between 1.6 and 11.4 % dry weight of the lichen thallus (Hale 1983). Carotenoids are metabolic products of both symbionts and are in the range 1.5–24 mg/g dry weight of the thallus. Among the carotenoids in the lichens, thalli were identified in β-carotene epoxide, α-cryptoxanthin, lutein, astaxanthin, mutatoxanthin. Polysaccharides and related compounds are present in the lichen in an amount of 3–5 % dry weight of the thallus (Culberson 1970). Among vitamins, lichen contains ascorbic acid, biotin, α-tocopherol, nicotinic acid, pantothenic acid, riboflavin, thiamine and folic acid. Vitamins were identified as metabolic products which biosynthesise alga, while the mushrooms are poor sources of these compounds (Hale 1974).

The majority of organic compounds found in lichens are secondary metabolites. Lichens may contain substantial amounts of secondary metabolites, usually between 0.1 and 10 % of the dry weight, but sometimes up to 30 % (Galun and Shomer-Ilan 1988; Stocker-Wörgötter 2008; Solhaug et al. 2009). More than 800 secondary metabolites are known from lichens, most are unique to these

organisms and only a small minority occurs in the other fungi or higher plants. All of the secondary substances in lichens are of fungal origin. These substances are the crystals deposited on the surface of the hyphae, which are poorly soluble in water, and usually can be isolated from the lichens by organic solvents (Bačkorová et al. 2012).

Once formed, the lichen secondary metabolites appear to be extremely stable. Very old herbarium specimens of lichens show no significant reduction in concentrations of lichen substances (Rundel 1978). In addition, the production of secondary compounds is genetically controlled (Culberson and Culberson 2001) and, in some instances, is correlated with the morphology and geography in individuals at the species and genus levels (Egan 1986; Zhou et al. 2006).

Histologically, lichen secondary metabolites are deposited in either the cortex or, more commonly, the medulla. The most usual cortical compounds are usnic acid and atranorin, but anthraquinones, pulvinic acid derivates and xanthones may also occur here. All of these, with the exception of atranorin, are typically pigmented. The great majority of lichen substances are deposited in the medulla. Lichen substances are often expressed differentially in the layers of lichen thallus, and typical cortical substances can be distinguished from compounds usually found only in the medulla. This could be linked with their biological function: the cortical compounds are regarded as a kind of light filter, which is apparently not a function of compounds below the algal layers (Marques 2013).

Lichens synthesise significant amounts of substances only in permissive physiological stages. As a consequence, the production in axenic cultures can differ substantially from that in nature. Mycobionts grown without their photobionts synthesise specific secondary lichen compounds under certain conditions (Culberson and Armaleo 1992; Mattsson 1994; Stocker-Wörgötter and Elix 2002; Fazio et al. 2007; Hager et al. 2008) but can also produce substances that are different from the metabolites found in symbiosis (Yoshimura et al. 1994; Brunauer et al. 2007). For example, natural lichen *Lecanora dispersa* contains 2,7-dichlorolichexanthone as the major secondary compound, but cultured spore isolates, growing without the alga, produced pannarin and related depsidones instead (Leuckert et al. 1990). Pannarin could not be confirmed of the natural source lichen, but the biosynthetic potential for this depsidone was proven for the species by a herbarium survey. Another similar result is the production of atranorin in *Usnea hirta*, when cultured in a modified LB medium (Kinoshita 1993), since this compound is not present throughout the entire genus *Usnea*.

Each lichen mycobiont prefers specially adapted culture conditions (such as nutrient medium, added sugars or polyols, pH, temperature, light, stress) to produce the specific secondary metabolites (Hager et al. 2008). Similarly, lichen "tissue" cultures, in many cases, can produce secondary substances (Yamamoto et al. 1985, 1993), but the chemistry is usually different from the chemosyndrome of the corresponding natural lichen thalli (Yamamoto et al. 1993). Lichenised *Basidiomycotina* do not contain lichen substances (Lumbsch 1998).

The distribution patterns of secondary metabolites are usually taxon specific and, therefore, have been widely used in lichen taxonomy and systematics (Nordin

et al. 2007; Fehrer et al. 2008; Nelsen and Gargas 2008). However, it has been shown that the similarities in secondary chemistry may not necessarily indicate close phylogenetic relationships (Nelsen and Gargas 2008). For example, *Xanthoparmelia* and *Neofuscelia*, two closely related genera of the *Parmeliaceae* family, differ primarily in the pigments of their upper cortex: while *Xanthoparmelia* contains crystallised usnic acid, the closely related genus *Neofuscelia* is characterised by melanin-like cortical pigments. Moreover, other members of the *Parmeliaceae* family may exclusively contain atranorin in the cortex.

Secondary metabolites are not absolutely essential for the survival and growth of lichens (Bentley 1999), and the functions of these compounds in the lichen symbioses are still poorly understood (Hager et al. 2008). However, it is important that they may help to protect the thalli against herbivores, pathogens, competitors and external abiotic factors, such as high UV irradiation. Also, lichens have impact interactions with their environment.

Production of secondary metabolites in lichens is complex and variously influenced by environmental factors, including light, UV exposure, elevation, temperature fluctuations and seasonality.

Although numerous studies have attempted to correlate the variation of metabolite concentrations in lichens under different light levels, the results have often been contradictory. Armaleo et al. (2008) reported a positive correlation between the amount of light available to *Parmotrema hypotropum* annually and the concentration of atranorin in the thallus; however, there was a decrease in norstictic acid concentration under the same conditions. In contrast, Stephenson and Rundel (1979) did not find any correlations between atranorin levels and light intensity for *Letharia vulpina*. Bjerke and Dahl (2002) reported a positive correlation between thallus concentration of usnic acid and light intensity in *Ramalina siliquosa* under culture conditions, but there was no correlation between light intensity and salazinic acid concentration in the same species. Reasons for conflicting patterns in the production of lichen substances in response to varying light conditions in lichens remain unclear. Bjerke et al. (2004) and Bjerke et al. (2005) have suggested that these conflicting results may be due, at least in part, to the interactive effects of multiple variables across differing habitat conditions.

Understanding how lichen metabolite concentrations vary in response to UV exposure may provide important insights into how lichens thrive in these harsh environments. One of the primary functions of lichen secondary compounds is to protect the algal layer from intense light levels, especially in the ultraviolet range (Waring 2008). For example, lichen-derived phenolic compounds including depsides, depsidones, usnic acid and pulvinic acid derivates are effective UV-absorbing compounds. Extensive field experiments have assessed the effects of UV on the concentration of lichen phenolics with pattern of increasing concentrations under high UV-A conditions (BeGora and Fahselt 2001). On the other hand, UV-B radiation appears to have a negative effect on lichen metabolite concentrations (BeGora and Fahselt 2001). Increased levels of lichen metabolites under high UV-A conditions may be due to greater production of photosynthates which in turn

support increased production of lichen compounds (BeGora and Fahselt 2001), while lower production of lichen compounds in the presence of UV-B may be due to the production of extracellular degradative enzymes or electronic transitions within molecules caused by exposure to UV-B (Fessenden and Fessenden 1986).

Various studies have shown that differences in geographic location and changing seasons can influence the concentration of secondary metabolites in some lichen species. Bjerke et al. (2004) showed variation in the usnic acid content of *Flavocetraria nivalis* thalli collected from 25 sites in northwestern Spitsbergen, Norway. In addition to the influence of geographic location, the phenolic content of lichens has also been reported to vary by season. For example, BeGora and Fahselt (2001) reported the lowest levels of usnic acid for *Cladonia mitis* during the spring and summer with the highest concentrations occurring in the late winter. The lower levels of usnic acid reported for summer months may be due to drought and heat stress which have a depressing effect on metabolic activity (Bjerke et al. 2005). Another reason could be a function of higher levels of UV-A or the ratio of UV-B to UV-A during the summer. However, Bjerke et al. (2005) found increased levels of usnic acid in *Flavocetraria nivalis* in the late spring and early summer and generally lower levels during the autumn and winter which they believe may be due to higher levels of precipitation during the spring and summer months.

Studies have also demonstrated that the production and/or content of secondary metabolites in lichens varied with changes in elevation. For example, it has been reported for *Umbilicaria americana* that lichen secondary metabolite production decreases with increasing elevation (Swanson et al. 1996). In contrast, Rubio et al. (2002) found increasing levels of rhizocarpic acid in *Acarospora schleicheri* with increasing elevation. The direct effects of elevation on the production of phenolic compounds by lichens may in fact be complicated by the dynamic interaction of various environmental factors.

Production of lichen compound may fluctuate with temperature changes. For example, the concentration of salazinic acid in *Ramalina siliquosa* was positively correlated with mean annual temperature (Hamada 1982). Under seminatural conditions, the amount of gyrophoric acid and methyl gyrophorate was reported to increase by an average of 6.7–22.3 times, respectively, in *Peltigera didactyla* with an increase in temperature of 3 °C (Bjerke et al. 2003). Hamada (1991) observed a steady increase in the concentration of usnic acid and 4-*O*-dimethylbarbatic acid in mycobiont cultures from *Ramalina siliquosa* when the temperature of the culture was increased to 12 °C for usnic acid and 15 °C for 4-*O*-dimethylbarbatic acid, with higher temperatures resulting in a decrease in the content of both components. In contrast, the content of 4-*O*-dimethylbarbatic acid in intact lichen increased with temperature up to 19 °C (Hamada 1984), suggesting that the optimal temperature for the production of lichen substances is generally lower in the isolated mycobiont when compared with intact lichen. Increased concentrations in lichen phenolics may be due to increased photosynthetic activity related to increased temperature. Culberson et al. (1983) found the concentrations of some phenols in *Cladonia cristatella*, in a controlled phytotron experiment, were higher at lower temperature. Tundra lichens are typically metabolically active at near-zero

or subzero temperature. Similarly, Bjerke et al. (2004) found that in *Flavocetraria nivalis*, the concentration of usnic acid was highest at the sites with the lowest temperatures. Based on the above-mentioned, it can be concluded that the variation in temperature shows important influence on the levels of secondary metabolites in lichens.

1.3 Secondary Metabolite Production in Lichens: The Important Pathways

Lichen secondary metabolites are derived from three chemical pathways: **acetate–polymalonate pathway, shikimic acid pathway and mevalonic acid pathway** (Fig. 1.3).

Acetate–polymalonate pathway includes the most common lichen compounds such as

- Secondary aliphatic acids, esters and related derivates
- Mononuclear phenolic compounds
- Depsides, tridepsides and benzyl esters
- Depsidones and diphenyl esters
- Depsones
- Dibenzofurans, usnic acids and derivates
- Anthraquinones and biogenetically related xanthones
- Chromones
- Naphthoquinones
- Xanthones

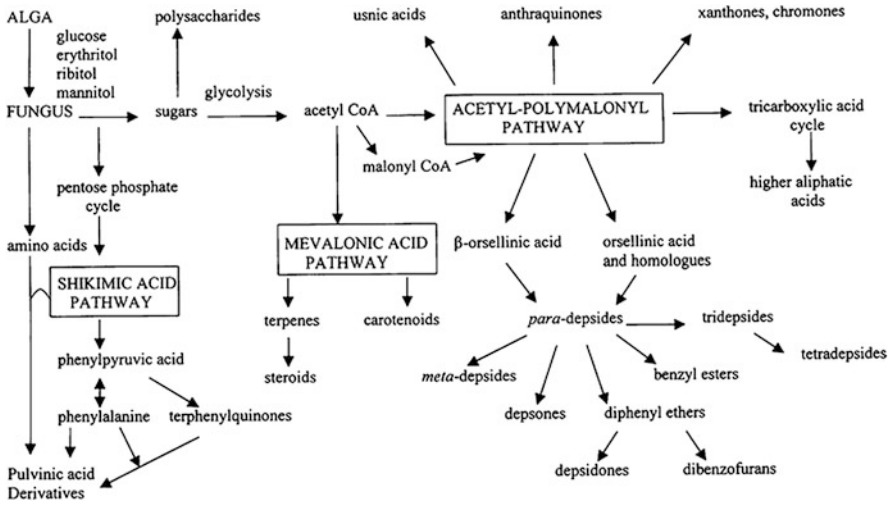

Fig. 1.3 Biosynthetic pathways of lichen secondary metabolites

Mevalonic acid pathway includes

- Di-, sester- and triterpenes
- Steroids

Shikimic acid pathway includes

- Terphenylquinones
- Pulvinic acid derivates

Apart from compounds derived from these common pathways, which are found throughout all major lichen groups, there are also some unusual compound classes among these organisms, for example, arthogalin, a cyclic depsipeptide (Huneck and Himmelreich 1995), and other amino acid-derived compounds such as the cytotoxic scabrosin esters isolated from *Xanthoparmelia scabrosa* (Ernst-Russell et al. 1999). Uncommon features are also detected in residues of common substance classes, in the form of other intramolecular arrangements, or in the binding with other compounds such as sugars. Recently, Řezanka and Guschina (1999) described many unusual compounds as brominated depsidones, brominated acetylenic fatty acids (Řezanka and Dembitsky 1999) and monotetrahydrofuranic acetogenin derivates (Řezanka et al. 2004). A series of g-lactonic aliphatic acid glycosides were also identified (Řezanka and Guschina 2000, 2001a, b), some of them forming a macrolactone ring (gobienines: Řezanka and Guschina 2001c). Glycosides are not frequently encountered in lichens, yet recent reports revealed the presence of xanthone glucosides (umbilicaxanthosides: Řezanka et al. 2003) and the mycosporine collemin A from *Collema cristatum* (Torres et al. 2004).

1.3.1 Lichen Secondary Metabolites of the Acetate–Polymalonate Pathway

Acetate–polymalonate pathway utilises acetyl-CoA and malonyl-CoA which are derivates of coenzyme A. Of the lichen secondary metabolites, which are formed in this pathway, aromatic products are especially well represented, the most characteristic being formed by the bonding of two or three orcinol or β-orcinol-type phenolic units through ester, ether and carbon–carbon linkages. The large majority of depsides, depsidones, dibenzofurans, usnic acid and depsones all appear to be produced by such mechanisms and all are peculiar to lichens. Other aromatic compounds such as the chromones, xanthones and anthraquinones are probably formed by internal cyclisation of a single, folded polyketide chain and are often identical or analogous to products of non-lichen-forming fungi or higher plants. In addition to the compounds of known chemical structure, many of the unknown structure are given common names and assigned to compound class, because they are frequently encountered and easily recognised by microchemical methods.

1.3.1.1 Aromatic Products Arising from Intermolecular Esterification or Oxidative Coupling

The most common phenolic acid units derived by the acetate–polymalonate pathway and combined to form the characteristic lichen substances are of two types: the orcinol-type units and β-orcinol-type units. While compounds formed from these two types of units are similar in many ways, differences in their structure and especially in their distribution among the lichens suggest that the usual tendency to consider the orcinol and β-orcinol compounds separately probably has a biosynthetic justification.

The orcinol series The most common fate of acetate–polymalonate-derived phenolic acids in lichens is intermolecular esterification of two or three similar or identical units. For example, the carboxylic acid of one unit is joined to the hydroxyl *para* to the carboxylic acid of the second unit. Such esterifications lead to the *para*-depsides. A second esterification reaction leads to tridepsides. If an ester linkage joins the first unit to a position *meta* to the carboxylic acid of the second ring, a *meta*-depside results.

Known compounds of *para*-depsides are anziaic acid, confluentic acid, diploschistesic acid, erythrin, evernic acid, glomelliferic acid, hiascic acid, imbricaric acid, tumidulin, microphyllinic acid, obtusatic acid, olivetoric acid, perlatolic acid, planaic acid, sphaerophorin, divaricatic acid and lecanoric acid. Known *meta*-depsides are boninic acid, cryptochlorophaeic acid, sekikaic acid, homosekikaic acid, merochlorophaeic acid, novochlorophaeic acid, paludosic acid, ramalinolic acid, scrobiculin and thamnolic acid. Tridepsides include tenuiorin, umbilicaric acid and gyrophoric acid.

The most known orcinol-type depsidones have an *a*- or a β-keto group in the side chain of the first ring. It is well known that this functional group has a strong effect upon the ester linkage between the two rings, since enol lactones form readily. Oxidative cyclisation of depsides to depsidones usually joins the 2-hydroxyl of ring A and the 5-position of ring B.

The best known depsidones are physodic acid, lobaric acid, norlobaridone, grayanic acid, alectoronic acid, diploicin, 4-*O*-methylphysodic acid, α-collatolic acid, lividic acid and variolaric acid.

Depsone (picrolichenic acid) has a methylated 2-hydroxyl, and carbon–carbon coupling links the 1-position of the A ring and the 5-position of the B ring.

β-orcinol series The orcinol-type compounds discussed in the previous section form a closely related series of substances in which changes in the length and oxidation state of the 6-alkyl substituents are major sources of variation among phenolic units. The compounds synthesised by various combinations of these units show secondary modifications attributable to O-methylation, chlorination, decarboxylation and lactonisation. The β-orcinol compounds may undergo all of the same secondary reactions, but the most common variation is in the oxidation state of the C_1 substituents at the 3- and 6-positions of the phenolic acid units.

Of the 16 units theoretically derivable by changing the oxidation states of the C_1 substituents, only eight are represented among the known compounds. Six *para*- and three *meta*-depsides are derived from only three β-orcinol units, all of which have the original fully reduced methyl group at the 6-position and either a methyl, an aldehydic, or more rarely a carboxylic acid group at the 3-position. All the *para*-depsides have the same B ring substituted with methyls at both the 3- and the 6-positions. The C_1 substituents of the B rings of *meta*-depsides and depsidones are usually more highly oxidised than those of *para*-depsides.

The best known *para*-depsides are atranorin, baeomycesic acid, barbatic acid, chloroatranorin, diffractaic acid, squamatic acid and 4-*O*-demethylbarbatic acid, while *meta*-depsides include decarboxythamnolic acid, haemathamnolic acid, hypothamnolic acid and thamnolic acid.

Except in gangaleoidin, the A ring of all β-orcinol depsidones has either a 3-aldehydic substituent like the A ring of the *para*-depside atranorin or a 3-methyl substituent like the B ring of atranorin. The B rings of depsidones are much more variable, however, and some show oxidation of the 6-methyl substituent, a condition never observed among the phenolic acid units of the known *para*- and *meta*-depsides. Known compounds of depsidones are fumarprotocetraric acid, galbinic acid, gangaleoidin, hypoprotocetraric acid, myriocarpic acid, norstictic acid, pannarin, physodalic acid, protocetraric acid, psoromic acid, salazinic acid, stictic acid, vicanicin and virensic acid.

One benzyl ester, barbatolic acid, is known in three usneaceous genera producing a number of the usual β-orcinol esters previously described. The A ring of barbatolic acid is like the A ring of atranorin, but the B ring is substituted at the 6-position with the hydroxymethyl group involved in the ester linkage.

Dibenzofurans The phenolic units involved in the production of the true lichen dibenzofurans are derived by the orsellinic acid-type cyclisation. The dibenzofurans appear to form by carbon–carbon coupling and cyclodehydration of two such acetate–polymalonate-derived phenolic acid units. Two types of lichen dibenzofurans can be distinguished by the orientation of the aromatic rings. The first type is presently known only in *Cladoniaceae*; the second type is known in the *Lecideaceae*, *Lecanoraceae* and *Roccellaceae* and the genus *Lepraria*. In all cases, the 5- and 4-positions of one phenolic unit, which becomes ring C in the dibenzofuran structure, are involved in the furan ring. The lichen dibenzofurans are dendroidin, didymic acid, pannaric acid, porphyrilic acid, schizopeltic acid and strepsilin.

Usnic acid The best known lichen products are the usnic acid, yellow pigments produced in the upper cortex of many species from phylogenetically widely separated families. These pigments are known only in lichens. The usnic acids appear, therefore, to be among the compounds which are most characteristic of lichens, are apparently not synthesised in cultures of the isolated fungal components and are formed by reactions uniting two or three phenolic units.

It was first isolated by German scientist W. Knop in 1844 and first synthesised between 1933 and 1937 by Curd and Robertson. Usnic acid was identified in many genera of lichens including *Usnea*, *Cladonia*, *Lecanora*, *Ramalina*, *Evernia*, *Parmelia* and *Alectoria*.

Usnic acid possesses a wide range of interesting biological properties. It is a potent antibiotic effective against Gram-positive bacteria, including *Staphylococcus*, *Streptococcus* and *Pneumococcus*, other bacteria such as *Mycobacterium tuberculosis* and some pathogenic fungi. It also exhibits antiviral, antiprotozoal, antimitotic, anti-inflammatory and analgesic activity. Other characteristics, like ultraviolet absorption, preserving properties, antigrowth, antiherbivore and anti-insect properties, have also been demonstrated.

Cyclisation to the aromatic ring of the methylphloroacetophenone precursor of the usnic acids is basically different from the orsellinic acid cyclisations leading to precursors of dibenzofuran derivates, but the oxidative coupling and cyclodehydration reactions of pairs of units are similar in the two cases. With the usnic acids, a C-methyl substituent prevents rearomatisation of one ring. The structurally isomeric usnic acids are related by the orientation of the A ring about the carbon–carbon bond. In the two types of dibenzofuran derivates, the second unit also remains fixed but the A ring is reoriented with respect to the ether linkage.

The orientation of the second phenolic unit in the usnic acids resembles that of all the known dibenzofuran derivates in that the position involved in the carbon–carbon coupling is *meta* to a carbonyl substituent and *ortho* and *para* to O-substituted positions. It differs from the dibenzofurans in having a second ortho-hydroxyl substituent instead of an alkyl group. Dibenzofuran derivates could form from the methylphloroacetophenone precursor of usnic acid by a change in the orientation of the C ring with respect to the ether linkage, and such an orientation would still allow a *para*-quinoid radical.

Until recently, it was thought that two usnic acid pigments were produced in lichens, (+)-usnic acid and its optical isomer. Now structural isomers have also been found in which the carbon–carbon bond remains the same, but the ether linkage forms by dehydrative cyclisation at a different hydroxyl group of the A ring. Both optical isomers of these isousnic acids have been found. Since the structures of usnic acid and isousnic acid are very similar, it is probable that the pairs of compounds rotating polarised light in the same direction also have the same configuration about the asymmetric carbon.

The isousnic acids are known only in *Cladonia*, but extensive surveys for the distribution of these compounds have not yet been reported. In *Cladonia*, both (+)- and (−)-usnic acids occur, but in this genus as in others, both optical isomers are never found in a single plant. Some genera produce only one optical isomer. It is probable that the methylphloroacetophenone unit of usnic acid is a common aromatic lichen product for which mechanisms of coupling and cyclodehydration have arisen evolutionarily many times.

Diquinone Only one lichen compound, pyxiferin, has been identified as a diquinone. If pyxiferin were formed by oxidative coupling of acetate–polymalonate-derived units, the first ring would arise by decarboxylation,

hydroxylation and oxidation of orsellinic acids. The second ring would also require oxidative removal of the C_1 substituent. The tentative structure of this compound requires a considerable biosynthetic departure from pathways leading to most of the orsellinic acid derivates in lichens.

1.3.1.2 Aromatic Products Derived from a Single Polyacetyl Chain

Aromatic products derived from a single polyacetyl chain include monoaryl products related to phenolic acids, chromones, xanthones and anthraquinones.

Lichen monoaryl derivates are relatively rare in lichens. All of the lichen monoaryl derivates have lost their free carboxylic acid groups either by esterification or by decarboxylation. These compounds might be synthesised in the lichen from coenzyme A derivates of the initially formed phenolic acid units or by biological decomposition of depsides.

Xanthones are known in free-living fungi, and recent studies indicate that they are rather common in lichens too. Unlike the fungal xanthones, many lichen xanthones have one or more nuclear chlorine substituents. The fundamental structure of the known lichen xanthones could be derived directly by linear condensation of seven acetate and malonate units with one orsellinic acid-type cyclisation. The two rings are joined by a ketonic carbon and by an ether-oxygen arising from cyclodehydration. Xanthones include arthothelin, concretin, 2,4-dichloronorlichexanthone, 2,7-dichloronorlichexanthone, lichexanthone, norlichexanthone, thiophanic acid, thiophaninic acid, thuringione and vinetorin.

The most widespread **anthraquinone** in lichens is parietin (physcion), which is also known in fungi and higher plants. Other known anthraquinones are chiodectonic acid, chrysophanol, 1,3-dihydroxy-8-methoxy-2-chloro-6-methylanthraquinone, emodin, endocrocin, fallacinal, fragilin, 1-hydroxy-3,8-dimethoxy-2-chloro-6-methylanthraquinone, mysaquinone, nephromin, norsolorinic acid, parietin, parietinic acid, rhodocladonic acid, rugulosin, skyrin, solorinic acid, teloschistin, 1,3,8-trihydroxy-2-chloro-6-methylanthraquinone, 1,3,8-trihydroxy-2,4-dichloro-6-methylanthraquinone and xanthorin.

In addition, three **chromones** are known in lichens: siphulin, sordidone and lepraric acid.

1.3.1.3 Aliphatic Products Derived from a Single Polyacetyl Chain: Higher Aliphatic Acids

The fatty acids of lichens show some resemblance to those in non-lichen-forming fungi, but none are identical. Aliphatic acids, much rarer in lichens than in other plant groups, appear to be formed in a very different manner through the tricarboxylic acid cycle. Known higher aliphatic acids and related substances are acaranoic acid, acarenoic acid, (+)-aspicilin, (−)-caperatic acid, (−)-lichesterinic acid, linoleic acid, (−)-nephromopsinic acid, (+)-nephrosteranic acid, (+)-nephrosterinic

acid, (+)-nonrrangiformic acid, oleic acid, (−)-protolichesterinic acid, (+)-protolichesterinic acid, (+)-*allo*-protolichesterinic acid, (+)-pseudononrrangiformic acid, (+)-rangiformic acid, (+)-roccellaric acid, (+)-roccellic acid, 9,10,12,13-tetrahydroxyheneicosanoic acid, tetrahydroxytricosanoic acid, ventosic acid and unidentified aliphatic acids.

1.3.2 Secondary Products of the Mevalonic Acid Pathway: Terpenes, Steroids, Carotenes

All of the acetate-derived aliphatic products described to this point have arisen by head-to-tail condensations leading to unbranched products. Compounds derived by the mevalonic acid pathway involve consideration of an acetyl coenzyme A unit at the methylenic carbon of an acetoacetyl coenzyme A unit to produce a branched precursor, *β*-hydroxy-*β*-methylglutaryl coenzyme A, which is then reduced to mevalonic acid. Compounds derived by the mevalonic acid pathway resemble those formed by the acetate–polymalonate pathway by requiring acetate and coenzyme A, but malonate units formed by carboxylation of acetate units are not involved and the mode of condensation differs. Compounds derived by the mevalonic acid pathway includes **carotene** (*β*-carotene, γ-carotene, violoxanthin, xanthophyll, unspecified carotenoid pigments), **sterols** (ergosterol, fungisterol, *β*-sitosterol, steroid of unknown structure) and **terpenes** which are divided into diterpenes ((−)-16α-hydroxykaurane, nephrin) and triterpenes (7*β*-acetoxy-22-hydroxyhopane, 16*β*-*O*-acetylleucotylic acid, 6α-*O*-acetylleucotylin, 6-deoxy-16*β*-*O*-acetylleucotylin, 6-deoxyleucotylin, 6-deoxy-16*β*-di-*O*-acetylleucotylin, 15α,22-dihydroxyhopane, dolichorrhizin, durvilldiol, durvillonol, eulecanorol, friedelin, *epi*-friedelinol, leucotylic acid, leucotylin, pyxinic acid, retigeranic acid, retigerdiol, taraxerene, triterpene C, triterpene D, triterpene N-1, ursolic acid, zeorin).

1.3.3 Secondary Products of the Shikimic Acid Pathway: Terphenylquinones and Pulvinic Acid Derivates

The **terphenylquinones** and **pulvinic acid** pigments are well-documented examples of secondary lichen products derived by the shikimic acid pathway. The genera of lichens best known for their production of pulvinic acid pigments belong to the *Stictaceae* family in which the algal symbionts are frequently blue-green rather than green. Most families of lichens containing only green algae are best known for producing typical acetate–polymalonate-derived aromatic esters and coupled products.

Lichens with green algae generally show a very low nitrogen content unless they grow on high-nitrogen substrates. Although nitrogen metabolism in lichens is poorly understood, the small number of secondary substances containing nitrogen and the complete absence of alkaloids in lichens suggest that in many species the supply of available nitrogen to the fungus is severely limited. Some lichens show a strong habitat preference for barnyards, rookeries or other places high in organic nitrogen. In contrast to the green algae, many blue-green algae show a much-enhanced nitrogen content compared to that of their substrates.

Not all lichens with blue-green algae contain pulvinic acid derivates, and many lichens with pulvinic acid pigments or terphenylquinones do not contain nitrogen-fixing algae. Although phenylalanine can serve as a precursor to pulvinic acid derivates in the species studied, the compounds other than rhizocarpic acid and epanorin do not contain nitrogen in the molecule. They might arise from non-nitrogenous, prephenic acid derivates or by some mechanisms allowing a small concentration of nitrogen to be constantly recycled.

Only two terphenylquinones, polyporic acid and thelephoric acid, are known, while pulvinic acid derivates are more numerous (calycin, epanorin, leprapinic acid, leprapinic acid methyl ether, pinastric acid, pulvinic acid, pulvinic dilactone, rhizocarpic acid, stictaurin, vulpinic acid).

1.4 Methods for Determining Lichen Secondary Metabolites

The following methods are used for the identification of lichen compounds:

- Classic spot tests
- Microcrystallography
- Paper and thin-layer chromatography
- High-performance liquid chromatography (HPLC)
- Chemical methods
- Gas chromatography and mass spectrometry

Spot test This test has been used universally as rapid, non-specific means for detecting the presence of certain unspecified lichen substances. This test is most convenient and simple to perform, even under field conditions. However, this is only a preliminary step in the process of identification of lichens or its substances. In order to identify accurately the secondary metabolite present in the lichen thallus, one has to perform more sensitive test such as TLC or HPLC. Spot test is carried out by placing a small drop of reagent on the lichen thallus, either directly on the upper surface (cortex) or on the medulla. In the later case, the cortex is scraped or superficially cut with the help of a blade. The reagents used are 10 % aqueous KOH solution (K), saturated aqueous solution of bleaching powder ($NaOCl_2$) or calcium hypochlorite ($Ca(OCl)_2$) (called as C) and 5 % alcoholic

p-phenylenediamine solution (PD). The colour changes at the reagent application point of the thallus are noted as + or −. These colour changes take place due to the presence of particular secondary metabolite in the thallus, which is termed as spotting (Nash 1996; Karunaratne 1999; Karunaratne et al. 2005; Molnár and Farkas 2010; Shukla et al. 2013).

Microcrystallography Some of the secondary metabolites form characteristic crystals when a crystallising reagent is added and gently warmed. The test is conducted on the glass slide. The lichen compound is extracted using acetone. Glycerol, ethanol and glacial acetic acids are some of the chemicals used in different combination to make the reagent. Microcrystallography has been largely superseded by the more sensitive and reliable method such as TLC. But the technique is still useful for a number of lichen compounds which are difficult to identify in TLC due to the same Rf class (or value) or spot colour (e.g. lecanoric acid and gyrophoric acids, barbatic and diffractaic acids). However, mixture of substances may be difficult to identify with this method and also minor substances may be undetectable (Nash 1996; Karunaratne 1999; Karunaratne et al. 2005; Molnár and Farkas 2010; Shukla et al. 2013).

Paper chromatography Paper chromatography is used for the separation of amino acids. Paper is used as the support or adsorbent, but partition probably plays a greater part than adsorption in the separation of the components of mixtures, as the cellulose fibres have a film of moisture around them even in the air-dry state. The technique is therefore closely allied to column partition chromatography, but whereas the latter is capable of dealing with a gram of more material, the former requires micrograms. It is therefore an extremely sensitive technique of enormous value in chemical and biological fields (Nash 1996; Karunaratne 1999; Karunaratne et al. 2005; Molnár and Farkas 2010; Shukla et al. 2013).

Thin-layer chromatography (TLC) It is a relatively simple and inexpensive technique which can be performed by anyone with access to basic laboratory facilities. Lichen substances are extracted in acetone and the extract is spotted on to glass or aluminium plates coated with silica gel. The plate is placed in a sealed tank so that the base of the plate is immersed in a shallow layer of a specific mixture or organic solvents. The different lichen substances present in the sample are separated from each other during the passage of solvent through the silica gel layer and are later made visible by spraying with sulphuric acid. The resulting spots are provisionally identified by their colour and relative position in comparison to the control sample (Nash 1996; Karunaratne 1999; Karunaratne et al. 2005; Molnár and Farkas 2010; Shukla et al. 2013).

High-performance liquid chromatography (HPLC) This method was applied to identify and quantify characteristic substances in commercially available oakmoss products. This technique provides a powerful complement to the established TLC method. The bonded reverse phase columns are used here, and all the aromatic lichen products are suitable for analysis with this method. Samples are dissolved in methanol and injected in to the appropriate portion column, through which an

appropriate solvent or sequence of solvents is passed under high pressure. The substance separates and is detected using UV detector. The retention time (Rt or time of passage) and peak intensity are recorded by a chart recorder. HPLC is also used to measure either absolute or relative concentrations of lichen compounds, because the peak intensity (area under curve) is proportional to the concentration. Most workers use HPLC to detect lichen compounds and combine this technique with TLC and/or mass spectrometry to verify the identification of the peaks (Nash 1996; Karunaratne 1999; Karunaratne et al. 2005; Molnár and Farkas 2010; Shukla et al. 2013).

Chemical methods As with many areas of natural product chemistry, new impetus in the chemistry of lichen substances is provided by the more rapid and improved methods for detecting, isolating and purifying these compounds and in determining their structure. The techniques of preparative TLC, radial chromatography and preparative HPLC provide rapid and efficient methods for the purification of lichen substances and developments in mass spectrometry, proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy and X-ray analysis greatly aid structural studies.

The more classical chemical procedures of degradation and total synthesis also developed apace with the use of newer reagents and synthetic methods. For instance, the use of condensing reagents trifluoroacetic anhydride and dicyclohexylcarbodiimide makes the preparation of lichen depsides a relatively straightforward procedure, so that total synthesis is now a common means of structural confirmation.

Gas chromatography and mass spectrometry Xanthones, anthraquinones, dibenzofurans, terpenes and pulvinic acid derivates which lack thermolabile ester groups can be studied by gas chromatography with mass spectrometry (GCMS). Xanthones in lichens were studied by injecting a lichen extract directly into a mass spectrometer. More recently, the main terpenoid components of the lichens of the family Pyxinaceae have been studied by GMCS (Nash 1996; Karunaratne et al. 2005).

1.5 Application of Lichen Secondary Metabolites in Medicine and Pharmacy

To date, many lichens have proved to be a source of important secondary metabolites for pharmaceutical industries (Huneck 1999; Oksanen 2006) and still hold a considerable interest as alternative treatments in various parts of the world (Richardson 1991). A wide spectrum of biological potential is shown by the lichens, but they have been long neglected by mycologists and overlooked by pharmaceutical industry because of their slow-growing nature and difficulties in their artificial cultivation and have scarcely been studied from a biochemical perspective (Crittenden and Porter 1991; Yamamoto et al. 1998; Behera et al. 2003, 2004).

There is a growing interest in the pharmaceutical properties of compounds derived from lichens. However, relatively few lichen substances have been screened in detail for biological activity and therapeutic potential, due principally to difficulties in obtaining them in quantities and purities sufficient for structural elucidation and pharmacological testing. Additionally, precise lichen determination is essential and requires taxonomic expertise (Boustie and Grube 2005).

Lichen secondary metabolites exhibit antimicrobial, antioxidant, anti-inflammatory, cytotoxic, analgesic, antipyretic and antiviral properties (Table 1.1) and could be potential sources of pharmaceutically useful chemicals. Several examples are described below.

The depsidones, physodic and physodalic acids, prevent the formation of reactive metabolites by blocking the oxidation systems present in the hepatic microsomal fraction (Osawa et al. 1991). The *para*-depside gyrophoric and diffractaic acids also inhibit the proliferation of human keratinocyte cells (Kumar and Muller 1999). Ursolic acid inhibited the growth of human epidermoid carcinoma cells by affecting tyrosine kinase activity (Hollosy et al. 2000). The use of ursolic acid for the manufacture of an anticancer agent for suppressing metastasis has been patented (Ishikawa et al. 1997). Hidalgo et al. (1994) reported the antioxidant activity of some depsides, such as atranorin (isolated from *Placopsis* sp.) and divaricatic acid (isolated from *Protousnea malacea*), and depsidones, such as pannarin (isolated from *Psoroma pallidum*) and 1′-chloropannarin (isolated from *Erioderma chilense*). All of these secondary compounds inhibited rat brain homogenate autoxidation and β-carotene oxidation, and depsidones were found to be the most effective. Russo et al. (2008) found that both sphaerophorin (depside) and pannarin (depsidone) inhibited superoxide anion formation in vitro.

Species of *Usnea* contains high amount of usnic acid, a very active lichen substance used as tumour inhibitor and as analgesic. Atranorin (from *Physcia aipolia*), fumarprotocetraric acid (from *Cladonia furcata*), gyrophoric acid (from *Umbilicaria polyphylla*), lecanoric acid (from *Ochrolechia androgyna*), physodic acid (from *Hypogymnia physodes*), protocetraric acid (from *Flavoparmelia caperata*), stictic acid (from *Xanthoparmelia conspersa*) and usnic acid (from *Flavoparmelia caperata*) showed relatively strong antimicrobial effects against numerous bacteria and fungi, among which were human pathogens (Ranković and Mišić 2008; Ranković et al. 2008). (+)-Usnic acid was found to be a strong hepatotoxic agent against monogastric murine hepatocytes, due to its ability to uncouple and inhibit the electron transport chain in the mitochondria and induce oxidative stress in cells (Han et al. 2004). The (−)-enantiomer of usnic acid (isolated from *Cladonia convoluta*) induced apoptotic cell death in murine lymphocytic leukaemia cells and was moderately cytotoxic to various cancer cell lines, such as murine Lewis lung carcinoma, human chronic myelogenous leukaemia, human brain metastasis of a prostate carcinoma, human breast adenocarcinoma and human glioblastoma (Bézivin et al. 2004). Usnic acid also decreased the proliferation of human breast cancer cells and human lung cancer cells without any DNA damage (Mayer et al. 2005). Finding cancer therapies that do not have DNA-damaging effects and that do not cause the development of secondary malignancies later in life is of great interest. Accordingly, usnic

Table 1.1 Literature sources mentioning data for some lichen substances with different pharmaceutical activity

Lichen compounds	Studied activities	References
Lecanoric acid	Antitumour, antioxidant, antibacterial, antifungal	Gomes et al. (2003), Lopes et al. (2008), Ranković and Mišić (2008), Honda et al. (2010), Bogo et al. (2010)
Atranorin	Antimicrobial, antioxidant, anti-inflammatory, anticancer	Kumar et al. (1999), Yilmaz et al. (2004), Turk et al. (2006), Ranković et al. (2008, 2014), Melo et al. (2011)
Zeorin	Antioxidant, antibacterial, antifungal	Behera et al. (2008), Kosanić et al. (2010)
Gyrophoric acid	Antimicrobial, anticancer, antioxidant	Candan et al. (2006), Ranković et al. (2008), Burlando et al. (2009)
Protocetraric acid	Antibacterial, antifungal, antioxidant, anticancer	Tay et al. (2004), Ranković and Mišić (2008), Honda et al. (2010), Manojlović et al. (2012)
Fumarprotocetraric acid	Antibacterial, antifungal, antioxidant, anticancer	Yilmaz et al. (2004), Ranković and Mišić (2008), Kosanić et al. (2014)
Stictic acid	Antioxidant, antimicrobial, anticancer	Lohézic-Le Dévéhat et al. (2007), Ranković and Mišić (2008)
Salazinic acid	Antitumour, antibacterial, antifungal, antioxidant	Candan et al. (2007), Burlando et al. (2009), Manojlović et al. (2012)
Usnic acid	Antiviral, antitumour, antioxidant, antibacterial, antifungal, antipyretic, analgetic, anti-inflammatory, hepatotoxic, antiviral	Lauterwein et al. (1995), Tay et al. (2004), Ranković et al. (2008, 2014), Paudel et al. (2010), Perry et al. (1999), Odabasoglu et al. (2006), Bazin et al. (2008), Burlando et al. (2009), Ramos and Almeida da Silva (2010)
Vulpinic acid	Antimicrobial, anticancer	Lauterwein et al. (1995), Burlando et al. (2009)
Evernic acid	Antifungal, antibacterial, antioxidant, anticancer	Halama and Van Haluwin (2004), Kosanić et al. (2013)
Lobaric acid	Antibacterial, antifungal, anticancer	Ingolfsdottir et al. (1998), Piovano et al. (2002), Sundset et al. (2008)
Physodic acid	Antibacterial, antifungal, antioxidant, anticancer	Turk et al. (2006), Ranković et al. (2008, 2014), Kosanić et al. (2013)
Protolichesterinic acid	Antitumour, antibacterial, anticancer	Ingolfsdottir et al. (1998), Turk et al. (2003), Bucar et al. (2004)
Norstictic acid	Antimicrobial, antioxidant, anticancer	Tay et al. (2004), Honda et al. (2010), Ranković et al. (2014)
Ramalin	Antioxidant, antibacterial	Paudel et al. (2008, 2010)
Barbatic acid	Antioxidant, antimicrobial	Verma et al. (2011), Martins et al. (2010)

(continued)

Table 1.1 (continued)

Lichen compounds	Studied activities	References
Divaricatic acid	Antioxidant, antimicrobial	Hidalgo et al. (1994), Kosanić et al. (2010)
Pannarin	Antioxidant, anticancer	Hidalgo et al. (1994), Russo et al. (2008)
Umbilicaric acid	Antioxidant, antimicrobial	Buçukoglu et al. (2013)
Variolaric acid	Antioxidant, anticancer	Brisdelli et al. (2013)
Homosekikaic acid	Antioxidant, antibacterial	Sisodia et al. (2013)
Sekikaic acid	Antioxidant, antibacterial	Verma et al. (2012), Sisodia et al. (2013)
Benzoic acid	Antioxidant	Sisodia et al. (2013)
Diffractaic acid	Analgetic, antiproliferative, antioxidant, antipyretic	Brisdelli et al. (2013), Atalay et al. (2011), Kumar and Muller (1999), Okuyama et al. (1995)
Vicanicin	Antioxidant, anticancer	Brisdelli et al. (2013)
2,4-Dihydroxy-6-propyl	Antioxidant	Sisodia et al. (2013)
1′-Chloropannarin	Antioxidant	Hidalgo et al. (1994)

acid may represent a novel source for a natural non-genotoxic anticancer drug (chemotherapeutic agent).

Russo et al. (2008) reported that the depside sphaerophorin (isolated from *Sphaerophorus globosus*) and the depsidone pannarin (isolated from *Psoroma pholidotoides*, *P. pulchrum* and *P. pallidum*) inhibited the growth of M14 human melanoma cells, triggering apoptotic cell death. The anticancer activities of these lichen metabolites are promising in the treatment of this aggressive, therapy-resistant skin tumour. An ethyl acetate-soluble fraction (ET4) of the crude methanolic extract of *Ramalina farinacea* was found to be a broad-spectrum antiviral agent against RNA (respiratory syncytial virus and HIV-1) and DNA (adenovirus and herpes simplex virus type 1) viruses (Esimone et al. 2009). Anti-HIV effects of ET4 target both the entry and post-entry stages in the viral replication cycle. Usnic acid (isolated from the aposymbiotic mycobionts of *Ramalina celastri*) exhibited specific antiviral activity against the *Junin virus* (*Arenaviridae*), which is the agent of Argentine hemorrhagic fever in humans, as well as against Tacaribe virus, a nonpathogenic arenavirus (Fazio et al. 2007). Parietin (isolated from the aposymbiotic mycobionts of *Teloschistes chrysophthalmus*) showed virucidal effects against the same viruses.

Norstictic acid, physodic acid, evernic acid, usnic acid, salazinic acid, fumarprotocetraric acid, protocetraric acid, atranorin and zeorin isolated from different lichen species are relatively strong antioxidant, antimicrobial and anticancer agents (Kosanić et al. 2010, 2013, 2014; Manojlović et al. 2012; Ranković et al. 2014).

Some lichen substances have been shown to relieve pain effectively or reduce fever and inflammation in various mammals, and it is reasonable to assume that these compounds also could be effective in humans. Vijayakumar et al. (2000)

reported that (+)-usnic acid, isolated from *Roccella montagnei*, showed significant, dose-dependent anti-inflammatory activity in rats, reducing carrageenan-induced paw oedema. Diffractaic and usnic acids have an analgesic effect in mice in vitro (Okuyama et al. 1995), and usnic acid also is an antipyretic against lipopolysaccharide-induced fever.

Lichens and other natural products remain as an untapped reservoir of potentially useful chemical compounds not only as drugs but also as unique templates that could serve as a starting point for synthetic analogues. Over 50 % of all modern clinical drugs are of natural product origin, and natural products play an important role in the drug development programmes in pharmaceutical industries.

Here we want to show that lichen secondary substances exhibit a huge array of remarkable pharmaceutical activities and that these important properties of lichen substances make them possible pharmaceuticals. Lichens and their secondary metabolites exhibit many pharmaceutical roles, primarily including antimicrobial, antioxidant, antiviral, anticancer, antigenotoxic, anti-inflammatory, analgesic and antipyretic properties. This study suggests that the lichens can be productively used in the pharmaceutical area because of its possible activities reported.

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Chapter 2
Lichens Used in Traditional Medicine

Stuart D. Crawford

Abstract Lichens are used in traditional medicines by cultures across the world, particularly in temperate and arctic regions. Knowledge of these medicinal uses is available to us because of the contributions of traditional knowledge holders in these cultures.

The traditional medicinal uses of 52 lichen genera are summarized in this paper. Cultures in different regions of the world tend to emphasize different lichen genera in their traditional medicines, with *Usnea* being the most widely used genus. The folk taxonomy of lichens within a given culture is not synonymous with the scientific taxonomy and reflects the cultural value of those lichens and the traditional method of their identification. Even within western science the identity and taxonomy of lichens have not remained constant throughout history.

Lichens in traditional medicine are most commonly used for treating wounds, skin disorders, respiratory and digestive issues, and obstetric and gynecological concerns. They have been used for both their secondary metabolites and their storage carbohydrates. The European uses of lichens have been exported worldwide and sometimes influence the use of lichens by other cultures. These European uses started in the fifteenth and sixteenth centuries and arose from interpretations of Ancient Greek uses, as well as the application of the doctrine of signatures.

2.1 Introduction

Lichens are important traditional medicines in many different cultures. This information has been made available to us from the contributions of hundreds of traditional knowledge holders in communities across the world. It is our responsibility to respect and value the knowledge that has been given to us. This paper is a tribute to the wealth of traditional knowledge that exists about lichens.

There have been a few previous reviews on the traditional uses of lichens for medicine. The traditional uses of lichens in Europe were reviewed by Smith (1921),

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B. Ranković (ed.), *Lichen Secondary Metabolites*,
DOI 10.1007/978-3-319-13374-4_2

with later contributions by Llano (1948) and Richardson (1974). Sharnoff (1997) compiled the first global review lichen uses, which was added to by Crawford (2007). Upreti and Chatterjee (2007) reviewed the medicinal uses of lichens in India and republished Sharnoff's (1997) database on medicinal uses elsewhere. Wang and Qian (2013) recently reviewed the medicinal uses of lichens in China. The current paper includes all the medicinal uses recorded by these previous authors, as well as many additional records. It is the most comprehensive review to date, but it is still far from complete.

2.2 Cultures That Use Lichens

There are records of medicinal uses of lichens in cultures in Africa, Europe, Asia, Oceania, North America, and South America. The majority of these uses are in North America, Europe, India, and China, but this is most likely because that is where the majority of the ethnographic work has been done. Interestingly, no records have been found for any traditional use of lichens in Australia.

It is difficult to determine the prevalence of lichens in traditional medicine across the world. Most ethnobotanists and ethnographers have ignored cryptogams, both historically and currently.

If the ethnographic literature on a culture does not mention lichens, it might be because that culture does not utilize lichens. However, it might also be because the ethnographer's culture does not value lichens, and the ethnographer therefore did not notice and record the value of lichens in the culture that they were documenting. In the cultures for which traditional uses of lichens have been recorded, there are usually between one and three medicinal lichens. There are more records of lichen use among cultures in temperate and arctic areas and less in the tropics. This probably represents the relative dominance of lichens in these zones.

A few ethnobotanists have recognized the cultural value of lichens, and their work has been invaluable in documenting lichens in traditional medicines. These workers include, among others, N. J. Turner (Canada), M. R. González-Tejero (Spain), L. S. Wang (China), and D. K. Upreti (India). As a result, there is an overrepresentation of these geographic areas in this current analysis.

2.3 The Lichens That Are Used in Traditional Medicine

This paper documents a total of 52 different genera of lichens that are used in traditional medicines. The most commonly used genus of lichen is *Usnea*, which is used across the world for medicine, although it is often used synonymously with other arboreal hair lichens. Despite its worldwide importance, *Usnea* is not traditionally one of the dominant medicinal lichens in Europe. Numerous other genera of lichens have particular importance in certain parts of the world, as is shown in Table 2.1.

Table 2.1 Lichen genera commonly used in traditional medicine

Lichen genus	Main area of use
Usnea	Worldwide (except Australia)
Evernia and *Pseudevernia*	Europe and North Africa
Letharia	North America
Lethariella	China
Cetraria	Europe
Parmotrema and *Everniastrum*	India
Xanthoparmelia	North America and Africa
Cladonia and *Cladina*	N. America, Europe, and Asia
Thamnolia	Asia
Ramalina	N. America, Europe, and Asia
Lobaria and *Peltigera*	N. America, Europe, and Asia
Umbilicaria	North America and Asia

2.3.1 The Folk Taxonomy of Lichens

All cultures develop a folk taxonomy of living organisms that allows people to make sense of the world around them. Folk taxonomies are unique to a specific culture and usually reflect its particular environment and values. Some cultures have a very detailed folk taxonomy for lichens. The traditional taxonomy of the Saami recognizes lichens as being a distinct life form from mosses and divides lichens into three different generic taxa and numerous specific taxa (Nissen 1921). Other cultures placed less value on lichens, which is reflected in a much more simplistic folk taxonomy for lichens. European botanists in the fifteenth century lumped all lichens, and many other cryptogams, into a single life form category of ***moss***.

Folk taxonomies can be very accurate, but they are often different than the scientific taxonomy. This mismatch between folk and scientific taxonomies is particularly prevalent in lichens. For instance, the Saami folk genera of ***jægel*** includes *Cetraria*, *Cladina*, and *Stereocaulon*, but excludes *Parmelia*, which is placed in the folk genera ***gadna***. The scientific taxonomy would lump *Parmelia* and *Cetraria* together in Parmeliaceae and exclude *Cladina* and *Stereocaulon*. Another example is the common practice within folk taxonomies of classifying lichens according to their substrate. There is often a folk genera that includes all arboreal hair lichens (and sometimes mosses), which are then divided into different species depending on what type of tree they are growing on.

One of the biggest challenges in ethnolichenology is that a folk taxon of lichens that has cultural significance may not be synonymous with any scientific taxon. This means that if a culturally important lichen is identified according to the scientific taxonomy without understanding the folk taxonomy, it may be recorded as the wrong lichen. For example, a botanist recorded that the Saami used *Usnea plicata* for blisters, but maybe the lichen that he saw only happened to be *U. plicata*,

and the Saami actually used any species of *Alectoria*, *Bryoria*, or *Usnea* that was growing on a birch tree.

Folk taxonomies of lichens are intrinsically linked with the traditional methods of identifying lichens. It is very common to identify lichens based on where they are found. Lichens are often thought to imbibe their desirable properties from the substrate on which they are growing. For example, Nuxalk consider alectoroid lichens to be better medicine if growing on alder, the Gitga'at consider *Lobaria oregana* to be better if on fir, and the Ancient Greeks thought that *Evernia* was better if growing on cedar. The medicinal properties of a lichen species may change depending on where it is growing. However, this may also be a clever aid for identification. Many lichens have specific microhabitat preferences, and selecting lichens from only a specific substrate will result in preferentially selecting certain species.

Another interesting identification method is employed by the Quichua of Saraguro, Ecuador, who have determined that an effective medicine requires seven different colors of rock lichens. It is possible that there is a synergistic effect between the different lichen species. It is also possible that collecting seven different species makes it much more likely to collect the correct one.

2.3.2 Development of Lichen Taxa in Western Science

The meaning of the word ***lichen*** has changed over time, which can make it complicated to identify culturally important lichens in old documents. ***Lichen*** comes from the Ancient Greek **Λειχήν** (***leikhēn***), the first record of which is from Theophrastus in 300 B.E. (Richardson 1974). Theophrastus was probably referring to thalloid liverworts, but subsequent Ancient Greek authors may have used that name for a lichen (see Ancient Greek use of *Ramalina* spp.). Early European botanists lumped together a variety of cryptogams into the same taxon, usually including lichens, mosses, liverworts, fungi, seaweed, and sometimes even coral. de Tournefort (1694) was the first European author to distinguish lichens by the name ***lichen***, but he also included some thalloid liverworts in his taxon and excluded some lichens. It was Dillenius (1742) who reorganized the ***lichen*** taxon to make it synonymous with our modern concept.

The taxonomy and names of lichens have changed radically since Dillenius and are continuing to change in contemporary times. This can make it difficult to determine what lichen is being discussed in ethnographic literature. To add further complications, most authors know very little about lichens and thus frequently use names that are outdated or even just completely wrong.

The genus *Usnea* was created by Dillenius (1742). Linnaeus (1753) described five *Usnea* species, but lumped them all together in his all-encompassing genus *Lichen*. They were moved to the *Usnea* genus by Weber and Wiggers (1780). Four of the original species are often mentioned in ethnographic literature: *Usnea barbata*, *U. florida*, *U. hirta*, and *U. plicata*. The number of *Usnea* species has

now increased to around 350 species (Thell et al. 2012), so any reference to one of the original *Usnea* species in old herbals or ethnographies is suspect. Of the original five, only *Usnea hirta* occurs in North America (Esslinger 2014). References to *Usnea barbata* are particularly ambiguous, as the taxonomy of this species is still confusing and still being determined (Articus 2004).

The pendant *Bryoria* species were originally all lumped together as *Lichen jubatus* (Linnaeus 1753), which became *Alectoria jubata* (Acharius 1810). The taxonomy of *Bryoria* was not well understood until Brodo and Hawksworth (1977) created the genus *Bryoria*, so references to specific *Bryoria* species prior to that are ambiguous.

The Parmeliaceae is a large and diverse family of lichens that includes many culturally significant lichens. This family currently contains around 80 genera and over 2,000 species (Thell et al. 2012). Five culturally significant genera of Parmeliaceae were described before 1810: *Usnea*, *Parmelia*, *Cetraria*, *Alectoria*, and *Evernia*. By 1903, *Letharia* and *Pseudevernia* had been split from *Evernia*, and *Parmotrema* and *Hypogymnia* had been split from *Parmelia*, although historically not all authors have recognized these genera. The taxonomy of Parmeliaceae remained relatively constant until 1965, when the genus *Cetraria* began to be split into numerous different genera. The genus *Parmelia* was also split up starting in 1974. This splitting was mostly completed by the early 1990s, by which time there were over 80 genera in the family (Thell et al. 2004). Recent molecular work has resulted in some genera being lumped and others split, such that Thell et al. (2012) recognize 79 genera. Currently, the original genus *Parmelia* is divided into 32 genera and *Cetraria* into 22 genera.

For practical reasons, lichenologists sometimes lump the morphologically similar genera that were previously included in *Parmelia* and *Cetraria* back together into the categories of parmelioid (Hale and DePriest 1999) and cetrarioid lichens (Randlane et al. 2013). These morphological groupings are not entirely monophyletic (Thell et al. 2012), but they can still be useful. A third morphological grouping of Parmeliaceae lichens that is often used is the alectorioid lichens, which include several similar-looking genera of hair lichens that were previously lumped together in the genus *Alectoria*. The genus *Usnea* is sometimes included in this category.

One result of the profusion of genera within Parmeliaceae is that any reference to an unidentified species of *Parmelia* or *Cetraria* in an older ethnographic work is very ambiguous. The categories of parmelioid, cetrarioid, and alectorioid lichens are very useful when dealing with folk taxonomies of lichens, so they will be utilized in the current work.

2.4 The Medicinal Uses of Lichens

Lichens are used for many different medicinal purposes, but there are some general categories of use that reoccur across the world. Lichens are often used externally for dressing wounds, either as a disinfectant or to stop bleeding. Other common topical

uses are for skin infections and sores, including sores in the mouth. This importance of this use is apparent in the name ***lichen*** (from ***leikhēn***, 'what eats around itself'), which comes from the Ancient Greek practice of using a cryptogam to cure a skin disease.

Lichens are often drunk as a decoction to treat ailments relating to either the lungs or the digestive system. This is particularly common in Europe, but is also found across the world. Many other uses of lichens are related to obstetrics or treating gynecological issues. This may be related to the common use of lichens for treating sexually transmitted infections and ailments of the urinary system. Two other uses of lichens that are less common, but reoccur in several different cultures, are for treating eye afflictions and for use in smoking mixtures.

Many of the traditional medicinal uses of lichens are probably related to their secondary metabolites, many of which are known to both be physiologically active and to act as antibiotics. However, some of the traditional uses of lichens also rely on the qualities of lichen carbohydrates. In particular, the lichenins [β-(1→3)-(1→4)-linked D-glucans] are common in the Parmeliaceae and have a remarkable ability to absorb water and form a gel (Crawford 2007). Many of the traditional uses of lichens involve boiling the lichen to create a mucilage which is drunk for lung or digestive ailments or applied topically for other issues. Other lichen carbohydrates which may be important are the isolichenins and galactomannans, which are taxonomically widespread, and the pustulins that are found in Umbilicariaceae.

2.4.1 Medicinal Lichens of Europe

Lichens are used in traditional medicine across the world, and many cultures outside of Europe have traditional uses for lichens that are completely unrelated to Europe. However, European uses of lichens have been exported worldwide, and there are numerous instances where the European use for a lichen appears to be associated with its traditional use in a different culture. This dispersal of European uses of lichens is related to the general dispersal of other aspects of European culture across the world. One specific source of this bias may be that most ethnographers that recorded traditional uses of lichens are from a European background, and their personal cultural bias can affect what they have documented. Another source is that most literature on lichens is from a European background, and if it features any uses of lichens, those uses are generally European.

An understanding of the traditional use of lichens in Europe can therefore be important for understanding traditional uses elsewhere. The origins of the medicinal use of lichens in Europe dates back to the fourth and third century B.E., when medicinal lichens were recorded by the Ancient Greek scholars Hippocrates and Theophrastus (Lebail 1853). The use of lichens continued to be recorded by various scholars throughout the rest of the classical era, including Pedanius Dioscorides and Pliny the Elder (Rome, first century C.E.), Galen of Pergamon (Greece, second century C.E.), Paul of Aegina (Greece, seventh century C.E.), and Serapion the

Younger (a twelfth or thirteenth century compilation). These authors discuss at least three different cryptogams that might be lichens, but the most important for subsequent pharmacopoeias was an arboreal fruticose lichen called ***splanchnon*** ("intestine"). According to the original writings of Dioscorides, ***splanchnon*** was not only a powerful medicine, it was also sweet-smelling and used as a perfume (López Eire et al. 2006).

In the middle ages, various Persian scholars like Rhazes (tenth century) and Avicenna (eleventh century) wrote about the medicinal properties of ***splanchon***, and it was adopted into Unani medicine under the name ***ushna***. This lichen is currently interpreted as being *Usnea* spp.

At the start of the modern era (~ fifteenth century), herbalism flourished in Western Europe, with many authors adopting Greek herbal knowledge. These Europeans lumped together all fruticose arboreal lichens into one taxon, which they called ***usnea*** (borrowing from the Arabic ***ushna***), ***tree moss***, or ***oak moss*** (Dorstenius 1540; L'Obel 1576; Gerarde 1597; Ray 1686; Quincy 1724; Culpeper 1788). This taxon was considered to be synonymous with the Ancient Greek ***splanchon***, with all of its medicinal and perfume qualities. Parkinson (1640) accurately distinguished between numerous genera, but considered them all types of ***oak moss*** and attributed the same medicinal values to all of them.

It was not until the late 1700s that a distinction was made between the different genera of ***oak moss***, at which time the name *Usnea* was only applied to our modern genus. From this time onwards, most authors decided that the medicinal values of ***splanchon*** were referring to *Usnea* (Lightfoot 1777; Willemet 1787; Adams 1847; Lebail 1853), although the same medicinal properties were sometimes applied to *Evernia prunastri* (Willemet 1787; Lebail 1853).

Oak moss was used to make a popular scented hair powder called Cyprus powder in Europe in the late 1600s (Bauhin and Cherler 1650; Zwelfer 1672). By the time European botanists could distinguish different genera, Cyprus powder was found to contain a variety of lichen genera, including *Usnea*, *Pseudevernia*, and other arboreal lichens (Amoreux 1787). At this time *Evernia prunastri* was the preferred lichen to use for perfumes in France (Amoreux 1787). In more recent times, ***oak moss*** refers to only *Evernia prunastri* and ***tree moss*** to *Pseudevernia furfuracea*, and these are the two lichen species harvested for perfume (Moxham 1986).

When Europeans first adopted Ancient Greek herbal knowledge, they were confused as to the identity of ***splanchon***, but eventually decided that it was *Evernia*/*Pseudevernia* when used for perfume and *Usnea* when used for medicine. Dioscorides' description of ***splanchon*** is ambiguous and its identity cannot be determined with certainty, but Richardson (1974) suggests that it is referring to *Evernia prunastri* and *Pseudevernia furfuracea*. He may be correct, as these lichens were used medicinally in Europe and North Africa from ancient times to present. Europeans have added medicinal properties to *Usnea* that were not originally associated with ***splanchon*** by the Ancient Greeks. Perhaps these medicinal uses for *Usnea* existed in Europe independent of the Ancient Greek writings, and this

caused Europeans to wrongly associate the Ancient Greek medicinal uses of *Evernia/Pseudevernia* with *Usnea*.

Starting in the 1500s, the doctrine of signatures was an ubiquitous concept in European medicine. It was thought that plants looked like the organ or ailment that they cured and various lichens were adopted into the European pharmacopoeia as a result. The main medicinal lichens in early modern era Europe were *Cetraria islandica*, *Cladonia pyxidata*, *Peltigera canina*, *Peltigera aphthosa*, *Usnea* spp., *Lobaria pulmonaria*, *Xanthoria parietina*, and *Evernia prunastri*. For more details, refer to these lichens in the tables below. The widespread use of these lichens had been mostly abandoned by 1800, with the exception of *Cetraria islandica*, which has persisted as a medicinal lichen in parts of Europe until today.

2.5 Known Records of Lichens Used in Traditional Medicine

The following tables document all of the traditional medicinal uses of lichens for which the author has found records. Tables 2.2 and 2.3 provide a list of the different genera and an index to the table where they can be found. Tables 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 2.10, 2.11, 2.12, 2.13, 2.14, 2.15, 2.16, 2.17, 2.18, 2.19, 2.20, 2.21, 2.22, 2.23, 2.24, 2.25, 2.26, and 2.27 are organized taxonomically by lichen family and provide the details on each traditional use.

Table 2.2 Lichen genera used in traditional medicine

Alectoria, Alectorioid	*Lecanora*, Lecanoraceae	*Pseudevernia*, Parmeliaceae
Anaptychia, Physciaceae	*Leptogium*, Collemataceae	*Pseudocyphellaria*, Lobariaceae
Anzia, Parmeliaceae	*Letharia*, Parmeliaceae	*Punctelia*, Parmelioid
Aspicilia, Megasporaceae	*Lethariella*, Parmeliaceae	*Ramalina*, Ramalinaceae
Bryoria, Alectorioid	*Lobaria*, Lobariaceae	*Rhizoplaca*, Lecanoraceae
Cetraria, Cetrarioid	*Masonhalea*, Cetrarioid	*Roccella*, Roccellaceae
Cetrelia, Cetrarioid	*Mycoblastus*, Mycoblastaceae	*Siphula*, Icmadophilaceae
Cladina, Cladoniaceae	*Nephroma*, Nephromataceae	*Stereocaulon*, Stereocaulonaceae
Cladonia, Cladoniaceae	*Nephromopsis*, Cetrarioid	*Sticta*, Lobariaceae
Dermatocarpon, Verrucariaceae	*Niebla*, Ramalinaceae	*Sulcaria*, Alectorioid
Dictyonema, Hygrophoraceae	*Ophioparma*, Ophioparmaceae	*Teloschistes*, Teloschistaceae
Evernia, Parmeliaceae	*Parmelia*, Parmelioid	*Thamnolia*, Icmadophilaceae
Everniastrum, Parmelioid	*Parmotrema*, Parmelioid	*Umbilicaria*, Umbilicariaceae
Flavocetraria, Cetrarioid	*Peltigera*, Peltigeraceae	*Usnea*, Alectorioid
Flavoparmelia, Parmelioid	*Pertusaria*, Pertusariaceae	*Vulpicida*, Cetrarioid
Heterodermia, Physciaceae	*Physcia*, Physciaceae	*Xanthoparmelia*, Parmelioid
Hypogymnia, Parmeliaceae	*Polycauliona*, Teloschistaceae	*Xanthoria*, Teloschistaceae
Lasallia, Umbilicariaceae		

Table 2.3 Index to tables of lichen families used in traditional medicine

Table 2.4 Cladoniaceae used in traditional medicines around the world

Culture and ***folk name***	Traditional use
Cladina spp. Nyl.	
Den'ina (Alaska, USA) ***k'udyi***	Decoction used for diarrhea (Kari 1987)
Upper Tanana (AK, USA)	A "liquor" prepared from plant was drunk for colds (McKennan 1959)
Aleut (Alaska, USA) ***kinadam aiyukax***	Drunk as a tea for chest pains. Hunters who are climbing hills chew the lichen to maintain their wind (Bank 1953; Smith 1973)
Nganasans (Siberia)	Remedy for scurvy
Saami (Scandinavia) ***ullo-jægel*** ("wool lichen")	Decoction for unspecified medicine (Nissen 1921; Eidlitz 1969)
Cladina arbuscula (Wallr.) Burgaz	
China	Used for dizziness, hypertension, pulmonary tuberculosis, fever, trauma with pus formation, and skin infections due to external injury (Wang and Qian 2013)
Cladina rangiferina (L.) Nyl.	
Ojibwe (MN and WI, USA) ***asa' gûniñk'***	Boil and use water to wash a newborn baby (Smith 1932)
Whapmagoostui Cree (Quebec, Canada) ***whapskumuk***, ***epshatuk***	Used to treat inflammation associated with diabetes (Fraser 2006)
Finland	Remedy for coughs and tuberculosis. Boil in water and drink (Richardson 1974)
China	Used for fever, headaches, cuts, coughing up blood, jaundice, blurred vision, cloudy cornea, difficulty urinating, urinary tract infection, irritable depression, rheumatism, and phlegm due to dry throat. Drink decoction; or apply decoction or powdered lichen to affected area (Wang and Qian 2013)

(continued)

Table 2.4 (continued)

Culture and ***folk name***	Traditional use
Monpa (Arunachal Pradesh, India)	Remedy for kidney stones. Half teaspoon of sun-dried, ground lichen added to one cup boiling water. Drunk in morning on empty stomach for 1 month or until cured (Rout et al. 2005)
Cladina stellaris (Opiz) Brodo [*Cladina alpestris*]	
Nihithawak (SK, Canada) ***wāpiskastastkamihk*** or ***atikōmīciwin***	Drink to expel intestinal worms: either decoction or powdered lichen added to water (Leighton 1985)
Inuit (Nunavut, Canada) ***nirait***	Broth used for sickness and eye infections (Black et al. 2008)
Primorsky and Sakhalin (Russian Far East)	Powdered form used to treat wounds and some infections (Moskalenko 1986)
China 太白花 (***tai-bai-hua***)	Used for hypertension, headaches, nosebleeds, eye diseases, tuberculosis, menstrual disorders, and vaginal discharge. Drink decoction (Hu et al. 1980; Wang and Qian 2013)
Cladonia subtenuis (Abbayes) Mattick	
Cherokee (NC, USA)	Used to relieve the pain of insect stings. Lichen chewed and put on sting, sometimes mixed with tobacco (Garrett 2003)
Cladonia amaurocraea (Flörke) Schaer.	
China	Used for headaches and dizziness (Wang and Qian 2013)
Cladonia bellidiflora (Ach.) Schaerer	
Tlingit (Alaska, USA)	Treatment for eye disease when mixed with mother's milk (Garibaldi 1999)
Haida (BC, Canada)	Red ends dipped in mother's milk and applied to sore eyes (Turner 2004a)
Cladonia cervicornis (Ach.) Flot.	
China	Used for scalds, cuts, and coughing up blood. Drink decoction; or apply decoction or powdered lichen to affected area (Wang and Qian 2013)
Cladonia chlorophaea (Flörke ex Sommerf.) Sprengel	
Okanagan (BC, Canada) ***penpenemekxísxn̓***	Decoction used to wash sores which were slow to heal. Folk name means "liver on rock" (Turner et al. 1980)
Britain ***chalice-moss***; ***cup-moss***; or ***Our Lady's chalice***; ***cwpanau pas*** (Welsh)	Used like *C. pyxidata* for whooping cough, use has continued to contemporary times in Welsh counties of Merionethshire and Denbighshire. In Waterford (Ireland), used for same purpose boiled in new milk (Allen and Hatfield 2004)
Cladonia coccifera (L.) Willd.	
Europe (early modern era) ***cup moss***	Decoction used for fever and whooping cough in children, like *C. pyxidata* (Willemet 1787; Luyken 1809; Lindley 1838)

(continued)

Table 2.4 (continued)

Culture and ***folk name***	Traditional use
Cladonia cornuta (L.) Hoffm.	
Europe (early modern era) ***horn moss***	Used with *C. pyxidata* against persistent coughs in children (Watson 1756)
Cladonia fenestralis Nuno	
Tibetans (Sichuan, China)	Medicinal tea (Wang and Qian 2013)
Cladonia fruticulosa Kremp.	
China	Extract used for bacterial infections on skin (Wang and Qian 2013)
Cladonia gracilis (L.) Willd.	
China 太白鹿角 (***tai-bai-lu-jiao***)	Used for dizziness, difficult or painful urination, nose bleeding, impetigo, and pink eye. Drink decoction; or apply decoction or powdered lichen to affected area (Hu et al. 1980; Wang and Qian 2013)
Cladonia macroceras (Delise) Ahti	
China	Drunk as decoction to relieve blockage of urination, bring down swelling, and remove toxic substances (Wang and Qian 2013)
Cladonia miniata G. Meyer [*Cladonia sanguinea*]	
Brazil	Rubbed down with sugar and water, used as remedy for mouth ulcers (Lindley 1838)
Cladonia pleurota (Flörke) Schaer.	
China	To clear ***heat***, cool liver, dissolve ***phlegm***, and eliminate ***dampness*** (Wang and Qian 2013)
Cladonia pyxidata (L.) Hoffm.	
Europe (early modern era) ***cup moss***	Widely used for whooping cough in children (Quincy 1724; Gedner 1756; Lightfoot 1777; Willemet 1787). Also for fevers and kidney stones (Luyken 1809; Lindley 1838; Lebail 1853). In Finland taken with milk for pulmonary tuberculosis (Vartia 1973)
Cladonia scabriuscula (Delise) Nyl.	
Keyagana (Papua New Guinea) ***lanefa-kikinofa***	Heated and taken orally for vaginal discharge/bleeding (Jorim et al. 2012)

Table 2.5 Lecanoraceae used in traditional medicines around the world

Culture	Traditional use
Lecanora muralis (Schreb.) Rabenh. [*Parmelia saxicola*]	
Nishinam (CA, USA)	Made into a tea and used to treat colic (Powers 1877)
Rhizoplaca chrysoleuca (Sm.) Zopf.	
China	Used for tuberculosis, intestinal obstruction, trauma with pus formation, burns and scalds, skin infections, cancer, and pain relief. Used externally or orally (Wang and Qian 2013)

Table 2.6 Mycoblastaceae used in traditional medicines around the world

Culture	Traditional use
Mycoblastus alpinus (Fr.) Kernst.	
China	Used for stopping bleeding from external injury, draining pus, burns, and nocturnal seminal emission. Drink decoction or apply powder to affected area (Wang and Qian 2013)

Table 2.7 Alectorioid lichens (Parmeliaceae) used in traditional medicines

Culture and ***folk name***	Traditional use
Alectoria Ach. spp.	
Scandinavia	Decoction for bathing chapped skin on babies or the feet of adults. Same use for *Lobaria pulmonaria*, *Usnea* sp, and *Peltigera aphthosa* (Richardson 1974)
Alectoria ochroleuca (Hoffm.) A. Massal.	
Chugach (Alaska, USA)	Possibly same as Chugach use of *Bryoria trichodes* (Wennekens 1985)
Alectoria sarmentosa Ach.	
Haida (BC, Canada) ***k'aalts'idaa liisga*** or ***k'aalts'adaa liijaa*** ("crow's mountain goat wool")	Used to strain impurities out of hot pitch when making medicine, and for other unspecified medicines. Also used *Usnea longissima* (Turner 1998, 2004a)
Nuxalk (BC, Canada) ***suts'wakt*** or ***ipts-aak*** ("limb moss")	Warmed and applied to a broken boil or festering sore (if growing on red alder). Possibly *Usnea* spp. (Smith 1929; Turner 1973)
Ditidaht (BC, Canada) ***p'u7up***	Used for wound dressing, baby diapers, and sanitary napkins. Also used *Usnea* spp. (Turner et al. 1983)
Flathead (Montana, USA) ***sqalīō***	Mother drinks tea of ***sqalīō*** and *Matricaria discoidea* to make her deliver her placenta (Stubbs 1966). Possibly *Usnea* spp.
Umatilla, Cayuse (OR, USA) ***laxpt*** or ***mak'hl***	Boiled and applied as compress for open sores, arthritis, and ***achash-pama*** [an eye problem] (Hunn 2005). Possibly *Usnea* spp.
Pallars (Spain) ***cabellera de pi***	Drunk as tea for asthma and catarrh (Agelet and Vallès 2003)
Bryoria spp. Brodo & D. Hawksw.	
Atsugewi (California, USA)	Applied as poultice to reduce swellings. Either boiled or used dry (Garth 1953)
Tsilhqot'in (BC, Canada) ***texa***; ***taxa***	Burn ***texa*** with own hair and rub ashes on hair and scalp to stop hair from going gray (Kay 1995; Turner 2004b)
France (eighteenth century)	Used for healing skin abrasions, diarrhea, and vaginal discharge (Gedner 1756; Willemet 1787)

(continued)

Table 2.7 (continued)

Culture and ***folk name***	Traditional use
Bryoria asiatica (Du Rietz) Brodo & D. Hawksw.	
China	Used for kidney deficiency and general weakness, dizziness, heart palpitation, involuntary ejaculation, night sweats, difficulty urinating, edema, impetigo, draining pus, and improving eyesight. Drink decoction; or apply decoction or powdered lichen to affected area (Wang and Qian 2013)
Bryoria bicolor (Ehrh.) Brodo & D. Hawksw.	
China	Same as Chinese use of *B. asiatica* (Wang and Qian 2013)
Bryoria fremontii (Tuck.) Brodo & D. Hawksw.	
Sahaptin (OR and WA, USA) ***kunč***	Boiled and used as poultice for arthritis (Hunn 1990)
Nimi'ipuu (Montana, USA) ***ho.póp***	Good for upset stomach, indigestion, and diarrhea (Hart 1976; Marshall 1977)
Flathead (Montana, USA) ***caúmtemkan***, ***st'telu***, ***skolápkan***, ***skolké in***, ***sqatlo***, or ***šáwtəmqən***	Important food when baked with root vegetables; when baked alone it is more a tonic for the sick than a food. (Turney-High 1937; Stubbs 1966; Hart 1974)
Okanagan (BC, Canada) ***skwelíp***	Mixed with berry juices and melted into syrup: given to newly weaned babies for their health (Gabriel and White 1954). Dried, powdered, and mixed with grease: Rubbed on navel of newborn babies to protect against infection (Turner et al. 1980)
Nlaka'pamux (BC, Canada) ***wí7e***	Warts removed by cutting them off and covering the fresh wound with ***wí7e*** that had been heated on the fire (Teit and Boas 1900; Turner et al. 1990)
Bryoria trichodes (Michaux) Brodo & D. Hawksw. [*Alectoria americana*]	
Sugpiaq (Alaska, USA) ***nakuraartum nuyii*** or ***napam ungagua'i***	Piled on sick person in the steam bath to hold the heat on his body, also used to staunch blood from wounds. Might also use *Alectoria ochrolechia* (Wennekens 1985)
Sulcaria sulcata (Lév.) Bystrek	
China	Used for dizziness, kidney deficiency, general weakness, heart palpitation, involuntary excessive ejaculation, night sweating, edema, impetigo, and sores. Drink decoction or apply to affected area (Wang and Qian 2013)
Sulcaria virens (Tayl.) Bystr.	
China	Used for aching back and legs, traumatic bleeding, menstrual irregularities, uterus prolapse, vaginal discharge, epilepsy, paralysis, impotence, and dizziness. Drink decoction or apply to affected area (Wang and Qian 2013)

(continued)

Table 2.7 (continued)

Culture and ***folk name***	Traditional use
Usnea spp. Dill. ex Adans.	
Maasai (Kenya) ***intanasoito***	Used for stomachache, malaria, backache, fever, loss of appetite, and typhoid. Crush, boil in water, and sieve (Kiringe 2008)
Mt. Kilimanjaro (Tanzania)	Ingredient in herbal tea to relieve altitude sickness (Sharnoff 1997)
Unani medicine (India) ***ushna*** or ***shaibat-al-ajooz*** "old women's hair"	An important medicine used from ~1000 C.E. to present. Used for heart troubles, for reducing inflammation, for promoting digestion and improving appetite, as an antidote, as an astringent, and as an analgesic. Helps wounds heal and lactation in women if applied as a paste on breast. *Parmotrema* spp. is sometimes included as ***ushna***, perhaps resulting from confusion with ***shaileya*** of Ayurvedic medicine (Rauf et al. 2006; Yavuz and Çobanoğlu 2010; Rauf et al. 2011). See Unani use if *U. longissima*
Iran, Iraq ***lihayat-as-shāyib***	Taken to correct bad breath. Folk name means "old man's beard" (Hooper 1937)
Taplejung (Nepal) ***jhyau***	Fired powder of ***jhyau*** is mixed with water and taken for tonic, fever, and throat pain (Poudel 2008)
New Ireland (Papua New Guinea)	Used to induce menstruation (Lee et al. 1977)
Doi Inthanon (Chiang Mai, Thailand)	Used in a bath for women following childbirth, to aid parturition and prevent infection (Sharnoff 1997)
Maori (New Zealand) ***angiangi*** or ***kohukohu***	Steeped in water and placed on affected parts for venereal disease (Best 1905). Dried, powdered, and rubbed on skin for various skin afflictions (Kerry-Nicholls 1886; Goldie 1904). Crushed with hand and lightly bandaged onto wound to stop bleeding (Brooker and Cooper 1962; Macdonald 1974). Along with moss, used as sanitary napkin, as diaper, and to keep newborn babies warm (Goldie 1904)
Europe (early modern era) ***oak moss***, ***tree moss***, ***usnea***	The Ancient Greeks had important medicinal uses for a fruticose arboreal lichen called ***splanchon***, which was likely *Evernia prunastri* or *Pseudevernia furfuracea* (see Ancient Greek use of *E. prunastri*). This lichen entered European pharmacopoeias in the early 1500s and included all fruticose arboreal lichens. By the late 1700s it was only *Usnea* spp. Europeans added to the Ancient Greek uses of ***splanchon*** and used a decoction of *Usnea* spp. for a styptic, for drying skin lesions, as an antiinflammatory, as a skin moisturizer, and for nausea, diarrhea, whooping cough, smallpox, insomnia, umbilical hernias, and uterine medicine (Lebail 1853). It was also used for diseases of the scalp and to cure dandruff (Allen and Hatfield 2004) and as ***usnea cranii humani*** (see *Parmelia saxatilis*)

(continued)

Table 2.7 (continued)

Culture and ***folk name***	Traditional use
Kartitsch (Austria)	Gathered as a medicinal plant (Christanell et al. 2010)
Aragon (Spain)	Used for respiratory ailments (González-Tejero et al. 1995)
Valsugana Valley (Italy)	Shepherds put it in their shoes to prevent or treat blisters (Sharnoff 1997)
Scandinavia	Decoction for bathing chapped skin on babies and the feet of adults. *Alectoria* sp., *Lobaria pulmonaria*, and *Peltigera aphthosa* also used (Richardson 1974)
Saami (Scandinavia) ***lappo***	Powdered and sprinkled on external wounds, and on sores from long journeys. Also used for curing ringworm and scabies (Lebail 1853; Nissen 1921)
Finland	Put on fresh or infected wound, athlete's foot, and other skin eruptions. Taken orally for sore throat and toothache. *Alectoria* spp. also used (Vartia 1973)
Dalarna (Sweden)	Used to treat foot blisters (Ahmadjian and Nilsson 1963)
Nuxalk (BC, Canada)	Probably same as Nuxalk use of *Alectoria sarmentosa* (Turner 1973)
Ditidaht (BC, Canada)	Same as Ditidaht use of *Alectoria sarmentosa* (Turner et al. 1983)
Makah (WA, USA)	Used for boils (Gill 1983)
Nihitahawak (SK, Canada) ***mithāpākwan***	Fresh lichen inserted into the nostril to stop a nose bleed (Leighton 1985)
Wabasca (AB, Canada) ***miyapakwan***	Decoction used to wash sore or infected eyes. Possibly *U. hirta* (Siegfried 1994; Marles et al. 2000)
Flathead (Montana, USA)	Probably same as Flathead use of *Alectoria sarmentosa* (Stubbs 1966)
Umatilla, Cayuse (OR, USA)	Probably same as Umatilla and Cayuse use of *Alectoria sarmentosa* (Hunn 2005)
Navaho (Utah, USA) ***cin bidaɣai***	An infusion or poultice is used to treat frozen body parts. Folk name means "wood mustache" (Wyman and Harris 1951)
Quichua (Loja, Ecuador) ***musgo de arbol***	Used for inflated, sore stomach in children. Boiled in water with honey and drunk. Must not be collected from eucalyptus or pine (Abel 2009, pers. comm.)
Usnea sect. *Neuropogon* spp. (Nees & Flot.) Mont. [syn. *Neuropogon* spp.]	
Mapuche-Tehuelche (Argentina/Chile) ***barba de piedra***; ***flor de piedra***	Used for coughs. Medicine for unspecified gastrointestinal, respiratory, cardiovascular, obstetric-gynecological, and genitourinary afflictions, as well as cultural syndromes (Estomba et al. 2006; Molares and Ladio 2014)
Usnea aciculifera Vain.	
China	Used for bladder infection, painful urination, urinary retention, swelling, and edema in heart and kidneys (Wang and Qian 2013)

(continued)

Table 2.7 (continued)

Culture and ***folk name***	Traditional use
Usnea articulata L. Hoffm. [syn. *Usnea flavescens*]	
Iraqw (Tanzania) ***hewas***	Treatment for stomachache. A handful of ***hewas*** is chewed fresh and the juice swallowed, it is bitter but relieves the pain after a while. *U. gigas* is also used (Kokwaro 1976)
Usnea articulata (L.) Hoffm.	
Samoa	Used for wounds and shin bruises (Brooker et al. 1987)
Usnea atlantica Vain.	
Canary Islands ***barbas***	Used as a disinfectant, along with other *Usnea* spp. (Darias et al. 1986)
Usnea baileyi (Stirt.) Zahlbr.	
Ayurvedic medicine (India)	Occasional adulterant in ***chharila*** (see *Parmotrema nilgherrense*). Mixed with other aromatic herbs, such as *Valeriana jatamansi* for favoring and curing tobacco, along with *U. longissima*, *U. subsordida*, *Everniastrum nepalense*, *E. cirrhatum*, and *Ramalina inflata* (Shah 1998)
Usnea barbata (L.) Weber ex F.H. Wigg.	
Xhosa (South Africa)	Used to treat mammary infections in cattle, udder is washed several times with decoction of lichen. Used for indigestion in humans, tincture or decoction taken orally several times daily (Afolayan et al. 2002)
Nepal	Endangered medicinal lichen banned from raw export (Bhattarai 1999)
Ati (Philippines) ***tagahumok puti***	Used for wounds, chopped and mixed with coconut oil, spread over wound. Used for abdominal pain, drink decoction (Madulid et al. 1989)
West Malaysia	Used for colds and strengthening after confinement (Foxworthy 1922)
Europe (early modern era)	Probably synonymous with *Usnea* spp. in early modern era pharmacopoeias, which adopted the Ancient Greek uses for insomnia, nausea, and the uterus (see European use of *Usnea* spp.). Used for internal and external bleeding, whooping cough, jaundice, and growing hair (Lightfoot 1777; Willemet 1787; Luyken 1809)
Abejar (Spain)	Used as drying agent and antiseptic for cracks and irritations of the feet (Bustinza and Caballero 1947)
Mbya-Guarani (Brazil) ***memby rakú í ja*** ("master of the energy of creatures")	Liquid made from it is given to women to cure sterility (Cadogan 1949)
Usnea campestris R. Sant.	
Mendocina (Argentina) ***barba de piedra***	Unspecified medicine (Ruiz Leal 1972; Garcia et al. 1990)

(continued)

Table 2.7 (continued)

Culture and ***folk name***	Traditional use
Usnea ceratina Ach.	
China	Used for coughs, inflamed lungs, pulmonary tuberculosis, hepatitis, headache due to *heat*, infection due to injury, inflamed lymph channels, mastitis, and snakebites (Wang and Qian 2013)
Usnea densirostra Taylor	
Argentina ***yerba de la piedra***; ***barba de piedra***	Tea applied externally as astringent, antiseptic, and antiinflammatory. Also use *U. durietzii* (Bandoni et al. 1972; Garcia et al. 1990; Vitto et al. 1997; Correche et al. 2008)
Uruguay ***yerba de la piedra***	Unspecified medicine (Osorio 1982)
Usnea diffracta Vain.	
China 老君鬚 (***lao-jun-xu***), ***Lao Tzu's beard***, ***pine gauze***, or ***female gauze***	In herbals from 500 C.E., picked in 5th lunar month and dried in shade. Used for cough, tuberculosis of neck or lungs, headache, dizziness, sweating, dim vision, swelling, pus oozing from breasts or sores, burns and scalds, snakebite, traumatic injuries, bone fracture, bleeding from external injuries, vomiting blood, blood in feces, bleeding from uterus, menstrual disorders, vaginal discharge, swelling of female genitalia, urinary tract afflictions, and ascarid or schistosoma parasitic infections. Drink decoction; or apply decoction or powdered lichen to affected area (Hu et al. 1980; Sharnoff 1997; Wang and Qian 2013)
Tibet ***gser.skud*** ("gold thread")	Cures fevers of the lungs, liver, and channels and fever caused by poisoning (Clark 1995)
Korea 송낙 (***song-nag***)	Used to induce menstruation (Pusan) and treat tuberculosis of the neck (Gongju) (Lee 1966; Lee et al. 1977)
Usnea durietzii Mot. [syn. *Neuropogon durietzii*]	
San Luis (Argentina)	Same as Argentine use of *U. densirostra* (Vitto et al. 1997)
Usnea filipendula Stirt. [syn. *Usnea dasypoga*]	
Java	Unspecified medicinal use (Uphof 1959)
Primorsky and Sakhalin (Russian Far East)	Powdered form used to treat wounds and some infections (Moskalenko 1986)
Usnea florida (L.) F. H. Wigg.	
China	Used for aching in sinews and bones, stopping bleeding or infection from external injuries, skin diseases, painful urination, coughs, tuberculosis of lungs or neck, heart palpitations, and edema. Drink decoction; or apply decoction or powdered lichen to affected area (Wang and Qian 2013)
Europe (early modern)	Decoction used for colds and coughs (Willemet 1787)
Mapuche (Chile)	Infusion used for diarrhea (Houghton and Manby 1985)

(continued)

Table 2.7 (continued)

Culture and ***folk name***	Traditional use
Usnea gigas Motyka [syn. *Usnea africana*]	
Iraqw (Tanzania)	Same as Iraqw use of *U articulata* (Kokwaro 1976)
Usnea himalayana C. Bab.	
Japan ***nayonayo saruogase***	Burned as a "lichen cigarette" (Ohmura 2003)
Usnea hirta (L.) F. H. Wigg.	
Europe (early modern)	Used for heal wounds and to prevent hair loss (Willemet 1787)
Usnea laevis (Eschw.) Nyl.	
Venezuelan Andes ***barba de piedra*** or ***tusinya***	Used for infections, dermatosis, fungal infections, tuberculosis, and pneumonia (Marcano 1991; Marcano et al. 1999)
Usnea longissima Ach.	
Unani (India) ***ushna***	Used as a simple drug to stimulate menstruation or induce abortion, taken orally and inserted into the vagina (Razzack and Fazal 1993). See Unani use of *Usnea* spp.
Northern Anatolia (Turkey)	For treating cancer, tuberculosis, and ulcers (Yazici and Aslan 2003; Odabasoglu et al. 2006)
China 松蘿 (***song-luo***), ***sun-lo***	Same use in China as *U. diffracta* (Wang and Qian 2013). Also used as a decongestant and for local treatment of ulcers and tuberculosis (Vartia 1973; Richardson 1974; Hu et al. 1980)
Mongolia	Used medicinally (Laxinamu et al. 2013)
Baiga (Madhya Pradesh, India)	Used to treat bone fractures, along with other ingredients (Lal and Upreti 1995)
Indo-Tibetan Himalayas ***urmil***	Used to heal bone fractures. Washed, air-dried, soaked overnight in salted water, and placed over affected part (Sharma 1997)
Ayurvedic medicine (India)	Same as Ayurvedic use of *U. baileyi* (mixed in tobacco) and an occasional adulterant in ***chharila*** (see *Parmotrema nilgherrense*) (Shah 1998)
Haida (BC, Canada)	Same as Haida use of *Alectoria sarmentosa* (Turner 1998, 2004a)
Ditidaht (BC, Canada) ***p'u7up*** or ***Indian bandage***	All *Usnea* spp. and *Alectoria sarmentosa* used for wound dressing, but *U. longissima* is preferred. Wrapped around wound and left a while (Turner et al. 1983)
Usnea nidifica Tayl.	
China	Unspecified medicine (Wang and Qian 2013)
Raratongan (Cook Is.) ***'uru nū*** (Mangiai), ***remu nū*** (Mauke)	Online reference to medicinal usage on Mangiai: thallus chewed and applied to cuts (to stop bleeding) and stings (McCormack 2007). Whistler (1990) records ***remu*** as a general term for lichens, mosses, and seaweeds, but records no use

(continued)

Table 2.7 (continued)

Culture and ***folk name***	Traditional use
Usnea pectinata Tayl.	
China	Used for stopping bleeding from external injuries, relieving pain, bloody feces, and swelling (Wang and Qian 2013)
Usnea plicata (L.) Weber	
Tripolitania (Libya) ***scíba***	Ingredient in medicinal decoction called ***scíba***, along with *Pseudevernia furfuracea*, *Ramalina calicaris*, and *R. farinacea* (Natale and Pollio 2012)
Saami (Scandinavia)	Put on sores on feet after walking long distances (Linnaeus 1737)
Europe (early modern)	An astringent for internal and external use (Lightfoot 1777), for whooping cough (Lindley 1838), jaundice, strengthening stomach and abdominal cavity, and restraining abortion (Luyken 1809). Also recommend *U. barbata*. See European use of *Usnea* spp.
Usnea sikkimensis Biswas sp. nov.	
Sikkim and Darjeeling (India) ***darimataghosa*** (Bengali)	Used for lung troubles, hemorrhages, and asthma; powdered and used to strengthen hair (Biswas 1956) (may be a European use)
Lepchas (Dzongu, Sikkim, India)	Used to bandage surface wounds, skin eruptions, and boils; inserted into nostril to stop nose bleeds; put in shoes to prevent or treat blisters (Pradhan and Badola 2008)
Usnea strigosa (Ach.) Eaton	
Kimi (Amusa, Papua New Guinea) ***oleazu***	Concoction taken orally for headaches (Jorim et al. 2012)
Usnea subfloridana Stirt.	
Leitrim (Ireland)	Treatment for sore eyes. Mixed with tobacco and butter, boiled, cooled, and applied as lotion to eyes (Allen and Hatfield 2004)
China	Used for painful and reddened eyes, bleeding from external injuries, and swelling (Wang and Qian 2013)
Usnea subsordida Stirt.	
Ayurvedic medicine (India)	Same as Ayurvedic use of *U. baileyi* (mixed in tobacco) and an occasional adulterant in ***chharila*** (see *Parmotrema nilgherrense*) (Shah 1998)
Usnea trichodeoides Vain.	
China	Used for coughs; pulmonary tuberculosis; headaches; blurred vision; inflamed cornea; swellings, sores, and pus discharge; bleeding from external injuries; bloody feces; uterine bleeding; menstrual disorders; and vaginal discharge. Drink decoction; or apply decoction or powdered lichen to affected area (Wang and Qian 2013)

Table 2.8 Cetrarioid lichens (Parmeliaceae) used in traditional medicines

Culture and ***folk name***	Traditional use
Cetraria islandica (L.) Ach.	
Europe (1600s to present)	Medicinal lichen in European pharmacopoeias from the 1600s to present. Common throughout Europe and Greenland, mostly for pulmonary and digestive uses. Used for salves and as a mild mucilaginous tonic. Used for pulmonary tuberculosis, coughing blood, asthma, chronic congestion, a laxative, indigestion, and dysentery. Has also been recommended for uterine cysts, kidney stones, edema, wounds, and scurvy (Ray 1686; Linnaeus 1737; Scopoli 1760; Cramer 1780; Willemet 1787; Withering 1801; Lindley 1838; Anonymous 1845; Rink and Lindorff 1856; Fink 1906; Kartnig 1980)
Estonia	Tea taken as anticancer remedy (Sak et al. 2014)
Venezia Giulia (Italy)	Used for congestion and for recovery after tuberculosis (Lokar and Poldini 1988)
Ubaye Valley (France)	Decoction used for lung ailments and as an emollient (Novaretti and Lemordant 1990)
Pallars (Spain) ***liquen de bosc***	Drunk as tea for congestion, tuberculosis, asthma, inflammation, and high blood pressure (Muntané 1991; González-Tejero et al. 1995; Agelet and Vallès 2003)
Sweden ***islandslav***	Used for whooping cough, colds, congestion, asthma, other chest ailments, appetite stimulation, diabetes, nephritis, and tuberculosis. Either decoction or infusion made from dried shredded lichen in either water or milk and drunk either warm or cold. Honey or chocolate sometimes added (Ahmadjian and Nilsson 1963)
Ket (Siberia)	Decoction for coughs (Eidlitz 1969)
China	Decoction drunk to strengthen stomach and improve digestion (Wang and Qian 2013)
Dehcho (NWT, Canada)	Decoction used to treat tuberculosis. Boiled in water 0.5–1 h, until liquid is red, and one third cup is taken 3 times daily (Lamont 1977)
Cetrelia pseudolivetorum (Asahina) W.L. Culb. & C.F. Culb.	
China	Same as Chinese use of *Anzia opuntiella* (Wang and Qian 2013)
Flavocetraria cucullata (Bellardi) Kärnefelt & A. Thell	
Pallars (Spain)	Drunk as tea to treat symptoms of asthma (Agelet and Vallès 2003)
Flavocetraria nivalis (L.) Kärnefelt & Thell	
Europe	Although not as commonly used in Europe as *Cetraria islandica*, some practitioners thought it had similar properties (Tychsen 1799; Lindley 1838)
Kallawaya (Qollahuayas, Bolivia)	Prepared in tea for treatment of motion sickness and heart attacks (Bastien 1983)
Masonhalea richardsonii (Hook.) Kärnefelt [syn. *Cornicularia richardsonii*]	
Tlingit (Alaska, BC)	Used as a treatment for inflammation of the lungs (Garibaldi 1999)
Nephromopsis pallescens (Schaer.) Park	
China	Eaten, and has an unspecified medicinal use (Wang and Qian 2013)
Vulpicida canadensis (Räsänen) J.E. Mattsson & M. J. Lai	
Ulkatcho (BC, Canada) ***dahgha*** [“limb hair”]	Medicine for coughs and colds, drink tea made from a couple handfuls of ***dagha*** in 1 L water. Also chewed fresh to help the lungs (Hebda et al. 1996)

(continued)

Table 2.8 (continued)

Culture and ***folk name***	Traditional use
Vulpicida juniperinus (L.) J.E. Mattsson & M.J. Lai	
Scandinavia	Possibly used to poison wolves along with *Letharia vulpina* (Uphof 1959)
Vulpicida pinastri (Scop.) J.E. Mattsson & M.J. Lai	
Scandinavia	Possibly used to poison wolves along with *Letharia vulpina* (Smith 1921)
China	Used for pulmonary tuberculosis, wounds oozing pus, skin infections, cancer, and spasms (Wang and Qian 2013)

Table 2.9 Parmelioid lichens (Parmeliaceae) used in traditional medicines

Culture and ***folk name***	Traditional use
Unidentified parmelioid lichens	
Unspecified (Cape area, South Africa) ***klipbolm***	Infusion is drunk for syphilis in men, back pain, and kidney trouble; mouthwash for oral thrush and teething children (Laidler 1928; Van Wyk et al. 2008; De Beer and Van Wyk 2011). Used for cancer, women's problems, aiding fertility, and inducing abortion (Aston Philander 2011).
KhoiSan (Cape area, South Africa) ***klipblom***, ***klipmos***, or ***klipbuchu***	Used as a female medicine for ***maak baarmoeder skoon*** ("cleaning the womb"), treating general pains (especially back and kidneys), an ointment for burns and wounds, colds, and bladder diseases (De Beer and Van Wyk 2011). Infusion used for cough, sore throat, fertility, oral thrush in infants, abdominal pain, backache, and kidney and bladder diseases (van Wyk and Gericke 2000).
Nepal ***jhau***	Extract and decoction are applied to treat moles (Gaire and Subedi 2011)
Lucca (Italy)	Decoction for coughs, cleansing liver, and antiinflammatory (Pieroni 2000)
Piaroa (Amaz., Venezuela) ***odoche jupacua*** (iguana toe)	Used to treat gonorrhea or "painful urination." Boiled into a tea and drunk 3–4 times a day for a week (Azenha et al. 1998)
Guahibo (Amaz., Venezuela)	Boiled in water and applied to insect bites or cuts and wounds (Azenha et al. 1998)
Everniastrum nepalense (Taylor) Hale ex Sipman [syn. *Parmelia nepalensis*]	
Ayurvedic medicine (India)	Same as Ayurvedic use of *Usnea baileyi* (mixed in tobacco) and an occasional adulterant in ***chharila*** (see *Parmotrema nilgherrense*) (Shah 1998)
Taplejung (Nepal) ***jhyau***	Used like *Ramalina* spp. for antiseptic, burns, and wounds. Applied as powder in tincture of iodine after applying the leaf juice of *Artemisia dubia* or *Eupatorium adenophorum* (Poudel 2008). Banned from raw export (Bhattarai 1999)

(continued)

Table 2.9 (continued)

Culture and ***folk name***	Traditional use
Kathmandu (Nepal) ***kalo jhyau***	Used for toothache, sore throat, and pain (Kumar et al. 1996)
Everniastrum cirrhatum (Fr.) Hale ex Sipman [syn. *Parmelia kamtschadalis*]	
India	Same as Ayurvedic use of *Usnea baileyi* (mixed in tobacco) and an occasional adulterant in ***chharila*** (see *Parmotrema nilgherrense*) (Shah 1998). When burnt the smoke relieves headache and the powder is a good cephalic snuff (Biswas 1947; Nadkarni and Nadkarni 1955).
Flavoparmelia caperata (L.) Hale	
Tarahumar (Mexico) ***ŕeté cajéra***	Dried, crushed, and dusted on burns (Pennington 1963)
China	Decoction drunk to clear ***heat*** (Wang and Qian 2013)
Parmelia hyporysalea (Vain.) Vain	
Ayurvedic medicine (India)	Occasional adulterant in ***chharila*** (see *Parmotrema nilgherrense*) (Chanda and Singh 1971)
Parmelia omphalodes (L.) Ach.	
Britain ***crottle***, ***crotal***, ***dark crottle***, or ***fiasgag nan creag*** (Gaelic: "rock lichen")	In Scotland, they wore socks dyed with ***crottle*** if walking long distance; or sprinkled it on their hose to stop their feet from getting inflamed (Cameron 1900; MacIntyre 1999). Used for a soup to strengthen invalids in Ireland; and for a poultice for cuts, sores, and burns in Ireland and Scotland (McGlinchey 1986; Allen and Hatfield 2004). Probably used interchangeably with *P. saxatilis*
Europe (early modern era)	Used to stop bleeding and stop hemorrhage during surgery. Put into nose to stop nosebleeds (Gedner 1756; Willemet 1787)
Parmelia saxatilis (L.) Ach.	
China 石花 (***shi-hua***)	Used for blurred vision, vomiting blood, jaundice, bleeding from uterus, chronic dermatitis, and oral ulcers in children. Drink decoction in wine or apply powder to affected area (Hu et al. 1980; Wang and Qian 2013)
Dalarna (Sweden) ***stenlav***	Used to remove warts (Ahmadjian and Nilsson 1963)
Britain ***crottle*** or ***light crottle***	Probably used interchangeably with *P. omphalodes* as ***crottle***
Foula (Shetland Is, Britain) ***old man***	Mixed with tobacco and smoked in the eighteenth century, a practice still remembered in 1966 (Hawksworth 2003)

(continued)

Table 2.9 (continued)

Culture and ***folk name***	Traditional use
Europe ***usnea cranii humani***, ***muscus cranii humani***, or ***muscus ex cranio humano*** (Latin); ***moss of a dead man's skull*** (English); ***usnée humaine*** (French); ***muschio del cranio*** (Italian)	An important medicine as early as the late 1500s (Gerarde 1597) and throughout the 1600s (Parkinson and Marshall 1640; Ray 1686), but various authors think it quackery by the 1700s (Quincy 1724; Diderot et al. 1765). In early drawings it is distinctly *Usnea*-like, but later authors recognize two distinct types: *Usnea*-like or crust-like (James 1748). In modern times, it has been identified as either *Parmelia saxatilis* (Smith 1921) or *Physcia* sp. (Llano 1948), although it is likely any lichen or moss found on a skull (Modenesi 2009). When collected off the skull of criminals (alt. someone who died a violent death), it was very valuable as a cure for epilepsy, to stop bleeding, and (if powdered and given in sweet wine) for whooping cough in children. Also mixed with ***mumia*** (the exudate from a mummy) to make ***unguentum armarium***, a salve that was applied to a weapon to heal a wound that it had caused
Parmelia sulcata Taylor	
Metís (Alberta, Canada)	Rubbed on gums of teething babies to relieve discomfort (Marles et al. 2000)
Saanich (BC, Canada) ***smexdáles***	Medicinal properties depend on type of tree it is growing on. Possible the lichen traditionally used for birth control. Not differentiated from *Lobaria pulmonaria* (Turner and Hebda 2012)
Parmotrema abessinicum (Nyl. ex Kremp.) Hale	
Bellary District (India) ***rathipuvvu*** ("rock flower")	Eaten medicinally (Llano 1948)
Parmotrema nilgherrense (Nyl.) Hale	
Ayurvedic medicine (India) ***chharila*** (Hindi), ***shaileya*** or ***shilapushpa*** ("rock flower") (Sanskrit), ***shailaja*** (Bengali), ***chadila*** (Urdu), ***pathar phool*** (Gujarati), ***dagad phool*** (Gujarati and Marathi), ***kallu hoovu*** (Kannada), ***rati puvvu*** (Telugu), ***sheeleyam*** (Malayalam), ***kapashwe*** (Tamil)	An important drug in many old Ayurvedic texts that is still used today. The first record is in the Atharvaveda (1500 B.E.). Although in some areas of India, high-quality ***chharila*** is mostly *Parmotrema nilgherrense*, the lichen mixture can also contain *Parmotrema chinense*, *P. perforatum*, *P. perlatum*, *Everniastrum cirrhatum*, and *E. nepalense*, with the occasional adulterants *Ramalina farinacea*, *R. inflata*, *Usnea baileyi*, *U. longissima*, *U. subsordida*, *Parmelia hyporysalea*, *Anaptychia* spp., and *Leptogium* spp. It is used for indigestion, loss of appetite, flatulence, diarrhea, stomach disorders, kidney stones, painful urination, hemorrhoids, involuntary semen emission, lack of menstruation, painful menstruation, enlarged spleen,

(continued)

Table 2.9 (continued)

Culture and ***folk name***	Traditional use
	bronchitis, congestion, shortness of breath, excessive salivation, fevers, headaches, sore throats, toothaches, broken bones, musculo-skeletal pain, rheumatism, reducing swelling, leprosy, scabies, soothing irritated skin, and prenatal and postnatal care. Also used as an aphrodisiac, diuretic, sedative, astringent, antiseptic, antibiotic, and a demulcent to reduce inflammation. It is powdered and applied on wounds to promote healing, smoked to relieve headaches, used as incense, used as a cephalic snuff, used in medicated oils, applied as a poultice to renal and lumbar regions to induce urination, and applied as a liniment to the head for headaches. (Dutt 1877; Chanda and Singh 1971; Kumar and Upreti 2001; Karadi 2010; Prasad 2013) An ingredient in ***spemen***, which is used for treating infertility in men (Pardanani et al. 1976)
Ayurvedic medicine (other countries) ***jhoola*** (Nepal)	Nepal: soup as an aphrodisiac, paste applied externally for kidney stones. China: soup for male infertility, paste applied externally for kidney stones. Malaysia: soup as an aphrodisiac and for seminal weakness. Afghanistan: used for chest disorders, paste applied to wounds for healing. Saudi Arabia: cephalic snuff for headaches and as a pain killer (Kumar et al. 1996; Karadi 2010)
Kathmandu (Nepal) ***kalo jhyau***	Used for toothache, sore throat, and pain (Kumar et al. 1996)
Parmotrema perforatum (Jacq.) A. Massal.	
Ayurvedic medicine (India)	Commonly used as ***chharila*** (see *P. nilgherrense*) (Nadkarni and Nadkarni 1955; Chanda and Singh 1971). Imported for medicine (Younos et al. 1987) and used for diuretic treatments (Biswas 1947)
Parmotrema perlatum (Huds.) M. Choisy	
Ayurvedic medicine (India)	Commonly used as ***chharila*** (see *P. nilgherrense*) (Nadkarni and Nadkarni 1955; Chanda and Singh 1971)
Parmotrema reticulatum (Taylor) M. Choisy	
Tepehuan and mestizos (Chihuahua, Mexico) ***ódai yoošígai*** or ***flor de piedra*** ("rock flower")	Tea drunk to relieve discomfort from kidney disorders or venereal disease. The tea is commonly prepared in late afternoon and left for one night before being drunk (Pennington 1969)

(continued)

Table 2.9 (continued)

Culture and ***folk name***	Traditional use
Parmotrema sancti-angelii (Lynge) Hale	
Gond and Oran (Uttar Pradesh, India ***jhavila***	Salve used to treat skin disease called ***sem***. Burn 30–50 g of ***jhavila*** and mix ash with mustard or linseed oil (Lal and Upreti 1995)
Parmotrema subtinctorium (Zahlbr.) Hale	
China	Used for bleeding from external injury, localized swelling and pain (Wang and Qian 2013)
Parmotrema tinctorum (Nyl.) Hale	
China	Used for blurred vision, bleeding from uterus, bleeding from external injuries, sores and swelling, chronic dermatitis, and localized swelling. Drink decoction or apply powdered lichen to affected area (Wang and Qian 2013)
Parmotrema zollingeri (Hepp) Hale	
Ati (Philippines) ***kalas***	Used as medicine for children with high fever and suffering from convulsions. Burn ***kalas*** and let the child smell the fumes (Madulid et al. 1989)
Punctelia borreri (Sm.) Krog	
China	Used for blurred vision, bleeding from uterus, bleeding from external injuries, sores and swelling, and chronic dermatitis. Drink decoction or apply powdered lichen to affected area (Wang and Qian 2013)
Xanthoparmelia spp. (Vain.) Hale	
Navajo (AZ and NM, USA) New Mexico: ***tschétláat*** ("rock covering"), ***nihalá·d*** ("earth moss"), or ***célá·d*** ("rock moss") Arizona: ***owa'si*** ("rock flower") or ***owa'huru'suki*** ("rock manure")	New Mexico: Elmore (1943) records ***tschétláat*** as remedy for impetigo. Wyman and Harris (1941) record widespread use of ***nihalá·d*** or ***célá·d*** chewed for canker, swollen gums, decayed teeth, etc. (may include *Peltigera* sp.). Arizona: Whiting (1939) records an unidentified rock lichen called ***owa'si*** or ***owa'huru'suki*** used for sore mouth, gums, and toothache. See also Hopi use of yellow rock lichen; and Tewa use of rock and ground lichen
Xanthoparmelia conspersa (Ehrh. ex Ach.) Hale	
Xhosa (South Africa) ***ubulembu belitye***	To treat syphilis eruptions: powder and apply externally to eruptions (perhaps after they are scarified); may be also used internally (sources disagree). To treat both known and suspected snakebites: drink one tablespoon of lichen in cold water; also scarify bite and sprinkle powdered lichen on it to draw out a ***humour***. See also Xhosa use of "unidentified rock lichen" (Smith 1888; Watt and Breyer-Brandwijk 1962)

(continued)

Table 2.9 (continued)

Culture and ***folk name***	Traditional use
Iroquois (Ontario, Canada)	Used for inflamed gums and raw throat caused by fever. Mix in 1 cup cold water with the bark of the tree it was collected off, *Coptis trifolia*, and *Fraxinus nigra*. Take one teaspoon, leave in mouth until water is warm, and then swallow. Repeat for entire cup (Herrick 1995)
O'odham (Arizona, USA) ***jievut hiawsik*** or ***jewed hiósig*** ("earth flower")	Traditional use described by Curtin (1949). Lipp (1995) identified the lichen as *X. conspersa*, but Hawksworth (2003) disagrees. Carried as good luck charm, but overuse will make you sick. Mixed with tobacco and smoked to "make young men crazy." Also ground into a powder and sprinkled on sores or cuts, but not bound, as it would cause blisters. Applied over several days to heal rattlesnake bite
Xanthoparmelia convoluta (Kremp.) Hale [*Xanthomaculina convoluta*]	
Khoikhoi (Namibia)	Infusion taken as remedy for rheumatism and arthritis. See also Topnaar [a Khoikhoi tribe] use of *X. hottentotta* (Watt and Breyer-Brandwijk 1962)
Xanthoparmelia hottentotta (Ach.) A. Thell et al. [syn. *Xanthomaculina hottentotta*]	
Unspecified (Namibia)	Used to treat inflammation of udder for goats and sheep. Dried, roasted, and powdered; mixed with aromatic shrubs, fungal spores, and very fine quartz dust; then added to tail-fat to make an ointment for the udder (Epstein 1937)
Topnaar (Kuiseb, Namibia) **ǀ *ui* ǁ *khaob***; or ***uijkhao.b.***	Decoction drunk to cure coughs and to relieve stomach and chest pains (van Damme et al. 1992)
Xanthoparmelia scabrosa (Taylor) Hale	
New Age herbalism	Currently sold as "traditional Chinese medicine," as an aphrodisiac, and a cure for male impotence. No record of this use was found prior to 2007 (Tshiteya 2007)
Xanthoparmelia tinctina (Maheu & Gillet) Hale	
China	Used for blurred vision, bleeding from uterus, bleeding from external injuries, sores and swelling, and chronic dermatitis. Drink decoction; or apply decoction or powdered lichen to affected area (Wang and Qian 2013)

Table 2.10 Other Parmeliaceae lichens used in traditional medicines

Culture and ***folk name***	Traditional use
Evernia divaricata (L.) Ach.	
China	Used for coughs, pneumonia, hot flashes due to pulmonary tuberculosis, hepatitis, headaches, infection due to trauma, inflammation of the breasts, and snakebites (Wang and Qian 2013)
Evernia prunastri (L.) Ach.	
Ancient Greece **σπλάγχνον** (***splanchon***, "intestines") or **βρῦον** (***bryón***, "moss")	An arboreal lichen recorded by Dioscorides in Section 1.21 of De Materia Medica (50–70 C.E.) that is best if sweeter-smelling, whiter, and growing on cedar. Probably refers to *E. prunastri* and *Pseudevernia furfuracea*, with *E. prunastri* being preferred (Richardson 1974). Used in ointments for an astringent; decoction used either hot or cold for washing the vulva for diseases of the womb; and used as a remedy against fatigue (López Eire et al. 2006)
Europe (early modern era) ***oak moss***, ***mousse chêne***, or ***eichenmoss***	Ancient Greek uses adopted in Europe starting in 1500s, but originally applied to all fruticose arboreal lichens. By late 1700s these uses often applied to *Usnea* spp., but sometimes to *Evernia prunastri*. Used for uterine and anal prolapse and for preventing abortion (Quincy 1724; Willemet 1787; Luyken 1809). Also used for intestinal weakness, fevers, and pulmonary afflictions (Lindley 1838; Uphof 1959). Mixed with *Pseudevernia furfuracea* and *Hypogymnia physodes* as ***Lichen quercinus virdes***, a popular drug in early modern Europe (Senft 1911; Llano 1948)
Evernia mesomorpha Nyl.	
Chipewyan (SK, Canada) ***k'i tsa^{n}jú*** ("birch lichen")	Treatment for snow blindness. Harvest from birch, boil, cool, and drop decoction into eyes (Marles 1984)
China	Same use in China as *E. divaricata* (Wang and Qian 2013)
Pseudevernia furfuracea (L.) Zopf [syn. *Evernia furfuracea*, *Borrera furfuracea*]	
Ancient Egypt	Found in a vase in a tomb dated to the fourteenth to sixteenth century B.E., along with other medicinal plants (Müller 1881). Used, along with other botanicals, to stuff mummies (Baumann 1960). Does not currently grow in the area, potentially imported from elsewhere
Ancient Greece	See Ancient Greek use of *Evernia prunastri*
Tripolitania (Libya) ***scíba***	Ingredient in medicinal decoction called ***scíba*** from early 1900s, along with *Usnea plicata*, *Ramalina calicaris*, and *R. farinacea* (Natale and Pollio 2012)
Europe (early modern era) ***treemoss***, ***mousse d'arbre*** (French)	A substitute for quinine (Willemet 1787). Used for fevers and as an astringent (Lindley 1838). Mixed with *Evernia prunastri* and *Hypogymnia physodes* as ***lichen quercinus virdes***, a popular drug in early modern Europe (Senft 1911; Llano 1948)
Kutahya (Turkey)	Used for wounds, eczema, and hemorrhoids. Put in healing cream with clay (Güvenç et al. 2012)

(continued)

Table 2.10 (continued)

Culture and ***folk name***	Traditional use
Pallars (Catalonia, Spain) ***liquen***	Drunk as tea for asthma, congestion, and high blood pressure (Agelet and Vallès 2003)
Alfacar, Víznar (AN, Spain) ***musgo***	Used for respiratory ailments. Washed, boiled for a considerable time, and then drunk (González-Tejero et al. 1995)
Jaén (AN, Spain) ***líquen de pino*** or ***muedos***	In Villanueva del Arzobispo it is collected and sold for very good medicines (Fernández Ocaña 2000)
Letharia vulpina (L.) Hue [syn. *Evernia vulpina*]	
Sweden ***ulf-mossa***	Used for wolf poison in Sweden. Pulverized, mixed with fat and flesh, warmed in pan over fire, and then add fresh blood and cheese to create odor. Sometimes mix with powdered glass or strychnine. Put under skin of carcass, wolf will die within 24 h of ingestion. Older, drier lichen is more potent (Withering 1801; Schade 1954)
Niitsitapii (Alberta, Canada) ***e-simatch-sis*** ("yellow dye")	Infusion of the lichen and bone marrow for stomach disorders like ulcers. Lichen was blackened in a fire and rubbed on a rash, eczema, and wart sores (McClintock 1910; Hellson and Gadd 1974)
Okanagan (BC, Canada) ***kwarē'uk*** or ***kwernúkw***	Weak decoction drunk for internal problems and strong decoction used to wash external sores and wounds (Teit and Boas 1928; Turner et al. 1980)
Umatilla and Cayuse (Oregon, USA) ***laxpt*** or ***maqa'hl***	Boiled and then applied as a poultice for open sores, boils, bruises, swellings, arthritis, and eye problems. Also used for saddle sores on horses. Liquid also drunk for hemorrhaging (Hunn 1990, 2005)
Achomawi (California, USA)	Used for poison arrows. Tips imbedded in masses of wet lichen and left for up to a year, rattlesnake venom sometimes added (Merriam 1966)
Yuki and Wailaki (CA, USA) ***ōl-gät'-i***	Medicine for inflammation and to dry up running sores (Chesnut 1902; Mead 1972)
Lethariella cashmeriana Krog	
Naxi (nw Yunnan, China) ***luxingcha*** or ***hongxuecha***	Traditional Tibetan health-promoting tea for reducing blood pressure, body fat, and inflammation. Boiling water is added to dry thalli in a cup, and the infusion is drunk after 3–5 min. Also drunk non-medicinally (Wang et al. 2001; Fu et al. 2005)
Lethariella cladonioides (Nyl.) Krog	
China and Tibet (nw Yunnan) ***gangge*** (Tibet), ***jin shua ba*** (China), ***hongxuecha***	Used for health-promoting tea to tranquilize the mind and treat a decrease in vital energy, schizophrenia, and epilepsy. Also used for reducing inflammation, relieving pain, and burns and scalds. Drunk as decoction or tea; or powder applied to affected area (Zhang and Hu 1981; Fu et al. 2005; Wang and Qian 2013; Ju et al. 2013)
Lethariella sernanderi (Mot.) Obermayer	
Naxi (nw Yunnan, China)	Same as Naxi use of *L. cashmeriana* (Wang et al. 2001; Fu et al. 2005)

(continued)

Table 2.10 (continued)

Culture and ***folk name***	Traditional use
Lethariella sinensis Wei & Jiang	
Naxi (nw Yunnan, China)	Same as Naxi use of *L. cashmeriana* (Wang et al. 2001)
Lethariella zahlbruckneri (Du Rietz) Krog	
China ***hongxuecha***	Used for aching back and weak legs, paralysis, menstrual disorders, vaginal discharge, dizziness, impotency, and epilepsy. Drink decoction or make tea or wine; or apply powder to affected area (Fu et al. 2005; Wang and Qian 2013)
Hypogymnia physodes (L.) Nyl. [syn. *Parmelia physodes*]	
Europe (early modern era)	Mixed with *Evernia prunastri* and *Pseudevernia furfuracea* as ***lichen quercinus virdes***, a popular drug in early modern Europe (Senft 1911; Llano 1948).
Neshnabé (WI, USA) ***wa'kwûnûk*** ("egg bush")	Eaten raw as a cure for constipation (Smith 1933)
Hypogymnia hypotrypa (Nyl.) Rass.	
China	Used for dim vision, bleeding from uterus, bleeding from external injury, chronic dermatitis, and sores. Drink decoction with 3–9 g lichen one time; or apply decoction or powdered lichen to affected area (Wang and Qian 2013)
Anzia opuntiella Müll. Arg.	
China	Used for blurred vision, bleeding from uterus, traumatic bleeding, sores, and chronic psoriasis. Drink decoction; or apply decoction or powdered lichen to affected area (Wang and Qian 2013)
Anzia ornata (Zahlbr.) Asahina	
China	Same as Chinese use of *A. opuntiella* (Wang and Qian 2013)

Table 2.11 Physciaceae used in traditional medicines around the world

Culture and ***folk name***	Traditional use
Anaptychia spp. Körber	
Ayurvedic medicine (India)	Occasional adulterant in ***chharila*** (see *Parmotrema nilgherrense*) (Chanda and Singh 1971)
Heterodermia diademata (Taylor) D. D. Awasthi	
Nepali (Sikkim, India) ***dhungo ku seto jhua***	Used for cuts and injuries. Leaves of *Ageratina adenophora* are made into paste and put on cuts and then plastered with paste of lichen thalli to protect it from water and any other infection (Saklani and Jain 1994)
Physcia spp. (Schreber) Michaux	
Europe (early modern era) ***usnea cranii humani***	See European use of ***usnea cranii humani*** under *Parmelia saxatilis* (Llano 1948)

Table 2.12 Ramalinaceae used in traditional medicines around the world

Culture and ***folk name***	Traditional use
Niebla bourgeana (Mont. ex Nyl.) Rundel & Bowler	
Almeria (Spain) ***flor de piedra*** ("stone flower")	Decoction used as diuretic to treat renal lithiasis. One cup taken daily until patient is better (González-Tejero et al. 1995; Martínez-Lirola et al. 1996)
Niebla flaccescens (Nyl.) Rundel & Bowler	
Quechua (Pampallacta: Peru) ***papel-papel***	Drink infusion for coughs. Thallus also chewed with coca leaves for magic rituals (Velasco-Negueruela et al. 1995)
Ramalina spp. Ach.	
Ancient Greece **Λειχήν** (***leikhēn***) or **βρύον** (***bryón***, "moss")	A cryptogam growing on wet rocks is recorded by Dioscorides in Section 4.53 of De Materia Medica (50–70 C.E.). Could be any saxicolous lichen or bryophyte. Early European herbals interpret it as a thalloid liverwort (e.g., L'Obel 1576). Recent interpretation is as *Ramalina* sp., as this matches with the original drawing (López Eire et al. 2006; Yavuz 2012). Applied as a poultice, it stops bleeding, relieves inflammation, and cures ***lichen*** (the skin disease). Mixed with honey it cures jaundice, and smeared on the mouth and tongue, it relieves colds and congestion. Pliny records a similar cryptogam in his Naturalis Historia (77 C.E.) that is dry, is white, and grows on rocks near streams. It is put on wounds to stop bleeding and used to cure jaundice and impetigo. It has been interpreted as a thalloid liverwort, *Peltigera canina*, or *Ochrolechia parella* (Bostock and Riley 1855; Yavuz 2013)
Taplejung (Nepal)	Same as Taplejung use of *Everniastrum nepalense* (topical antiseptic) (Poudel 2008)
Kanikkars (Tamil Nadu, India) ***kalchadai***	Used in combination with dried elephant milk, silt stone, and *Cuminum cyminum* extract to cure small pox (Nagendra Prasada et al. 1996)
Ramalina calicaris (L.) Fr.	
Tripolitania (Libya) ***scíba***	Ingredient in medicinal decoction called ***scíba***, along with *R. farinacea*, *Usnea plicata*, and *Pseudevernia furfuracea* (Natale and Pollio 2012)
Ramalina capitata (Ach.) Nyl.	
Pallars (Spain) ***liquen***	Drunk as tea to relieve symptoms of asthma (Agelet and Vallès 2003)
Ramalina conduplicans Vain.	
Yi, Dai, and Han (s. Yunnan, China) ***shouxu***, ***shikuacai***, or ***shuhua***	Cold dish served at marriage banquets, couples who eat it will love each other more and never separate. Boiled in water with soda for 10–20 min, soaked in new water for 1–2 days, and served with chili powder, salt, and other seasonings (Wang et al. 2001). Medicine to reduce inflammation (Wang and Qian 2013)
Ramalina farinacea (L.) Ach.	
Ayurvedic medicine (India)	Occasional adulterant in ***chharila*** (see *Parmotrema nilgherrense*) (Shah 1998)
Tripolitania (Libya) ***scíba***	Ingredient in medicinal decoction called ***scíba***, along with *R. calicaris*, *Usnea plicata*, and *Pseudevernia furfuracea* (Natale and Pollio 2012)

(continued)

Table 2.12 (continued)

Culture and ***folk name***	Traditional use
Nigeria	Aqueous extract for treating mental disorders. Tinctures for treatment of ringworm tinea (Esimone and Adikwu 1999)
Ramalina inflata Hooker f. & Taylor	
Ayurvedic medicine (India)	Same as Ayurvedic use of *Usnea baileyi* (mixed in tobacco) and an occasional adulterant in ***chharila*** (see *Parmotrema nilgherrense*) (Shah 1998)
Ramalina menziesii Taylor	
Pomo (California, USA)	Used as baby diapers (Goodrich et al. 1980)
Ramalina roesleri (Hochst.) Hue	
China	Used for traumatic injuries, bleeding, and swelling (Wang and Qian 2013)
Ramalina sinensis Jatta	
Yunnan (China)	Same as Chinese use of *R. conduplicans* (Wang et al. 2001)

Table 2.13 Stereocaulaceae used in traditional medicines around the world

Culture and ***folk name***	Traditional use
Stereocaulon exutum Nyl.	
China	Same as Chinese use of *S. paschale* (Wang and Qian 2013)
Stereocaulon himalayense Asahina & I.M. Lamb	
Lepchas (Darjeeling, India) ***dhungo-ku-jhau***	Thalli pounded and boiled in water; take 100 ml twice daily after meals for burning sensation when urinating or other urinary trouble; decoction also used for tongue blisters (Saklani and Jain 1994)
Indo-Tibetan Himalayas ***chanchal***	Decoction used to treat urinary infections. Entire lichen boiled in water or goat's milk (Sharma 1997)
Stereocaulon paschale (L.) Hoffm.	
Mistissini Cree (Quebec) ***wapskirnok***	Used to treat rheumatism/arthritis associated with diabetes (Fraser 2006; Leduc et al. 2006)
China 石寄生 (***shi-ji-sheng***)	Used for spontaneous external bleeding, other bleeding, and dizziness. Drink decoction (Hu et al. 1980; Wang and Qian 2013)
Stereocaulon vulcani (Bory) Ach.	
Réunion ***fleur de roche*** or ***fleur galet***	Boiled to treat ulcers. Roasted and used to treat cankers. Mixed with sulfur, *Hubertia ambavilla* [endemic shrub], and coconut oil to make an ointment for wounds. Used in a drink to stop vaginal discharges. Boiled in water with handful of *Hylocereus undatus* roots [cactus], *Tribulus cistoides*, and a piece of *Argemone mexicana* root [poppy] and drunk to treat syphilis (Lavergne 1989)

Table 2.14 Collemataceae used in traditional medicines around the world

Culture	Traditional use
Leptogium spp. (Ach.) Gray	
Ayurveda (India)	Occasional adulterant in ***chharila*** (see *Parmotrema nilgherrense*) (Chanda and Singh 1971)

Table 2.15 Lobariaceae used in traditional medicines around the world

Culture and ***folk name***	Traditional use
Lobaria spp. (Schreber) Hoffm. [partial syn. *Sticta* spp.]	
Bhutan	Pulverized and made into a paste to cure skin diseases (Søchting 1999)
Northwest Yunnan (Tibet) ***qingwapi***	Whole plant used to treat indigestion (Ju et al. 2013)
Gitksan (BC, Canada) ***gwilalh ganaaw*** ("frog blankets")	Used as arthritis medicine, a tonic, and a spiritual health-promoting and purification treatment. Aqueous infusion used as tea or a bath (Johnson 1997)
Haida (BC, Canada) ***kayd gyaa'ad*** ("tree blanket")	Ingredient in several different medicinal mixtures. Also called ***hlk'inxa kwii'awaay*** ("forest cloud") or ***xil kwii.awaa*** ("cloud leaves") (Turner 2004a)
Nuxalk ***sts'wakt-aak***	Used for stomach pains, but not diarrhea, constipation, or vomiting. Only collected from *Cornus stolonifera* [dogwood] or *Pyrus diversifolia* [crabapple], boiled, and five cups of hot decoction are drunk daily. Decoction also used as an eyewash. Also, plant is pulverized and applied to skin (Smith 1929; Turner 1973)
Makah (Washington, USA) ***didi'dichia*** ("growing on rocks")	When found on rocks it is used for running sores that are hard to heal, especially sores on the leg caused by bruises from walking among rocks (Densmore 1939). The identity of this lichen is uncertain: most *Lobaria* and *Sticta* species grow on trees
Lobaria isidiosa (Müll. Arg.) Vain.	
China 老龍皮 (***lao-long-pi***)	Used for indigestion, reducing inflammation, relieving pain, burns and scalds, edema due to kidney inflammation, and malnutrition in children (Hu et al. 1980; Wang and Qian 2013)
Lobaria kurokawae Yoshim.	
China	Same as Chinese use of *L. pulmonaria*, but not used for severe itching of skin (Wang and Qian 2013)

(continued)

Table 2.15 (continued)

Culture and ***folk name***	Traditional use
Lobaria orientalis (Asahina) Yoshim.	
China	Same as Chinese use of *L. pulmonaria* (Wang and Qian 2013)
Lobaria oregana (Tuck.) Müll. Arg.	
Gitga'at (BC, Canada) ***nagaganaw*** ("frog dress")	Boiled with juniper and used as medicine for sore throats. Best for medicine if collected off *Abies lasiocarpa* [fir] (Turner and Thompson 2006)
Lobaria pulmonaria (L.) Hoffm.	
Europe (early modern era) ***muscus pulmonarius*** (Latin); ***lungwort***, ***lungs of oak***, or ***oak lung*** (English); ***hazelraw*** (Scotland); ***crotal coille*** (Ireland)	Its use for lung ailments goes back at least as far as the 1500s (L'Obel 1576) and was widespread throughout Europe during the 1600s (Parkinson and Marshall 1640; Ray 1686). Its popularity then waned, only being used in certain areas like the Scottish Highlands and New Forest (England), but many authors remained convinced of its efficacy (Watson 1756; Withering 1801; Wise 1863; Cameron 1900; de Crespigny and Hutchinson 1903). It was mainly used in lung ailments (e.g., tuberculosis, asthma, coughs, spitting blood), but also for liver diseases, as an appetite stimulant, for diarrhea, for heavy menstrual flow, and to stop bleeding. It was usually boiled with water or milk and drunk or made into an ointment for external use. It was also used for lung ailments in livestock in England, Germany, and Sweden (De Grey 1639; Willemet 1787; Drummond 1861)
Molise (Italy)	Applied to cuts as an antiseptic and healing agent (Guarrera et al. 2008)
India ***golmataghosa*** (Bengal)	Used for hemorrhages, lung troubles, asthma, and strengthening hair. The hill men use it for curing eczema on the head and cleaning hair (Biswas 1956)
Afghanistan ***gul-i-sang*** ("stone flowers")	Applied to newborn child's navel to dry and heal wound. Used as contraceptive, 4 different methods: (1) consume the lichen with water during menstrual period (Kabul); (2) dry, grind, and pop the resulting power into the mouth like snuff for 3 days during menstrual period (Kunduz); (3) grind and consume 24 h after giving birth (Kabul); (4) men consume the lichen (Kabul) (Hunte et al. 1975)

(continued)

Table 2.15 (continued)

Culture and ***folk name***	Traditional use
China 哈螞七 (***ha-ma-qi***)	Used for indigestion, malnutrition in children, abdominal distension, ascarid infestation, burns and scalds, edema due to kidney inflammation, local swelling, reducing inflammation, relieving pain, and severe itching of skin. Drink decoction or apply powder to affected area (Hu et al. 1980; Wang and Qian 2013)
Nlaka'pamux (BC, Canada) ***ʔes-tə/kʷl'-ít tək p'ə·/p'éy'łe tək /qʷzém*** ("yellowish frog moss")	Previously used medicinally, details forgotten (Turner et al. 1990)
Coast Tsimshian (BC, Canada)	Used medicinally (Johnson 2006)
Hesquiat (BC, Canada) ***ƛ̓acƛ̓astuphc̓um***	Applied to the faces of children when their skin is peeling. Also used as medicine for coughing up blood (Turner and Efrat 1982)
Saanich (BC, Canada)	Same as Saanich use of *Parmelia sulcata* (possible birth control) (Turner and Hebda 2012)
Lobaria quercizans Michaux [syn. *Sticta glomulifera* in N.A.]	
Menomini (Wisconsin, USA) ***wakùn***	Eaten as a tonic and as medicine for run-down systems. Only picked off hard maple or hemlock trees and cooked in soups (Smith 1923)
Lobaria retigera (Bory) Trevis.	
China 老龍皮 (***lao-long-pi***)	Same as Chinese use of *L. pulmonaria* (Hu et al. 1980; Wang and Qian 2013)
Lobaria sublaevis (Nyl.) Yoshim.	
China	Used for indigestion, edema, inflammation, and pain relief (Wang and Qian 2013)
Lobaria yunnanensis Yoshim.	
China	Same as Chinese use of *L. pulmonaria* (Wang and Qian 2013)
Lobaria virens (With.) J.R. Laundon [syn. *Lobaria laetevirens*]	
Europe (early modern era)	Occasionally listed in old European pharmacopoeias (Gioanetto 1993)
Pseudocyphellaria aurata (Ach.) Vain.	
Ambavaniasy (Madagascar)	Used as tea to treat indigestion (Sharnoff 1997)
Sticta spp. (Schreber) Ach.	
Makah (Washington, USA)	See Makah use of *Lobaria* spp. (Densmore 1939)
Nuxalk	See Nuxalk use of *Lobaria* spp. (Smith 1929)
Sticta wrightii Tuck.	
China	Used for indigestion; and edema from kidney inflammation (Wang and Qian 2013)

Table 2.16 Nephromataceae used in traditional medicines around the world

Culture and ***folk name***	Traditional use
Nephroma arcticum (L.) Torss.	
Yup'ik (Alaska) ***kusskoak***	Infusion with hot water is fed to a person in weak condition to make him strong, a very effective medicine (Oswalt 1957)

Table 2.17 Peltigeraceae used in traditional medicines around the world

Culture and ***folk name***	Traditional use
Peltigera spp. Willd.	
Dena'ina (Alaska, USA) ***k'udyika'a***	Decoction drunk for tuberculosis and prolonged bleeding. *Umbilicaria* spp. are also used (Kari 1987)
Haida (BC, Canada) ***hlk'inxa kwii'awaay*** ("forest cloud") or ***xil kwii.awaa*** ("cloud leaves")	Ingredient in several different medicinal mixtures (Turner 2004a)
Oweekeno (BC, Canada) ***x̌xwp̓igà***	Thallus pounded, mixed with spruce pitch, and used to dress wounds (Compton 1993)
Ditidaht (BC, Canada) ***λ̓aλ̓x̣ʔa·ʔaq*** ("flat against the rock") or ***λ̓i·λ̓i·dqʷaqsibakk̓ʷ*** ("resembles baleen whale")	A gray *Peltigera* growing on rocks that was used to induce urination. Picked, washed, squashed, and eaten (Turner et al. 1983)
Navajo (NM, USA) ***nihalá·d*** ("earth moss")	May be chewed like *Xanthoparmelia* sp. for cankers, swollen gums, and decayed teeth (Wyman and Harris 1941)
Peltigera aphthosa (L.) Willd.	
China	Used to improve digestion (Wang and Qian 2013)
Europe (early modern era)	As early as the 1700s, it was used as medicine thrush (mouth ulcers) in children. Make an infusion in milk and drink. It induces vomiting in large doses. Also used to expel worms (Gedner 1756; Strandman 1769; Willemet 1787; Withering 1801; Luyken 1809; Lindley 1838)
Tlingit (Alaska, USA)	Dried, powdered, and used to treat burns and scalds (Emmons 1991)
Ditidaht (BC, Canada) ***t̓it̓idiččʔa·*** ("rocks growing on rocks")	Chewed and eaten for tuberculosis. Also used as poultice for sores on legs (Turner et al. 1983)

(continued)

Table 2.17 (continued)

Culture and ***folk name***	Traditional use
Nlaka'pamux (BC, Canada) ***p'ə·/p'éy'łe təkˇ /qʷzém*** ("frog moss") or ***p'ə·/p'éy'łeh=éy'st*** ("frog's rocks")	Used to rub on beestings (Turner et al. 1990)
Peltigera britannica (Gyelnik) Holt.-Hartw. & Tønsberg	
Ditidaht (BC, Canada)	Possibly same as Ditidaht use of *P. aphthosa* (Turner et al. 1983)
Peltigera canina (L.) Willd.	
Britain ***lichen cinereus terrestris*** (Latin); ***dog lichen*** or ***ash-coloured ground liverwort*** (English); ***lus ghoinnich*** or ***gearan*** (Gaelic, from Cameron 1900)	A plant called ***the star of the earth*** was used as a cure of rabies in Britain as early as the 1600s. In the oldest record, this is definitely a vascular plant (De Grey 1639), but later authors decided that it was *P. canina* (Gourdon 1687; Dampier and Sloane 1698). The remedy was popularized in 1720 by Dr. Mead and enjoyed a short period of renown (Mortimer 1735; Hartley et al. 1737), before people began to become suspicious of its efficacy (Steward 1738; Ranby and Peters 1744; Layard 1757; Lightfoot 1777). Still being used in some areas in Wales in early 1800s (Trevelyan 1909; Allen and Hatfield 2004). Dried lichen and black pepper were pulverized and mixed into warm milk. This remedy was called ***pulvis antilyssus***
India and China ***patamataghosa*** (Bengali)	Used for rabies and jaundice in India (Biswas 1956) and China (Wang and Qian 2013)
Himalayas (India)	Tonic and medicine for liver complaints (Subramanian and Ramakrishnan 1964)
Hesquiaht (BC, Canada)	Unspecified medicine (Turner and Efrat 1982)
Peltigera membranacea (Ach.) Nyl. [syn. *Peltigera canina* var. *membranacea*]	
Kwakwaka'wakw (BC, Canada) ***tl'extl'ekw'és*** ("seaweed of the ground")	Used as a love charm (Boas 1921)
Peltigera polydactylon (Neck.) Hoffm. [syn. *Peltigera polydactyla*]	
Lepchas (Sikkim, India) ***jhau***	Used as antiseptic and to stop bleeding. Thalli made into paste and put on cuts (Saklani and Jain 1994)
Indo-Tibetan Himalayas ***sharda***	Lichen is washed, pounded, and boiled in goat's milk; the resulting mash is soaked in cow's urine to be used as an antiseptic over cuts and bruises (Sharma 1997)
China	Used for traumatic injuries and to strengthen the constitution (Wang and Qian 2013)
Iroquois (Ontario, Canada)	Tea used to induce vomiting and as an anti-love medicine. Either makes loved one return or unbewitches you (Herrick 1995)

Table 2.18 Teloschistaceae used in traditional medicines around the world

Culture	Traditional use
Polycauliona candelaria (L.) Frödén, Arup, & Søchting [syn. *Xanthoria candelaria*]	
Europe (early modern era)	Boiled with milk to treat jaundice, along with *Xanthoria parietina* (Tonning 1769).
Teloschistes flavicans (Sw.) Norm.	
China	Used to clear ***heat*** in lung and liver and to remove toxins (Wang and Qian 2013)
Xanthoria parietina (L.) Th. Fr.	
Andalucia (Spain) ***flor de piedra*** ("stone flower") or ***rompepiedra*** ("stone breaker")	Decoction in wine for menstrual complaints (Campohermoso). Decoction in water for kidney disorders (Barranquete, Cueva de los Medinas, Joya, Pozo de los Frailes, and Puebloblanco). Decoction in water for toothaches (Fernan Pérez and Joya). An analgesic for several pains (Fuente del Escribano). Ingredient in a cough syrup with *Ceratonia siliqua, Ficus carica*, and *Prunus amygdalus* fruits; *Olea europaea* and *Origanum vulgare* leaves and flowers; and lots of sugar or honey (San Isidro Jiménez) (González-Tejero et al. 1995)
Europe (early modern era)	Boiled with milk to treat jaundice, along with *Polycauliona candelaria* (Tonning 1769). Used for diarrhea (Luyken 1809), for intermittent fevers (Lindley 1838), for hepatitis (Gioanetto 1993), for diarrhea and dysentery (Willemet 1787), and as a quinine replacement for malaria (Lebail 1853)
China	Used medicinally as an antibacterial (Wang and Qian 2013)

Table 2.19 Roccellaceae used in traditional medicines around the world

Culture and ***folk name***	Traditional use
Roccella sp.	
Ancient Greece **φῦκος θαλάσσιον (*phŷkos thalássion*,** "marine phycos"), ***ballaris***, ***irane***, or ***gnomeusilum***	A cryptogam growing on seashore rocks is recorded by Dioscorides in Section 4.99 of De Materia Medica (50–70 C.E.). Recommended for inflammations and gout in the feet that needs to be reduced (López Eire et al. 2006). Possibly a marine algae, but identified by Richardson (1974b) as *Roccella* sp.
Sicuani (Peru)	Two *Roccella* sp. sold in indigenous market: one for coughs and one for fever (Sharnoff 1997)
Roccella babingtonii Mont.	
Seri (Sonora, Mexico) ***heecoj***	Tea: shortness of breath and fever. Ground, moistened, and strained: filtrate put on a burn or sore. Ground with clay and water: fever and diarrhea. Ground and mixed with water: bathe child with fever (Felger and Moser 1985)

(continued)

Table 2.19 (continued)

Culture and ***folk name***	Traditional use
Roccella fuciformis (L.) DC.	
Pondicherry (India) ***mathaghasa*** ("to rub on skull")	Used to clean hair and cure eczema on the skull and back or the ear (Biswas 1947)
Roccella phycopsis Ach. [*Roccella tinctoria*]	
France ***orseille***, ***orchal***	Remedy for tickling in the throat (France). Used in Mauritius for a medicinal broth (may refer to all fruticose lichens) (de Candolle 1816; Lebail 1853)
Madras (India)	Unspecified drug (Biswas 1947)

Table 2.20 Ophioparmaceae used in traditional medicines around the world

Culture	Traditional use
Ophioparma lapponica (Räs.) R. W. Rogers & Hafellner	
China	Used externally to stop bleeding from external injury, relieve pain (Wang and Qian 2013)
Ophioparma ventosa (L.) Norman	
China	Same Chinese use as *O. lapponica* (Wang and Qian 2013)

Table 2.21 Umbilicariaceae used in traditional medicines around the world

Culture and ***folk name***	Traditional use
Lasallia papulosa (Ach.) Llano [syn. *Umbilicaria papulosa*]	
Ekuanitshit (Quebec, Canada) ***uâkuanâpishku***	Tea used for urinary problems (Clément 1990; Uprety et al. 2012)
Umbilicaria spp. Hoffm.	
Dena'ina (Alaska, USA) ***qalnigi jegha*** ("rock ear")	Decoction drunk for tuberculosis and prolonged bleeding. Also used *Peltigera* spp. (Kari 1987)
Inuit (Quebec, Canada)	Used as a tea to treat tuberculosis (Stevens et al. 1984; Sharnoff 1997)
Umbilicaria esculenta (Miyoshi) Minks [syn. *Gyrophora esculenta*]	
Japan ***iwa-take***	An esteemed food that promotes longevity when eaten (Kawagoe 1925; Sato 1968)
Kyeong Gi Do (Korea) 석의버섯 (***seog-eui-beo-seod***)	Used to treat dysentery (Lee 1966)
China 石耳 (***shi-er***, "stone ear")	Used for tuberculosis, spontaneous external bleeding, intestinal bleeding, rectal hernia into the vagina, bloody and cloudy urination, vaginal discharge, snakebites, and cuts. Drink decoction; or apply externally to affected area (Hu et al. 1980; Wang and Qian 2013)

(continued)

Table 2.21 (continued)

Culture and ***folk name***	Traditional use
Umbilicaria hypococcinae (Jatta) Llano	
China	Used for indigestion, distention and pain in stomach duct and abdomen, dysentery, and malnutrition in children. Drink decoction (Wang and Qian 2013)
Umbilicaria mammulata (Ach.) Tuck.	
Attikamekw (Quebec, Canada) ***asine-wakunik***	During difficult childbirth the lichen is boiled and placed on woman's stomach (Raymond 1945)
Nihitahawak Cree (Saskatchewan, Canada) ***asinīwākon***	Made into soup as nourishment for sick person, as it will not upset the stomach. Lichen cleaned, broken into small pieces, and very hot water poured over it and water discarded. Lichen then added to fish broth and cooked 5–10 min, soup thickened as it cooled (Leighton 1985)
Umbilicaria muhlenbergii (Ach.) Tuck. [syn. *Actinogyra muhlenbergii*]	
Chipewyan (Saskatchewan, Canada) ***thetsı̨***	Used to expel tapeworms. Lichen is burned slightly in a frying pan, mashed well, and then boiled to make a syrup which is drunk. It can be chewed for the same purpose (Marles 1984; Marles et al. 2000)
Cree (Manitoba, Canada) ***asinīwāhkona, wakoonak***, or ***asinīwākon***	Decoction given to someone with a stomachache to "clean out the stomach." (Marles et al. 2000)
Tłı̨chǫ (NWT, Canada) ***kwechi***	Soup eaten as a tonic and for breathing problems (Rebesca et al. 1994; Uprety et al. 2012)
Umbilicaria nanella Frey and Poelt	
China	Used for indigestion, stomachache, dysentery, malnutrition in children, expelling ascarid parasites, vaginal discharges, glomus tumors, and reducing swelling. Drink decoction (Wang and Qian 2013)
Umbilicaria vellea (L.) Ach.	
China	Used for eye infections, bloody feces, and rectal hernia into the vagina (Wang and Qian 2013)

Table 2.22 Icmadophilaceae used in traditional medicines around the world

Culture and ***folk name***	Traditional use
Siphula sp. Fr.	
Northern Peru ***pelo de piedra***	Unspecified medicine. Oral aqueous application (Bussmann 2006)
Thamnolia subuliformis (Ehrh.) W. Culb.	
Naxi (nw Yunnan, China) ***xuecha***, ***baixuecha***, or ***snow tea***	Used for inflammation. Boiling water added to dry thalli in cup and infusion is drunk after 3–5 min. May be same as Naxi use of *T. vermicularis* (Wang et al. 2001; Fu et al. 2005)
Thamnolia vermicularis (Sw.) Ach. ex Schaerer [syn. *Cladonia vermicularis*]	
Naxi (nw Yunnan, China) ***xuecha***, ***baixuecha***, or ***snow tea***	Used for sunstroke, eye irritation, coughs, sore throat, inflammation, high blood pressure, fevers, epilepsia, and a decrease in vital energy. Boiling water added to dry thalli in cup and infusion is drunk after 3–5 min (Wang et al. 2001; Jiang et al. 2002; Fu et al. 2005; Wang and Qian 2013)
Northwest Yunnan (Tibet) ***xiare***	A widely recognized medicinal plant, tea used to tranquilize the mind and clear ***heat*** (Byg et al. 2010; Ju et al. 2013)
Ayurvedic (Uttarakhand and Himachal Pradesh, India) ***swarn***	Germicide to preserve milk and other dairy products. Lichen is dried and burned, and milk is exposed to the smoke (Sharma 1997)
Bhotia (Uttarakhand, India) ***chhai dhoop***	Used to preserve butter milk. A handful of lichen is put in a wide cup containing burning coal and the smoke directed into the milk. It kills the 1–2 mm long white worms that grow in milk (Upreti and Negi 1996)
South America ***contrayerba blanca***	Used to stimulate the stomach (Lindley 1838)

Table 2.23 Megasporaceae used in traditional medicines around the world

Culture and ***folk name***	Traditional use
Aspicilia esculenta (Pall.) Flagey	
Tehran (Iran) شیر زاد (***shīr-zāda***); ***chir zadi***; or ***agalactie***	Ingredient in wine and medicinal compounds in ninth to thirteenth centuries Arabic writings (Crum 1993). Used to increase the flow of human milk (Hooper 1937)

Table 2.24 Pertusariaceae used in traditional medicines around the world

Culture	Traditional use
Pertusaria albescens (Hudson) M. Choisy and Werner [syn. *Variolaria discoidea*]	
Europe (early modern era)	Used to treat intermittent fevers, along with *P. amara* (Lindley 1838)
Pertusaria amara (Ach.) Nyl. [syn. *Variolaria faginea*]	
Europe (early modern era)	Used to treat intermittent fevers, along with *P. albescens* (Lindley 1838)
Pertusaria pertusa (Weigel) Tuck. [syn. *Pertusaria communis*]	
Europe (early modern era)	Cure for intermittent fever, more effective for men. Also used for intermittent toothache, and powdered and used to kill worms (Lebail 1853)
Pertusaria velata (Turner) Nyl.	
China	Used to stop bleeding and relieve pain. External use only (Wang and Qian 2013)

Table 2.25 Verrucariaceae used in traditional medicines around the world

Culture	Traditional use
Dermatocarpon miniatum (L.) W. Mann	
China	Used for high blood pressure, as a diuretic, for expelling parasites, for malnutrition in children, for dysentery, for improving digestion, and for abdominal distention. Drink decoction or eat as soup (Wang and Qian 2013)

Table 2.26 Hygrophoraceae used in traditional medicines around the world

Culture and ***folk name***	Traditional use
Dictyonema huaorani Dal-Forno, Schmull, Lücking & Lawrey	
Huaorani (Amazon, Ecuador) ***nẹ̀nẹ̀ndapẹ̀***	Mixed with other bryophytes, made into an infusion, and drunk by shaman to cause hallucinations and call on malevolent spirits to curse people. Also causes sterility (Davis and Yost 1983; Schmull et al. 2014)

Table 2.27 Unidentified lichens used in traditional medicines around the world

Culture and ***folk name***	Traditional use
Xhosa (South Africa) ***mthafathafa***	An unidentified rock lichen is used to treat gonorrhea. Fresh lichen is crushed and mixed with water, and infusion is drunk. Lichen also dried over fire and crushed, and powder is applied to wound's infected area (Matsiliza and Barker 2001). See also Cape area use of unidentified parmelioid lichen
Trentepohlia jolithus [*Lepraria iolithus*]	A non-lichenized algae considered a lichen in early literature. Used for small pox and measles (Luyken 1809)
New Forest (England) ***brighten***	An unidentified lichen is recommended for weak eyes (Wise 1863)
Slieve Aughty (Ireland) ***dub-cosac***	An unidentified lichen is good for heart trouble (Allen and Hatfield 2004)
Brahuis (Balochistan, Pakistan)	An unidentified rock lichen that is extremely bitter is used medicinally in diseases of languor and oppression of the life force. The lichen is dried and crushed. They swallow the powder, and then drink water (Masson 1842; Hooper 1937)
Rotuma (Fiji) ***rimi***	A gray lichen found on coconut tree trunks is used to make medicine used in treating high fevers and/or convulsions (McClatchey 1993)
Dena'ina (Alaska, USA) ***sheh tsadn nde***	A large foliose lichen is used for coughs, tuberculosis, and general sickness. Boil and drink decoction. Also used for bleeding that won't stop (Garibaldi 1999)
Tlingit (Alaska, USA)	Lichens from the ground in the woods are used for sores. Crushed and then heated on rocks with seal oil and mountain goat tallow (de Laguna 1972)
Chipewyan (Alberta, Canada)	White crustose lichens on aspen bark, along with the dead tree periderm, are scraped off and put on cuts and deep wounds to stop bleeding (Marles et al. 2000)
Niitsitapii (Alberta, Canada)	Mixed with kinnikinnick leaves and shredded willow bark to make a smoking mixture (Russell 1973). Cited by Siegel (1989) who added the claim that it was narcotic (Siegel 2013 pers. comm.) and was then cited by Pollan (2001) who added the claim that it was hallucinogenic
Nihitahawak Cree (Saskatchewan, Canada)	White crustose lichens on aspen bark, along with the dead tree periderm, are scraped off and used to stop bleeding and to treat venereal disease (Leighton 1985)
Algonquin (Quebec, Canada)	White crustose lichens on birch bark used for diaper rash and other skin rashes (Black 1980)
Tewa (California, USA) ***k̂uk'owà*** ("rock skin"); ***nǎŋ'a*** ("earth clothing")	***k̂uk'owà*** is pulverized and applied to lips for cold sores, rubbed on sores about a child's mouth, and put into the cavity of a decayed tooth to stop pain. ***nǎŋ'a*** is applied to teeth and gums to cure toothache (Robbins et al. 1916). See also Hopi use of *Xanthoparmelia* sp.
N. Paiute (Nevada, USA) ***tuh-botza-yo-caw-son*** or ***lizard semen***	Black, orange, and yellow lichens on rocks are used as important antibiotics and fungicides. Powdered material is applied as a healing agent to sores, especially mouth sores of children (Train et al. 1941; Sharnoff 1997)

(continued)

Table 2.27 (continued)

Culture and ***folk name***	Traditional use
Western Shoshone (Nevada, USA) ***timbe-boon-goo***	Black, orange, and green lichens on rocks. Diarrhea medicine: soak overnight in water and drink the solution. Smallpox medicine: powder and boil with *Purshia* leaves and dried mountain rat urine; drink half cup of solution morning and night (Train et al. 1941)
Hopi (Arizona, USA)	Yellow lichens on rocks are applied to cheeks to reduce swelling and relieve toothache (Beaglehole and Beaglehole 1935). See also Hopi use of *Xanthoparmelia* sp.
Kewa Pueblo and Hispanics (New Mexico, USA) ***yerba de la piedra*** (Spanish)	Gray lichens are boiled until green and given to one who talks and laughs to himself. Also good for headaches (Kewa). Also rubbed on gums as cure for inflamed gums or powdered and applied on any kind of sore or injury (Hispanics) (Curtin 1965)
Ka'igwu (Oklahoma, USA)	Lichens on north side of tree trunk are dried, powdered, and applied to sore gums for abscesses and teething infants. Also mixed with smoking tobacco for a mildly soporific effect (Vestal and Schultes 1939)
Seri (Sonora, Mexico) ***hast yamása*** ("rock lichen")	Gray foliose and orange crustose lichens on rocks are taken as a tea to induce vomiting (Felger and Moser 1985)
Huastec (Mexico) ***tsakam k'uthay***	An unidentified arboreal lichen is used as an unspecified obstetrical-gynecological medicine and for bleeding. Its name means "little *Tillandsia usneoides*" (Alcorn 1984)
Lacadone (Chiapas, Mexico)	Unidentified lichens are invoked in magical healing of skin eruptions (Sharnoff 1997)
Quichua (Loja, Ecuador) ***musgo de piedra***	There are 7 different colors of lichens on rocks. If all 7 colors are boiled in a drink, it will cure a person with a chronic illness who is about to die (Abel 2009 pers. comm.)
Loja (Ecuador)	An unidentified lichen is used for an unspecified medicine (Bussmann and Sharon 2006)
Denís and Kinja (Amazonas, Brazil) ***baduhu-tsinã*** ("deer snuff")	An unidentified pyrenocarpous lichen on trees is used as a snuff. Yellow powder is collected off the surface of lichen for snuff. Used frequently and induces sneezing (Prance 1972; Milliken et al. 1992)
White crustose lichen Witoto/Bora (Loreto, Peru)	An unidentified white crustose lichen growing on *Rinorea racemosa* is sometimes used (along with other botanicals and ash) to add to the resin of *Virola sebifera* or *V. elongata* to make ***oo'-koey***, a hallucinogenic orally ingested paste (Mckenna et al. 1984; UBC 2014)
Chácobo (Beni, Bolivia)	Five unidentified lichens are used to treat chest and appendix pain, headache, liver problems, and rheumatism (Boom 1987)
Aymara (Titicaca, Bolivia) ***pampa untu*** (wild llama fat)	An unidentified lichen is given to babies as an infusion if they are constipated (La Barre 1948)

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Chapter 3
Lichen Secondary Metabolites as Potential Antibiotic Agents

Marijana Kosanić and Branislav Ranković

Abstract It is well known that pathogenic microbes pose serious threats to human health and are increasing in prevalence in institutional health-care settings due to the growing resistance that infectious agents have developed against antibiotics. Therefore, new alternatives for combating the spread of infection through antibiotic-resistant microbes are necessary for keeping pace with the evolution of "super" pathogens. Natural products are proposed as a therapeutic alternative to conventional antimicrobial treatment. Among them, lichen-derived product and their antibiotic properties are of special interest to scientists as up to 50 % of all lichens have been reported to possess antibiotic activities. A great number of reports concerning the antimicrobial screening of lichens have appeared in the literature. According to published data, the lichens and their secondary metabolites exhibited the activity against a great number of microorganisms. Therefore, the present study represents lichens as very interesting source of bioactive compounds which provide unlimited opportunities for new antimicrobial agents.

3.1 Needing for New Antibiotics from Nature

Before the introduction of antibiotics in the 1940s, infections were rare, but rapidly increased in frequency as the use of antibiotics increased. In fact, most antibiotics that were first used in the 1940s and 1950s are no longer used clinically because nowadays the resistance of infectious beings to these antibiotics is very common. Over time they have been developing new antibiotics, and with the introduction of each, new drug-resistant bacteria appeared rapidly. The process of resistance is augmented by short generation times of bacteria enabling rapid mutation and selection of resistant strains and a horizontal transfer of resistance genes. Bacterial pathogens resistant to more than one, or even most clinically used antibiotics, have become common. Today, we moved the mode of use and prescription of antibiotics

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B. Ranković (ed.), *Lichen Secondary Metabolites*,
DOI 10.1007/978-3-319-13374-4_3

in order to try to slow the relentless pace of bacterial evolution but not yet found a solution to this problem. Microbiologists continue to study how bacteria evolve so that we can predict how they will respond to medical treatment and so we can better manage the evolution of infectious diseases.

Bacteria are able to resist the effects of antimicrobials through preventing intracellular access, immediately removing antimicrobial substances through efflux pumps, modifying the antimicrobial agent through enzymatic breakdown, or modifying the antimicrobial targets within the bacterial cell to render the substance ineffective. Successful development of resistance often results from a combination of two or more of these strategies (Sheldon 2005).

The first antibiotic resistance mechanism described was that of penicillinase. Its presence and activity were first reported by Abraham and Chain in 1940 shortly after its discovery (Abraham and Chain 1940).

Antimicrobial resistance traits are genetically coded and can either be intrinsic or acquired. Intrinsic resistance is due to innately coded genes which create natural "insensitivity" to a particular antibiotic. Innate resistance is normally expressed by virtually all strains of that particular bacterial species. Acquired resistance is gained by previously susceptible bacteria either through mutation or horizontally obtained from other bacteria possessing such resistance via transformation, transduction, or conjugation. Acquired resistance is limited to subpopulations of a particular bacterial species and may result from selective pressure exerted by antibiotic usage.

The drug resistance of human and animal pathogens is one of the best documented in biological evolution and a serious problem in both developed and developing countries. The consumption of more than one ton daily antibiotics in some European countries has resulted in resistance to bacterial populations, thus causing a serious public health problem. In view of this scenario, the search for new antimicrobial substances from natural sources, including lichens, has gained importance in pharmaceutical companies.

Since lichens produce a variety of substances with antimicrobial properties, it is expected that screening programs discover candidate compounds for the development of new antibiotics. However, scientific research to determine the therapeutic potential of lichens is limited, and there is a lack of scientific studies that confirm the possible experimental antibiotic properties of a large number of lichens. It is expected that compounds that reach targets different from those used by known antibiotics may be active against resistant pathogens.

3.2 Antimicrobial Activity and Probable Mechanisms of Action of Lichens

Antimicrobial activity of lichens is understood as their ability to eliminate microorganisms or to inhibit their growth.

The antimicrobial properties of lichen extracts and their secondary metabolites are known for long (Burkholder and Evans 1945; Stoll et al. 1950; Vartia 1973; Piovano et al. 2002; Ranković et al. 2007a; Paudel et al. 2008; Schmeda-Hirschmann et al. 2008; Micheletti et al. 2009) and still assess the mechanisms of their effects.

The probable mechanisms of antimicrobial action of lichens are:

Inhibition of cell wall synthesis The peptidoglycan layer is important for cell wall structural integrity, being the outermost and primary component of the wall. Inhibitors of cell wall synthesis act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls and thus come to degradation of the cell wall.

Inhibition of protein synthesis (translation) Protein synthesis inhibitors act at the ribosome inhibiting the synthesis of proteins of the pathogen to occur, misreading the sequence of amino acids, and thus inhibit the functioning of the pathogenic cells.

Alteration of cell membranes Injury bacterial plasma membranes lead to cell death through leakage of the cell contents and associated disruption of the cross-membrane potential (which essentially are ion concentration gradients).

Inhibition of nucleic acid synthesis Nucleic acid inhibitors act by inhibiting the production of nucleic acids (DNA and RNA).

Antimetabolite activity Antimetabolites prevent a cell from carrying out a metabolic reaction. Antimetabolites function by competitive inhibition of enzymes and by erroneous incorporation into nucleic acids. In both cases, the cells become unable to normally function.

The secondary metabolites of the lichen are active substances against pathogenic microorganisms. Most known lichen substances with antimicrobial activity are usnic acid, phenolic compounds, triterpenes, steroids, anthraquinones, depsides, depsidones, and dapsones, and most of them are known mechanisms of their antibiotic action.

Usnic acid Usnic acid, a compound produced by various lichen species, has been demonstrated previously to inhibit growth of different bacteria and fungi; however, the mechanism of its antimicrobial activity has not been fully explained.

The mechanisms of the antibiotic activity of usnic acid against Gram-positive bacteria were attributed to its protonophoric properties as an uncoupler of oxidative phosphorylation (Abo-Khatwa et al. 1996). This effect was remarkably more pronounced than that produced by dinitrophenol, a well-known uncoupler agent that increases the permeability of mitochondria to protons, reducing the electrochemical potential and thus inhibiting adenosine triphosphate synthesis. Two research groups found that usnic acid played an active role in the transport of protons through the membranes of isolated mouse liver mitochondria, uncoupling the electron flow through the respiratory electron transport chains from the generation of an acidic gradient and thereby inhibiting the synthesis of adenosine

triphosphate (Abo-Khatwa et al. 1996; Bouaid and Vicente 1998). The ability of usnic acid to shuttle protons through the membranes was confirmed by studies with artificial phospholipid membranes (Bačkor et al. 1998). A model for the function of usnic acid in the control of the intracellular pH of lichens was recently proposed (Hauck and Jurgens 2008) that involves two hypotheses: (1) at the optimum pH near the pK_{a1} value of usnic acid (4, 4), buffering is assumed to be compensated by the usnic acid-mediated proton transport into the cell and (2) at low pH (<3.5), the equilibrium between usnic acid and usneate shifts toward the usnic acid, and protons are increasingly shuttled into the cells, considering that usnic acid dissociates to usneate at cytosolic pH 7.4. This implies that more protonated molecules would be able to cross the membrane and release protons into the cell. As a result, the intracellular pH would decrease and lead to the death of the cells (Hauck and Jurgens 2008).

In addition, Maciąg-Dorszyńska et al. (2014) assume that inhibition of RNA synthesis may be a general mechanism of antibacterial action of usnic acid, with additional direct mechanisms, such as impairment of DNA replication in *B. subtilis* and *S. aureus*.

Usnic acid has been used as antibiotic (e.g., Binan, Usno) and is still available as a topical antiseptic in some products (e.g., Gessato shaving treatment from Italy and Camillen 60 Fudes spray and nail oil from Germany). It is suggested for application in medical devices, since usnic acid inhibits bacterial biofilm formation on polymer surfaces (Francolini et al. 2004). This compound or derivatives are valuable active compounds against serious pathogens such as vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus* (Elo et al. 2007), mycobacteria (Ingolfsdottir et al. 1998), or *Listeria monocytogenes* (Tomasi et al. 2006).

Phenols Most lichen substances with antibiotic activity are phenolic metabolites. Phenols are one of the largest classes of secondary biomolecules, which are characterized by the presence of aromatic rings with hydroxyl group bonded directly to an aromatic hydrocarbon group. Although they are firstly identified in plants (Cowan 1999), their presence was also observed in lichens (Odabasoglu et al. 2004; Kekuda et al. 2011; Ranković et al. 2010, 2014; Mitrović et al. 2011; Kosanić and Ranković 2011; Manojlović et al. 2012). In recent years, there was a causal relationship between the total contents of these compounds with biological activities recorded in a large number of lichens, which include anti-inflammatory, antiallergic, anticancer, antihypertensive, antirheumatic, and antibacterial activity. Antimicrobial properties of phenolics are explained by the presence of phenol hydroxyl groups, the number of which is in correlation with their toxicity toward microorganisms (Cowan 1999). The possible mechanisms of their action include inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth, or direct action on microbial metabolism through inhibition of oxidative phosphorylation, by sulfhydryl groups and some nonspecific interactions (Cowan 1999).

A correlation between phenolic constituents and antimicrobial activity has been established. Gulluce et al. (2006) found that the content of total phenolic of the extracts of *Parmelia saxatilis*, *Platismatia glauca*, *Ramalina pollinaria*, *Ramalina polymorpha*, and *Umbilicaria nylanderiana* was strongly related with their antimicrobial activity. A positive correlation was seen between the phenolic content and antibiotic activity of *Lasallia pustulata*, *Parmelia sulcata*, *Umbilicaria crustulosa*, and *Umbilicaria cylindrica* (Ranković et al. 2007a). Ranković et al. (2007b) found a high correlation between antimicrobial efficacy of macrolichens *Cladonia furcata*, *Parmelia caperata*, *Parmelia pertusa*, *Hypogymnia physodes*, and *Umbilicaria polyphylla* and their phenolic content. Many other studies also have shown a direct correlation between the phenolic content and the antimicrobial activity (Vartia 1973; Ingolfsdottir et al. 1998; Ranković et al. 2009, 2011).

Terpenes In recent years, more data indicate the presence of terpenes in numerous representatives of lichens (Culberson 1970; Rundel 1978; Abdullah et al. 2007). One of the many functions of these compounds is their antimicrobial activity, but the mechanism of action of terpenes on microorganisms is not fully understood (Cowan 1999). According to their lipophilic nature, it is assumed to act by disrupting membrane functions of microbial cells (Cowan 1999), and some authors believe that they may cause nonspecific cell membrane permeability increase for the antibiotic molecule (Byron et al. 2003).

Steroids These compounds are highly often present in lichens. Steroids have been reported to have antibacterial properties; the correlation between membrane lipids and sensitivity for steroidal compound indicates the mechanism in which steroids specifically associate with the membrane lipid and exert its action by causing leakages from liposomes (Epand et al. 2007; Mohammed 2013).

Depsides, **tridepsides**, and **tetradepsides** consist of two, three, and four hydroxybenzoic acid residues linked by ester groups. These are the most numerous classes of secondary metabolites in lichens. More than one hundred lichen compounds are depsidones, which have an additional ether bond between aromatic rings. **Depsidones** in lichen are believed to arise by oxidative cyclization of depsides. It has been found that depsidone and depside compounds such as atranorin, divaricatic acid, lecanoric acid, evernic acid, salazinic acid, physodic acid, and stictic acid possess important antimicrobial activity (Manojlović et al. 2012; Kosanić et al. 2013, 2014a; Ranković et al. 2014).

Anthraquinones and **xanthones** are also important constituents of many lichens. Anthraquinones such as parietin, parietinic acid, emodin, fallacinol, and fallacinal were shown to have a high antimicrobial effect (Manojlović et al. 2002). Manojlovic et al. (2010) found that the antimicrobial activity of lichen *Laurera benguelensis* is mainly related to the presence of lichexanthone.

No clear mechanisms have been identified that specifically indicate how depsides, tridepsides, tetradepsides, depsidones, anthraquinones, and xanthones target microbial invasion.

3.3 Methods to Detect Antimicrobial Activity of Lichens

Methods to detect antimicrobial activity are in vitro procedures used to detect antimicrobial resistance in individual microbial isolates.

For lichens, antimicrobial activity can be examined both for their extracts and for their secondary metabolites.

Extraction is a process of separation of active compounds from plant material using different solvents. Extract can be prepared using various methods, such as sonification, heating under reflux, Soxhlet extraction, maceration, and others. Different solvent systems are available to extract the bioactive compound from natural products. The solvent systems used in extraction are selected on the basis of their capacity to dissolve the maximum amount of desired active constituents and the minimum amount of undesired constituents. The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol, or ethyl acetate. For extraction of more lipophilic compounds, dichloromethane and a mixture of dichloromethane/methanol are used (Sasidharan et al. 2011). Due to the fact that extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities, their separation to obtain pure compounds using different separation techniques such as TLC, column chromatography, flash chromatography, Sephadex chromatography, and HPLC is still required. The pure compounds are then used for the determination of structure and antimicrobial activity.

Currently, several methods have been applied to measure the in vitro antimicrobial activity of lichens, such as disk diffusion, agar dilution, and broth microdilution (Fig. 3.1).

The **disk diffusion** method allows for the simultaneous testing of a large number of antimicrobials in a relatively easy and flexible manner. In this method, the bacterial inoculum is adjusted to certain concentration, inoculated onto the entire surface of a Mueller-Hinton agar (MHA) plate with a sterile cotton-tipped swab to form an even lawn. The paper disks impregnated with diluted antibiotic solution were placed on the surface of each MHA plate using a sterile pair of forceps. Then the plates were incubated aerobically and the diameter of zone inhibition was measured by a ruler or caliper. Based on the diameter of the inhibition zone, the results are then assigned to three categories, susceptible, intermediate, or resistant. The bigger the diameter of the inhibition zone, the more susceptible is the microorganism to the antimicrobial.

Agar dilution is a quantitative susceptibility testing method because MIC values can be obtained using the method. In this method, twofold serial dilutions of an antibiotic made in MHA medium and then bacterial suspensions were inoculated on the MHA using a Cathra replicator with 1 mm pins. It has been studied extensively as a method for the bacteria growing aerobically. The advantages of agar dilution include the ability to simultaneously test the susceptibility of a number of bacteria in one plate and the ability to test susceptibility of fastidious organisms since the agar with supplements is able to adequately support the bacterial growth. However,

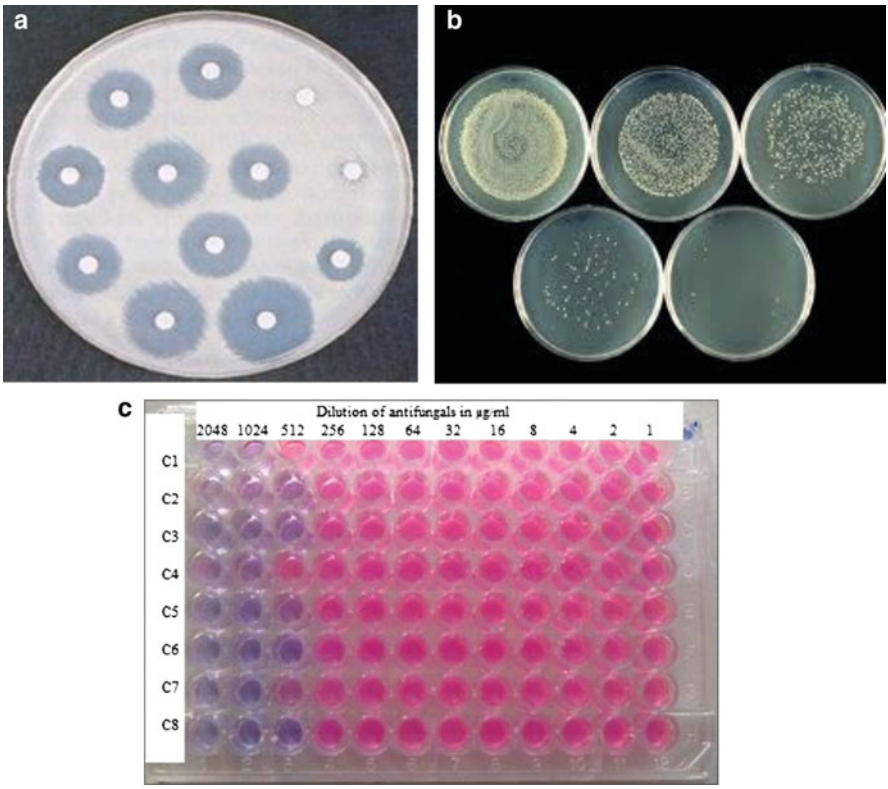

Fig. 3.1 Disk diffusion (**a**), agar dilution (**b**), and broth microdilution (**c**) methods to measure the in vitro antimicrobial activity of test samples

agar dilution is not commonly used in most microbiology laboratories because it is time-consuming and labor-intensive.

Broth microdilution is another quantitative reference method routinely used in clinical laboratories. In this method, susceptibility panel in 96-well microtiter plates was containing various concentrations of antimicrobial agents. Then, standardized numbers of bacteria were inoculated into the wells of 96-well microtiter and incubated overnight at 35 °C. This method is often used to obtain minimal inhibitory concentration (MIC) values. The MIC value was observed as the lowest concentration where no viability was observed in the wells of 96-microwell plates after incubation. It is a widely utilized method, allowing for the simultaneous testing of multiple antimicrobials with ease particularly when commercially prepared microtiter trays are used. Compared with agar-based method, broth microdilution can decrease much labor and time. However, limitations of the method primarily are associated with the lack of or poor growth of many anaerobic microorganisms. Testing some fastidious anaerobes gives inconsistent and

unreliable results because of poor growth of strains due to excessive exposure to oxygen during the setup procedure (CLSI 2009).

Among the three methods, disk diffusion seems to be the most popular method used to examine the antimicrobial activity of natural antimicrobials including lichens. Although the method is relatively inexpensive and easy to perform, there are several disadvantages. Since disk diffusion measures the inhibition zone size which is then converted to categories of susceptible/intermediate/resistant, this method is unable to obtain MIC values (Dickert et al. 1981). Also, it has been reported (Klancnik et al. 2010) that this method is not always reliable for determining the antimicrobial activity of natural antimicrobials, i.e., lichen extract, because the polarity of the natural compounds can affect the diffusion of compounds onto the culture medium. Compounds with less polarity diffused slower than more polar ones (Moreno et al. 2006). Due to these concerns, disk diffusion may not be a suitable one to determine the antimicrobial activity of natural compounds. Besides, similar to other agar-based methods, disk diffusion is labor-intensive and time-consuming (Klancnik et al. 2010).

In contrast, agar dilution and broth microdilution methods are able to overcome some of the limitations of the disk diffusion method. Not only are they more convenient for routine antimicrobial susceptibility testing of bacteria in clinical laboratories, they are capable of drawing quantitative conclusions by determining the MIC values for antimicrobials, as opposed to qualitative data generated by the disk diffusion method (Kim and Kim 2007).

3.4 Lichen Extracts as Potential Antibacterial and Antifungal Agents

The screening of lichen extracts has been of great interest to scientists for the discovery of new compound effective in the treatment of microbial infection. There are various reports on the antimicrobial activity of crude lichen extracts.

The first study on the antibiotic properties of lichens was carried out by Burkholder et al. (1944). He tested 42 lichens for antibiotic property and 27 were reported to inhibit growth of bacteria. A number of lichen extracts were screened for antibacterial activity in the 1950s, and in many cases activity was confirmed against mycobacteria and Gram-positive organisms (Stoll et al. 1950). A review of the work performed during this period is presented in Vartia (1973). More recent reports include a study describing the antimicrobial screening of lichen extracts and subsequent isolation of compounds with a broad spectrum of activity against filamentous fungi, yeast, as well as Gram-positive and Gram-negative bacteria.

Extracts of Andean lichens *Protousnea poeppigii* and *Usnea florida* demonstrated antimicrobial activity against the pathogenic fungi *Microsporum gypseum*, *Trichophyton mentagrophytes*, and *T. rubrum*. Gram-positive *Staphylococcus aureus* and *Bacillus subtilis* were sensitive to methanolic extracts of four different

Antarctic lichen species (Paudel et al. 2008). Ranković et al. (2007a, b) tested aqueous, acetone, and methanol extracts of *Cladonia furcata*, *Parmelia caperata*, *Parmelia pertusa*, *Hypogymnia physodes*, *Umbilicaria polyphylla*, *Lasallia pustulata*, *Parmelia sulcata*, *Umbilicaria crustulosa*, and *Umbilicaria cylindrica* from Serbia on six species of bacteria and ten species of fungi. The strongest activity was observed with methanol extracts of *Parmelia pertusa* and *Parmelia sulcata*, and the weakest activity was manifested by *Parmelia caperata* and *Umbilicaria cylindrica*. Aqueous extracts of all tested lichen species were inactive. *Bacillus mycoides* was the most sensitive bacterial species tested, whereas *Candida albicans* was the most sensitive fungal species examined. Other studies monitored *Ramalina farinacea* and 69 species of lichens from New Zealand and showed their inhibitory effect against a lot of bacteria such as *Bacillus*, *Pseudomonas*, *E. coli*, *Streptococcus*, *Staphylococcus*, *Enterococcus*, and *Mycobacterium* (Esimone and Adikwn 1999; Perry et al. 1999). Behera et al. (2005) reported that acetone, methanol, and light petroleum extracts of lichen *Usnea ghattensis* were effective against *Bacillus licheniformis*, *B. megaterium*, *B. subtilis*, and *S. aureus*. Also, Karagoz et al. (2009) evaluated aqueous and ethanol extracts of 11 different species from Turkey and determined potent antibacterial activity of aqueous extract of *Peltigera polydactyla* and ethanol extract of *Ramalina farinacea*. Recently, Mitrović et al. (2011) studied antibacterial and antifungal activity of methanol extracts of five lichen species (*Parmelia sulcata*, *Flavoparmelia caperata*, *Evernia prunastri*, *Hypogymnia physodes*, and *Cladonia foliacea*). The analysis of their antibacterial potential was performed on 15 strains of bacteria and revealed the strongest inhibitory effect, especially on Gram-positive bacteria, of *Hypogymnia physodes* and *Cladonia foliacea*. In the case of fungi, *Evernia prunastri* exerted the best effect on yeasts, while *Hypogymnia physodes* were better on filamentous fungi. Similarly, acetone extracts of the lichens *Cladonia furcata*, *Lecanora atra*, and *Lecanora muralis* were studied for their antimicrobial potential (Ranković et al. 2011). The antimicrobial activity was estimated by determination of the minimal inhibitory concentration by the broth microdilution method against six species of bacteria and ten species of fungi. The extract of *Cladonia furcata* was the most active antimicrobial agent with minimum inhibitory concentration values ranging from 0.78 to 25 mg/ml, while the lowest activity was shown by *Lecanora muralis*. In similar research, antifungal activity of hexane, ethyl acetate, and methanol extracts of *Parmelia reticulata* was evaluated against soilborne pathogenic fungi, namely, *Sclerotium rolfsii*, *Rhizoctonia solani*, *R. bataticola*, *Fusarium udum*, *Pythium aphanidermatum*, and *P. debaryanum* by Goel et al. (2011). Maximum antifungal activity was exhibited by hexane and ethyl acetate extracts against most of the test pathogens.

Acetone, diethyl ether, and ethanol extracts of the lichen *Cetraria aculeata* for their antimicrobial activity have been evaluated. The extracts were found active against *Escherichia coli*, *Staphylococcus aureus*, *Aeromonas hydrophila*, *Proteus vulgaris*, *Streptococcus faecalis*, *Bacillus cereus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Listeria monocytogenes*. However, no antimicrobial activity against the fungi was detected (Türk et al. 2003). The lichen extract almost

increased by twofold in the presence of the stock solution of the colloidal silver concentrate. The ointment containing the extract of lichen *Ramalina farinacea* exhibited antimicrobial activities against *Escherichia coli*, *Salmonella typhi*, *Aspergillus niger*, *and Candida albicans* (Ofokansi and Esimone 2005).

The aqueous and ethanol extracts prepared from some lichens species were evaluated for antibacterial activity against six standard strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*) and (*Aeromonas*) that were isolated from different lakes. The aqueous and ethanol extracts showed a variable range of antibacterial activity to both standard strains and environmental strains. Similarly the aqueous extract of *Peltigera polydactyla* and the ethanol extract of the *Ramalina farinacea* exhibited potent antibacterial activities (Karagoz et al. 2009).

The antimicrobial activity of the acetone, diethyl ether, and ethanol extracts of the lichen *Cetraria aculeata* tested against different pathogenic bacteria and fungi showed only with bacteria but not with fungi. In a related study, *Roccella belangeriana* were extracted from different solvents like acetone, methanol, diethyl ether, ethanol, ethyl acetate, petroleum ether, chloroform, and aqueous extracts and tested against 12 bacterial strains. A maximum antibacterial activity was observed from chloroform extracts against *Enterococci* sp., and minimum activity was observed from ethyl acetate extract against *Klebsiella pneumoniae*, *Enterococci* sp., *Salmonella* sp., and *Shewanella* sp. (Karthikaidevi et al. 2009). In addition, antibacterial and antifungal activity of the acetone, methanol, and aqueous extracts of the lichens *Lecanora frustulosa* and *Parmeliopsis hyperopta* have been screened in vitro against the *Bacillus mycoides*, *Bacillus subtilis*, *Staphylococcus aureus*, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Botrytis cinerea*, *Candida albicans*, *Fusarium oxysporum*, *Mucor mucedo*, *Paecilomyces variotii*, *Penicillium purpurascens*, *Penicillium verrucosum*, and *Trichoderma harzianum*. Tested lichen species also showed strong activity against both bacteria and fungi (Kosanić et al. 2010). This would perhaps indicate that the lichens would be used in the treatment of various diseases.

According to Schmeda-Hirschmann et al. (2008), dichloromethane and methanol extracts of *Protousnea poeppigii* had strong antifungal effects against the fungal pathogens *Microsporum gypseum*, *Trichophyton mentagrophytes*, and *T. rubrum*. The extracts were also active against the yeasts *Candida albicans*, *C. tropicalis*, and *Saccharomyces cerevisiae* and the filamentous fungi *Aspergillus niger*, *A. flavus*, and *A. fumigatus* but with much higher strength. In the same assay, extracts of *Usnea florida* also showed strong antifungal properties. Methanol extracts of five lichens from Antarctica (*Caloplaca regalis*, *Caloplaca* sp., *Lecanora* sp., *Ramalina terebrata*, *Stereocaulon alpinum*) exhibited target-specific antibacterial activity, especially strong against Gram-positive bacteria, compared to previously described lichens (Paudel et al. 2008). Whiton and Lawrey (1982) reported that ascospore germination of *Sordaria fimicola* was significantly inhibited by evernic and vulpinic acids. Aqueous, ethanol, and ethyl acetate extracts of *Alectoria sarmentosa* and *Cladonia rangiferina* were found to have moderate antifungal action against different species of fungi, including human pathogens (Ranković and Mišić 2007),

ethanol extracts showing the highest activity. Halama and Van Haluwin (2004) reported that acetone extracts of *Evernia prunastri* and *Hypogymnia physodes* showed a strong inhibitory effect on the growth of some plant pathogenic fungi, i.e., *Phytophthora infestans*, *Pythium ultimum*, and *Ustilago maydis*.

Antimicrobial features of acetone, methanol, and aqueous extracts of lichens of *Cladonia furcata*, *Parmelia caperata*, *Parmelia pertusa*, *Hypogymnia physodes*, and *Umbilicaria polyphylla* were investigated by Ranković et al. (2009) by two different methods at the same time. Testing of antimicrobial activities of extracts from five species of lichens was performed by disk diffusion test in relation to Gram-positive and Gram-negative bacteria and fungal organisms and through determination of minimal inhibitory concentration (MIC) by broth tube dilution method. They found that acetone and methanol extracts of all investigated lichens in different concentrations manifested selective antibacterial and antifungal activity. That activity was more evident in relation to Gram-positive than Gram-negative bacteria and fungal organisms. Acetone and methanol extracts of lichens *Parmelia pertusa*, *Hypogymnia physodes*, and *Umbilicaria polyphylla* inhibited the growth of all tested microorganisms, most of all of lichens *Cladonia furcata* and *Parmelia caperata*. Although the methanol extracts were generally the most active against the test organisms, the lowest MIC value was measured for acetone extract of species *Cladonia furcata* 0.39 mg/ml in relation to bacterium *Bacillus subtilis*. Aqueous extracts of investigated lichens were inactive against all tested organisms.

Santiago et al. (2010) examined antibacterial activity of 63 lichens collected from different sites in Luzon Island, Philippines. Lichen crude extracts were then tested against Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) using the paper disk diffusion assay. Their results showed that all 45 tested extracts inhibited at least one of the test bacteria. However, only 38 extracts were found to be very active (>19 mm zone of inhibition) against Gram-positive bacteria. Similarly, Santos et al. (1964) observed that 30 of the 38 lichen extracts tested inhibited at least one of the 12 test microorganisms, particularly the Gram-positive test bacteria. Other studies also confirmed several lichens to be active against Gram-positive microorganisms. For example, Saenz et al. (2006) found that four species of lichens, namely, *Ramalina canariensis*, *R. subfarinacea*, *Cladonia firma*, and *Lecanora muralis*, were most active against Gram-positive bacteria.

In vitro antifungal activity of acetone, methanol, and chloroform extracts of *Parmotrema tinctorum* was investigated against ten plant pathogenic fungi, viz., *Aspergillus niger*, *A. flavus*, *A. fumigatus*, *Alternaria alternata*, *Fusarium oxysporum*, *F. solani*, *F. roseum*, *Ustilago* sp., *Albugo candida*, and *Penicillium citrinum*, with reference to commercially available synthetic antifungal drug ketoconazole (positive control) using disk diffusion assay (Tiwari et al. 2011). Methanol extract was most effective against all investigated fungi followed by acetone and chloroform extract. Principal component analysis (PCA) concluded that though ketoconazole was effective against five of the investigated fungi, the extracts of *Parmotrema tinctorum* were more effective against the rest of the five broad-spectrum plant pathogenic fungi (*A. fumigatus*, *F. solani*, *F. roseum*, *P. citrinum*, and *Ustilago* spp.).

In a study described by Karthikaidevi et al. (2011), antimicrobial activity of the mangrove lichen Roccella belangeriana collected from the Gulf of Mannar Biosphere Reserve area was tested. The lichen was extracted in different solvents, acetone, methanol, diethyl ether, ethanol, ethyl acetate, petroleum ether, chloroform, and water and tested against 14 bacterial strains and trifungal strains by well diffusion assay. Regarding antibacterial activity, the maximum zone of inhibition was recorded in methanol extracts against *Vibrio cholerae*, and the minimum zone of inhibition was in ethyl acetate extract against *Klebsiella pneumoniae*, *Enterococci* sp., *Salmonella* sp., and *Shewanella* sp. Regarding the antifungal activity, the maximum zone of inhibition was recorded against *Aspergillus niger*, and the minimum was noted against *Rhizopus* sp.

Five common lichens (*Cladonia* sp., *Everniastrum* sp., *Parmelia* sp., *Stereocaulon* sp., and *Usnea* sp.) of Darjeeling hills were extracted from different solvents like ethanol, methanol, petroleum ether, chloroform, and aqueous extracts and tested against four Gram-positive and four Gram-negative bacterial strains. Ethanol extracts exerted stronger inhibitory action followed by methanol extracts. Aqueous extracts manifested less activity to the tested microorganisms (Sharma et al. 2012).

Srivastava et al. (2013) investigated antimicrobial activity of the acetone, methanol, and ethanol extracts of some common lichen species such as *Usnea longissima*, *Everniastrum cirrhatum*, *Peltigera polydactylon*, and *Sulcaria sulcata* which were screened in vitro against six clinically important pathogenic bacteria, *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Escherichia coli* by the Kirby-Bauer technique of disk diffusion method. Minimum inhibitory concentration was taken out by broth microdilution method according to the NCCLS guidelines. It was found that acetone, methanol, and ethanol extracts of the investigated lichens showed relatively strong antimicrobial activity against all the Gram-positive bacteria and two Gram-negative bacteria. The lowest MIC value was observed to be as low as 6.25 μg/ml against *B. cereus* and *U. longissima*.

Antibiotic properties of acetone and methanol extracts from 34 North American lichens were screened against four pathogenic bacteria by Shrestha et al. (2014). The microwell dilution method was used to determine the minimum inhibitory concentration. Most of the lichen extracts demonstrated inhibitory effects against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and methicillin-resistant *S. aureus* with MIC values ranging from 3.9 to 500 μg/ml. In addition, extracts from three species, *Letharia columbiana*, *Letharia vulpina*, and *Vulpicida canadensis* (MIC = 125–500 μg/ml), were also effective against *Escherichia coli*. Generally, acetone extractions were found to be more effective than methanol extractions.

In the study described by Vivek et al. (2014), antibacterial potential of three *Parmotrema* species, viz., *P. tinctorum*, *P. grayanum*, and *P. praesorediosum* from India, was tested against three Gram-positive and five Gram-negative bacteria by agar well diffusion assay. The lichen extracts showed dose-dependent antibacterial activity. Overall, the lichen extracts were more inhibitory to Gram-positive bacteria than Gram-negative bacteria. *P. grayanum* displayed high inhibitory activity

against test bacteria. Balaji and Hariharan (2007) reported marked antimicrobial efficacy of dichloromethane extract of *P. praesorediosum*. Kumar et al. (2010) showed the antibacterial activity of methanol extract of *P. pseudotinctorum*. Sinha and Biswas (2011) reported the antibacterial efficacy of solvent extracts of *P. reticulatum* from Sikkim, India. Verma et al. (2011) found antibacterial efficacy of solvent extracts of *P. nilgherrensis* and *P. sanctiangelii* collected from Karnataka, India. Chauhan and Abraham (2013) showed the inhibitory effect of methanol extract of *Parmotrema* sp. against clinical isolates of bacteria. In addition, Javeria et al. (2013) showed the inhibitory efficacy of solvent extracts of *P. nilgherrense* against drug-resistant bacteria.

In the study described by Ranković et al. (2012), acetone lichen extracts obtained from *Usnea barbata* showed a moderate antibacterial and antifungal activity. It inhibited the microorganisms tested at concentrations from 0.125 to 12.5 mg/ml. The acetone extract from *T. candida* inhibited all the tested microorganisms, but at higher concentrations. In related research, *Evernia prunastri* and *Pseudoevernia furfuraceae* lichens were screened for their antimicrobial effects by Kosanić et al. (2013) who found varying antimicrobial success in the inhibition of Gram-positive and Gram-negative bacteria and fungi, and *Pseudoevernia furfuraceae* was found to be the most effective.

Kosanić et al. (2014a, b) extracted with acetone the three *Cladonia* species (*C. furcata*, *C. rangiferina*, and *C. pyxidata*) in order to investigate their antimicrobial effect. As test organisms in this study, *Bacillus mycoides*, *B. subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, *Aspergillus flavus*, *A. fumigatus*, *Candida albicans*, *Penicillium purpurascens*, and *P. verrucosum* were used. They obtained results showing that extracts from *C. furcata* and *C. rangiferina* showed similar antibacterial and antifungal activity. They inhibited the microorganisms tested at concentrations from 0.78 to 25 mg/ml, while extracts from *C. pyxidata* inhibited all the tested microorganisms, but at higher concentrations.

Lecanora muralis, *Parmelia saxatilis*, *Parmeliopsis ambigua*, *Umbilicaria crustulosa*, and *Umbilicaria polyphylla* were tested for their antibacterial and antifungal activity (Kosanić et al. 2014a, b). The antimicrobial activity was estimated by determination of the minimal inhibitory concentration by the broth microdilution method against six species of bacteria and ten species of fungi, and it has been found that of the lichens tested, *Umbilicaria polyphylla* had the largest antimicrobial activity with minimum inhibitory concentration values ranging from 0.78 to 1.56 mg/ml.

3.5 Studies on Antimicrobial Activities of Lichen Secondary Metabolites

Such as the abovementioned, there are many studies on the antimicrobial activity of crude lichen extracts. However, studies on antimicrobial activity of lichen compounds are scarce and scattered. Lichens have been found to contain a variety of secondary lichen substances with strong antimicrobial activity (Table 3.1).

Table 3.1 List of lichen secondary metabolites used to evaluate antimicrobial activity

Lichen compounds	References
Lecanoric acid	Gomes et al. (2003), Ranković and Mišić (2008), Honda et al. (2010)
Atranorin	Kumar and Müller (1999), Yilmaz et al. (2004), Turk et al. (2006), Ranković et al. (2008, 2014), Kosanić et al. (2014a, b)
Zeorin	Kosanić et al. (2010)
Gyrophoric acid	Candan et al. (2006), Ranković et al. (2008)
Stenosporic acid	Candan et al. (2006)
Protocetraric acid	Tay et al. (2004), Ranković and Mišić (2008), Manojlović et al. (2012)
Fumarprotocetraric acid	Yilmaz et al. (2004), Ranković and Mišić (2008), Kosanić et al. (2014a, b)
Stictic acid	Ranković and Mišić (2008)
Salazinic acid	Candan et al. (2007), Manojlović et al. (2012)
Usnic acid	Lauterwein et al. (1995), Perry et al. (1999), Yilmaz et al. (2003), Ivanova et al. (2004), Tay et al. (2004), Ranković et al. (2008, 2012, 2014), Schmeda-Hirschmann et al. (2008), Ranković and Mišić (2009), Paudel et al. (2010), Ramos and Silva (2010)
Vulpinic acid	Whiton and Lawrey (1982), Lawrey (1986), Lauterwein et al. (1995)
Evernic acid	Whiton and Lawrey (1982), Lawrey (1986), Halama and Van Haluwin (2004), Kosanić et al. (2013)
Lobaric acid	Ingolfsdottir et al. (1998), Piovano et al. (2002), Sundset et al. (2008)
Physodic acid	Turk et al. (2006), Ranković et al. (2008, 2014), Kosanić et al. (2013)
Protolichesterinic acid	Ingolfsdottir et al. (1998), Türk et al. (2003)
Norstictic acid	Tay et al. (2004), Honda et al. (2010), Ranković et al. (2014)
Ramalin	Paudel et al. (2008, 2010)
Barbatic acid	Martins et al. (2010)
Divaricatic acid	Piovano et al. (2002), Kosanić et al. (2010)
Diffractaic acids	Piovano et al. (2002), Honda et al. (2010)
Umbilicaric acid	Buçukoglu et al. (2013)
Homosekikaic acid	Sisodia et al. (2013)
Sekikaic acid	Sisodia et al. (2013)
Parietin	Manojlović et al. (2002, 2005)
Parietinic acid	Manojlović et al. (2002)
Emodin	Manojlović et al. (2002)
Fallacinal	Manojlović et al. (2002)
Fallacinol	Manojlović et al. (2002)
Isodivaricatic acid	Schmeda-Hirschmann et al. (2008)
Divaricatinic acid	Schmeda-Hirschmann et al. (2008)
Hirtusneanoside	Renzaka and Sigler (2007)
Neuropogonines A, B, and C	Ivanova et al. (2002)
Hypostictic acid	Honda et al. (2010)
Norstictic acid	Honda et al. (2010)
Secalonic acid	Honda et al. (2010)
Psoromic acid	Tasdemir and Franzblau (2007)
Vulpic acid	Tasdemir and Franzblau (2007)
Usimines A, B, and C	Paudel et al. (2010)

Literature sources mentioning data for numerous lichen substances with examined antimicrobial activity are mentioned below.

Atranorin (from *Physcia aipolia*), fumarprotocetraric acid (from *Cladonia furcata*), gyrophoric acid (from *Umbilicaria polyphylla*), lecanoric acid (from *Ochrolechia androgyna*), physodic acid (from *Hypogymnia physodes*), protocetraric acid (from *Flavoparmelia caperata*), stictic acid (from *Parmelia conspersa*), and usnic acid (from *Flavoparmelia caperata*) showed relatively strong antimicrobial effects against six bacteria and ten fungi, among which were human, animal, and plant pathogens, mycotoxin producers, and food-spoilage organisms (Ranković and Mišić 2008; Ranković et al. 2008). Usnic acid was found to be the strongest antimicrobial agent (comparable to streptomycin) and physodic and stictic acids the weakest.

The antifungal activity of ten depsidones and five depsides was evaluated, as well as the antibacterial of these compounds and three additional depsides and one diarylether; all of them were isolated from lichens growing in Chile (Piovano et al. 2002). Obtained results showed, in general, negative activity against yeast and filamentous fungi at concentrations of 250 mg/ml. Nevertheless, divaricatic and diffractaic acids, and to a lesser degree lobaric acid, presented a moderate but significant activity against *Microsporum gypseum*, *Trichophyton mentagrophytes*, *T. rubrum*, and *Epidermophyton floccosum*, all of them being dermatophyte fungi which cause skin infections. Regarding antibacterial activity, results indicated that against Gram-negative bacteria, the 19 compounds are inactive. In contrast against Gram-positive bacteria, a marked action can be observed for seven compounds.

Anthraquinones (parietin, parietinic acid, emodin, fallacinal, and fallacinol) from *Caloplaca schaereri* were tested for antimicrobial activity using *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas fluorescens*, *Candida albicans*, *Trichoderma harzianum*, *Aspergillus niger*, and *Penicillium verrucosum* (Manojlović et al. 2002). All the anthraquinones tested showed potent antibacterial activity against *B. subtilis*, *S. aureus*, and *P. fluorescens* (MIC 20–320 μg/ml), but only parietinic acid showed any activity against *E. coli* (MIC 160 μg/ml). Their effects are generally most potent on *B. subtilis* and *P. fluorescens*. Fallacinol was most potent against *S. aureus*. Fallacinol was the most active (potent) of the isolated compounds against all the fungi tested but was particularly active against *T. harzianum*, *A. niger*, and *P. verrucosum* (MIC 10–40 μg/ml). Potent antifungal effects on the fungi tested also showed parietinic acid (MIC 20–80 μg/ml), while parietin had MIC values of 80, 40, and 20 μg/ml for *C. albicans*, *P. verrucosum*, *A. niger*, and *T. harzianum*, respectively. Emodin showed MIC values of 20–40 μg/ml for *A. niger*, *T. harzianum*, and *P. verrucosum* but was much less effective against *C. albicans* (MIC 80 μg/ml).

According to Schmeda-Hirschmann et al. (2008), isodivaricatic acid, divaricatinic acid, and usnic acid, the main lichen metabolites in *Protousnea poeppigii*, displayed antifungal action against *Microsporum gypseum*, *Trichophyton mentagrophytes*, and *T. rubrum*, usnic acid being less active. Divaricatic acid and zeorin constituents of *Lecanora frustulosa* and *Parmeliopsis hyperopta* have been screened in vitro against the *Bacillus mycoides*, *Bacillus*

subtilis, *Staphylococcus aureus*, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Botrytis cinerea*, *Candida albicans*, *Fusarium oxysporum*, *Mucor mucedo*, *Paecilomyces variotii*, *Penicillium purpurascens*, *Penicillium verrucosum*, and *Trichoderma harzianum*. Divaricatic acid and zeorin showed strong activity against both bacteria and fungi (Kosanić et al. 2010). *Bacillus subtilis*, *B. cereus*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Proteus vulgaris*, *Listeria monocytogenes*, *Aeromonas hydrophila*, *Candida albicans*, and *Candida glabrata* growth were inhibited by usnic acid, atranorin, and fumarprotocetraric acid constituents of *Cladonia foliacea* (Yilmaz et al. 2004). Similarly, anti-Gram-positive activities have been reported for evernic acid, vulpinic acid, and hirtusneanoside (Renzaka and Sigler 2007).

Antimicrobial activity of salazinic acid isolated from *Parmelia sulcata* has been screened against 28 foodborne bacteria and fungi, and it has been found that these compounds showed activity against *Pseudomonas aeruginosa* and *Salmonella typhimurium* as well (Candan et al. 2007). Antimicrobial activity of fumarprotocetraric acid, lecanoric acid, protocetraric acid, and stictic acid isolated from the lichen of *Cladonia furcata*, *Ochrolechia androgyna*, *Parmelia caperata*, and *Parmelia conspersa* was studied in relation to *Bacillus mycoides*, *Bacillus subtilis*, *Staphylococcus aureus*, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Botrytis cinerea*, *Candida albicans*, *Fusarium oxysporum*, *Mucor mucedo*, *Paecilomyces variotii*, *Penicillium purpurascens*, *Penicillium verrucosum*, and *Trichoderma harzianum* (Ranković and Mišić 2008). The antimicrobial activity was estimated by determining the minimal inhibited concentration by the broth tube dilution method. The researched lichen components inhibited the growth of all the tested microorganisms. The lowest MCI value (0.031 mg/ml) was measured for the fumarprotocetraric acid related to the *Klebsiella pneumoniae* species. The weakest antimicrobial activity was found in stictic acid, which inhibited most of the tested microorganisms at significantly higher concentrations.

Whiton and Lawrey (1982) reported that ascospore germination of *Sordaria fimicola* was significantly inhibited by evernic and vulpinic acids. Usnic acid, evernic acid, and vulpinic acid inhibited the growth of the Gram-positive bacteria *Staphylococcus aureus*, *Bacillus subtilis*, and *Bacillus megaterium*, but had no effect on the Gram-negative bacteria *Escherichia coli* or *Pseudomonas aeruginosa* (Lawrey 1986). Three new depsidones (neuropogonines A, B, and C) isolated from a *Neuropogon* showed a moderate activity on a *Mycobacterium vaccae* strain (Ivanova et al. 2002). Two years later, the same authors from this lichen isolated usnic acid and further investigated for antimicrobial activity against pathogenic Gram-positive and Gram-negative bacteria as *Streptococcus pyogenes*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Klebsiella pneumoniae*, and *Salmonella typhimurium* (Ivanova et al. 2004).

Manojlovic et al. (2005) reported antifungal activity of the anthraquinone parietin isolated from *Caloplaca cerina*. A potent fungitoxic compound, lecanoric acid, was isolated from *Parmotrema tinctorum* lichen and tested against the fungus

Cladosporium sphaerospermum (Gomes et al. 2002). In addition, MIC values obtained from monoaromatic phenols (methyl beta-orsellinate and methyl and ethyl orsellinates) derived from various Icelandic lichen species were found equal or higher than usual preservatives (methyl- and propyl-*p*-hydroxybenzoates, *o*-cresol) (Ingólfsdóttir et al. 1985).

The antimicrobial activity of gyrophoric acid and stenosporic acid constituents of the *Xanthoparmelia pokornyi* lichen has been screened against some foodborne bacteria and fungi. Both acids showed antimicrobial activity against *Aeromonas hydrophila*, *Bacillus cereus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Proteus vulgaris*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Yersinia enterocolitica*, *Candida albicans*, and *Candida glabrata* while were inactive against the tested filamentous fungi (Candan et al. 2006).

Tasdemir and Franzblau (2007) investigated four commercially available lichen metabolites, (+)-usnic acid, evernic acid, psoromic acid, and vulpic acid, for their in vitro antitubercular effects. $H_{37}Rv$ strain of *Mycobacterium tuberculosis* and the well-established microplate alamar blue assay were used for the determination of the MIC values. (+)-Usnic acid proved to be the most potent antituberculotic agent (MIC = 5.2 μg/ml), followed by psoromic acid (MIC = 44 μg/ml) and vulpic acid (MIC = 140 μg/ml). Evernic acid was found to be inactive at highest concentrations tested (MIC > 200 μg/ml). Honda et al. (2010) described the extraction and identification of several classes of phenolic compounds from the lichens *Parmotrema dilatatum*, *Parmotrema tinctorum*, *Pseudoparmelia sphaerospora*, and *Usnea subcavata* and determined their antitubercular activity. The depsides (atranorin, diffractaic, and lecanoric acids), depsidones (protocetraric, salazinic, hypostictic, and norstictic acids), xanthones (lichexanthone and secalonic acid), and usnic acid, as well as seven orsellinic acid esters, five salazinic acid 8′,9′-*O*-alkyl derivatives, and four lichexanthone derivatives, were evaluated for their activity against *Mycobacterium tuberculosis*. Diffractaic acid was the most active compound (MIC value 15.6 mg/ml, 41.6 mM), followed by norstictic acid (MIC value 62.5 mg/ml, 168 mM) and usnic acid (MIC value 62.5 mg/ml, 182 mM). Hypostictic acid (MIC value 94.0 mg/ml, 251 mM) and protocetraric acid (MIC value 125 mg/ml, 334 mM) showed moderate inhibitory activity. The other compounds showed lower inhibitory activity on the growth of *M. tuberculosis*, varying from MIC values of 250–1,370 mM.

Cladonia verticillaris lichen lectin was evaluated by Ramos et al. (2014) for its antimicrobial potential, and it showed activity against Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*, and *Enterococcus faecalis*) and Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) assayed strains, with greater inhibitory effect on the growth of *E. coli* (MIC of 7.18 μg/ml). The lowest minimum bactericidal concentration (MBC, 57.4 μg/ml) was detected against *E. faecalis*. The antifungal assay performed with *Trichophyton mentagrophytes*, *Microsporum gypseum*, *Trichophyton rubrum*, *Trichosporon cutaneum*, and *Trichosporon asahii*. *Cladonia verticillaris* lichen lectin was the most active against *T. rubrum* with an inhibition percentage of 35 % compared to negative control.

A total of five compounds, usnic acid, usimine A, usimine B, usimine C, and ramalin, were isolated by bioactivity-guided fractionation of the methanol extract of *Ramalina terebrata* (Paudel et al. 2010). The qualitative antibacterial activities of the isolated compounds were determined by the disk diffusion method, while the minimum inhibitory concentration (MIC) determination assay gave the quantitative strength of the test samples. All the test samples showed antibacterial activity against *Bacillus subtilis*, and usnic acid showed antibacterial activity against *Staphylococcus aureus*. The MIC values of the isolated compounds against *B. subtilis* were in the range of 1–26 μg/ml.

Manojlović et al. (2012) investigated antibacterial and antifungal activities for protocetraric acid from *Parmelia caperata* lichen and depsidone salazinic acid from *Parmelia saxatilis* species. Antioxidant activities of these isolated metabolites were evaluated against *Bacillus mycoides*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Candida albicans*, *Penicillium purpurascens*, and *Penicillium verrucosum*. As a result of the study, salazinic acid and protocetraric acid showed similar antimicrobial activity, but antibacterial activity was stronger than antifungal activity for both components.

In the study described by Ranković et al. (2012), lichen compounds norstictic acid isolated from *Toninia candida* and usnic acid from *Usnea barbata* demonstrated very strong antimicrobial activity. The MIC for different components relative to the tested microorganisms ranged from 0.0008 to 1 mg/ml. The strongest antimicrobial activity was found in usnic acid, which in extremely low amounts inhibited all species of bacteria and fungi. Similarly, 2 years later, antimicrobial activities of major lichen metabolites in *Hypogymnia physodes* lichen (physodic acids, atranorin, and usnic acid) were studied by Ranković et al. (2014). They found that usnic acid and physodic acid showed very strong and similar antimicrobial activity, followed by atranorin. Antibacterial activity was stronger than antifungal activity for all compounds.

Evernic acid and physodic acid from *Evernia prunastri* and *Pseudoevernia furfuraceae* lichens were screened for their antimicrobial effects by the broth microdilution method (Kosanić et al. 2013), and physodic acid was found to be the most effective. One year later, in a related experiment, Kosanić et al. (2014a, b) investigated antimicrobial activity for atranorin and fumarprotocetraric acid isolated from *Cladonia* lichen. Antimicrobial activity was studied in relation to five species of bacteria and five species of fungi. The isolated lichen components demonstrated very strong antimicrobial activity. The MIC for different components relative to the tested microorganisms ranged from 0.015 to 1 mg/ml. The strongest antimicrobial activity was found in fumarprotocetraric acid, which in extremely low amounts inhibited all the species of bacteria and fungi.

The results obtained in studies related to the antimicrobial activity of lichens indicate differences in activity between extracts depending on the species of lichen and as a function of the type of extracting solvent. These results are in agreement with the suggestion of Oloke and Kolawole (1998) that bioactive components of any medical plant have different solubility in different extracting solvents.

Numerous researchers found lower antimicrobial activity of aqueous extracts in comparison with acetone, methanol, or other organic solvent extracts (Land and Lundstrom 1998; Madamombe and Afolajan 2003; Ranković et al. 2008; Kosanić et al. 2010). The reason for the weak activity of aqueous extracts is that active substances present in the thalli of lichens are insoluble or poorly soluble in water (Kinoshita et al. 1994).

In general, in studies regarding antimicrobial activity of lichens, fungi and Gram-negative bacteria were more resistant than Gram-positive ones. The difference of sensitivity between Gram-positive and Gram-negative bacteria and fungi can be ascribed to morphological differences between these microorganisms, above all to differences in permeability of the cell wall (Nostro et al. 2000). The cell walls of Gram-positive bacteria are made of peptidoglycans and teichoic acids, while those of Gram-negative bacteria are made of peptidoglycans, lipopolysaccharides, and lipoproteins (Heijenoort 2001; Ranković et al. 2008). The cell walls of fungi are poorly permeable and consist of polysaccharides such as hitchin and glucan (Farkaš 2003).

Since microorganisms have developed resistance to many antibiotics, pharmacologists need to pursue new sources for antimicrobial agents. All these results suggest that lichens and their metabolites yield significant new bioactive substances for the treatment of various diseases caused by microorganisms. New compounds are to be described from poorly studied lichens, and even in species that are considered chemically well known, new chemical strains are still detected (e.g., Stocker-Worgotter et al. 2004). Many lichens are known which contain structurally unknown lichen products. Here we opened the entrance door of a vast and interesting field of research.

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Chapter 4
Studies on Antioxidant Properties of Lichen Secondary Metabolites

Marijana Kosanić and Branislav Ranković

Abstract At the present time, it is suspected that much used synthetic antioxidants have toxic and carcinogenic effects. Consequently, there is a growing interest towards finding new antioxidants of natural resources without any undesirable effect. Numerous in vitro studies on plants, micro- and macroalgae, macromycetes and lichens strongly support the fact that their constituents with antioxidant capacity are capable of exerting protective effects against oxidative stress in biological systems. Therefore, it is of prime importance to utilise natural antioxidants for their protective effect against oxidative stress and physiological dysfunctions. In the quest for novel natural antioxidant sources, our prime interest has focused on lichens. In recent time, numerous studies point to the importance of lichens in the neutralisation of free radicals. Lichens are rich in the secondary metabolites, primarily phenols, which are well known for its antioxidant properties. Because of that, the present chapter focuses on the role of lichens and their secondary metabolites in combating danger posed by overproduced free radicals.

4.1 Free Radicals, Oxidative Stress and Antioxidants

Free radicals (reactive oxygen species, such as the hydroxyl radical, superoxide anion and hydrogen peroxide, and reactive nitrogen species, such as nitric oxide and peroxynitrite) (Fig. 4.1) play an important role in many chemical processes in the cells. At low or moderate concentrations, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are necessary for the maturation process of cellular structures and can act as weapons for the host defence system (Halliwell 1995; Squadriato and Pelor 1998; Young and Woodside 2001; Droge 2002; Sangameswaran et al. 2009; Huda-Faujan et al. 2009). Under normal conditions, the balance between the generation and diminution of ROS is controlled by the antioxidant defence system. However, under certain pathological conditions such as

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B. Ranković (ed.), *Lichen Secondary Metabolites*,
DOI 10.1007/978-3-319-13374-4_4

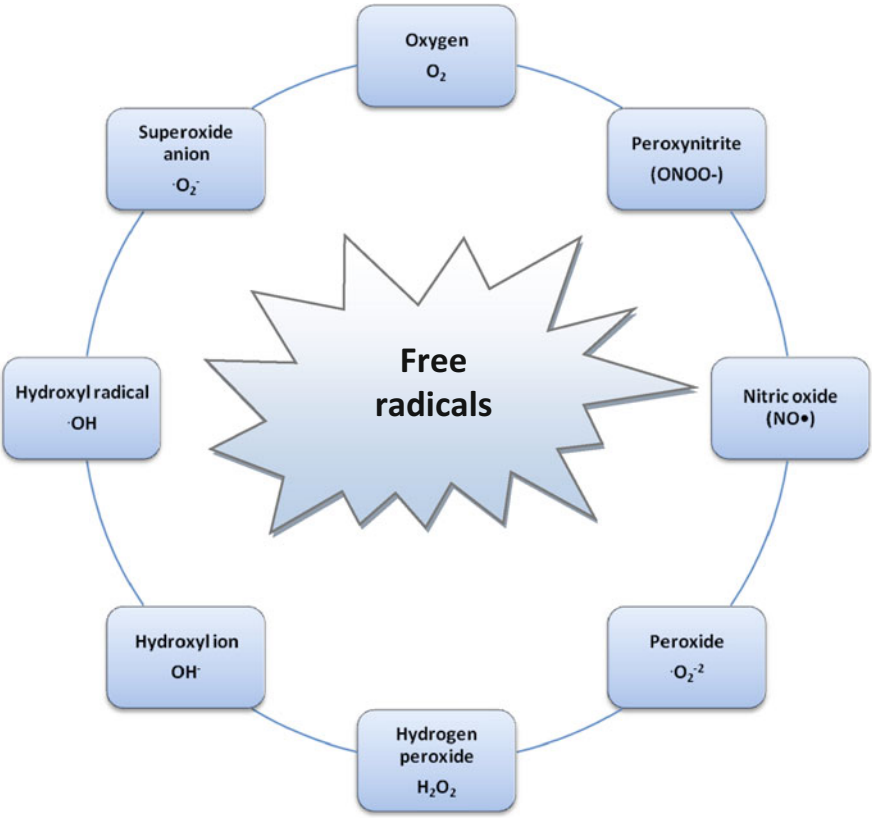

Fig. 4.1 Free radicals (reactive oxygen species and reactive nitrogen species)

drought, salinity, chilling, metal toxicity and UV-B radiation as well as pathogens, when ROS are not effectively eliminated by the antioxidant defence system, the dynamic balance between the generation and diminution of ROS is broken (Lobo et al. 2010).

When produced in excess, free radicals and oxidants generate a phenomenon called oxidative stress, a deleterious process that can seriously alter the cell membranes and other structures such as proteins, lipids, lipoproteins and deoxyribonucleic acid (Betteridge 2000). If not regulated properly, oxidative stress can induce a variety of chronic and degenerative diseases such as Alzheimer's disease, atherosclerosis, emphysema, hemochromatosis, many forms of cancer (e.g. melanoma, Parkinson's disease and schizophrenia) as well as the ageing process (Fig. 4.2) (Sangameswaran et al. 2008; Sachindra et al. 2010).

Antioxidants are substances which possess the ability to protect the body from damage caused by oxidative stress (Souri et al. 2008). The body has several mechanisms to counteract oxidative stress by producing antioxidants, either naturally generated in situ (endogenous antioxidants) or externally supplied through foods (exogenous antioxidants) (Fig. 4.3).

Fig. 4.2 Diseases induced by oxidative stress

In addition, in order to protect biomolecules against the attack of ROS and/or to suppress the resultant damage, synthetic antioxidants have been used for industrial processing in recent years. The most extensively used synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) (Ramachandra et al. 2012). They are used widely in the food industry because of their effectiveness and generally being less expensive than natural antioxidants. However, since synthetic antioxidants are often carcinogenic, finding natural substitutes is of great interest (Zhang et al. 2009). Concerns regarding toxicological effects and carcinogenic potential of synthetic antioxidants have prompted the need for natural alternatives in the last few decades (Lu and Foo 2001; Aligiannis et al. 2003; Vagi et al. 2005; Es-Safi et al. 2006). Therefore, the importance of search for the exploitation of effective natural antioxidants has greatly increased in recent years (Pokorny et al. 2001; Gulcin et al. 2004; Naveena et al. 2008).

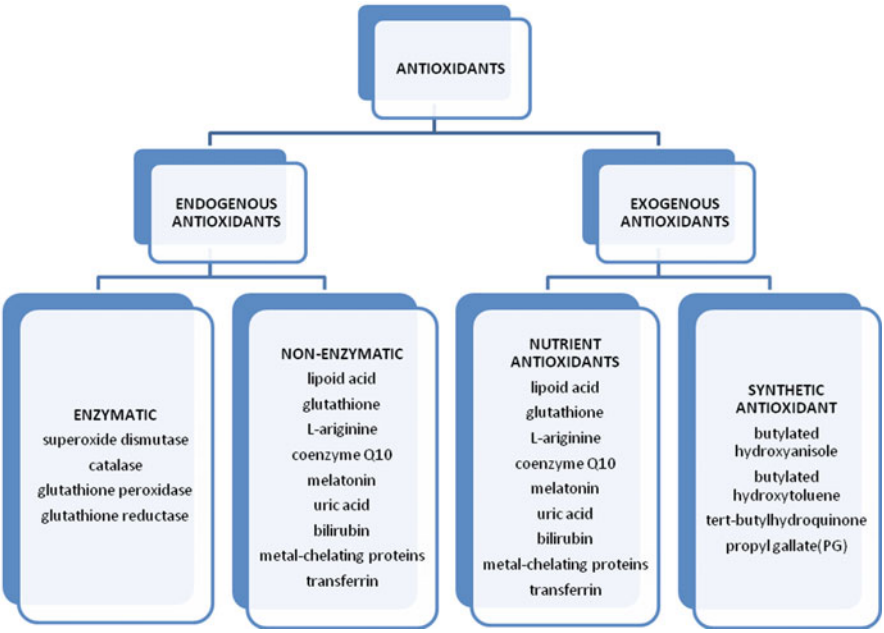

Fig. 4.3 Endogenous antioxidants and exogenous antioxidants for protecting the body from damage caused by oxidative stress

4.2 Antioxidant Properties of Lichens

Lichens are a good source of natural antioxidants. Although mainly hydrophobic, the phenolic nature of the major secondary metabolites of lichens is expected to afford antioxidant properties. Phenolics are the major secondary metabolites from lichens that play crucial function in the regulation of lichen growth and development in stressful and unfavourable climatic conditions. Lichens grow in extreme environmental conditions which in turn could cause up-regulated pathways of secondary metabolite synthesis and increased production of polyphenolics that have protective effects against oxidative stress owing to their antioxidant capacities (Kumar et al. 2014). Lichens produce many phenolic compounds including depsides, depsidones, dibenzofurans and pulvinic acid derivatives (Paudel et al. 2012).

Phenolic compounds are widespread products of secondary metabolism of lichen, and antioxidant activity is most frequently associated with their presence. Lichen phenolics are mainly depsides, depsidones and dibenzofurans, whereas vascular plant phenolics include tannins, lignins and flavonoids. Orsellinic acid is the basic unit in the biosynthesis of lichen phenolics. Lichen phenols are generally secreted by the fungal partner and deposited as crystals on the surface of the cell wall of the fungal hyphae. Lichen phenols are primarily acetate-polymalonate derived with the exception of pulvinic acid derivates which are synthesised via

the shikimic acid pathway. Lichen phenolics are composed of two monocyclic phenols joined either by an ester bond as in depsides or by both ester and ether bonds in depsidones or a furan heterocycle bond as found in dibenzofurans, such as usnic acid (Watson 2014).

Phenolic compounds contain in its structure an aromatic ring by one or more hydroxyl groups. It is believed that the antioxidant activity of the phenol is primarily a result of their ability to be the donor of hydrogen atoms and eliminate the free radicals to form less reactive phenoxyl radicals (Sawa et al. 1999).

$$\mathrm{Ph-OH + ROO \rightarrow Ph-O^{\bullet} + ROOH}$$

$$\mathrm{Ar-OH + OH \rightarrow H2O + ArO^{\bullet}}$$

It has been shown that with the increase of the number of hydroxyl groups in the molecule, as well as the extension of the side chain of an antioxidant increases the activity of these compounds, probably due to the possibility of stabilisation free radical forms the side chain conjugation (Yanishlieva et al. 1999). Because of the large number of potential reactive centres, the phenol molecule has the ability to react with the radicals of the same time.

Phenols possess ideal structural chemistry for antioxidant activity and have been shown to be more effective in vitro than vitamins E and C on molar basis (Michalak 2006). As described by Bors et al. (1990), there are three structural features that are important determinants for the antioxidant potential of phenols:

(a) The *orto* 3′,4′-dihydroxy structure in the B ring
(b) The 2,3-double bond in conjunction with the 4-oxo group in the C ring (which allows conjunction between the A and B ring or electron delocalisation)
(c) The presence of a 3-OH group in C ring and a 5-OH group in the A ring

Among them, the 3-OH group is the most significant determinant of electron-donating activity.

Mainly, the antioxidant activity of phenolic compounds is affected by their chemical structure. The antioxidant activity of phenols also depends on the type and polarity of the extracting solvent, the isolation procedures, purity of active compounds, as well as the test system and substrate to be protected by the antioxidant (Moure et al. 2001).

When antioxidant capacities of the lichens are compared with their phenolic constituents, it could be concluded that antioxidative nature of the lichens might depend on their phenolics. There are many reports which correlate the total phenolic content of lichens and their antioxidant activity. For example, for the methanol extract of *Lobaria pulmonaria*, there was a strong correlation between antioxidant activity and total phenolic content (Odabasoglu et al. 2004). Kekuda et al. (2012) found highly positive relationship between total phenols and antioxidant activity in methanol extract of a foliose macrolichen *Everniastrum cirrhatum*.

Pavithra et al. (2013) found a high correlation between antioxidant efficacy of a macrolichen *Usnea pictoides* and its phenolic content. A positive correlation was seen between the phenolic content and total antioxidant activity of *Anaptychia ciliaris*, *Nephroma parile*, *Ochrolechia tartarea* and *Parmelia centrifuga* having correlation coefficient values (r) of 0.990 (Ranković et al. 2010a). Kosanić and Ranković (2011a) found that content of total phenolic of the extracts of *Cetraria islandica*, *Lecanora atra*, *Parmelia pertusa*, *Pseudevernia furfuracea* and *Umbilicaria cylindrica* was strongly related with DPPH radical scavenging activity ($r = 0.966$), with reducing power ($r = 0.944$) and with superoxide anion radical scavenging ($r = 0.823$). A certain correlation also was established between the antioxidant activity and the total phenol content for the *Toninia candida* methanol, chloroform and petrol ether extracts (Manojlović et al. 2012).

Many other studies have shown a direct correlation between the phenolic content and the antioxidant activity (Tilak et al. 2004; Coruh et al. 2007; Rekha et al. 2012; Poornima et al. 2012).

However, the total phenolic content and antioxidant potency did not always correlate. Odabasoglu et al. (2005) determined the total antioxidant activity, total phenolic content and the reducing power of methanol and water extracts of four lichen species, *Bryoria fuscescens*, *Dermatocarpon intestiniformis*, *Peltigera rufescens* and *Pseudevernia furfuracea*. Water and methanol extracts of *P. rufescens* showed the highest antioxidant activity. However, there was no correlation between antioxidant activity and total phenolic content of the extracts. Although the methanol extract of *P. furfuracea* had the highest total phenolic contents, it exhibited low antioxidant activity. In contrast, there was a strong correlation between reducing power and total antioxidant activity of the extracts. Stanly et al. (2011) evaluated the antioxidant activity and total phenol content of four lichen species belonging to the genus *Ramalina*, *Parmotrema*, *Bulbothrix* and *Cladia* collected from Malaysia. There was no correlation between total phenolic content and radical scavenging activity of methanol extracts of all the tested species. For example, the antioxidant properties of some isolated phenols are not so impressive. For instance, two depsidones were found only slightly more active than the commercial quercetin in a superoxide scavenging assay with IC50 # 600 lM (Lohézic-Le Dévéhat et al. 2007), and none of the orcinol or orsellinate derivatives were as active as the commercial gallic acid to reduce DPPH (Lopes et al. 2008).

These results suggest that the antioxidant activity of some tested extracts might be attributed to the presence of non-phenolic compounds. Nevertheless, it should be taken into consideration that individual phenolics may have distinct antioxidant activities; there may be antagonistic or synergistic interactions between phenolics and other compounds like carbohydrates, proteins, etc. (Odabasoglu et al. 2005).

4.3 Assays to Determine Antioxidant Capacities of Lichens

Methods to assess the antioxidant activity of lichens are based on hydrogen atom transfer (HAT) and others on electron transfer (ET).

HAT-based assays measure the capability of phenolic antioxidants to quench free radicals (generally, peroxyl radicals considered to be biologically more relevant) by H-atom donation. The HAT mechanisms of antioxidant action in which the hydrogen atom (H) of a phenol (Ar–OH) is transferred to a $ROO^{\bullet}$ radical can be summarised by the reaction

$$ROO^{\bullet} + AH/ArOH \rightarrow ROOH + A^{\bullet}/ArO^{\bullet}$$

where the aryloxy radical ($ArO^{\bullet}$) formed from the reaction of antioxidant phenol with peroxyl radical is stabilised by resonance. The AH and Ar–OH species denote the protected biomolecules and phenolic antioxidants, respectively. Effective phenolic antioxidants need to react faster than biomolecules with free radicals to protect the latter from oxidation. Since, in HAT-based antioxidant assays, both the fluorescent probe and antioxidants react with $ROO^{\bullet}$, the antioxidant activity can be determined from competition kinetics by measuring the fluorescence decay curve of the probe in the absence and presence of antioxidants, integrating the area under these curves and finding the difference between them (Huang et al. 2005; Prior et al. 2005).

HAT-based assays include oxygen radical absorbance capacity (ORAC) assay, TRAP assay using R-phycoerythrin as the fluorescent probe, crocin bleaching assay using 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) as the radical generator and β-carotene bleaching assay, although the latter bleaches not only by peroxyl radical attack but by multiple pathways (Huang et al. 2005; Prior et al. 2005).

In most **ET-based assays**, the antioxidant action is simulated with a suitable redox-potential probe, namely, the antioxidants react with a fluorescent or coloured probe (oxidising agent) instead of peroxyl radicals. Spectrophotometric ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes colour when reduced. The degree of colour change (either an increase or decrease of absorbance of the probe at a given wavelength) is correlated to the concentration of antioxidants in the sample. 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)/trolox-equivalent antioxidant capacity (TEAC) and 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) (Brand-Williams et al. 1995; Bondet et al. 1997; Sanchez-Moreno et al. 1998) are decolourisation assays, whereas in Folin total phenols assay (Folin and Ciocalteu 1927; Singleton et al. 1999), ferric reducing antioxidant power (FRAP) (Benzie and Strain 1996; Benzie and Szeto 1999) and cupric reducing antioxidant capacity (CUPRAC) (Apak et al. 2004), there is an increase in absorbance at a prespecified wavelength as the antioxidant reacts with the chromogenic reagent (i.e. in the latter two methods, the lower valencies of iron and copper, namely, Fe(II) and Cu(I), form charge transfer complexes with the corresponding ligands, respectively).

ET-based assays generally set a fixed time for the concerned redox reaction and measure thermodynamic conversion (oxidation) during that period. Although the reducing capacity of a sample is not directly related to its radical scavenging capability, it is a very important parameter of antioxidants (Apak et al. 2013).

4.4 Antioxidant Activities of Lichen Extracts

Lichens are proven to be good source of antioxidants, and a plenty of literatures have supported the antioxidant action of these organisms. Few reports concerning the antioxidative nature of pure lichen metabolites are available in the literature; most of the publications describe the antioxidant activities of crude lichen extracts.

There was a first report in 1993 (Yamamoto et al. 1993) on antioxidation activity in lichens by the method using SOD. Thereafter, antioxidant activities of many lichen species were assessed by numerous researchers. For example, Aslan et al. (2006) investigated antioxidant activity of methanol extract of *Evernia divaricata*, *Evernia prunastri*, *Cladonia foliacea*, *Dermatocarpon miniatum* and *Neofuscella pulla* by scavenging of free radical DPPH and the inhibition of linoleic acid oxidation. They found that extracts of *Cladonia foliacea*, *Evernia divaricata*, *Evernia prunastri* and *Neofuscella pulla* did not exert any activity in both assays, whereas those of *Dermatocarpon miniatum* provided 50 % inhibition at 396.1 μg/ml concentration in the former and gave 49 % inhibition in the latter. Mitrović et al. (2011) also studied antioxidant activity of methanol extract of *Parmelia sulcata*, *Flavoparmelia caperata*, *Evernia prunastri*, *Hypogymnia physodes* and *Cladonia foliacea* and found than they exhibited DPPH radical scavenging activity.

The antioxidant activity of ethanol extract of *Sanionia uncinata* was evaluated by Bhattarai et al. (2008a) by analysing its reducing power, superoxide scavenging activity, ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) cation scavenging activity and DPPH free radical scavenging activity. They found that tested lichen species could be an important source of natural antioxidant agents. Kumar et al. (2010b) reported significant antioxidant activity for methanol extracts of *Ramalina hossei* and *Ramalina conduplicans*; Yucel et al. (2007) for chloroform, methanol and aqueous extracts of *Cladonia rangiformis*; and Odabasoglu et al. (2005) for methanol and aqueous extracts of *Bryoria fuscescens*, *Dermatocarpon intestiniformis*, *Peltigera rufescens* and *Pseudevernia furfuracea*. Strong antioxidant effect was also found in the methanol extract of *Usnea ghattensis* (Verma et al. 2008), in methanol extract of *Parmotrema pseudotinctorum* (Kumar et al. 2010a, b), as well as in acetone, methanol and aqueous extracts of lichens *Cladonia furcata*, *Hypogymnia physodes*, *Lasallia pustulata*, *Parmelia caperata* and *Parmelia sulcata* (Kosanić et al. 2011).

Sisodia et al. (2013) evaluated antioxidant activity for different extract of *Ramalina roesleri* species and found that the DPPH radical scavenging activity of extracts ranged from 29.42 to 87.90 %. Sharma et al. (2012) examined the antioxidant activity of two common lichens, namely, *Parmotrema reticulatum* and *Usnea*

sp. from Darjeeling hills. The antioxidant assay of different concentration of ethanolic and methanolic extracts of lichens was determined with respect to five parameters, i.e. DPPH radical scavenging activity, total antioxidant activity, reducing power ability and flavonoid and phenolic content. The DPPH radical scavenging ranged from 10 to 31.5 % for methanol extracts of *Parmotrema reticulatum* and *Usnea* sp., respectively, and for reducing power measured values of absorbance varied from 0.376 to 0.514. In addition, total phenolic content of the extracts was high, and total flavonoid content was moderate.

The methanol extracts of 24 lichen species were tested for antioxidant activities in vitro. It was found that in DPPH assay, three species *Peltigera* sp., *Cladonia* sp. and *Canoparmelia* sp. showed comparable activity with commercial standard, BHA. In ABTS+ assay, extracts of *Parmotrema* sp., *Ramalina* sp., *Peltigera* sp. and *Cladonia* sp. showed stronger activity than ascorbic acid. The observed data indicated that the high altitude lichens contain stronger antioxidant constituents (Paudel et al. 2012).

In the study described by Mastan et al. (2014), the secondary metabolites of lichens *Cladonia fimbriata*, *Parmeliopsis ambigua*, *Punctelia subrudecta* and *Evernia mesomorpha* were extracted in the two solvents methanol and water. The lichen extracts showed comparable and strong antioxidant activity and exhibited higher DPPH and hydroxyl radical scavenging activity. Among the tested lichen extracts, water extract of *Evernia mesomorpha* gave the highest reducing power, although the reducing activity was lower than the standard ascorbic acid. These findings provided evidence that crude aqueous and organic solvent extracts of lichens contain antioxidant important compounds.

Gulcin et al. (2002) found that the aqueous extracts of *Cetraria islandica* had a strong inhibition on peroxidation of linoleic acid, reducing power, superoxide anion radical scavenging and free radical scavenging activities. Similar results were reported by Behera et al. (2005) for different extracts from the lichen *Usnea ghattensis* which was found their strong effect on DPPH radical, superoxide anion radical, nitric oxide and the strong inhibition of lipid peroxidation. Kekuda et al. (2009) find strong antioxidant activity for the extracts of the lichen *Parmotrema pseudotinctorum* and *Ramalina hossei*. Manojlovic et al. (2010) explored antioxidant properties of *Laurera benguelensis* by scavenging of DPPH radical and found that this lichen provided a weak radical scavenging activity.

Anaptychia ciliaris, *Nephroma parile*, *Ochrolechia tartarea* and *Parmelia centrifuga* were screened for antioxidant activity by Ranković et al. (2010a, b). They found that the methanol extract of the *Parmelia centrifuga* showed a strong antioxidant activity, in comparison to the extracts from *Anaptychia ciliaris*, *Nephroma parile* and *Ochrolechia tartarea* which were relatively weaker. *Lecanora muralis*, *Parmelia saxatilis*, *Parmeliopsis ambigua*, *Umbilicaria crustulosa* and *Umbilicaria polyphylla* were tested for DPPH radical scavenging, superoxide anion radical scavenging and reducing power (Kosanić et al. 2014a), and it has been found that of the lichens tested, *Umbilicaria polyphylla* had the largest antioxidant activities.

Antioxidant activity of petroleum ether, chloroform, ethyl acetate and methanol extracts of *Usnea pictoides* lichen was determined by DPPH free radical scavenging assay and ferric reducing assay (Pavithra et al. 2013). The scavenging potential of methanol extract was higher than other extracts, and also, in ferric reducing assay, methanol extract showed stronger reducing power than other extracts.

Usnea pictoides was found to be a strong antioxidant agent by Pavithra et al. (2013). The lichen was powdered and extracted sequentially using solvents of increasing polarity, viz., petroleum ether, chloroform, ethyl acetate and methanol. Antioxidant activity of solvent extracts was determined by DPPH free radical scavenging assay and ferric reducing assay. A dose-dependent scavenging of DPPH radicals by solvent extracts was observed. The scavenging potential of methanol extract was higher than other extracts. In ferric reducing assay, methanol extract showed stronger reducing power than other extracts. Overall, extracts containing high phenolic contents exhibited stronger antioxidant activity. A positive correlation was observed between total phenolic content and the antioxidant activity of lichen extracts.

In related study, Odabasoglu et al. (2004) determined the antioxidant activities, reducing powers and total phenolic contents of methanol and water extracts of three lichen species, *Usnea longissima*, *Usnea florida* and *Lobaria pulmonaria*. Of the extracts tested, the methanol extracts of *Lobaria pulmonaria* and *Usnea longissima* showed potent antioxidant activities. The methanol extract of *Lobaria pulmonaria* also had the highest total phenolic contents (87.9 mg/g lyophilisate). For the methanol extract of this species, there was also a strong correlation between antioxidant activity and total phenolic contents. However, a similar correlation was not observed for *Usnea longissima*. Although the methanol extract of *Usnea longissima* had a lower phenolic content (38.6 mg/g lyophylisate), it exhibited potent antioxidant activity. On the other hand, there was a strong correlation between the reducing powers and the total phenolic contents of the extracts. The highest reducing power was determined for the methanol extract of *Lobaria pulmonaria*.

Ranković et al. (2010a, b) evaluated methanol extracts of the lichens *Cetraria pinastri*, *Cladonia digitata*, *Cladonia fimbriata*, *Fulgensia fulgens*, *Ochrolechia parella* and *Parmelia crinite* for their antioxidant activity. They found that the methanol extract of the *Cetraria pinastri* showed a strong antioxidant activity, whereas the extracts of the species *Fulgensia fulgens*, *Cladonia fimbriata* and *Parmelia crinite* showed the moderate one and the extract of the species *Ochrolechia parella* and *Cladonia digitata* the weak one. The methanol extract of the lichen *Cetraria pinastri* had the biggest total phenol content (32.9 mg/g of the dry extract). A certain correlation was established between the antioxidant activity and the total phenol content for the researched lichen extracts.

Acetone, methanol and aqueous extracts of the lichens *Cetraria islandica*, *Lecanora atra*, *Parmelia pertusa*, *Pseudevernia furfuracea* and *Umbilicaria cylindrica* were found to possess effective antioxidant activities (Kosanić and Ranković 2011a). Antioxidant activities of the tested extracts were studied by DPPH radical scavenging, superoxide anion radical scavenging and reducing

power. The DPPH radical scavenging activity for studied species ranged from 32.68 to 94.70 %. For reducing power, measured values of absorbance varied from 0.016 to 0.109. The superoxide anion scavenging activity for different extracts was 7.31–84.51 %. In addition, the high contents of total phenolic compounds suggest that phenols might be the major antioxidant compounds in tested extracts. The same authors also found relatively strong antioxidant activity for acetone, methanol and aqueous extracts of the lichens *Cladonia furcata*, *Hypogymnia physodes* and *Umbilicaria polyphylla* (Kosanić and Ranković 2011b).

Antioxidant effect of methanol, chloroform and petrol ether extracts from the lichen *Toninia candida* was assayed for their antioxidant activity by Manojlović et al. (2012). The lichen extracts showed comparable and strong antioxidant activity and exhibited higher DPPH and hydroxyl radical scavenging, chelating activity and inhibitory activity towards lipid peroxidation.

Stereocaulon paschale, *Parmeliopsis ambigua*, *Parmelia pertusa*, *Parmelia caperata*, *Parmelia sulcata*, *Parmelia saxatilis*, *Hypogymnia physodes*, *Cladonia furcata*, *Lecanora atra*, *L. muralis*, *Umbilicaria crustulosa*, *Umbilicaria cylindrica* and *Umbilicaria polyphylla* were studied for their antioxidant capacity by Serbian group of scientists (Ranković et al. 2011, 2014b; Kosanić et al. 2012a, b). They found that the tested lichens have effective free radical scavenging activity, reducing power and superoxide anion radical scavenging, and based on strong relationships between total phenolics contents and the antioxidant effect of tested species, it could be concluded that antioxidative nature of these lichens depends on their phenolics.

The *n*-hexane, methanol and water extracts of 14 saxicolous lichens from trans-Himalayan Ladakh region were evaluated for their antioxidant capacities by Kumar et al. (2014). The ferric reducing antioxidant power (FRAP), 2,29-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide (NO) radical scavenging capacities and b-carotene-linoleic acid bleaching property exhibited analogous results where the lichen extracts showed high antioxidant action. The lichen extracts were also found to possess good amount of total proanthocyanidin, flavonoid and polyphenol as the main antioxidant components in extracts. The methanolic extract of *Lobothallia alphoplaca* exhibited the highest FRAP value. Methanolic extract of *Xanthoparmelia stenophylla* showed the highest ABTS radical scavenging capacity. The *n*-hexane extract of *Rhizoplaca chrysoleuca* exhibited the highest DPPH radical scavenging capacity. The highest antioxidant capacity in terms of b-carotene linoleic acid bleaching property was observed in the water extract of *Xanthoria elegans*. Similarly, *Melanelia disjuncta* water extract showed the highest NO scavenging capacity. Among *n*-hexane, methanol and water extracts of all lichens, the methanolic extract of *Xanthoparmelia mexicana* showed the highest total proanthocyanidin, flavonoid and polyphenol content.

In the study reported by Vivek et al. (2014), radical scavenging potential of three *Parmotrema* species, viz., *Parmotrema tinctorum*, *Parmotrema grayanum* and *Parmotrema praesorediosum* from Maragalale and Guliguli Shankara, Western Ghats of Karnataka, India, was determined. The powdered lichen materials were

extracted using methanol. Radical scavenging activity of lichen extracts was determined by DPPH free radical scavenging assay. Total phenolic content of lichen extracts was estimated by Folin–Ciocalteu reagent method. Scavenging of DPPH radicals by lichen extracts was concentration dependent. Among the lichen species, *Parmotrema grayanum* showed higher scavenging potential as indicated by lower IC50 value. Total phenolic content was also high in *Parmotrema grayanum*.

Antioxidant capacity of many other lichen extracts was confirmed by other researchers (Ranković et al. 2012; Kosanić et al. 2013a, b, 2014b).

4.5 Antioxidant Activities of Lichen Secondary Metabolites

Lichens have been found to contain a variety of secondary lichen substances with strong antioxidant activity. There are reports concerning the antioxidative nature of pure lichen metabolites that are available in the literature (Table 4.1).

Methanol–water (90:10 v/v) extracts of five polar lichen species, namely, *Stereocaulon alpinum*, *Ramalina terebrata*, *Caloplaca* sp., *Lecanora* sp. and *Caloplaca regalis*, from King George Island were analysed using thin layer chromatography (TLC) followed by a DPPH (2,2-diphenyl-1-picrylhydrazyl) spray technique. The experimental data showed that 33–50 % of the major constituents of the test extracts were active antioxidants (Bhattarai et al. 2008a, b).

Hidalgo et al. (1994) reported the antioxidant activity of some depsides, such as atranorin (isolated from *Placopsis* sp.) and divaricatic acid (isolated from *Protousnea malacea*), and depsidones, such as pannarin (isolated from *Psoroma pallidum*) and 1′-chloropannarin (isolated from *Erioderma chilense*). All of these secondary compounds inhibited rat brain homogenate auto-oxidation and β-carotene oxidation, and depsidones were found to be the most effective. Russo et al. (2008) found that both sphaerophorin (depside) and pannarin (depsidone) inhibited superoxide anion formation in vitro, pannarin being more efficient, confirming Hidalgo et al. (1994). Similarly, de Barros Alves et al. (2013) found high the antioxidant power of fumarprotocetraric acid produced by the lichen *Cladonia verticillaris* evaluated using the thiobarbituric acid reactive species assay in mouse lung tissue.

Thadhani et al. (2011) were assessed antioxidant activity of several classes of lichen metabolites in the in vitro superoxide radical (SOR), nitric oxide radical and 2,2-diphenyl-1-picrylhydrazil radical scavenging assays. The despsides sekikaic acid and lecanoric acid showed promising antioxidant activity in SOR assay with IC_{50} values of 82.0 ± 0.3 μmol and 91.5 ± 2.1 μmol, respectively, while the depsidone lobaric acid exhibited an IC_{50} value of 97.9 ± 1.6 μmol, all relative to the standard, propyl gallate ($IC_{50} = 106.0 \pm 1.7$ μmol). One of the most abundant mononuclear phenolic compounds, methyl-β-orcinol carboxylate, was found to be a potent NO scavenger ($IC_{50} = 84.7 \pm 0.1$ μmol), compared to the standard rutin ($IC_{50} = 86.8 \pm 1.9$ μmol).

Table 4.1 Literature sources mentioning data for lichen substances responsible for antioxidant activity of lichens

Lichen substances	References
Lecanoric acid	Jayaprakasha and Rao (2000), Lopes et al. (2008), Thadhani et al. (2011), Verma et al. (2011), Buçukoglu et al. (2013)
Atranorin	Hidalgo et al. (1994), Jayaprakasha and Rao (2000), Melo et al. (2011), Sisodia et al. (2013). Ranković et al. (2014a, b), Kosanić et al. (2014a, b)
2,4-Dihydroxy-6-propyl	Sisodia et al. (2013)
Ramalin	Paudel et al. (2008)
Barbatic acid	Verma et al. (2011)
Divaricatic acid	Hidalgo et al. (1994)
Pannarin	Hidalgo et al. (1994), Russo et al. (2008)
Zeorin	Verma et al. (2011)
Gyrophoric acid	Buçukoglu et al. (2013)
Umbilicaric acid	Buçukoglu et al. (2013)
Protocetraric acid	Manojlović et al. (2012)
Fumarprotocetraric acid	de Barros Alves et al. (2014), Kosanić et al. (2014a, b)
Stictic acid	Lohézic-Le Dévéhat et al. (2007)
Salazinic acid	Verma et al. (2012), Manojlović et al. (2012)
Usnic acid	Odabasoglu et al. (2006), Manojlović et al. (2012), Ranković et al. (2012), Verma et al. (2012), Sisodia et al. (2013), Ranković et al. (2014a, b)
Variolaric acid	Brisdelli et al. (2013)
2-Hydroxy-4-methoxy-6-propyl benzoic acid	Sisodia et al. (2013)
Evernic acid	Kosanić et al. (2013a, b)
Erythrin	Choudhary et al. (2009)
2,4-Dihydroxy-3,6-dimethyl benzoate	Sisodia et al. (2013)
Lobaric acid	Thadhani et al. (2011), Brisdelli et al. (2013)
Physodic acid	Kosanić et al. (2013a, b), Ranković et al. (2014a, b)
Protolichesterinic acid	Sisodia et al. (2013)
Norstictic acid	Ranković et al. (2012)
Cuculloquinone	Stepanenko et al. (2002)
Homosekikaic acid	Sisodia et al. (2013)
Sekikaic acid	Thadhani et al. (2011), Verma et al. (2012), Sisodia et al. (2013)
Benzoic acid	Sisodia et al. 2013
Diffractaic acid	Brisdelli et al. (2013)
Vicanicin	Brisdelli et al. (2013)
Sphaerophorin	Russo et al. (2008)
Isidiophorin	Atalay et al. (2011)
Rhizonaldehyde	Atalay et al. (2011)
Rhizonyl alcohol	Atalay et al. (2011)
Pulmonarianin	Atalay et al. (2011)

(continued)

Table 4.1 (continued)

Lichen substances	References
Hypotrachynic acid	Papadopoulou et al. (2007)
Deoxystictic acid	Papadopoulou et al. (2007)
Cryptostictinolide	Papadopoulou et al. (2007)
8′-Methylconstictic acid	Papadopoulou et al. (2007)
8′-Methylstictic acid	Papadopoulou et al. (2007)
8′-Methylmenegazziaic acid	Papadopoulou et al. (2007)
Ethylstictic acid	Papadopoulou et al. (2007)

Brisdelli et al. (2013) investigated the effects of six lichen metabolites (diffractaic acid, lobaric acid, usnic acid, vicanicin, variolaric acid, protolichesterinic acid) on reactive oxygen species (ROS) level towards three human cancer cell lines, MCF-7 (breast adenocarcinoma), HeLa (cervix adenocarcinoma) and HCT-116 (colon carcinoma). All tested lichen compounds did not exhibit free radical scavenging activity using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The lichen metabolites did not significantly increase the intracellular ROS level and did not prevent oxidative injury induced by *t*-butyl hydroperoxide in tested cells.

Choudhary et al. (2009) studied the superoxide radical scavenging activity assay. They found that the para depsides lecanoric acid, erythrin and the meta depside sekikaic acid showed exceptionally high percentage of radical scavenging activity in the SOR assay along with the depsidone lobaric acid. The common structural feature in all of the above compounds is two aromatic rings connected by an ester linkage, an ortho to the carbonyl bearing carbon of ring A and an oxygen atom which may act as the electron acceptor from the antibonding orbitals of superoxide radical leading to molecular oxygen. The electron thus obtained could be stabilised due to extended conjugation available in such compounds. In the case of depsidone lobaric acid, the electron accepted by C-2-O could be stabilised by both aromatic rings. The SOR activity of both the depsides lecanoric acid and erythrin was lost on permethylation suggesting that when C-2-O is methylated, the molecule loses its ability to accept electrons. Importantly, the IC_{50} values of the sekikaic acid, lecanoric acid and lobaric acid were lower than the propyl gallate standard.

Atalay et al. (2011) determined the lipid peroxidation inhibition potential for nine lichen compounds in two lipid peroxidation test systems (liposome and emulsion systems). They found that isidiophorin, rhizonaldehyde, rhizonyl alcohol and pulmonarianin retarded lipid peroxidation at in both test systems. However, stictic acid and ergosterol peroxide exhibited antioxidant activity in only the liposome test system. Usnic acid and diffractaic acid were not antioxidants in either system, while stictic acid was not lipid peroxidation inhibitor in the emulsion test system. All compounds, which inhibited lipid peroxidation in both test systems, were also DPPH radical scavengers.

Odabasoglu et al. (2006) investigated gastroprotective effect of usnic acid isolated from *Usnea longissima* in the indomethacin-induced gastric ulcers in

rats. They found that the gastroprotective effect of usnic acid can be attributed to its reducing effect on the oxidative damage. Namely, all tested doses of usnic acid showed a significant increase in the levels of superoxide dismutase (SOD), glutathione peroxidase (GPx) and reduced glutathione (GSH) and a reduction in the lipid peroxidation (LPO) level in tissues.

For some stictic acid derivates, Lohézic-Le Dévéhat et al. (2007) found moderate antiradical activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and very high superoxide anion scavenging activity.

Methanolic extracts and lichen acids (gyrophoric acid, lecanoric acid and umbilicaric acid) obtained from six *Umbilicaria* species were tested for their antioxidant activity (Buçukoglu et al. 2013). The antioxidant ability was measured using a free radical scavenging activity assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH). The methanolic extracts showed moderate DPPH radical scavenging activity. Among the lichen acids, umbilicaric acid showed the highest antioxidant activity with 68.14 % inhibition.

Jayaprakasha and Rao (2000) extracted with benzene and acetone the lichen *Parmotrema stuppeum* in order to investigate its antioxidant effect. Both the extracts were fractionated on 1 % oxalic acid impregnated silica gel column to obtain methyl orsenillate, orsellinic acid, atranorin and lecanoric acid, respectively. Antioxidant activities of benzene extract, acetone extract and isolated compounds were evaluated in a carotene–linoleate model system. The obtained results showed that the pure compounds and extracts have moderate antioxidant activity.

Four new β-orcinol metabolites, hypotrachynic acid, deoxystictic acid, cryptostictinolide and 8′-methylconstictic acid along with the metabolites 8′-methylstictic acid, 8′-methylmenegazziaic acid, stictic acid, 8′-ethylstictic acid and atranorin that have been previously described, were isolated for the first time from the tissue extracts of the lichen *Hypotrachyna revoluta* (Papadopoulou et al. 2007). The structures of the new metabolites were elucidated on the basis of extensive spectroscopic analyses. Radical scavenging activity of the metabolites isolated in adequate amounts was evaluated using luminol chemiluminescence. The evaluation of the lichen metabolites by the chemiluminescence method showed most of them to possess noteworthy antioxidant activity, with the highest levels being exhibited by compound 8′-methylmenegazziaic acid, which was only seven times less potent than the standard antioxidant Trolox® that was used for comparison reasons. The results showed that compounds 8′-methylmenegazziaic acid and atranorin that posses an additional hydroxyl group on the aromatic ring are the most active ones and the activity is reduced by half when the hydroxyl of C-3 is replaced by an aldehyde moiety. Finally, the scavenging activity of the metabolites possessing an aldehyde group on C-3 seems to be drastically reduced when the methylene of the γ-lactone ring is substituted by a hydroxy or methoxy moiety, as observed in the cases of metabolites deoxystictic acid, stictic acid and 8′-methylstictic acid.

For cuculloquinone, a bisnaphthoquinone of *Flavocetraria cucullata* was found to inactivate DPPH to an 80 % extent, while the BHT that was used as a standard antioxidant was twofold less active (Stepanenko et al. 2002).

Lopes et al. (2008) found that lecanoric acid and some depside isolated from a *Parmotrema tinctorum* were very active DPPH radical scavengers.

In various antioxidant assays, Paudel et al. (2008) used to evaluate the antioxidant capacities of the ramalin isolated from the methanol–water extract of the Antarctic lichen *Ramalina terebrata*. The experimental data showed that ramalin was five times more potent than commercial butylated hydroxyanisole (BHA) in scavenging l-diphenyl-2-picryl-hydazil (DPPH) free radicals, 27 times more potent in scavenging 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid free radicals (ABTS*+)) than the vitamin E analogue, trolox, and 2.5 times more potent than BHT in reducing Fe3+ to Fe2+ ions. Similarly, ramalin was 1.2 times more potent than ascorbic acid in scavenging superoxide radicals and 1.25 times more potent than commercial kojic acid in inhibiting tyrosinase enzyme activity, which ultimately leads to whitening of skin cells. Furthermore, ramalin was assessed to determine its antioxidant activity in vivo. One microgram per millilitre ramalin significantly reduced the released nitric oxide (NO) and 0.125 μg/ml ramalin reduced the produced hydrogen peroxide in lipopolysaccharide (LPS)-stimulated murine macrophage Raw264.7 cells. Considering all the data together, ramalin can be a strong therapeutic candidate for controlling oxidative stress in cells.

Melo et al. (2011) evaluated free radical scavenging activities and antioxidant potential of atranorin using various in vitro assays for scavenging activity against hydroxyl radicals, hydrogen peroxide, superoxide radicals and nitric oxide. Besides, the total reactive antioxidant potential and total antioxidant reactivity indexes and in vitro lipoperoxidation were also evaluated. They found that atranorin exerts differential effects towards reactive species production, enhancing hydrogen peroxide and nitric oxide production and acting as a superoxide scavenger; no activity towards hydroxyl radical production/scavenging was observed. Also, total reactive antioxidant potential and total antioxidant reactivity analysis indicated that atranorin acts as a general antioxidant, although it demonstrated to enhance peroxyl radical-induced lipoperoxidation in vitro.

Atranorin, protolichesterinic acid, usnic acid, 2-hydroxy-4-methoxy-6-propyl benzoic acid, homosekikaic acid, sekikaic acid, benzoic acid, 2,4-dihydroxy-6-propyl and 2,4-dihydroxy-3,6-dimethyl benzoate isolated from the hexane extract from *Ramalina roesleri* were assayed for antioxidant activity by 1,1-diphenyl-2-picrylhydrazyl (Sisodia et al. 2013). Maximum DPPH radical scavenging activity was exhibited by sekikaic acid followed by homosekikaic acid.

Verma et al. (2012) found high scavenging of radicals of salazinic acid, sekikaic acid and usnic acid isolated from three terrestrial natural lichen species *Ramalina celastri*, *Ramalina nervulosa* and *Ramalina pacifica*. On the contrary, Brisdelli et al. (2013) investigated the effects of six lichen metabolites (diffractaic acid, lobaric acid, usnic acid, vicanicin, variolaric acid, protolichesterinic acid) on reactive oxygen species (ROS) using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. They found that lichen compounds did not exhibit free radical scavenging activity and did not significantly increase the intracellular ROS level.

Manojlović et al. (2012) investigated antioxidant activities for protocetraric and usnic acids from *Parmelia caperata* lichen and depsidone salazinic acid from

Parmelia saxatilis species. Antioxidant activities of these isolated metabolites were evaluated by free radical scavenging, superoxide anion radical scavenging and reducing power. As a result of the study, usnic acid had stronger antioxidant activity than salazinic acid and protocetraric acid in all used tests.

In the study described by Ranković et al. (2012), lichen compounds, norstictic acid isolated from *Toninia candida* and usnic acid from *Usnea barbata*, exhibited high antioxidant potential in vitro, but it should be noted that the norstictic acid had a larger antioxidant capacity.

Evernia prunastri and *Pseudevernia furfuracea* lichens and their major metabolites evernic acid and physodic acid were screened for their antioxidant effects by Kosanić et al. (2013a) who found varying antioxidant success in free radical scavenging, superoxide anion radical scavenging and reducing power, and physodic acid was found to be the most effective.

Antioxidant activities of major lichen metabolites in *Hypogymnia physodes* lichen (physodic acids, atranorin and usnic acid) were studied by Ranković et al. (2014b). An physodic acid was found to be the most effective antioxidant in free radical and superoxide anion scavenging, as well as in reducing power assays among tested lichen metabolites.

Kosanić et al. (2014a) investigated antioxidant activities of acetone extracts of the lichens *Cladonia furcata*, *Cladonia pyxidata* and *Cladonia rangiferina* and their atranorin and fumarprotocetraric acid constituents. Antioxidant activities were evaluated by free radical scavenging, superoxide anion radical scavenging and reducing power. As a result of the study, isolated components had larger antioxidant activity than tested extracts. Atranorin had the largest free radical scavenging activity with IC_{50} values 131.48 μg/ml. Moreover, atranorin had the most effective reducing power and superoxide anion radical scavenging.

This work reveals that the lichens can be an interesting source of new antioxidative agents, with a potential use in different fields (food, cosmetics, pharmaceutical). Further work should be focused on the isolation of new pure compounds from lichens and investigation of their antioxidant activity.

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Chapter 5
Investigations of Lichen Secondary Metabolites with Potential Anticancer Activity

Tatjana Stanojković

Abstract Cancers figure among the leading causes of morbidity and mortality worldwide. In the past half a century, natural products have served us well in combating cancer. The main sources of these compounds are microorganisms, plants and marine organisms. Lichens as chemically significant biota represent a large group of symbiotic organisms of fungi (mycobiont) and algae (photobiont) comprising about 17,000 species, and are a source of diverse secondary metabolites.

This chapter focuses primarily on the anticancer properties of lichen secondary metabolites. We have reviewed various publications related to anticancer activity emphasizing results about specific lichen compounds. We have shown that various isolated lichen compounds often demonstrate significant inhibitory activity against various cancer cell lines at very low concentrations. Although lichens are a source for excellent anticancer active compounds, only a small number have been tested for their biological significance. This is our effort just another attempt to expand and deepen research in this area, especially on compounds that have shown promising results.

5.1 Introduction

Throughout history, natural products have afforded a rich source of compounds that have found many applications in the fields of medicine, pharmacy, and biology. Within the sphere of cancer, a number of important new commercialized drugs have been obtained from natural sources, by structural modification of natural compounds or by the synthesis of new compounds, designed following a natural compound as model. The search for improved cytotoxic agents continues to be an important line in the discovery of modern anticancer drugs. The huge structural diversity of natural compounds and their bioactivity potential have meant that several products isolated from plants, marine flora, and microorganisms can serve

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B. Ranković (ed.), *Lichen Secondary Metabolites*,
DOI 10.1007/978-3-319-13374-4_5

as “lead” compounds for improvement of their therapeutic potential by molecular modification. Over 70 % of anticancer compounds are either natural products or natural product-derived substances. On the other hand conjugation of toxic natural products to monoclonal antibodies or polymeric carriers can lead to more efficacious targeted therapies. Since less than 15 % of higher plants have been systematically investigated, the natural product research towards chemotherapy requires further attention and multi-scientific collaboration (Karikas 2010). Also, some herbal compounds have been subjected to clinical trials. This chapter focuses only on those herbal compounds originating from lichens whose anticancer effect was investigated. The aim of this chapter is to highlight the importance of lichen secondary metabolites with potential anticancer activity.

5.2 Anticancer Secondary Metabolites of Lichens

5.2.1 Lichens: Significance of Lichens and Lichen Secondary Metabolites

Generally, lichen metabolites can be divided into two groups: primary and secondary. Primary metabolites are proteins, lipids, carbohydrates, and other organic compounds involved in lichen’s metabolism and structure. Secondary metabolites, also known as lichen substances, are produced mainly by the fungus and secreted onto the surface of the lichen’s hyphae either in amorphous forms or as crystals. They often accumulate in the upper cortex or in specialized structures such as fruiting bodies (Fahselt 1994; Elix 1996). Lichens synthesize a great variety of secondary metabolites, many of which are unique. Approximately 1,050 secondary compounds have been identified to date (Stocker-Wörgötter 2008). They are produced by the mycobiont (Elix 1996; Huneck 1999) and accumulate in the cortex (such as atranorin, parietin, usnic acid, fungal melanins) or in the medullary layer (such as physodic acid, physodalic acid, protocetraric acid) as extracellular tiny crystals on the outer surfaces of the hyphae. The photobiont might also have an influence on the secondary metabolism of the mycobiont (Brunauer et al. 2006, 2007; Yamamoto et al. 1993; Yoshimura et al. 1994; Molnár and Farkas 2010).

Lichens had to evolve diverse biosynthetic pathways to produce such complex arrays of secondary metabolites: polyketide, shikimic acid, and mevalonic acid pathways. Most of the lichen substances are phenolic compounds. Polyketide-derived aromatic compounds, depsides, depsidones, dibenzofurans, xanthones, and naphthoquinones, are of great interest. Compounds from other pathways are esters, terpenes, steroids, terphenylquinones, and pulvinic acid (Fahselt 1994; Cohen and Towers 1995; Müller 2001; Brunauer et al. 2006, 2007; Stocker-Wörgötter and Elix 2002; Johnson et al. 2011; Manojlovic et al. 2012). So, many lichens and lichen products have proved to be a source of important secondary metabolites for food and pharmaceutical industries (Huneck 1999; Oksanen 2006)

and still hold a considerable interest as alternative treatments in various parts of the world (Richardson 1991). Also, we know more about these substances through experimental studies, but the functions of these compounds in the lichen symbioses are still poorly understood (Hager et al. 2008). They may impact biotic and abiotic interactions of lichens with their environment. In addition, they may help to protect the thalli against herbivores, pathogens, competitors, and external abiotic factors, such as high UV irradiation. A wide spectrum of biological potential is shown by the lichens, but they have been long neglected because of their slow-growing nature and difficulties in their artificial cultivation and have scarcely been studied from a biochemical perspective (Crittenden and Porter 1991; Yamamoto et al. 1995; Yamamoto 2000; Behera et al. 2003, 2004). Industrial-scale harvests of lichens are neither ecologically sensible nor sustainable and for many species are not feasible. The new technologies in molecular biology come in light for the direct access of lichen genomes to reveal and eventually to harvest the production of novel secondary metabolites (Miao et al. 2001).

Furthermore, lichen substances exhibit a great diversity of biological effects, including antimicrobial, antiinflammatory, analgesic, antipyretic, and antiproliferative and cytotoxic activities (Boustie and Grube 2005). However, relatively few lichen substances have been screened in detail for biological activity and therapeutic potential, principally due to difficulties in obtaining them in quantities and purities sufficient for structural elucidation and pharmacological testing (Muggia et al. 2009).

5.2.2 *Lichen Secondary Metabolites as Potential Anticancer Drugs (Some Earlier Studies)*

As noted above, over 1,050 secondary metabolites have been reported for lichens and cultured aposymbiotically mycobionts (Molnár and Farkas 2010). Among them a relatively small number of these secondary products (50–60) occur in non-lichenized fungi or higher plants (Elix and Stocker-Wörgötter 2008). One example is the anthraquinone parietina which is present in other fungi like *Aspergillus* and *Penicillium*, as well as in the vascular plant genera *Rheum*, *Rumex*, and *Ventilago* (Romagni and Dayan 2002). This metabolic diversity is largely due to the symbiotic relationship between the lichen partners (Lawrey 1986), and lichen secondary products can comprise up to 20 % of the dry thallus weight, but in most lichens the amount varies from 5 to 10 %. Many lichen secondary metabolites exhibit cytotoxic properties and could be potential sources of pharmaceutically useful chemicals. The purpose of this study was to provide insights regarding the anticancer properties of lichen secondary metabolites and also to provide information regarding the mode of action of lichen compounds against cancer cells. However, so far a limited number of studies were published where the mechanism of action against cancer cell lines had been explored (Molnár and Farkas 2010). The

molecular mechanism of cell death by lichen compounds includes cell cycle arrest, apoptosis, necrosis, and inhibition of angiogenesis (Brisdelli et al. 2013). There is clearly an urgent need for expanding research in this area of study, including studies of those compounds which have shown promising results as well as a strong focus on identifying specific mechanisms of action and extensive clinical trials using the most promising lichen-based drug therapies followed by large-scale production of the best of those compounds. A large number of representatives of this group have already been tested and have been the source of pharmaceutically important anticancer drugs, but there still remains a vast potential reservoir of untapped possibilities. Among the more promising possibilities are lichenized fungi with their more than 1,000 identified secondary chemicals. The use of lichen secondary products as anticancer drugs dates back to the late 1960s when the activity of lichen polysaccharides against tumor cells was initially explored (Fukuoka et al. 1968; Shibata et al. 1968). Similarly, in early studies of Kupchan and Kopperman (1975), they first reported the tumor inhibitor activity of usnic acid extracted from *Cladonia* sp. against Lewis lung carcinoma. They reported a 35–52 % increase in the life span of treated mice versus the control group using a dose range of 20–200 mg/kg of usnic acid. The butyrolactone, protolichesterinic acid, was also found active as an antiproliferative against leukemia cells K-562 (IC50 ¼ 20 mg/ml) and against Ehrlich solid tumor, while nephrosteranic acid derivatives have a poor activity (Hirayama et al. 1980). Polyporic acid (a terphenylquinone) and derivatives (Cain 1966); a depsidone, physodalic acid (Shibamoto and Wei 1984); and lichen glucans (Nishikawa et al. 1969, 1979; Hirayama et al. 1980; Nishikawa and Ohno 1981) including lichenin derivatives (Demleitner et al. 1992) have also been investigated in this way.

5.3 Overview of the Most Investigated Lichen Secondary Metabolites

5.3.1 Usnic Acid

Usnic acid, the most extensively studied lichen metabolite since its first isolation in 1844, exhibited an antiproliferative effect on human leukemia cells (K562) and endometrial carcinoma (HEC-50) cells (Cardarelli et al. 1997; Ingólfsdóttir 2002; Kristmundsdóttir et al. 2002). Therefore, the usnic acid is one of the most interesting lichen metabolites for the study of their antitumor effects. The cytotoxicity, the in vitro antitumor effects, and the mechanism of action of usnic acid need to be investigated in greater detail in order to reach clinical trials and to allow further applications (Table 5.1).

Usnic acid and usnic acid-amine derivatives showed in vitro antiproliferative/cytotoxic activity against a wide variety of murine and human cancer cell lines (Takai et al. 1979; Cardarelli et al. 1997; Bézivin et al. 2004; Mayer et al. 2005;

Table 5.1 Overview of recent literature related to in vitro anticancer activity of lichen secondary metabolites (according to Shrestha and St. Clair 2013)

Lichen metabolites/lichen species	Cell lines tested	References
Usnic acid (commercial) Atranorin (commercial) Parietin (*X. parietina*) Gyrophoric acid (*Umbilicaria hirsuta*)	Human ovarian carcinoma A2780 Human colon adenocarcinoma HT-29	Bačkorová et al. (2012)
Diffractaic acid (*Protousnea magellanica*) Vicanicin (*Psoroma pallidum*) Lobaric acid (*Stereocaulon alpinum*) Variolaric acid (*Ochrolechia deceptionis*) Protolichesterinic acid (*Cornicularia aculeate*) Usnic acid (*Cladonia lepidophora*)	Human breast adenocarcinoma MCF-7 Human colon adenocarcinoma HCT-116 Human cervix adenocarcinoma HeLa	Brisdelli et al. (2012)
Atranorin (*Bacidia stipata*) Diffractaic acid (*P. magellanica*) Divaricatic acid (*Protousnea malacea*) Vicanicin (*Psoroma malacea*) Protolichesterinic acid (*R. melanophthalma*)	Human prostate cancer androgen responsive (LNCaP) Human prostate cancer Androgen nonresponsive DU-145	Russo et al. (2012)
Usnic acid (commercial) Atranorin (commercial) Parietin (*X. parietina*) Gyrophoric acid (*U. hirsuta*)	Human ovarian A2780 Human breast MCF-7 Human colon HT-29 Human T cells, Jurkat Human cervix HeLa Human breast SK-BR-3 Human colon wild-type p53 HCT-116 p53+/+ Human colon p53 null HCT-116 p53−/−	Bačkorová et al. (2012)
Lecanoric acid and its orsellinate derivatives	Larynx carcinoma HEP-2 Breast carcinoma MCF-7 Kidney melanoma cell B16-F10 Vero cell	Bogo et al. (2010)
(+) Usnic acid (*C. arbuscula*) (−) Usnic acid (*Alectoria ochroleuca*)	Breast cancer cell line T-47D Pancreatic cancer cell line Capan-2	Einarsdóttir et al. (2010)
Retigeric acid A and retigeric acid B (*Lobaria kurokawae*)	Human Pca LNCaP PC-3, DU 145 Human epidermoid KB and vincristine Resistant KB (KB/VCR) Human ovarian 3-AO and cisplatin resistant 3-AO (3-AO/CDDP) Human benign prostate Epithelial RWPEI Human hTERT-RPEI Human breast MCF-7	Liu et al. (2010)

(continued)

Table 5.1 (continued)

Lichen metabolites/lichen species	Cell lines tested	References
	Human osteosarcoma U2OS and Saos2	
Olivetoric acid (*Pseudevernia furfuracea*)	Rat adipose tissue endothelial cells	Koparal et al. (2010)
Usnic acid (commercial)	Breast cancer cell lines MCF-7 (estrogen dependent, wild-type p53) Lung cancer cell line H1299 (null for p53)	O'Neill et al. (2010)
(+) Usnic acid (*Xanthoparmelia somloensis*) Salazinic acid (*X. somloensis*) Vulpinic acid (*L. vulpina*) Gyrophoric acid (*Lasallia pustulata*) Evernic acid (*E. prunastri*)	Malignant mesothelioma cells MM98 Vulvar carcinoma cells A431 Keratinocytes HaCaT	Burlando et al. (2009)
16-*O*-Acetyl-leucotylic acid Leucotylic acid (both from *Myelochroa aurulenta*)	Human leukemia cells HL-60	Tokiwano et al. (2009)
Usnic acid and its 9 derivatives	Lymphocytic leukemia L 1210 Murine Lewis lung 3LL Chronic myelogenous leukemia K-562 Brain metastasis of prostate DU 145 Breast MCF 7 Glioblastoma U251 Hamster cell lines: CHO and CHO-MG	Bazin et al. (2008)
Sphaerophorin (*Sphaerophorus globosus*) Pannarin (*Psoroma* spp.)	Human melanoma cells M14	Russo et al. (2008)
(+) Usnic acid (*R. farinacea*) (−) Usnic acid (*Cladonia foliacea*)	Chinese hamster lung fibroblast V79 Human lung V79 Human lung carcinoma A549	Koparal et al. (2006)
Sphaerophorin (*S. globosus*) Pannarin (*Psoroma* spp.) Epiphorellic acid-1 (*Cornicularia epiphorella*)	Human prostrate carcinoma DU 145 Normal human prostatic epithelial cells	Russo et al. (2006)
Usnic acid (commercial)	Breast cancer MCF-7 (estrogen dependent, wild-type p53) Breast cancer cell lines MDA-MB-231 (estrogen independent, mutant p53) Lung cancer cell line H1299	Mayer et al. (2005)
(−) Usnic acid Fumarprotocetraic acid 90-(*O*-methyl) protocetraric acid	Murine leukemia L1210 Murine Lewis lung 3LL Chronic myelogenous leukemia U215	Bézivin et al. (2004)

(continued)

Table 5.1 (continued)

Lichen metabolites/lichen species	Cell lines tested	References
	Human brain metastasis of a prostate DU 145 Human breast MCF 7 Human glioblastoma RCB-0461	
Depsidones—vicanicin, pannarin, 1-chloropannarin, salazinic acid, stictic acid, variolaric acid, psoromic acid, fumarprotocetraric acid, lobaric acid Depsides—atranorin, sphaerophorin, divaricatic acid, diffractaic acid gyrophoric acid, usnic acid	Hepatocytes from rat	Correché et al. (2004)
Pannarin, 10 chloropannarin Salazinic acid, psoromic acid Fumarprotocetraic acid, lobaric acid Vicanicin, stictic acid Variolaric acid, atranorin Sphaerophorin, divaricatic acid Diffractaic acid, gyrophoric acid	Lymphocytes from rat spleens	Correché et al. (2002)
(+) Usnic acid Methyl a-orcinolcarboxylate Ethyl hematommate Diffractaic acid (+) Protolichesterinic acid	Human keratinocyte cell line HaCaT	Kumar and Muller (1999)
Lobaric acid (*S. alpinum*) Protolichesterinic acid (*C. islandica*)	Breast cancer cell T-47D and ZR-75-1 Erythroleukemia K-563	Ogmundsdottir et al. (1998)
Usnic acid derivatives	Lewis lung carcinoma L1210	Takai et al. (1979)

Bazin et al. 2008; Sahu et al. 2011; Bačkorová et al. 2011; Burlando et al. 2009). The toxicity of usnic acid was associated with increased P450 activity and oxidative stress in human hepatoblastoma cells (Sahu et al. 2011); with mitochondrial dysfunction in HepG2 cells (Sahu et al. 2011), in the breast cancer T-47D cell line and in the pancreatic cancer Capan-2 cell line (Einarsdóttir et al. 2010); and with apoptotic induction in murine leukemia L1210 cells (Bézivin et al. 2004; Bazin et al. 2008).

(+) Usnic acid was found to be a strong hepatotoxic agent against monogastric murine hepatocytes (Han et al. 2004). Also, Correché et al. (2004) investigated the cytotoxic and apoptotic effects of usnic acid obtained from Continental (Chilean) and Antarctic lichens in primary cultures of rat hepatocytes.

The (−) enantiomer of usnic acid (isolated from *Cladonia convoluta*) was moderately cytotoxic to various cancer cell lines, such as murine Lewis lung carcinoma, human chronic myelogenous leukemia, human brain metastasis of a prostate carcinoma, human breast adenocarcinoma, and human glioblastoma (Molnár and Farkas 2010: Bézivin et al. 2004). Usnic acid also decreased

proliferation of human breast cancer cells and human lung cancer cells without any DNA damage (Mayer et al. 2005). Finding cancer therapies that do not have DNA-damaging effects and that do not cause the development of secondary malignancies later in life is of great interest. Accordingly, usnic acid may represent a novel source for a natural non-genotoxic anticancer drug. Usnic acid from the lichen *Usnea barbata* (Rankovic et al. 2012) induced a significant cytotoxic effect on the tested human melanoma Fem-x and human colon carcinoma LS174 cell lines, which was stronger than the lichen extracts. Then, as shown by numerous data, there is a significant antitumor activity of usnic acid in vitro. Here are some of them. Usnic acid activated programmed cell death in A2780 and HT-29, probably through the mitochondrial pathway (Bačkorová et al. 2012).

Lichen compounds showed differential sensitivity to various cancer cells. Usnic acid was highly effective against the whole spectrum of cell lines (HeLa, MCT-7, A2780, HT-29, Jurkat, SK-BR-3, and HCT-116). Similar to cytotoxicity, usnic acid also significantly inhibited the clonogenic ability of all the tested cell lines. Also, usnic acid demonstrated strong pro-apoptotic action associated with the altered cell cycle distribution and accumulation of cells in S phase (Bačkorová et al. 2011).

Somewhat earlier (Einarsdóttir et al. 2010) it was announced that both (+) and (−) usnic acids are effective inhibitors of DNA synthesis, with IC50 values of 4.2 and 4.0 μg/ml against T-47D (breast cancer cell line) and 5.3 and 5.0 μg/ml against Capan-2 (pancreatic cancer cell line). There was a reduction in cell size and both acids inhibit cell entry into the S phase. Regarding the mechanism of action, staining with the mitochondrial dye JC-1 demonstrated a dose-dependent loss of mitochondrial membrane potential following treatment with usnic acid in both cell lines. A study on the effects of usnic acid on MCF-7 (estrogen dependent, wild-type p53) indicated no morphological changes in microtubules or increase in the mitotic index. This suggests that the antineoplastic activity of usnic acid is not related to alterations in the formation and/or stabilization of microtubules (O'Neill et al. 2010).

In usnic acid from *Xanthoparmelia somloensis*, Burlando et al. (2009) have investigated its cytotoxic effect towards malignant mesothelioma cells (MM98), vulvar carcinoma cells (A431), and keratinocytes (HaCaT). Usnic acid showed high cytotoxicity for all three cell lines. Further, both types of usnic acid showed dose- and time-dependent cytotoxicity against V79 (Chinese hamster lung fibroblast) and A549 (human lung carcinoma) cell lines. Cytotoxicity was more pronounced in A549 than V79 with cell viability more diminished in A549 versus V79 after 2 days of treatment (Koparal et al. 2006). In order to investigate the mechanism of action of usnic acid, elevated levels of the p53 and p21 proteins were confirmed following treatment with usnic acid, but there was no p53 transcriptional activity, suggesting that the accumulation of p21 was not secondary to p53 transactivation (Mayer et al. 2005). They concluded that usnic acid has antiproliferative activity against wild-type p53 (MCF-7) and nonfunctional p53 (MDA-MB-231) breast cancer cells, as well as against the H1299 lung cancer cell line, which is null for p53. Usnic acid is therefore a non-genotoxic anticancer agent that works in p53-independent manner. In another study (Bézivin et al. 2004), usnic acid also induced L1210 (murine

lymphocytic leukemia) in apoptosis in a dose- and time-dependent manner as fluorescence microscopy revealed condensation of nuclear chromatin, nuclear fragmentation, and formation of apoptotic bodies.

5.3.2 Depsides and Depsidones

There are several studies about antitumor activity of depsides and depsidones and especially on atranorin. Bačkorová and colleagues reported that antiproliferative/cytotoxic effects of atranorin efficiently induced apoptosis and inhibited cell proliferation in various cancer cell lines tested. Similar with usnic acid, atranorin demonstrated strong pro-apoptotic action. Moreover, the same authors reported on the sensitivity of up to nine human cancer cell lines (A2780, HeLa, MCF-7, SK-BR-3, HT-29, HCT-116, p53 (+/+), HCT-116, p53 (−/−), HL-60, and Jurkat) to the antiproliferative/cytotoxic effects of same typical secondary metabolites of lichens (parietin and gyrophoric acid). Further, the analysis of cell cycle distribution also revealed an accumulation of cells in S phase. This study has confirmed a differential sensitivity of cancer cell lines to lichen secondary metabolites (Bačkorová et al. 2011). In addition, atranorin, diffractaic acid, and divaricatic acid were found to be active against prostate cancer cells (human prostate cancer androgen-responsive (LNCaP) and human prostate cancer androgen-nonresponsive DU-145 cells) only in high concentration (Russo et al. 2006, 2012). This study for the first time showed that apoptosis induced by the compounds appeared to be mediated, at least in part, via the inhibition of Hsp70 expression. Also, depsides—atranorin, sphaerophorin, divaricatic acid, diffractaic acid, and gyrophoric acid—and depsidones, vicanicin, pannarin, 1-′chloropannarin, salazinic acid, stictic acid, variolaric acid, psoromic acid, fumarprotocetraric acid, and lobaric acid, were evaluated for their cytotoxic activity towards hepatocytes from rat and lymphocytes from rat spleens (Correché et al. 2002, 2004). The research has shown that salazinic acid, stictic acid, and psoromic acid showed apoptosis of hepatocytes in a dose-dependent manner with stictic acid showing the strongest apoptotic activity. Ogmundsdottir and associates show that lobaric acid and protolichesterinic acid towards breast cancer cells T-47D and ZR-75-1 as erythroleukemia K-563 cells caused a significant reduction in DNA synthesis. Significant cell deaths in three malignant cell lines (T-47D and ZR-75-1 from breast carcinomas and K-562 from erythro-leukaemia), were observed at concentrations of 20 and 30 μg/ml of protolichesterinic acid and lobaric acid, respectively (Ogmundsdóttir et al. 1998). Similarly, gyrophoric acid, usnic acid, and diffractaic acids were reported as potent antiproliferative agents which inhibited cell growth at IC_{50} values of 1.7, 2.1, and 2.6 μM on human keratinocyte cell line HaCaT (Kumar and Muller 1999). Also, in the work of Pejin and associates, it is shown that the results suggest a moderate anticancer activity towards malignant HT-29 (IC50 value was 29.29 μg/ml) and a low growth inhibition on nonmalignant MRC5 cells (IC50 value was 2,478.40 μg/ml) of stictic acid (Pejin et al. 2013). This may indicate that stictic acid can be

considered as a promising lead compound for the design of novel human colon adenocarcinoma drugs. Generally, depsidones showed stronger cytotoxic activity than depsides. The strong biological activity of some depsidones may be due to the strong hydrogen bond between the aldehyde group at C3 and the hydroxyl group at C4. Similarly, the cytotoxic activity of depsides may be in part due to the presence of a COOH group on C′1 and an OH group on C′2. Likewise, Manojlovic and associates reported the strong cytotoxic effect of depsidone salazinic acid as well as phenolic compound protocetraric acid against Fem-x (human melanoma) and LS174 (human colon carcinoma) cell lines (Manojlovic et al. 2012). Pannarin, a depsidone, was shown to inhibit growth of DU-145 prostate carcinoma and M14 human melanoma cells (Russo et al. 2006; Brandão et al. 2013) Also, in the purpose of identifying novel agents with antigrowth and pro-apoptotic activity on prostate cancer cells, Russo et al. (2012) evaluated the effect of lichen secondary metabolites; the depsides atranorin, diffractaic, and divaricatic acids; as well as the depsidone vicanicin on cell growth in the androgen-sensitive (LNCaP) and androgen-insensitive (DU 145) human prostate cancer cells. The depsides resulting from decarboxylation of baeomycesic and squamatic acids showed antiproliferative effects on PC-3 prostate cancer cells (50 % growth inhibitory concentration (GI50) 70.06, 79.37 μm, respectively) (Guo et al. 2011). Also, olivetoric acid as di-depside displayed dose-dependent antiangiogenic activities, inhibited cell proliferation, and disrupted endothelial tube formation in rat adipose tissue endothelial cells (Koparal et al. 2010).

Very good cytotoxic activity against malignant Fem-x and LS174 cells also showed depsidone physodic acids and depside atranorin, which were identified from the lichen *H. physodes* growing in Serbia (Rankovic et al. 2014). Their IC_{50} values were in the range of 17.89 to 24.63 μg/ml, respectively, something less than the value of the IC_{50} of usnic acid. These authors examined the antitumor activity of evernic acid, which belongs to depsides, and also depsidone physodic acid isolated from the lichens *E. prunastri* and *P. furfuraceae* (Kosanic et al. 2013). The obtained results show that the tested compounds exhibited high cytotoxic activity against the target cells in vitro. The best cytotoxic activity was exhibited the physodic acid. The effect of tested samples on cell cycle progression was investigated also in Fem-x and LS174 cells. An increase in cells containing sub-G1 amounts of DNA was observed, indicating that the evernic and physodic acids were inducing cell death. Similarly, somewhat earlier Russo et al. (2008) reported that the depside sphaerophorin (isolated from *Sphaerophorus globosus*) and the depsidone pannarin (isolated from *Psoroma pholidotoides*) inhibited the growth of M14 human melanoma cells, triggering apoptotic cell death. The data obtained from cell culture show that these lichen metabolites inhibit the growth of melanoma cells, inducing their apoptotic cell death, demonstrated by the fragmentation of genomic DNA and by a significant increase of caspase-3 activity and correlated, at least in part, to the increase of ROS generation. The anticancer activities of these lichen metabolites are promising in the treatment of this aggressive, therapy-resistant skin tumor (Molnár and Farkas 2010). However, it is done in one new study (Brisdelli et al. 2013) of six lichen metabolites (diffractaic acid, lobaric acid, lips acid,

vicanicin, variolaric acid, protolichesterinic acid) wherein its effects on proliferation, viability, and reactive oxygen species (ROS) level towards three human cancer cell lines MCF-7 (breast adenocarcinoma), HeLa (cervix adenocarcinoma), and HCT-116 (colon carcinoma) were investigated. In this comparative study, lichen metabolites showed various cytotoxic effects in a concentration-dependent manner. Moreover, all tested lichen compounds did not exhibit free radical scavenging activity. The lichen metabolites did not significantly increase the intracellular ROS level. Further, the cytotoxic activities of depsidone, variolaric acid, and two other secondary metabolites of lichens, R-alectoronic acid and ergosterol peroxide, were evaluated against the murine B16 melanoma cell line. All the tested compounds showed a significant antitumor activity, especially variolaric acid and alectoronic acid, as compared to cisplatin as a positive control (Millot et al. 2007). Anziaic acid was also found to act as an inhibitor of human topoisomerase II but had little effect on human topoisomerase I (Cheng et al. 2013). This is the first report of a depside with activity as a topoisomerase poison inhibitor and demonstrates the potential of this class of natural products as a source for new antibacterial and anticancer compounds. Protolichesterinic acid showed an inhibitory effect against 12 cell lines, with IC_{50} values of 2.4–18.1 μg/mL (Russo et al. 2012). Also, in recent years (Bogo et al. 2010), it has been shown that lecanoric acid, (para-depside) a secondary metabolite of the lichen *Parmotrema tinctorum*, has moderate antitumor activity against some malignant cell lines (MCF-7 breast carcinoma, 786-0 kidney carcinoma, and B16-F10 murine melanoma) tested. Similarly, some cytotoxic activity in vitro of lecanoric acid and orsellinic acid methyl ester, orcinol, and usnic acid isolated from the lichen *Parmelia subrudecta* is shown by other authors (Ivanova et al. 2010). The depsides resulting from decarboxylation of baeomycesic and squamatic acids showed antiproliferative effects on PC-3 prostate cancer cells (Haraldsdóttir et al. 2004; Brandão et al. 2013). Also, Brandao and his research group have reported the results of the evaluation of some lichen compounds (depsides atranorin and diffractaic, divaricatic, and perlatolic acids; the depsidones psoromic, protocetraric, and norstictic acids) tested against UACC-62 and B16-F10 melanoma cell lines and 3T3 normal fibroblast cells (Brandão et al. 2013).

5.3.3 Naphthoquinones

Naphthazarin and its derivates were isolated from *Cetraria islandica*. This naphthoquinone demonstrates in in vitro experiments strong cytotoxic effect to human epidermal carcinoma cells. Dimer of this naphthoquinone, hybocarpone, was isolated from *Lecanora hybocarpa* (Babula et al. 2009).

5.3.4 Anthraquinones

Emodin
Anthraquinones represent a large family of compounds having diverse biological properties. Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a naturally occurring anthraquinone present in the numerous lichens (Cohen and Towers 1995). Emodin, first assigned to be a specific inhibitor of the protein tyrosine kinase p65lck, has now a number of cellular targets interacting with it. Its inhibitory effect on mammalian cell cycle modulation in specific oncogene overexpressed cells formed the basis of using this compound as an anticancer agent. Identification of apoptosis as a mechanism of elimination of cells treated with cytotoxic agents initiated new studies deciphering the mechanism of apoptosis induced by emodin. At present, its role in combination chemotherapy with standard drugs to reduce toxicity and to enhance efficacy is pursued vigorously. Its additional inhibitory effects on angiogenic and metastasis regulatory processes make emodin a sensible candidate as a specific blocker of tumor-associated events (Srinivas et al. 2007).

Parietin
Parietin is derived from polyaromatic ring polyketides and is present in lichen of genera *Xanthoria* and *Teloschistes* in particular in the lichens *Teloschistes chrysophthalmus*, *Teloschistes spinosus*, and *Xanthoria parietina*. Among others, Bačkorová and the authors showed the certain cytotoxic potential of parietin by a series of cancer cell lines (Bačkorová et al. 2011).

5.3.5 Xanthones: Lichexanthone

Lichexanthone is one of the xanthones tested also in a recent study (Brandão et al. 2013) together with other secondary metabolites of lichens. This study revealed the following: lichexanthone was the least active substance tested, delineating a very distinct response relative to the other compounds and the standard doxorubicin.

5.3.6 Others: Some Specific Class of Compounds

Apart from compounds derived from common pathways, which are found throughout all major lichen groups, there are also some unusual compound classes among these organisms; for example, arthogalin, a cyclic depsipeptide (Huneck and Himmelreich 1995), and other amino acid-derived compounds such as the cytotoxic scabrosin esters isolated from *Xanthoparmelia scabrosa* (Ernst-Russell et al. 1999). Thus, Magaya and colleagues test the effects of arthogalin, a secondary metabolite of the lichen *Caloplaca inclinans*, on the growth of murine malignant prostate

sarcoma cells in vitro (Magaya et al. 2013). The results of this study showed that arthogalin is a potent inhibitor of growth of tested cancer cells. They also showed that arthogalin increases sensitivity of cells to radiation, and this effect is significant at a radiation intensity lower than the standard intensity of cancer radiotherapy. On the basis of this study, arthogalin shows promise for combined-modality cancer treatment. Also, earlier studies have shown that scabrosin esters (SEs), which have been isolated from the lichen *Xanthoparmelia scabrosa*, belong to the epipolythiodioxopiperazine (ETP) class of secondary metabolites characterized by possession of a reactive disulfide bond. Colony-forming assays, which were used in these studies, have shown that these compounds are active against human tumor cell lines at nanomolar concentrations. Colony-forming assays show that these toxins are active against human tumor cell lines at nanomolar concentrations (Moerman et al. 2003). These authors show that the typical scabrosin ester acetate butyrate induces early mitochondrial membrane hyperpolarization accompanied by apoptotic cell death. Here we will mention retigeric acid A (RA) and retigeric acid (RB), both a pentacyclic triterpenoids from the lichen species *Lobaria kurokawae*. Liu et al. (2010) showed cytotoxicity towards malignant cells at lower concentrations (>100 μM) of these compounds, but RB is more potent than RA. Specially, investigation on the effect of RB on PC-3 cells showed that RB caused a dose-dependent accumulation of cells in the S phase accompanied with decreases in cyclin B and increases in cyclin E and cyclin A. Both caspase-dependent and caspase-independent pathways were responsible for apoptosis in PC-3 cells. It should also be noted that 16-*O*-acetyl-leucotylic acid, a new triterpenic acid, exhibited potent antiproliferative activity against HL-60 with an EC50 value of 21 μM, while the leucotylic acid, derivative of 16-*O*-acetyl-leucotylic acid, has a higher EC50 value (72 μM) (Tokiwano et al. 2009).

5.4 Overview of Existing In Vivo Studies

Therefore, without any in vivo evaluation and clinical trials, no real efficacy in cancer therapy can be argued for any of the potential agents. Firstly, it is known that most of the antitumor in vivo studies on lichen extracts have been performed by Japanese scientists in the 1970s (Fujikawa et al. 1972; Hirayama et al. 1974). The allogeneic tumor S-180-forming ascites implanted into albino mice is generally used as the basic screening model. Few antitumor assays have been conducted on Ehrlich carcinomas. In each model, samples dissolved in distilled water were administered by i.p. injection for 10 consecutive days, starting 24 h after tumor implantation. After 30 days, the antitumor effect was evaluated through the inhibition ratio (IR), which is linked to the reduction of tumor weight, and the complete regression rate (CR), which is linked to complete recovery of grafted animals. However, with advances in the isolation and characterization of secondary metabolites of lichens, the growing interest for the in vitro testing of their antitumor effects, and in vivo studies. (Ribeiro-Costa et al. 2004). Only scarce in vivo assays

in mice have been attempted for some low molecular weight lichen compounds. One of the first lichen acids found with some activity on L-1210 and S-180 models in mice was polyporic acid, a dihydroxyquinone isolated from *Sticta coronata*. It was given in a dose of 60 mg/kg administered by intraperitoneal (i.p.) injection (Burton and Cain 1959). The well-known (−) usnic acid, a dibenzofuran, was proved to have a weak, if any, antitumoral effect against Lewis lung carcinoma and P-388 leukemia cells (Kupchan et al. 1975 cited in Takai et al. 1979). A series of 20 lichen compounds have been tested against Ehrlich carcinomas in mice, revealing some potential for the butyrolactones (+) protolichesterinic acid and nephrosterinic acid (50 and 70 % tumor growth inhibition, respectively) (Hirayama et al. 1980). A significant in vivo antineoplastic activity (murine leukemia P-388, tested/control × 100 (T/C) = 40 % at 160 μg/kg) is reported for ambewelamide A, an original diketopiperazine dione (Williams et al. 1998). This scabrosin ester and derivatives isolated from two lichen species have shown potent in vitro cytotoxic activities (IC_{50} within the μM to nM range for P = 388, P-815 and MCF-7) (Williams et al. 1998; Ernst-Russell et al. 1999). Another rare compound, hybocarpone isolated from a mycobiont culture of *Lecanora hybocarpa* (IC_{50} = 0.27 μM) (Ernst-Russell et al. 1999), is also a relevant compound to be investigated further in terms of anticancer studies. In 2004 R. M. Ribeiro-Costa and colleagues investigated the in vitro and in vivo properties of usnic acid encapsulated into PLGA microspheres. Microparticles will probably play a promising role in the future of chemotherapy. These polymeric delivery systems are capable of maximizing the therapeutic activity while reducing side effects of anticancer agents. In this study, poly(lactic-*co*-glycolic acid) (PLGA) microspheres contain a usnic acid from *Cladonia substellata*. The antitumor assay was performed in mice against sarcoma-180 tumor (UA 15 mg/kg weight body/day) during 7 days. Animals were then sacrificed, and tumor and organs were excised for histopathological analysis. A maximum release of 92 % was achieved at the fifth day. The IC50 values for free and encapsulated usnic acid were 12 and 14 μg/ml, respectively. The encapsulation of usnic acid into microspheres promoted an increase of 21 % in the tumor inhibition as compared with the free usnic acid treatment. In summary, usnic acid was efficiently encapsulated into PLGA microspheres and the microencapsulation improved its antitumor activity (Ribeiro-Costa et al. 2004).

5.5 Lichen Secondary Metabolites as Potential Anticancer Drugs: Prospects and Promise

Chemotherapy is still the method most commonly used and most promising in the treatment of cancer patients. Also, chemotherapy holds the most promise for selectively eradicating cancer cells while at the same time minimizing collateral damage to surrounding tissues. Many of these chemical agents owe their origins to natural sources in the environment, whereas other anticancer chemotherapeutics are

wholly designed by pharmaceutical scientists based upon current knowledge of cancer onset mechanisms. Selectivity for cancer cell destruction without harming healthy cells is the central focus of these treatment protocols, and chemotherapy's well-known side effects (hair loss, nausea, immunodeficiency, etc.) are a continuing reminder that much room for progress remains. Natural products and their derivatives represent more than 50 % of all the drugs in clinical use of the world. Almost 60 % of drugs approved for cancer treatment are of natural origin (Fakim 2006). Whether the promise of fully selective anticancer medicines will be realized in our lifetime remains unknown, but exciting developments from the investigation of lichen secondary metabolites lend credibility to the proposition that the best is yet to come. Therefore, increasing research on lichen natural resource may provide good results for exploiting and developing valuable natural products which benefit for human. For the past 20–30 years, some studies with lichen, even with the limited screening effort, have indicated the frequent occurrence of metabolites with antitumor properties (Ren et al. 2009; Rankovic et al. 2011; Kosanic et al. 2012a, b; Rankovic et al. 2014). All of this supports efforts to the development of new anticancer drugs that have as a starting point lichen secondary metabolites. The potential of lichen secondary metabolites as a possible source of anticancer drugs is certainly large and visible. As we know, the structure of more than 700 lichen substances is available, but due to the slow growth of lichen, their availability is insufficient in quantity and has difficulty in large-scale industrial production; lichens were frequently ignored by pharmaceutical industries. However, the secondary metabolites of lichen which are deposited on the surface of mycelium were usually produced by fungi; therefore, it becomes possible that cultured mycobionts could replace the lichen. Although many of lichen metabolites are not likely to become therapeutics, the information gained from studying them is likely to lead to the development and understanding of novel molecular targets and chemical synthesis or chemical modification of natural metabolites, which in turn may lead to the development of new classes of therapeutic agents. On the other hand, powerful new technologies such as combinatorial chemistry, high-throughput screening, bioinformatics, proteomics, and genomics have emerged and are being integrated widely in the field of pharmaceutical discovery research. These technologies have enormous potential to make use of the chemical diversity of natural products (Lahlou 2013). All this, including compound library design, protein 3D structures, NMR-based screening, 3D QSAR in modern drug design, and computer-aided prediction of drug toxicity and metabolism, may help in the development of new agents modeled on the basis of secondary metabolites of lichens. Finally, a multidisciplinary collaboration among lichenologists, chemists, pharmacologists, and biologists should be crucial in the development of potential anticancer drugs from secondary metabolites of lichens.

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Chapter 6
Antigenotoxic Effect of Some Lichen Metabolites

Hülya Zeytinoğlu Sivas

Abstract Naturally occurring compounds can have protective effects towards mutagens and carcinogens as shown by numerous studies. Several lichen species have taken quite much the attention of researchers since their extracts and compounds have been used on traditional medicine to cure different diseases such as ulcer, arthritis, tuberculosis and cancer throughout the ages. Although a wide variety of scientific investigations on the biological activities of lichen extracts and their constituent have been performed, there are quite less research on their genotoxicity/antigenotoxic activity. Up to date, most results for genotoxic/antigenotoxic activities of lichens have been obtained for lichen extracts using the Ames/*Salmonella*/microsome, the *Escherichia coli* WP2 microsome, chromosome aberration, micronucleus, sister chromatid exchange and the single-cell gel electrophoresis assays. In the present chapter, findings on the antigenotoxic/genotoxic activities and its mechanisms will be evaluated. By using the most common bacterial and nonbacterial assays, extracts of various lichen species have been shown to have promising antigenotoxic activity with quite less genotoxic activity. Lichen extracts may have a possible therapeutic potential and therefore this must be further investigated by other multiple in vitro bioassays for the development of therapeutic agents.

Abbreviations

2-AF	2-Aminofluorene
4-NPD	4-Nitrophenylenediamin
8-oxo-dG	8-Oxo-2′-deoxyguanosine, 8-hydroxy-2′-deoxyguanosine
9-AA	9-Aminoacridine
AFB1	Aflatoxin B_1
BrdU	Bromodeoxyuridine
CA	Chromosome aberration
CBS	Colloidal bismuth subcitrate
COMET	Single-cell gel electrophoresis

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B. Ranković (ed.), *Lichen Secondary Metabolites*,
DOI 10.1007/978-3-319-13374-4_6

HPL	Human peripheral blood lymphocytes
IMA	Imazalil
MI	Mitotic index
MMC	Mitomycin C
MMS	Methyl methanesulfonate
MN	Micronucleus
MNNG	*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine
SCE	Sister chromatid exchange
SCGE	Single-cell gel electrophoresis

6.1 Introduction

Naturally occurring organic compounds from a variety of organisms including medicinal plants can act as inhibitors of genotoxicity (Ipek et al. 2003, 2005; Jayaprakasha et al. 2007; Zeytinoglu et al. 2008; Kayraldız et al. 2010; Hoshina and Marin-Morales 2014). Investigation of biological activities of natural extracts or their fractions using a series of in vitro and in vivo bioassays is very important and becoming a popular area to develop new therapeutic agents. Numerous studies on the biological potential of several classes of natural agents, dietary constituents, hormones and vitamins have shown to act as genotoxicity inhibitors as well as cytostatic or environmental carcinogen protectors (Okai et al. 1996; Scarpato et al. 1998; Ingolfsdottir et al. 2000; Mersch-Sundermann et al. 2004). Also investigation of possible genotoxicity of such agents takes the attention of researchers because of their use in folk medicine or possible application potential. The most of medicinal plants used traditionally have never been subjected to toxicological tests such as that required for modern pharmaceutical compounds. However, research has shown that quite many plants which are used in traditional medicine or other area may have genotoxic or carcinogenic properties (Santos et al. 2009; Nieminen et al. 2002). Therefore, it becomes very important to search compounds or extracts derived from plants which contain a variety of compounds for their nontoxic, antigenotoxic or genotoxic properties.

Lichen species have taken quite much the attention of researchers since their extracts and compounds have been used in traditional medicine in Europe, Asia and Northern America (Richardson 1988; Cabrera 1996; Tilford 1997). Although extracts of lichens have been subjected to many scientific investigations for their several biological activities such as immunostimulating, analgesic, antiulcerogenic, antipyretic, antimicrobial, antioxidative and antitumour (Kumar and Müller 1999; Ingolfsdottir et al. 2000; Ingolfsdottir 2002; Türk et al. 2003; Tay et al. 2004; Yılmaz et al. 2004; Halici et al. 2005; Karunaratne et al. 2005; Behera et al. 2006; Zeytinoglu et al. 2008), there are quite less research on their genotoxic/antigenotoxic activity. Scientific investigation of antigenotoxic and genotoxic properties of lichens includes in vitro and in vivo studies, mostly using their extracts. Up to date, most results for genotoxicity/antigenotoxicity of lichens

come from using the aqueous, methanol, acetone or *n*-hexane extracts. In the present chapter, findings on the antigenotoxic/genotoxic activity of lichen extract or secondary metabolites and the mechanisms will be evaluated.

6.2 Bioassays for the Antigenotoxicity/Genotoxicity of Lichens

The Organization for Economic Co-operation and Development (OECD 2012) and the European Centre for the Validation of Alternative Methods (ECVAM 2012) have largely investigated the validation of mutagenicity tests. A set of assays are recommended to determine the genotoxicity of a test agent. The methods most frequently used for the assessment of genotoxic/antigenotoxic activity of lichen extracts or its components based on bacterial short-term assays and mammalian test system are recommended by the OECD and the ECVAM. The Ames/Salmonella/microsome (Ames) and the *Escherichia coli* WP2 tryptophan reverse mutation (WP2) assays are the most common bacterial systems, and MN, CA, SCE and COMET are the most common nonbacterial systems used up to date.

6.2.1 Bacterial Short-Term Assays

The Ames and the WP2 assays are short-term bacterial reverse mutation assays specifically designed to detect a wide range of chemicals or other agents which can produce genetic damage. The Ames employs several histidine-dependent *Salmonella* strains, each carrying different mutations in various genes in the histidine operon, pointing different mutagen acting mechanisms. The recommended combinations of *S. typhimurium* strains by OECD in the Ames test are given in Table 6.1 (reviewed by Mortelmans and Zeiger 2000). When the *Salmonella* strains carrying

Table 6.1 Genotype of the most commonly used *Salmonella* tester strains

Strains/allele	Mutation type	DNA target	Reversion event
TA97/hisD6610	Deletion	-C-C-C-C-C-C-	Frameshifts
TA98 TA1538/*hisD3052*	Deletion	-C-G-C-G-C-G-C-G-	Frameshifts
TA100 TA1535/*hisG46*	Deletion	-G-G-G-	Base pair substitution
TA102 TA104/hisG428	Wild type Deletion	TAA (ochre)	Transition/transversion
TA1537/hisC3076	Deletion	+1 frameshift (near -C-C-C-run of Cs)	Frameshifts

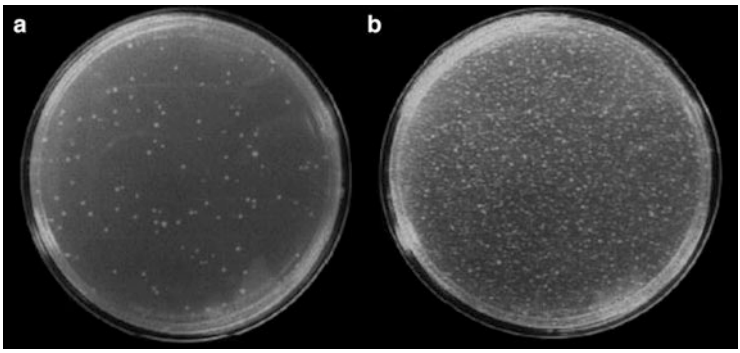

Fig. 6.1 Ames test plates of TA100 strain of *Salmonella typhimurium*. (**a**) Control: spontaneous revertants; (**b**) a mutagenic dose response to sodium azide (from Mortelmans and Zeiger 2000)

mutations in *his* gene are grown on a minimal media agar plate with a trace of histidine, only those bacteria that revert to histidine independence are able to form colonies (Fig. 6.1). When a mutagen is added to the plate, the number of revertant colonies per plate is increased (Maron and Ames 1983; Mortelmans and Zeiger 2000). Ames assay provides a very sensitive study of potentially mutagenic pathways for the metabolism of compounds in both the absence and the presence of a rat liver microsomal system (S9 mix).

Base pair substitution (A:T to G:C or G:C to A:T) and frameshift mutations (deletions) in *S. typhimurium* strains are represented to identify both types of mutation caused by a test compound. Therefore, differences in the activity of a test compound acting in these strains may yield some insight into how the compounds interact with the DNA of bacteria. Additionally, some genetic markers have been developed to make the strains more sensitive to certain types of mutagens.

The WP2 assay detects trp(−) to trp(+) reversion at a site blocking a step in the biosynthesis of tryptophan prior to the formation of anthranilic acid. The different auxotrophic WP2 strains all carry the same A:T base pair at the critical mutation site within the *trpE* gene. The most widely used *E. coli* WP2 strains, each carrying the *trpE* mutation, areWP2 (wild type for DNA repair), WP2 (pKM101), WP2 *uvrA*, WP2 *uvrA* (pKM101) and WP2 (pKM101) (Mortelmans and Riccio 2000). The assay is currently used by many researchers in conjunction with the Ames assay for screening chemicals for their mutagenicity. The Ames assay procedures are the same as for WP2 assay with the exception that limited histidine instead of limited tryptophan is used. International guidelines have been established for performing these mutagenicity assays. These assays are used worldwide as an initial screen to determine the mutagenic/antimutagenic potential of new chemicals, drugs or natural product from plants or animals.

Conversely, the antimutagenicity of a compound against a selected positive mutagen can be investigated when the two chemicals are co-administered to the bacteria in both test systems. Using known mutagenic compounds as “positive controls”, it is possible to study whether tested components can reduce DNA damage.

6.2.2 Nonbacterial Short-Term Assays

At present, several antigenotoxicity/genotoxicity assays which include the chromosome aberration (CA), micronucleus (MN), somatic mutation and recombination test (SMART), sister chromatid exchange (SCE) and the single-cell gel electrophoresis (SCGE) or COMET assays are available, and they are recommended to be used as a set for investigations.

According to literatures, the antigenotoxic/genotoxic potential of lichens has been evaluated, commonly MN, SCE, CA, COMET, 8-oxo-2-deoxyguanosine (8-oxo-dG) in mammalian cell and MI in plant cell assays. The purpose of the MN test is to examine the structural and numerical chromosomal damage which formed small membrane-bound DNA fragments or micronuclei in the cytoplasm of interphase cells caused by a tested agent or by clastogens and aneugens. Micronuclei can be formed by chromosome fragments lacking a centromere or whole chromosomes which are unable to migrate during cell division. The MN test can be conducted in the presence or in the absence of cytochalasin B, which is used to block cell division and generate binucleated cells (Fig. 6.2a). The cytokinesis-block micronucleus assay is a sensitive, comprehensive and simple methodology for measuring DNA damage, cytostasis and cytotoxicity which can be scored easily in a variety of systems, in vitro and in vivo (Fenech 2007; Kirsch-Volders et al. 2011). The assay is being applied successfully for biomonitoring of in vivo genotoxin exposure, for in vitro genotoxicity testing and in diverse research fields such as nutrigenomics and pharmacogenomics.

SCE assay is another short-term test and useful for the detection of reciprocal exchanges of DNA between two sister chromatids of a duplicating chromosome in mammalian and also non-mammalian cells. Various cytomolecular protocols have been used to perform the SCE assay (Bakkali et al. 2008). SCEs result from the interchange of DNA replication products and involve DNA breakage and reunion (Wilson and Thompson 2007). Detection of SCEs requires the differential staining of sister chromatids, which can be achieved generally by the incorporation of bromodeoxyuridine (BrdU) into chromosomal DNA for two cell cycles (Fig. 6.2b). After labelling, treatment of cells with a spindle inhibitor such as colchicine is required to accumulate cells in a metaphase-like stage of mitosis (Perry and Evans 1975; Ipek et al. 2003).

The short-term in vitro mammalian cell chromosome aberration (CA) test measures the frequency of asymmetrical structural chromosome aberrations after exposure to test chemicals or mutagens. The in vitro chromosomal aberration test may employ cultures of established cell lines or primary cell cultures. Procedures involve the stimulation of generally human peripheral blood lymphocytes (HPL) by cyclophosphamide, to divide in whole blood cultures. Cells in metaphase are analysed for the presence of chromosomal aberrations (Fig. 6.2c) (Clare 2012).

The COMET assay is used to detect the DNA strand breaks in eukaryotic cells and named due to the shape of DNA distribution seen which bears resemblance to a celestial comet. This well-established, highly sensitive, rapid and simple

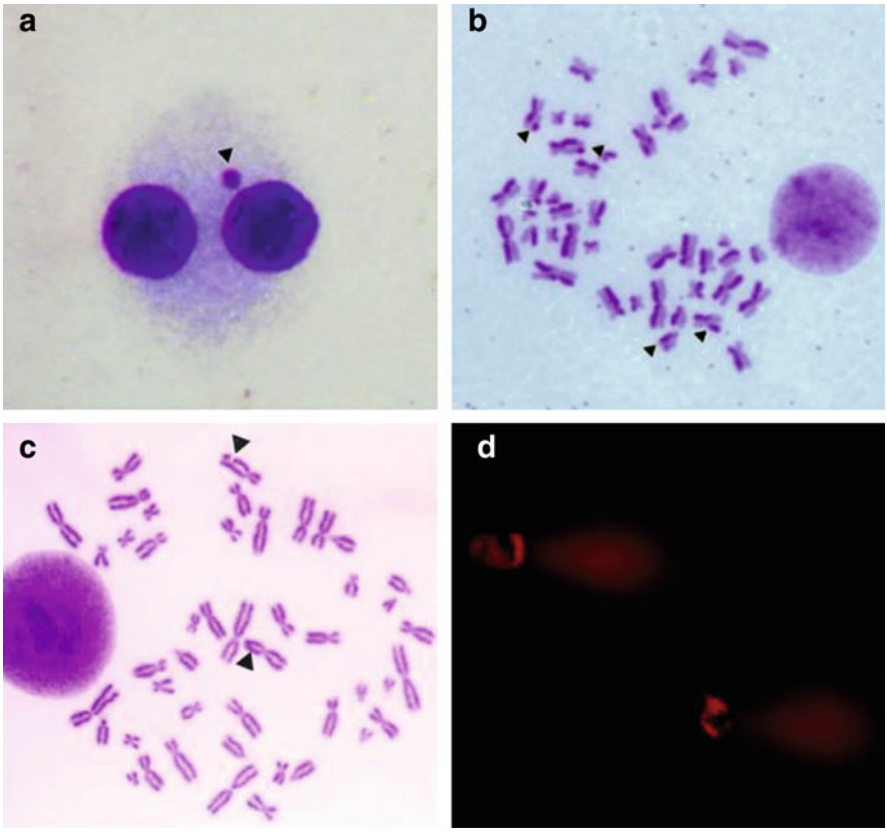

Fig. 6.2 Photomicrographs for some genotoxicity assays. (**a**) A mitogen-stimulated cytokinesis-block lymphocyte containing one MN; Giemsa staining of BrdU-incorporated chromosomes in human lymphocytes for SCE (**b**), *arrowheads* show chromosome breaks and sister union; and for CA (**c**) sister chromatids stained at different density (photograph kindly provided by Dr. B. Ayaz Tuylu). (**d**) COMET tails of chromosomes visualised by an epifluorescence microscope (photograph kindly provided by Dr. A. T. Koparal)

genotoxicity test is based on the lysing of cells embedded in agarose on a microscope slide to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Then electrophoresis at high pH results in structures resembling comets, observed by epifluorescence microscopy (Fig. 6.2d). The intensity of the comet tail relative to the head reflects the number of DNA breaks (Singh et al. 1988; Collins 2004; Speit et al. 2009). Depending on experimental conditions, the migrating DNA reflects the amount of single- or double-strand breaks, alkali-labile sites, including incomplete excision repair sites, but also DNA–DNA and DNA–protein cross-links (Santos et al. 2009; Verschaeve et al. 2010). A broad spectrum of DNA damage can then be detected either by visual classification of comet morphologies or from morphological parameters obtained by image analysis.

8-Oxo-2′-deoxyguanosine (8-oxo-dG) is a frequently used biomarker of oxidative DNA damage caused by free radicals and other reactive species constantly generated in vivo. Later, 8-oxo-dG is removed from DNA by the base excision repair pathway and subsequently transported into body fluids such as saliva, urine and plasma. Such oxidative damage to DNA is probably the contributor of the age-related development of diseases such as cancer. Agents that decrease oxidative DNA damage should thus decrease the risk of cancer development. Thus, the measurement of 8-oxo-dG is the commonest method of assessing DNA damage (Halliwell 2000; Türkez et al. 2012a). An assay for the measurement of 8-oxo-dG has been developed by using a monoclonal antibody specific to 8-oxo-dG (N45.1), and an ELISA (The enzyme-linked immunosorbent assay) has been well established (Toyokuni et al. 1997).

The mitotic index (MI) as a parameter for the evaluation of cytotoxic agents is the ratio of the number of cells, in a cell population, undergoing mitosis to the number of cells not undergoing mitosis. Mutagens can be detected cytologically by cellular inhibition, disruption in metaphase, induction of chromosomal aberrations and chromosomal fragmentation and disorganisation of the mitotic spindle and consequently of all subsequent dependent mitotic phases. MI is used as an indicator of adequate cell proliferation which can be measured by various plant test systems. Cytotoxicity tests, using plant test systems in vivo, such as *Allium cepa* and *Zea mays*, are validated by several researchers, who jointly performed with other organisms testing for genotoxicity (Agar et al. 2010; Gökbayrak and Sivas 2011; Aslan et al. 2012b).

6.3 Antigenotoxic/Genotoxic Potential of Lichen Extracts

Several researches have been performed on the antigenotoxicity/genotoxicity of lichens in just about the last 10 years. The studies up to date are summarised in two separate tables according to the activity assays. In Table 6.2, lichen species tested for only their antigenotoxicity or both genotoxicity and antigenotoxicity were listed. The lichen species which were tested for only their genotoxicity were listed in Table 6.3.

As indicated in Table 6.3, the earliest research for the genotoxicity of lichens has been performed using Ames mutagenicity assay for the secondary metabolites of *Hypogymnia enteromorpha* (Ach.) Nyl. by Shibamoto and Wei (1984). Then, the first report describing the therapeutic potential of lichens against drug genotoxicity was from Geyikoglu et al. (2007) (Table 6.2). Aqueous extracts of four common lichen species collected from Giresun Province in Turkey, *Dermatocarpon intestiniforme*, *Pseudevernia furfuracea*, *Parmelia pulla*, *Ramalina capitata* and *Rhizoplaca melanophthalma*, were tested for their genotoxic and antigenotoxic potentials. *Dermatocarpon intestiniforme*, *Pseudevernia furfuracea*, *Parmelia pulla* and *Ramalina capitata* were found to be antigenotoxic at 5–10 μg/ml concentration against colloidal bismuth subcitrate (CBS)-induced SCE and MN formation in human peripheral lymphocytes (HPL) in vitro. However, one other species *Rhizoplaca melanophthalma* was not antigenotoxic. The order of

Table 6.2 Lichen species tested for only their antigenotoxicity and genotoxicity

Species/extract	Against	Assay	Cell types	Genotoxic	Anti-genotoxic	References
Cetraria aculeatea/ aqueous	4-NPD 2-AF NaAz	Ames	TA98 TA100	No	Yes	Zeytinoglu et al. (2008)
	MMC	MN	HPL	No	No	
Cetraria islandica/ methanol	AFB1	SCE MN	HPL	NP	Yes	Kotan et al. (2011)
	9-AA NaN_3	Ames	TA1535 TA1537	No	Yes	Aslan et al. (2012b)
		MI	*Zea mays*			
Cladonia foliacea/ methanol		Ames	TA1535 TA1537	No	Yes	Anar et al. (2013)
		WP2	*E. coli*			
	AFB1	SCE	HPL	NP	Yes	
Cladonia rangiformis/ methanol	AFB1	SCE MN	HPL	No	Yes	Kotan et al. (2013)
Dermotocarpon intestiniforme/ aqueous	CBS	SCE MN	HPL	No	Yes	Geyikoglu et al. (2007)
	$CdCl_2$	MN	HPL	No	Yes	Guner et al. (2012)
	$HgCl_2$	SCE MN	HPL	No	Yes	Türkez and Dirican (2012)
	Imazalil	CA MN	HPL	No	Yes	Türkez et al. (2012b)
Evernia prunastri/ methanol	NNNG	Ames	TA1535 TA1537	NP	Yes	Alpsoy et al. (2013)
	Acridin	WP2	*E. coli*			
	AFB1	SCE	HPL			
Lecanora muralis/ methanol	AFB1	SCE MN	HPL	NP	Yes	Alpsoy et al. (2011)
Parmelia pulla/ aqueous	CBS	SCE MN	HPL	No	Yes	Geyikoglu et al. (2007)
Peltigera rufescens (Weis) Humb./aqueous	Imazalil	CA MN	HPL	No	Yes	Türkez et al. (2012b)
Peltigera canica/ methanol	9-AA	Ames	TA1535 TA1537	No	Yes	Gormez et al. (2013)
		WP2	*E. coli*			
Pseudevernia furfuracea/ aqueous	CBS	SCE MN	HPL	No	Yes	Geyikoglu et al. (2007)
Pseudevernia furfuracea/methanol, acetone, hexane	AFB1	SCE MN	HPL	No	Yes	Türkez et al. (2010)

(continued)

Table 6.2 (continued)

Species/extract	Against	Assay	Cell types	Genotoxic	Anti-genotoxic	References
Pseudevernia furfuracea/ methanol	9-AA NaN3	Ames	TA1535 TA1537	NP	Yes	Aslan et al. (2012b)
		MI	*Zea mays*			
Ramalina capitata/aqueous	CBS	SCE MN	HPL	No	Yes	Geyikoglu et al. (2007)
Rhizoplaca melanophthalma/ aqueous	CBS	SCE MN	HPL	No	No	Geyikoglu et al. (2007)
	NaN(3)	MI	*Zea mays*	NP	Yes	Agar et al. (2010)
	9-AA	Ames	TA1537			
Rhizoplaca chrysoleuca/ methanol	AFB1	SCE MN	HPL	NP	Yes	Alpsoy et al. (2011)
	NaN(3)	MI	*Zea mays*	NP	Yes	Agar et al. (2010)
	9-AA	Ames	TA1537			
Usnea longissima/ methanol	AFB1	SCE, MN	HPL	NP	Yes	(Agar et al. 2011)
Umbilicaria vellea/methanol	AFB1	SCE, MN	HPL	NP	Yes	Aslan et al. (2012a)
Xanthoria elegans/aqueous	MMC	CA, MN SCE 8-oxo-dG	HPL	No	Yes	Aydin and Türkez (2011b), Türkez et al. (2012a)
Xanthoparmelia somloensis/ methanol	AFB1	SCE MN	HPL	NP	Yes	Aslan et al. (2012a)
Secondary metabolite						
Usnic acid	MMS	COMET	V79 cells	Yes	Yes	Leandro et al. (2013)
		MN		No		

CA Chromosome aberration, *MN* Micronucleus, *NP* Not performed, *HPL* Human peripheral blood lymphocytes, *CBS* Colloidal bismuth subcitrate, *MNNG N*-methyl-*N*′-nitro-*N*-nitrosoguanidine

antigenotoxicity efficacy against CBS was *Pseudevernia furfuracea*, *Dermatocarpon intestiniforme*, *Ramalina capitata* and *Parmelia pulla*. On the other hand, all lichen extracts tested were not genotoxic alone (Table 6.2).

After this work, fresh aqueous extract of *Cetraria aculeata* (Schreb.) Fr. which is one of the common species in Turkey was studied for its genotoxic/antigenotoxic activities in both Ames and mammalian cell systems (Zeytinoglu et al. 2008). The extract (at 0.1–500 μg/ml) exhibited strong antigenotoxic activity against three known mutagenic agents, 4-nitrophenylenediamin (4-NPD), 2-aminofluorene (2-AF) and sodium azide (NaN_3) in TA98 and TA100 strains of *Salmonella typhimurium* in the presence and absence of metabolic activation, without any

Table 6.3 Lichen species tested for only their genotoxicity

Species/extract/secondary metabolite	Assay	Cell types	Genotoxic	References
Aspicilia calcerea/aqueous	CA, MN	HPL	No	Aydin and Türkez (2011a)
Bryoria capillaris/aqueous	CA, MN	HPL	No	Aydin and Türkez (2011b)
Cetraria chlorophylla/aqueous	CA, MN	HPL	No	Aydin and Türkez (2011a)
Hypogymnia physodes/ methanol	CA, MN	HPL	Yes	Ari et al. (2012)
Hypogymnia physodes/aqueous	CA, MN	HPL	No	Türkez et al. (2012c)
Peltigera rufescens/aqueous	CA, MN	HPL	No	Aydin and Türkez (2011b)
Physcia aipolia/aqueous	CA, MN	HPL	No	Aydin and Türkez (2011a)
Ramalina polymorpha/aqueous	CA, MN	HPL	No	Türkez et al. (2012c)
Usnea florida/aqueous	CA, MN	HPL	No	
Secondary metabolite				
Physodic acid (from *Hypogymnia enteromorph*)	Ames	TA100	No	Shibamoto and Wei (1984)
Physodalic acid (from *Hypogymnia enteromorph*)			Yes	
Usnic acid	MNPCEs	Mouse PCEs	Yes	Al-Bekairi et al. (1991)
	MN	HPL	No	Koparal et al. (2006)
	CA, MN	HPL	No	Polat et al. (2013)

CA Chromosome aberration, *MN* Micronucleus, *HPL* Human peripheral blood lymphocytes, *PCEs* Polychromatic erythrocytes, *MNPCEs* Micronucleated PCEs

mutagenic activity (Table 6.1). Preincubation of bacteria with the extract prevented the mutagenic activity of 4-NPD in the higher range in both strains grown without metabolic activation than those grown with metabolic activation. It was suggested that the antigenotoxic potential of the extract was higher in the absence of metabolic system and in inhibiting frameshift mutations. Results indicate a direct and specific activation of the extracts. However, in a further investigation, the extract of *Cetraria aculeata* (Schreb.) Fr. does not have antigenotoxic activity against mitomycin C (MMC) in terms of MN formation in HPL. The extract was not also genotoxic alone in the mammalian system. According to the overall results, the extract of *C. aculeata* is significantly antigenotoxic in the bacterial system, whereas it is not capable of inhibiting MN formation in MMC-induced human peripheral blood cells, and that is pointing at different effects in two bioassay systems.

Recently, more investigations have been performed with an aqueous extract of *Dermatocarpon intestiniforme* in cultured HPL (Table 6.2). The extract at 25 and 50 ppm concentration conferred protection against cadmium chloride ($CdCl_2$)

(30 ppm)-induced MN formation despite its non-genotoxicity in the cells (Guner et al. 2012). It was also revealed that the SCE and MN rates induced by mercury chloride ($HgCl_2$) were alleviated in the cells treated with 50 μg/ml of the extract (Türkez and Dirican 2012). The extract was also antigenotoxic against imazalil (IMA)-induced CA and MN formation in cultured HPL. The lymphocytes were treated in vitro with varying concentrations of the lichen extract (25, 50 and 100 μg/ml) and tested in combination with imazalil (336 μg/ml). The extract alone was not genotoxic, and when combined with IMA treatment, it reduced the frequency of CAs and the rate of MNs (Türkez et al. 2012b). According to the overall results of MN, CA and SCE assays performed, the extract of *Dermatocarpon intestiniforme* is quite antigenotoxic against different types of clastogens or aneugens which cause the structural and numerical chromosomal damage.

One other aqueous extract of lichen species Peltigera rufescens and *Xanthoria elegans* (25, 50 and 100 μg/ml) has been assessed by four genotoxicity end points including CA, MN, SCE and 8-oxo-dG assays in HPL (Türkez et al. 2012a, d). Imazalil- and MMC-induced frequencies of four genotoxic indices were diminished by the extract, indicating its inhibitory effect on oxidative DNA damage of reactive agents beside the structural and numerical chromosomal damages. The extract and its secondary metabolites may have a potential to decrease the risk of cancer development.

The antimutagenic and antigenotoxic effects of methanol extracts of *Rhizoplaca chrysoleuca* and *Rhizoplaca melanophthalma* against known mutagens have been evaluated in two different organisms as a plant and bacteria using different assays (Agar et al. 2010). Extracts (5–40 μg/plate) prevented NaN_3-induced mitotic index partially in *Zea mays* seeds. Furthermore, they were antimutagenic against 9-aminoacridine (9-AA)-induced mutation in TA1537 strain at all tested concentrations (0.5–5 μg/plate) in Ames test. The inhibition rates ranged from 70.73 to 85.71 %.

Several investigators have been focused on the possible antigenotoxic potential of lichens against a well-known mutagen aflatoxin B_1 (AFB1). Türkez et al. (2010) reported the antigenotoxic activity of another lichen species *Pseudevernia furfuracea* using its three diverse extracts as methanol, acetone and *n*-hexane. All the lichen extracts did not induce a significant number of SCEs and MN in cytokinesis-blocked HPL. Moreover, their results indicated that AFB1-induced SCEs were inhibited by the application of 50 μM methanol or acetone extracts. The positive effect of methanol, acetone and ether extracts in decreasing the incidence of MN in comparison with an unprotected level was attained when cultures were treated simultaneously with AFB1 and the extracts. Agar et al. (2011) reported that methanol extracts obtained from *Usnea longissima* suppress the mutagenic effects of AFB1 in HPL examined by the SCE and MN tests. Kotan et al. (2011, 2013) also found that AFB1-induced genotoxicity has been suppressed by the methanol extract of another lichen species *Cetraria islandica* and *Cladonia rangiformis*. The results showed that the frequencies of SCE and MN level decreased when 5 and 10 mg/ml concentrations of the extract were added to AFB1-treated cultures. The methanol extracts of *Rhizoplaca chrysoleuca* and

Lecanora muralis, 5 and 10 μg/ml (Alpsoy et al. 2011), and *Umbilicaria vellea* and *Xanthoparmelia somloensis* (Aslan et al. 2012a) were antigenotoxic against AFB1-induced SCE and MN formation in HPL in vitro.

The methanol extract of *Evernia prunastri* (Huds.) Willd. was a strong antimutagenic on TA1537 and WP2 strains of *E. coli* with 37.70 % and 69.70 % inhibition rates against *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and acridine-induced mutagenicity, respectively. Co-treatments of HPL with the extract and AFB1 decreased the frequencies of SCE (Alpsoy et al. 2013).

The genotoxic and antigenotoxic effects of methanol extract of *Cladonia foliacea* (Huds.) Willd. were studied using WP2, Ames (TA1535 and TA1537) and SCE test systems by Anar et al. (2013). According to their results, 5 μM concentration of AFB1 changed the frequencies of SCE. When 5 and 10 μg/ml concentrations of extract were added to AFB1, the frequencies of SCE were decreased. On the other hand, the extract was not mutagenic in Ames (*Salmonella typhimurium* TA1535, TA1537) and *Escherichia coli* WP2 test systems, while it has antimutagenic activity.

Pseudevernia furfuracea and *Cetraria islandica* were tested using their methanol extracts for both their genotoxic and antigenotoxic activities. The extracts of two species were not mutagenic in Ames and *Zea mays* mitotic index test systems. Furthermore, some extracts showed significant antimutagenic activity against 9-AA in Ames test. Inhibition rates for 9-AA mutagenicity ranged from 25.51 % (*Pseudevernia furfuracea*, 0.05 μg/plate) to 66.14 % (*Cetraria islandica*, 0.05 μg/plate). In addition, all of the extracts were significantly antimutagenic against NaN_3, increasing the MI values of *Zea mays* (Aslan et al. 2012b). Gormez et al. (2013) showed that the methanol extract of *Peltigera canina* posses an antigenotoxic potential in Ames and WP2 tests.

Another eight lichen species collected from Erzurum and Artvin provinces in Turkey, *Aspicilia calcarea*, *Bryoria capillaris*, *Cetraria chlorophylla*, *Hypnogymnia physodes*, *Peltigera rufescens*, *Physcia aipolia*, *Ramalina polymorpha* and *Usnea florida*, have been tested for only their genotoxicity of the water extracts in cultured HPL as given in Table 6.3 (Aydin and Türkez 2011a, b; Türkez et al. 2012c). All tested lichen extracts up to 500 or 1,000 mg/l concentration had no genotoxic effects on the cell by the application of CA and MN assays, however exhibiting antioxidant properties. The methanol extract of *Hypogymnia physodes* (L.) Nyl. was studied for its genotoxicity using CA and MN tests in HPL culture. Relatively higher concentrations are required for its genotoxic activity (Ari et al. 2012).

6.4 Antigenotoxic/Genotoxic Potential of Lichen Secondary Metabolites

Lichen secondary metabolites exert various biological actions such as antitumour, antimicrobial, anti-inflammatory, apoptotic and cytotoxic activities (Ingolfsdottir et al. 1997; Vijayakumar et al. 2000; Huneck 2001; Tay et al. 2004; Yılmaz

et al. 2004; Mayer et al. 2005; Einarsdottir et al. 2010; Mitrovic et al. 2011; Molnar and Farkas 2010). Usnic acid is one of the most abundant lichen secondary metabolites studied for its biological activities as given above. It has been used widely in the pharmaceutical and cosmetic industry, due to its high antimicrobial activity (Ingolfsdottir 2002). Furthermore, usnic acid exhibited antiproliferative effect on human leukaemia cell (K562) and endometrial carcinoma (Ishikawa, HEC-50) cells (Carderelli et al. 1997; Kristmundsdottir et al. 2002).

A few findings present about the genotoxic/antigenotoxic activities of lichen secondary metabolites (Karunaratne et al. 2005). The earliest genotoxicity reports for the secondary metabolites of lichens come from Shibamoto and Wei (1984). They have tested usnic acid, physodic acid and physodalic acids isolated from *Hypogymnia enteromorph* (Ach.) Nyl. for their mutagenicity in the Ames assay (Table 6.3). Among them only physodalic acid exhibited significant mutagenicity against *Salmonella typhimurium* strain TA 100 with or without S9 mix in both plate-incorporation and preincubation assays. (+) -Usnic acid and (−) usnic acid isolated from *Ramalina farinacea* and *Cladonia foliacea*, respectively, have been found to be non-genotoxic due to the absence of MN induction in HPL (Koparal et al. 2006).

Recently, the genotoxic and antigenotoxic potentials of (+) usnic acid against methyl methanesulfonate (MMS)-induced chromosomal and genome damage have been evaluated in mammalian cells in vitro and in vivo (Leandro et al. 2013). Usnic acid alone induced DNA damage at concentrations of 60 and 120 g/ml determined by the COMET assay. However, it has not induced MN formation in V79 cells at the concentrations tested, and not any genotoxic effects were observed in vivo. The combined administration of usnic acid and MMS significantly reduced the frequencies of MN and DNA damage in vitro and in vivo when compared to treatment with MMS alone (Table 6.2). Polat et al. (2013) also showed the nonmutagenicity of usnic acid by two assays as CA and MN. Mice were treated orally with aqueous suspensions of (+) usnic acid in a single dose of either 100 or 200 mg/kg. The slight increase in the micronucleated polychromatic erythrocytes (MNPCEs) without affecting DNA synthesis was reported, and an effect of usnic acid on spindle apparatus was suggested (Al-Bekairi et al. 1991) (Table 6.3).

Usnic acid triggered the oxidative stress and disruption of the normal metabolic processes of breast cancer cell line MCF7 and lung cancer cell line H1299 (null for p53); however, it was not involved in DNA damage. It was suggested that the property of usnic acid as a non-genotoxic anticancer agent that works in a p53-independent manner makes it a potential candidate for novel cancer therapy (Mayer et al. 2005).

6.5 Conclusion

The methods most frequently used for the assessment of genotoxic and antigenotoxic activities of lichen extracts and products in vitro and in vivo are described above. These methods are not meant to be comprehensive of all existing

methods, but more must be in consideration for further investigation of the genotoxicity for their safety assessment or antigenotoxicity of especially secondary metabolite alone or in combination for their synergistic activities. Positive results of an in vitro/in vivo test indicate that the tested substance is genotoxic or antigenotoxic, and negative results indicate that the test substance is not genotoxic under the conditions of the assay performed. Genotoxicity and antigenotoxicity of lichens have appeared to be evaluated using several types of assays by detecting direct or indirect base substitution and frameshift mutagenicity (Ames and WP2), clastogenicity (chromosome breakage) and aneugenicity (chromosome lagging due to dysfunction of mitotic apparatus) (MN), numerical and structural DNA damage (CA) and DNA strand breaks (COMET).

Accumulating data from the short-term in vitro and in vivo studies showed that lichen extracts could possess antigenotoxic effects. There are a small number of results for extracts which do not have antigenotoxic effects. Generally used tests for this purpose were common bacterial tests as Ames and WP2 and human lymphocytes tests as MN and SCE. However, there is a gap in the data about the lichen genotoxicity/antigenotoxicity since some group studied only mutagenicity, others antigenotoxicity without genotoxicity. Most findings are extremely promising that lichens may have therapeutic potential at least for cancer because of their antigenotoxic activities without genotoxic activity. The extracts of nine species of lichens out of 16 species tested, *C. aculeata*, *C. islandica*, *C. foliacea*, *D. intestiniforme*, *P. pulla*, *P. canica*, *P. furfuracea*, *R. capitata* and *X. elegans*, have antigenotoxic activities, but they are not genotoxic (Table 6.1). The extracts of seven species as *C. islandica*, *E. prunastri*, *L. muralis*, *R. chrysoleuca*, *U. longissima*, *U. vellea* and *X. somloensis* are antigenotoxic, but not tested for their genotoxic activities. On the other side, the extracts of *C. aculeata* and *R. melanophthalma* are neither genotoxic nor antigenotoxic for the human peripheral blood lymphocytes. The extracts of other six lichen species tested are not also genotoxic except for *H. physodes* (Table 6.2).

There are minor evidences about the genotoxic and antigenotoxic activities of the secondary metabolites of lichens. Interestingly, usnic acid shows variation in its effects since it is either genotoxic or antigenotoxic according to the results of COMET assay, but not genotoxic according to MN assay; however, it is genotoxic in vivo. Although physodic acid is nonmutagenic, physodalic acid is mutagenic in the same assay system.

Also variation in the effective doses of the extract on different cells or test systems suggests the necessity of more in vitro and in vivo antigenotoxicity studies to know the exact potential of the extract, and then it may find an application for treatments. Further investigation to complete the gap and more data for other lichen species will be so useful for their possible therapeutic application.

The mechanisms of antigenotoxic action of all these lichen extracts are not completely known but appear to be due to antioxidative potentials of their secondary metabolites as described in Chap. 1. Because, most of the extracts have been investigated for their antigenotoxicity and antioxidant activities, also indicated quite strong antioxidative activity (Türkez et al. 2010; Aydin and Türkez 2011a, b; Kotan et al. 2011; Polat et al. 2013). The chemopreventive potential of

several lichen extracts or secondary metabolites against DNA damage induced by known compounds such as AFB1, MMS and CBS, strongly indicates that lichens can be a resource of new therapeutics.

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Chapter 7
Lichen Secondary Metabolites as Possible Antiviral Agents

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Abstract Section 7.1 will begin with broad and specific definitions of lichens and antiviral agents. Here, we will focus briefly on viruses and antiviral agents: their nature, characteristics, source, and effects. We shall also attempt to classify antiviral agents based on their mode of action and targeted virus cluster. Lichen as a broad group of useful source of phytochemical agents would be clearly described. Emphasis would be placed on the renewed interest and attention surrounding them and their unique niche in natural product research.

Section 7.2 deals with issues related to secondary metabolites (phytochemicals) from lichen. Lichen compounds and extractives because they possess some biological effects will form also the focus of this section. How they influence biological host and agents would be described briefly in the latter part of this section with proper X-ray of various reported agents of lichen origin that display antiviral activities. The exact sources of these compounds and extractives would be fully elucidated. Here, we will lightly also mention yet-to-be-validated lichen-derived compounds with speculated or rumored antiviral property. This perhaps may inspire further comprehensive screening of such to fully validate their claims.

Section 7.3 will focus on conclusion beginning with a summary of narrated up-to-date available data of lichen compounds currently undergoing preliminary and extensive research and development. How these agents have been applied or would be applied to biomedical and pharmaceutical utility would equally receive attention. The pharmaceutical industry involvement and development would be mentioned to show the extent of link between the classical research point and industry development endpoint and finally close by speculating on the future of lichen research and the positive expected outcomes of improved research interest in this unique group of plants agents.

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B. Ranković (ed.), *Lichen Secondary Metabolites*,
DOI 10.1007/978-3-319-13374-4_7

7.1 Antiviral Agents and Lichens

7.1.1 Antiviral Agents

Antiviral agents refer to chemicals, moiety, and therapeutic substances employed for the treatment of virus infections and diseases. Therefore, viruses which cause a number of infections and diseases in plant, animals, and humans are targeted by these agents. Viruses are very tiny parasite that cannot reproduce by their own. Following infection of a host (e.g., a cell), it can direct the cell machinery to produce more viruses. Most viruses have either RNA or DNA as their genetic material. This genetic material or nucleic acid may be either single or double stranded (designated ss or ds). The whole intact virus (virion) consists of the nucleic acid and an outer shell of protein.

Antiviral agents target different antigen of the respective virus (virion) or other host cell factor to terminate or control the putative viral infection/disease. Viruses in contrast to bacteria and fungi are notoriously intracellular parasites living inside the cell of their host and harness the replicative ability of their host cell to drive their own multiplication and proliferation within the host, thereby making intervention against them difficult. Consequently, antiviral agents have been shown to act by one or more of the following processes:

(a) Inactivating extracellular virus particles
(b) Prevent viral attachment and/or entry
(c) Prevent replication of viral genome
(d) Prevent synthesis of specific viral protein
(e) Prevent assembly or release of new infectious virions

Extensive screening efforts have been made to find antiviral agents from synthetic and natural sources. Natural products from plants traditionally have provided the pharmaceutical industry with one of its most important sources of lead compounds, and up to 40 % of modern drugs are derived from natural sources (Gautam et al. 2007; Harvey 2008; Jassim and Naji 2003). The bioactive molecules occur in plants as secondary metabolites and as defense mechanisms against predation, herbivores, fungal attack, microbial invasion, and viral infection. During the past decade, potent agents have become available against viral infections, and extensive studies have shown that medicinal plants of several parts of the world contain compounds active against viruses that cause human diseases (Kott et al. 1999; Semple et al. 1998; Sindambiwe et al. 1999). Not left out of these are lichens which are known as promising sources of diverse secondary metabolites, the majority of which exhibit various biological properties including extensive antiviral properties (Molnar and Farkas 2010; Stocker-Worgotter 2008; Pengsuparp et al. 1995; Cohen et al. 1996; Neamati et al. 1997).

7.1.2 *Lichens*

A lichen is a stable, ecologically obligate, self-supporting mutualism between an exhabitant fungus and one or more inhabitant, extracellularly located unicellular or filamentous photoautotrophic partners (Hawksworth and Honegger 1994; Farrar 1976). Thus, lichens are symbiotic organisms of fungi (mycobiont) and algae or cyanobacteria (photobiont) (Voss et al. 1983). Lichen thalli are complex ecosystems rather than organisms (Farrar 1976; Lumbsch 1998). Lichens form the largest mutualistic group among fungi especially given that about 21 % of all fungi are able to act as a mycobiont (Honegger 1991). It is estimated that about 40 fungi genera are involved as photobiont in lichen formation (Kirk et al. 2008). Lichens can be found in a wide range of habitats ranging from aquatic to terrestrial terrain (Brightman and Seaward 1977; Seaward 2008; Lisicka 2008; Nash 2008). They are able to survive in extreme environmental conditions (Nash 2008).

Lichens are known to synthesize a great variety of secondary metabolites, many of which are unique.

7.2 Lichen Secondary Metabolites

Lichens are known to produce tremendous amounts of relatively low molecular weight secondary metabolite compounds, many of which are chemically diverse, ranging from aliphatic to aromatic orders (Turk et al. 2003). In addition to their role in lichen chemotaxonomy and systematics, lichen secondary compounds have several possible biological roles, including antitumor, antibacterial, anti-herbivore, antioxidant, and antiviral actions.

7.2.1 *Some Biological Activities of Lichen Secondary Compounds*

Lichen secondary metabolites are known to display a wide array of biological activities. Some lichen extract derivatives have shown strong cytotoxic activities with possible utility against cancer cells. Usnic acid has been reported to decrease proliferation of human breast cancer cells and human lung cancer cells without any DNA damage (Mayer et al. 2005). The depside sphaerophorin isolated from *Sphaerophorus globosus* and the depsidone pannarin isolated from *Psoroma pholidotoides*, *P. pulchrum*, and *P. pallidum* also displayed anticancer activity against the M14 human melanoma cells (Russo et al. 2008).

It was reported by Bezivin et al. (2004) that (−)-usnic acid, the enantiomer of usnic acid isolated from *Cladonia convoluta*, showed obvious cytotoxic activities against cancer cell lines, such as murine Lewis lung carcinoma, human chronic

myelogenous leukemia, human brain metastasis of a prostate carcinoma, human breast adenocarcinoma, and human glioblastoma. Also, anticancer effect has also been reported for the following lichens: *Cladonia convoluta*, *C. rangiformis*, *Evernia prunastri*, *Flavoparmelia caperata*, *Parmotrema perlatum*, *Platismatia glauca*, *Ramalina cuspidata*, and *Usnea rubicunda* (Bezivin et al. 2003). Strong anti-larvicidal against the third and fourth instar larvae of the house mosquito (*Culex pipiens*) and against the polyphagous larvae of the herbivorous insect *Spodoptera littoralis* were observed with the two enantiomers of usnic acid and vipiric acids (Cetin et al. 2008; Emmerich et al. 1993).

Lichens have also been found to contain a variety of secondary lichen substances with strong antioxidant activity scavenging toxic free radicals. The common existence of phenolic groups among lichens could account for this.

The depsides atranorin isolated from *Placopsis* sp. and divaricatic acid isolated from *Protousnea malacea* and the depsidones pannarin isolated from *Psoroma pallidum* and 1′-chloropannarin isolated from *Erioderma chilense* displayed some antioxidant properties (Hidalgo et al. 1994). Similarly, both sphaerophorin (a depside) and pannarin (a depsidone) inhibited superoxide anion formation in vitro (Russo et al. 2008). Other lichen extracts possessing antioxidant activities include methanol extracts of *Lobaria pulmonaria* (Karakus et al. 2009), *Dolichousnea longissima* (Odabasoglu et al. 2004), *Caloplaca regalis*, *Caloplaca* sp., *Lecanora* sp., *Ramalina terebrata*, and *Stereocaulon alpinum* sourced from Antarctica (Bhattarai et al. 2008). It is therefore most likely that lichens represent such tremendous large source of secondary metabolites with diverse antioxidant properties.

Lichens are also known to provide sources of metabolites with notable antimicrobial properties. They include extracts from *Protousnea poeppigii* with effects against several fungal species. Moreover, compounds such as isodivaricatic acid, divaricatinic acid, and usnic acid isolated from *Protousnea poeppigii* also displayed antifungal action against *Microsporum gypseum*, *Trichophyton mentagrophytes*, and *T. rubrum* (Schmeda-Hirschmann et al. 2008). Also, moderate to strong antifungal action against several fungal species was displayed by extracts from *Alectoria sarmentosa* and *Cladonia rangiferina* (Ranković and Mišić 2007) and *Evernia prunastri* and *Hypogymnia physodes* (Halama and Van Haluwin 2004).

Additionally, it was reported (Ranković and Mišić 2008; Ranković et al. 2008) that physodic acid from *Hypogymnia physodes*, stictic acid from *Xanthoparmelia conspersa*, protocetraric acid from *Flavoparmelia caperata*, atranorin sourced from *Physcia aipolia*, fumarprotocetraric acid isolated from *Cladonia furcata*, gyrophoric acid from *Umbilicaria polyphylla*, lecanoric acid from *Ochrolechia androgyna*, and usnic acid from *Flavoparmelia caperata* all showed relatively strong antimicrobial effects against some bacteria and fungi pathogens. Similarly, Paudel et al. (2008) showed that methanol extracts of some lichens (*Caloplaca regalis*, *Caloplaca* sp., *Lecanora* sp., *Ramalina terebrata*, *Stereocaulon alpinum*) exhibited target-specific antibacterial activity, especially strong against Gram-positive bacteria.

Given this background of generalized biological effects of lichen-derived substances, antiviral activity of lichen-derived compounds should be anticipated.

7.2.2 *Antiviral Activities of Lichen Secondary Metabolites*

Lichens have proven a useful source of antiviral agents. Established antiviral properties have been attributed to some specific lichen secondary metabolites including usnic acid, parietin, anthraquinones, hypericin, lichenan, etc. For instance, lichenan, which is widely distributed in lichens, is demonstrated to inhibit the tobacco mosaic virus (Stubler and Buchenauer 1996).

At about same time, Cohen et al. (1996) reported the antiviral activity of some naturally occurring anthraquinones, bianthrones, and hypericin derivatives isolated from the lichens *Nephroma laevigatum* and *Heterodermia obscurata*. Although the occurrence of these compounds was previously described (Cohen and Towers 1995a, b), however, they were now being tested by Cohen et al. (1996) against the virus herpes simplex virus type 1 (HSV-1) with a resultant antiviral activity where 6 out of 13 compounds tested displayed complete inactivating property at $\leq$2 μg/ml against the HSV-1 in an endpoint cytopathic effect (CPE) assay. The six compounds are emodin, 7-chloroemodin, and 7-chloro-1-*O*-methylemodin, and 5,7-dichloroemodin being the most active anthraquinone (0.25 μg/ml). The other two hypericin derivatives 7,7′ dichlorohypericin and hypericin equally showed comparable inhibitory activity against HSV-1 with complete inactivation at less than 0.06 μg/ml. Thus, the potential of these compounds as possible tools for further development against HSV-1 is not in question. Interestingly, it would appear that some of these metabolites, hypericin, if tested against a wider array of viruses may be effective as is reflected in the study by Andersen et al. (1991) where hypericin and emodin isolated from *Hypericum perforatum* were inhibitory against vesicular stomatitis virus, herpes simplex virus types 1 and 2, parainfluenza virus, and vaccinia virus, thus being a good case in mind to argue for more broad-based extensive screening of potential antiviral metabolites against a wide array of viruses.

In a screening exercise involving a total of 69 lichen species from New Zealand by Perry et al. (1999), they identified the three species *Cladia retipora*, *Pseudocyphellaria glabra*, and *P. homoeophylla* with antiviral activity. These antiviral activities were later traced to the usnic acid constituent following a bioactivity-guided isolation process. Viruses strongly inhibited are the herpes simplex virus type 1 (HSV-1) and the polio type 1 virus. It is widely known that usnic acid has multifaceted biological activities. So its antiviral activity equally does not come as a surprise.

In another related study, investigation of some Icelandic lichens resulted in the identification of two compounds possessing antiviral activities against three viruses: respiratory syncytial virus (RSV), herpes virus 1, and herpes virus 2. Activity against herpes viruses 1 and 2 was less potent than activity against RSV. The

compounds were the depsidone salazinic acid from *Parmelia saxatilis* (L.) Ach. and the benzyl depside alectorialic acid from *Alectoria nigricans* (Ach.). The IC_{50} value for salazinic acid was 11.9 μg/ml while for alectorialic acid 17.0 μg/ml, and at these concentrations, lichen compounds were not cytotoxic (Omarsdottir et al. 2006). Later on in the year following, Fazio et al. (2007) reported their findings where they described the antiviral activity of parietin isolated from *Teloschistes chrysophthalmus* against some Junin and Tacaribe arenaviruses.

The search for lichen metabolites with antiviral continued with some investigations from African lichens. In some studies involving lichen species from West Africa, Esimone et al. (2007) tested some extracts of the lichen *Parmelia perlata* against some RNA viruses: yellow fever, infectious bursal disease virus, and polio virus. While the water and acetone extracts showed no activity against the viruses when tested at concentrations below the cytotoxic level, the crude polysaccharide fraction on the other hand showed activity against yellow fever virus with an IC_{50} of 15 μg/ml. It was also reported that the time of addition of the test extracts to the infected cells did not have significant effect on cytopathic effect inhibition. Following the outcome of further investigation for mechanism of action profile, they proposed that the target of the inhibitory activity is possibly mediated against the envelope of the tested RNA virus. Thus, the results showed that the crude polysaccharide fraction from *Parmelia perlata* possesses specific antiviral activity against yellow fever virus. It is postulated that a major mechanism of inhibition of yellow fever infection by the crude polysaccharide fraction of the lichen could be by attack on the viral envelope.

Esimone and coworkers, in their increasing study of West African lichen species, identified the utility of *R. farinacea* derivatives against lentiviruses and adenoviruses. Esimone et al. (2005) initially showed that the ethyl-acetate-soluble fraction (*ET4*) from the lichen *Ramalina farinacea* inhibited the infectivity of lentiviral and adenoviral vectors, as well as wild-type HIV-1. Recorded antiviral activity was about 20 μg/ml. Preliminary mechanistic studies based on the addition of the extracts at different time points in the viral infection cycle (kinetic studies) led to the suggestion that early steps in the lentiviral or adenoviral replication cycle could be the major target of ET4. Inhibition of wild-type HIV-1 was also observed at a tenfold lower concentration of the extract.

Later, in a cross-continental cooperation following subsequent further investigation, they reportedly (Esimone et al. 2009) determined the antiviral activity of *ET4* against other wild-type viruses, including the herpes simplex virus type 1 (HSV-1) and the respiratory syncytial virus (RSV). They found *ET4* to inhibit HSV-1 and RSV potently with IC_{50} of 6.09 and 3.65 μg/ml, respectively. Attempt to elucidate the possible mechanism of action of the *ET4* revealed through the time-of-addition studies suggests that both entry and postentry steps of the HIV-1 replication cycle and the entry step of the RSV replication cycle are targeted, respectively. Thus, *ET4* is expected to inhibit HIV-1 at some point around the viral envelope, while RSV is inhibited downstream beyond the viral envelope. Additionally, they reported that *ET4* inhibited HIV-1 reverse transcriptase with an IC_{50} of 0.022 μg/ml. Further activity-guided fractionation of *ET4* yielded two subfractions *rfO* and *rfM*

with varied activities. While *rfM* demonstrated activity against HSV-1 (DNA virus) but not against the lentiviral vector, *rfO* was active against lentiviral vector and HIV-1 (RNA viruses) but not against HSV-1.

In follow-up continuity study by Lai et al. (2013) involving a German-Nigerian cooperation, further chemical investigation involving the ethyl-acetate-soluble fraction (*ET4*) of *R. farinacea* led to the isolation of 13 phenolic compounds including one new hydroquinone depside (5-hydroxysekikaic acid) and one new orsellinic acid derivative (2,3-dihydroxy-4-methoxy-6-pentylbenzoic acid). All 13 compounds displayed varying degrees of antiviral activity against the respiratory syncytial virus (RSV) with one of them sekikaic acid showing the most potent inhibition towards a recombinant strain of the respiratory syncytial virus rgRSV (IC_{50} 5.69 μg/ml) and a clinical RSV A2 strain (IC_{50} 7.73 μg/ml). The time-of-addition assay for mechanistic elucidation revealed that sekikaic acid clearly interferes with viral replication at a viral postentry step.

7.2.3 Experimental Methods in the Discovery of Antiviral Lichen Compounds

Viral infection could be detected by direct examination through antigen detection by immunofluorescence, histological appearance by light microscopy, and viral genome detection by hybridization with specific nucleic acid probe polymerase chain reaction (PCR). They could also be indirectly detected by cytopathic effect (CPE), hemabsorption, immunofluorescence, hemagglutination, and, in the case of egg viral cultivation pocks on CAM, inclusion bodies. Enzyme-linked immunosorbent assay (ELISA), complement fixation tests (CFT), protein immunoblot (western blot), and other serological assays are equally useful towards viral detection. Moreover, disease or death could also confirm viral infection especially in animal models. Additionally, there are various tools and vehicles for antiviral screening.

Cell lines and medium Antiviral screening is largely done by employing the use of in vitro means requiring the use of cell lines of mammalian origin. Therefore, cell lines utilized are greatly widespread and commonly include Vero (a monkey kidney-derived primary cell line), Hep2 (of human airway epithelial lineage), Hela (human cervical carcinoma cell line), human tsA201 cell (a derivative of human embryonic kidney cell line 293), 293A cell line (a permanent line of primary human embryonal kidney transformed by sheared human adenovirus type 5 (Ad 5) DNA), and TZM-bl cell (a transformed cell line of cervical cancer origin expressing the classical HIV-1 entry receptors). Madin-Darby canine kidney (MDCK) cells and several other cell lines may be utilized in various antiviral screenings. These cells may for instance usually be grown in Dulbecco's Modified Eagle Medium (DMEM) consisting of high glucose and 2 mM L-glutamine and supplemented with heat-inactivated fetal calf serum and a mixture of penicillin and streptomycin (Pen-Strep) (Ternette et al. 2007; Esimone et al. 2008). There are other media

which are enriched to accommodate additional requirements of certain specialized cells utilized in the antiviral screening.

Viral particles and vectors Viruses are usually utilized in these screenings since the effects of antiviral compounds of lichen origin must be assayed in the presence of the target virus. In this case, the virus could be utilized live and unaltered (e.g., RSV), or the virus is slightly genetically modified without necessarily altering its infectivity while imparting maybe some sort of regulatory or reporter protein property (an example is the rgRSV expressing the green fluorescent protein) (Ternette et al. 2007). In an outright setting change, viruses can be wholly redesigned to produce a viral particle with clearly transformed property referred to as viral vectors. Viral vectors are designed to act as the virus and to effectively deliver the viral genome into susceptible host cell. They can be genetically engineered to either delete unwanted genes/antigen/structures or to introduce new foreign genes/antigen/structures from another virus to create pseudo-typed viruses (vectors) (Esimone et al. 2005, 2009). They found great utility in the evaluation of antiviral lichen compounds.

Antiviral assays A variety of assay types exist to enable the effective evaluation of antiviral agents. The preliminary stage of screening of novel antiviral compound from lichen may begin with an initial simple screening of crude or broad-based fraction of the lichen containing the supposed antiviral substance. Screening is completed through utilization of various bioassays assays presenting sometimes in the format of a bioassay-guided fractionation process.

Bioassay-guided fractionation Bioassay-guided isolation involves the use of some well-defined protocol for the isolation of pure compounds with defined biological activity from a crude extract of a natural product which could be plant or animal parts or a cocktail of mixture. It involves step-by-step separation of fractions/constituents contained within the crude extracts or mixture based on their physicochemical activities and through assaying for biological activity. Several rounds of separation of fractions/constituents and bioassay may be required to finally identify pure molecular hit compound(s).

Antiviral cytopathic and plaque reduction assays Screening of novel antiviral constituent requires the presence of the screened substance in flask or system containing appropriate mammalian cells either adhered or in suspension within cell culture medium including the investigated lichen antiviral substance (Cohen et al. 1996; Kott et al. 1998; Esimone et al. 2009; Lai et al. 2013). Antibiotics are normally included in these media to prevent contamination. Normally, routine screening could take between 24 h and several days following which plaques representing regions of virus activity/replication are counted and reported relative to control setup excluding the antiviral substance. Viral-induced cytopathy or plaque formation may be observed directly under the light microscope or may be done following specific/immune staining of responsible viral protein present in the infected cell (Kott et al. 1998; Esimone et al. 2008). Where a fluorescent protein has been introduced into virus or vector, then observation could also be analyzed using

fluorescent microscopy (Lai et al. 2013). Additional antiviral assays to further define certain specific properties of the identified compound could still be analyzed employing the viral plaque reduction assay, for instance, assays investigating the mechanism of antiviral activity in a broader sense (e.g., time point of optimal antiviral effect during viral infectivity cycle), interference of compound with some measurable viral or host cell enzymes, and protein systems.

Viral cytopathic effect measurement As with viral plaque reduction assays above, a generalized viral cytopathic effect reduction by investigated antiviral lichen compound could be carried out to characterize the antiviral activity of the substance. The experimental setup remains essentially the same as above, but measurement of antiviral activity may be done by analyzing for the presence of cell pathology-indicating enzymes in cell culture supernatant or the measurement for any reduction in substance-treated cell oxidative activity for sign of cell pathology (Semple et al. 1998).

Further antiviral assays to discover antiviral lichen compounds Additional antiviral assays to further define certain specific properties of the identified compound could still be analyzed employing the viral plaque reduction assay, for instance, assays investigating the mechanism of antiviral activity in a broader sense (e.g. time point of optimal antiviral effect during viral infectivity cycle) (Stubler and Buchenauer 1996; Lai et al. 2013), interference of compound with some measurable viral or host cell enzymes, and protein systems. In existence are other virological and immune-based assays that have been reportedly employed for the antiviral screening of lichen-derived compounds, which include virucidal effect (Andersen et al. 1991; Lai et al. 2013), protein immunoblot assay/western blot analysis (Neamati et al. 1991), enzyme-linked immunosorbent assay (ELISA) (Semple et al. 1998), DNA and RNA polymerase inhibition assays (Pengsuparp et al. 1995), and some other antiviral assays. Clearly, antiviral assays could always be developed to fill the gap for any specific analysis of the candidate lichen-derived constituents/compounds.

7.3 Conclusion

Secondary metabolites of lichens as explained so far (summarized in Table 7.1) constitute potent lead compounds for the development of novel antiviral agents. Thus, secondary metabolites of lichens represent strong sources of novel bioactive lead compounds for the medical and pharmaceutical industry in particular. Though many lichen compounds have been fully characterized and proved to be efficacious against certain viral strains, a lot still remain untapped.

Lichens have proven a useful source of antiviral agents. There seems to have been a recent upsurge in the recognition of the potential of lichens as useful source of secondary metabolites with varied biological activities. And this is partly due to the varied and fairly documented biological and antiviral properties of lichen

Table 7.1 Antiviral lichen secondary metabolites

Lichen	Promising secondary metabolites	Antiviral activity (viruses inhibited)	References
Several lichens	Lichenan	Tobacco mosaic virus	Stubler and Buchenauer (1996)
Nephroma laevigatum, *Heterodermia obscurata*	Emodin, 7-chloroemodin, 7-chloro-1-*O*-methylemodin, 5,7-dichloroemodin, 7,7′ dichlorohypericin, and hypericin	Herpes simplex virus type 1 (HSV-1)	Cohen et al. (1996)
Cladia retipora, *Pseudocyphellaria glabra*, *P. homoephylla*	Usnic acid	Herpes simplex virus type 1 (HSV-1), polio type 1 virus	Perry et al. (1999)
Parmelia saxatilis, *Alectoria nigricans*	Salazinic acid, alectorialic acid	Respiratory syncytial virus (RSV), HSV-1, HSV-2	Omarsdottir et al. (2006)
Teloschistes chrysophthalmus	Parietin	Junin and Tacaribe arenaviruses	Fazio et al. (2007)
Parmelia perlata	Crude polysaccharide fraction (extract)	Yellow fever virus	Esimone et al. (2007)
Ramalina farinacea	Ethyl-acetate-soluble fraction (*ET4*)	Lentiviral and adenoviral vectors, HIV-1	Esimone et al. (2005)
Ramalina farinacea	Ethyl-acetate-soluble fraction (*ET4*)	HSV-1, RSV	Esimone et al. (2009)
Ramalina farinacea	Sekikaic acid	RSV	Lai et al. (2013)

secondary metabolites. Additionally, some equally new compounds have been reported from lichens, thereby making them an attractive source of possible new biologicals and antivirals. Of another obvious advantage is the relative ease of their growth and survival. Given the varied opportunities offered by lichens, they would thus remain an interesting focus of our searchlight in our pursuit of useful antiviral remedies against the hordes of viral infections and diseases facing humanity.

In spite of all these, there still remain quite limited reports of antiviral agents of lichen origin. Whether proven cases are yet to be reported or yet to be proven, lichens widely exist only waiting for their exploration to discover, investigate, and establish their probable antiviral potential, or both case scenarios. In any case, currently available reports and data seem to present encouraging environment warranting further and continuous active search, discovery, and scientific investigation of lichens secondary metabolites with antiviral activities. So a good point here is that we have to speed up extensive screening of potential lichen candidates/metabolites. Screenings should equally be made broad based to discover even the remotest virus that could be covered by one single metabolite. The implication of this is the example of hypericin and emodin already discussed (Andersen et al. 1991; Cohen et al. 1996).

In most cases, there appears to be a lingering gap between some preliminary stage investigations and further advance nonclinical/clinical studies which are necessary milestone for translational research to occur to drive the putative benefits of these antiviral lichen secondary metabolites through the lengthened screen of the biomedical and pharmaceutical pipelines. While newer discoveries should be encouraged, investigations already done should not simply sublime into a hit-and-run effect but should strongly metamorphose into an increasingly serious translation into clinical relevance. Suffice to admit that sometimes not-too significant activity potencies may become undesirable clogs in the wheel of progress, it is also possible that some slight structural modification by the chemical scientist may reduce the impact by improving chemical potency in relation to compound-induced CPE effect thereby improving the therapeutic window of the promising compound.

Since lichens have proven a useful storehouse of antiviral armamentarium, it is our firm expectation that a continuous screening of existing and newer candidates may provide us with the much needed good source of antivirals of the current and near future.

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Chapter 8
Future Directions in the Study of Pharmaceutical Potential of Lichens

Neeraj Verma and Bhaskar C. Behera

Abstract Lichens are a stable self-supporting symbiotic organism, composed of a fungal and an algal partner. In this symbiotic form, lichens produce a number of unique secondary metabolites through various biosynthetic pathways, namely, acetyl polymalonyl, shikimic acid and mevalonic acid pathways. Most of the lichen substances are phenolic compounds and are reported to have wide variety of biological actions: antioxidant, antimicrobial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic effects. Acetyl polymalonyl-derived polyketide compounds, depsides, depsidones, dibenzofuranes, xanthones and naphthaquinones, are of great interest. Compounds from other pathways are esters, terpenes, steroids, terphenylquinones and pulvinic acid. Although manifold biological properties of lichen secondary metabolites have been recognized, their pharmaceutical potential has not been fully explored due to their slow growing nature and difficulties in their artificial cultivation. Many researchers are still working hard to discover and identify the novel lead compounds from lichens. In this chapter, attention has been given to bring in notice some pharmaceutically important lichens and their secondary metabolites and to provide a direction for the study of lichen prospect.

8.1 Introduction

Lichens are unusual organisms composed of a fungus (mycobiont) with at least one symbiotic photosynthetic partner (photobiont). Lichens are found in almost all terrestrial habitats from the tropics to the polar region. The worldwide lichen biodiversity is estimated about 17,000 species (Nash 2008; Petrzik et al. 2014). Lichens are proven as the earliest colonizers of terrestrial habitats on earth with a worldwide distribution (Taylor et al. 1995; Mitrovic et al. 2011a). The adaptability of these organisms often adds a colourful aspect to their habitat growing either in

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B. Ranković (ed.), *Lichen Secondary Metabolites*,
DOI 10.1007/978-3-319-13374-4_8

extreme ecological environments or polar latitudes or at extreme altitudes (up to 7,400 m) (Boustie and Grube 2005). The distinct colours of these lichens are due to the massive accumulation of diverse secondary compounds, the "lichen substances". These substances are stress compounds, which help the organism for self-defence and to grow in very adverse and harsh environmental conditions (vide Huneck 1999). These lichen secondary metabolites are mainly phenolic compounds, depsides, depsidones, dibenzofuranes and other accessory pigments (Nash 1996), which are not known to occur in any other organism in nature (Huneck and Yoshimura 1996).

Throughout the ages, lichens and their secondary compounds are used for different purposes for humans and animals. Irrespective of the advances in medical sciences, the tribal peoples still utilize these organisms for different purposes depending on their nutritive and medicinal, decorative, brewing, distilling, dying, cosmetic and perfumery properties (Richardson 1988; Upreti and Chatterjee 2007; Karagoz et al. 2009). During the last five decades, exhaustive ethnobotanical work on lichens has been carried out by several workers in different parts of the world.

In Europe decoction of *Cetraria islandica* (Iceland moss) was recommended as a tonic for convalescents (Schneider 1904), in France the decoction of this species is used as pectoral and emollient (Novaretti and Lemordant 1990), while in Spain the same species is used for the treatment of catarrh and asthma and to treat mitigate inflammation (vide Upreti and Chatterjee 2007), and in Sweden, it is used for the treatment of asthma, diabetes, nephritis, lung diseases, whooping cough and colds (Airaksinen et al. 1986). In Alaska boiled juice of *Cladonia* species is drunk as medicine for diarrhoea and taken as tea for internal chest pains (Smith 1973; Kari et al. 1987). In the west coast of Vancouver Island in Canada, *Lobaria pulmonaria* is used as medicine against coughing up blood. *Lobaria retigera* and *Parmelia saxatilis* are used in China as Chinese medicine (Hu et al. 1980). Many species of *Usnea* like *Dolichousnea longissima* (*Usnea longissima*) in Chinese medicine are used as an expectorant and in the treatment of ulcers (Chopra et al. 1958), in East Africa *U. africana* is used to treat stomach ache (Kokwaro 1976), powder of *U. filipendula* is used in Russia to treat wounds and protection from bacterial infection (Moskalenko 1986), and *U. articulata* is used for wound and skin bruises in Auckland (Brooker et al. 1987).

In Asian countries lichens are still used as a folk and alternative medicine. Some workers, Brij et al. (1985), Brij (1988), Saklani and Upreti (1992) and Brij and Upreti (1995), have reported the information on ethnobotanical uses of some species of lichens used by different tribal communities of India. Lichen species *Lobaria pulmonaria* is used in Sikkim region for curing eczema, lung troubles, haemorrhages and asthma (Biswas 1956). In the same region another lichen species *Heterodermia diademata* is used to protect the wounds and cuts from infection (Saklani and Upreti 1992). In many other parts of India, different lichen species belonging to the family Parmeliaceae viz. *Parmelia kamschadalis* are used to treat diarrhoea, dyspepsia, spermatorrhoea, amenorrhoea and dysentery, and the mixture of *Parmotrema sancti-angelii* ash and mustard/linseed oil is used for the treatment of ringworm-like skin disease (Brij and Upreti 1995). *Dolichousnea longissima*

(*Usnea longissima*) is used for treating bone fractures in Garhwal and Himalayan regions of India (Brij 1988).

The genetic and phenotypic diversity of the organisms involved in lichen symbiosis represents a valuable source for commercially interesting compounds. With modern technology, the potential of discovering and utilizing useful lichen metabolites has increased. Till now 1,050 lichen compounds are reported (including those found in cultures), out of which few of them have shown various biological actions: antibiotic, antimycobacterial, antiviral, antitumour, analgesic, antipyretic and enzyme inhibitory (Matsubara et al. 1997; Huneck 1999; Muller 2001; Behera et al. 2006a; Oksanen 2006; Lopes et al. 2008; Molnar and Farkas 2010).

8.2 Class and Pathways of Lichen Compounds

Lichens are highly specialized fungi containing algal or cyanobacterial colonies in their thalli as a source of carbohydrates (Schwendener 1868). The majority of organic compounds found in lichens are secondary metabolites of the fungal component, which are exported outside the fungal cells to be found on cell surfaces as crystals in different parts of the thallus. They often accumulate in the upper cortex or in specialized structures such as fruiting bodies (Fahselt 1994; Elix 1996; Oksanen 2006). These compounds are usually insoluble in water and can only be extracted with organic solvents. Most of the lichen substances are phenolic compounds which act as antibiotics, pigments, enzyme inhibitors, immune-modulating agents, toxins, pesticides and antitumour agents. They have a major effect on the health, nutrition and economics of our society. A number of secondary metabolites are produced by lichens through biosynthetic pathways, mainly acetyl polymalonyl, shikimic acid and mevalonic acid pathways. Polymalonyl-derived polyketide compounds, depsides, depsidones, dibenzofuranes, xanthones and naphthaquinones, are of great interest. Compounds from other pathways are esters, terpenes, steroids, terphenylquinones and pulvinic acid (Fahselt 1994; Cohen and Towers 1995; Elix 1996; Muller 2001; Brunauer and Stocker-Worgotter 2005; Stocker-Worgotter 2005; Oksanen 2006).

8.3 Potential Biological Activities of Lichens of Pharmaceutical Interest

8.3.1 Antibacterial Activity

The first study on antibiotic properties of lichen was reported by Burkholder in 1944 (Burkholder et al. 1944). Later on Vartia (1973) reported the antimicrobial activity of several lichen species. After wide screening of antimicrobial activities of lichen

extracts by various workers, it reflects that more than 50 % of lichen species that have been studied for various biological activities possess antimicrobial activity. Studies on lichen *Ramalina farinacea* and other 69 species of lichens from New Zealand showed inhibitory effect against *Bacillus*, *Pseudomonas*, *Escherichia coli*, *Streptococcus*, *Staphylococcus*, *Enterococcus* and *Mycobacterium* (Esimone and Adikwn 1999; Perry et al. 1999). Behera et al. (2005a) reported that acetone, methanol and light petroleum extracts of lichen *Usnea ghattensis* were effective against *Bacillus licheniformis*, *B. megaterium*, *B. subtilis* and *Staphylococcus aureus*. Rankovic et al. (2007a, b) tested aqueous, acetone and methanol extracts of *Cladonia furcata*, *Parmelia caperata*, *Parmelia pertusa*, *Hypogymnia physodes*, *Umbilicaria polyphylla*, *Lasallia pustulata*, *Parmelia sulcata*, *Umbilicaria crustulosa* and *Umbilicaria cylindrica* from Serbia on six species of bacteria and ten species of fungi. Many depsides, depsidones and dibenzofuranes have been isolated and characterized for antimicrobial activity. Mitrovic et al. (2011b) studied antibacterial activity of methanol extracts of five lichen species, *Parmelia sulcata*, *Flavoparmelia caperata*, *Evernia prunastri*, *Hypogymnia physodes* and *Cladonia foliacea*, against 15 bacterial strains. The results revealed the strongest inhibitory effect, especially on Gram-positive bacteria. In recent reports, Karagoz et al. (2009) evaluated aqueous and ethanol extracts of 11 different species from Turkey and determined potent antibacterial activity of aqueous extract of *Peltigera polydactyla* and ethanol extract of *Ramalina farinacea*. Chemical identification revealed evernic acid, vulpinic acid and hirtusneanoside as main components (Lawrey 1986; Rezanka and Sigler 2007). Verma et al. (2011) reported the bactericidal activity of lichens *Cladonia ochrochlora*, *Parmotrema nilgherrense*, *Parmotrema sancti-angelii* and their secondary compounds, alectoronic acid, atranorin, α-collatolic acid, fumarprotocetraric acid, hypoprotocetraric acid, lecanoric acid and protocetraric acid, with low MIC value against *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Proteus vulgaris*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Sarcina lutea* and yeast strains *Candida albicans* and *Cryptococcus* var. *diffluens* (Table 8.1).

8.3.2 Antioxidant Activity

The second most important biological activity of lichens reported so far is antioxidant activity. Lichens are the good source of natural antioxidants. They are highly resistant to potential damage caused by reactive oxygen species (ROS). Kranner et al. (2005) demonstrated the role of antioxidants in photoprotection and desiccation tolerance in lichen *Cladonia valcani* and its isolated symbionts. In the last few years, antioxidant activities of some depsides and depsidones isolated from several lichen species have been reported by many workers (Hidalgo et al. 1994; Gulcin et al. 2002; Odabasoglu et al. 2004; Choudhary et al. 2005; Devehat et al. 2007; Lopes et al. 2008; Paudel et al. 2008; Karunaratne et al. 2009). Several other biological activities of lichens have also been studied; Jayaprakash and

Table 8.1 Lichen species and biological activities of their secondary metabolites

Lichen species	Metabolite	Class of compound	Production pathways[a]	Category of use	References
Alectoria ochroleuca	Usnic acid Vulpinic acid	Dibenzofuranes Pulvinic acid derivative	APP SAP	Antifungal activity	Lauterwein et al. (1995)
Arthothelium awasthii	Barbatic acid	Depsides	APP	Antioxidant activity Antityrosinase activity	Verma et al. (2008b, c)
Asashinea chrysantha	Chrysophanol Emodin	Anthraquinones Anthraquinones	APP APP	Anticancer activity	Mishchenko et al. (1980) Koyama et al. (1989)
Bulbothrix setschwanensis	Salazinic acid	Depsidones	APP	Anti-tyrosinase activity Xanthine oxidase inhibition	Behera and Makhija (2002)
Caloplaca aurantia	Emodin	Anthraquinones	APP	Antifungal activity	Manojlovic et al. (1998)
Caloplaca species	Anthraquinone	Anthraquinones	APP	Antifungal, antibacterial activity	Manojlovic et al. (2005)
Canoparmelia eruptens	Atranorin Lecanoric acid	Depsides Depsides	APP APP	Probiotic activity	Gaikwad et al. (2014)
Cetraria ericetorum *Cetraria islandica*	Fumarprotocetraric acid Naphthazarin Protocetraric acid Protolichesterinic acid	Depsidones Anthraquinones Depsidones Aliphatic and cycloaliphatic	APP APP APP APP	Inhibition of HIV-1 reverse transcriptase Inhibition of 5-lipoxygenase Antiproliferative activity Cytotoxic activity Antimicrobial activity Antimycobacterial activity Inhibition of DNA polymerase activity Inhibitor of human keratinocyte cell line	Pengsuparp et al. (1995) Borkowski et al. (1964) Ingolfsdottir et al. (1994, 1997a, 1998, 2000) Ogmundsdottir et al. (1998) Turk et al. (2003) Haraldsdottir et al. (2004)

(continued)

Table 8.1 (continued)

Lichen species	Metabolite	Class of compound	Production pathways[a]	Category of use	References
					Stepanenko et al. (1997) Paull et al. (1976) Muller et al. (1997)
Cladonia arbuscula	Usnic acid	Dibenzofuranes	APP	Antibiotic potential Antimycobacterial activity	Vartia (1973) Ingolfsdottir et al. (1998)
Cladonia confusa f. confusa (*Cladonia leptoclada*)	Usnic acid	Dibenzofuranes	APP	Antitumour activity	Kupchan and Kopperman (1975)
Cladonia ochrochlora	Fumarprotocetraric acid Hypoprotocetraric acid Protocetraric acid	Depsidones Depsidones Depsidones	APP APP APP	Bactericidal activity	Verma et al. (2011)
Dolichousnea diffracta (*Usnea diffracta*)	Decarboxy stenosporic acid Diffractaic acid Usnic acid	Depsides Depsides Dibenzofuranes	APP APP APP	Anti-inflammatory activity Analgesic activity Antipyretic activity Antimicrobial activity	Okuyama et al. (1995) Otsuka et al. (1972) Yamamoto et al. (1998)
Dolichousnea longissima (*Usnea longissima*)	Evernic acid Lichesterinic Protolichesterinic acid Longissiminone A Usnic acid	Depsides Aliphatic and cycloaliphatic Aliphatic and cycloaliphatic Dibenzofuranes	APP APP APP APP APP	Plant growth inhibition Inhibition Epstein–Barr virus activation Photosystem II inhibition Anti-inflammatory activity Inhibition of leukotriene B_4 biosynthesis	Nishitoba et al. (1987) Yamamoto et al. (1995) Endo et al. (1998) Kumar and Muller (1999) Choudhary et al. (2005)
Everniastrum cirrhatum	Atranorin Salazinic acid	Depsides Depsidones	APP APP	Probiotic activity	Gaikwad et al. (2014)

Flavocetraria nivalis *Flavocetraria cucullata*	Usnic acid	Dibenzofuranes	APP	Inhibition of tyrosine phosphatase Cytotoxic activity Apoptosis Antibiotic activity Antibacterial activity Antitrypanosomal activity Antiviral activity	Francolini et al. (2004) De Carvahlo et al. (2005) Mayer et al. (2005) Elo et al. (2007) Fazio et al. (2007) Bazin et al. (2008) Burlando et al. (2009)
Flavoparmelia euplecta	Euplectin	Naphthopyrones	APP	Cytotoxic agent	Ernst-Russell et al. (2000)
Graphis guimarana *Graphis nakanishiana*	Constictic acid Norstictic acid Stictic acid	Depsidones Depsidones Depsidones	APP APP APP	Scavenging of super oxide radical Inhibition of tyrosinase activity Inhibition of xanthine oxidase activity	Behera et al. (2006a, b)
Heterodermia obscurata	7,7′-Dichlorohypericin 5,7-Dichloroemodin	Anthraquinones Anthraquinones	APP APP	Inhibitory activity against herpes simplex virus type 1	Cohen and Towers (1995) Cohen et al. (1996)
Heterodermia podocarpa	Atranorin Zeorin	Depsides Terpenoids	APP MAP	Antioxidant activity Antityrosinase activity	Verma et al. (2008b, c)
Heterodea muelleri	Barbatic acid Diffractaic acid Usnic acid	Depsides Depsides Dibenzofuranes	APP APP APP	UV-C stress and cold temperature stress	Hager et al. (2008)
Hypotrachyna revoluta	8′-Methylmenegazziaic acid	Depsidones	APP	Radical scavenging activity	Papadopoulou et al. (2007)
Lecanora hybocarpa	Naphthazarin	Anthraquinones	APP	Cytotoxic activity	Ernst-Russell et al. (1999a)
Lobaria linita	Tenuiorin	Depsides	APP	Inhibition of 5-lipoxygenase activity	Ingolfsdottir and Gudmundsdottir (2002)

(continued)

Table 8.1 (continued)

Lichen species	Metabolite	Class of compound	Production pathways[a]	Category of use	References
Lobaria pulmonaria	Gyrophoric acid Melanin	Depsides Anthraquinones	APP APP	Light screening pigments	McEvoy et al. (2007)
Nephroma laevigatum	Emodin	Anthraquinones	APP	Antifungal activity	Manojlovic et al. (1998)
Parmelia nepalensis	Protolichesterinic acid	Aliphatic and cycloaliphatic	APP	Inhibition of 5-lipoxygenase activity	Kumar and Muller (1999)
Parmotrema austrosinense	Atranorin Lecanoric acid	Depsides Depsides	APP APP	Probiotic activity	Gaikwad et al. (2014)
Parmotrema cetratum (*Rimelia cetrata*)	Atranorin Consalazinic acid Salazinic acid	Depsides Depsidones Depsidones	APP APP APP	Probiotic activity	Gaikwad et al. (2014)
Parmotrema nilgherrense	Alectoronic acid Atranorin α-Collatolic acid	Depsidones Depsides Depsidones	APP APP APP	Bactericidal activity	Verma et al. (2011)
Parmotrema sancti-angelii	Atranorin α-Collatolic acid Lecanoric acid	Depsides Depsidones Depsides	APP APP APP	Bactericidal activity	Verma et al. (2011)
Parmotrema tinctorum	Atranorin Lecanoric acid	Depsides Depsides	APP APP	Antioxidant activity Antityrosinase activity	Verma et al. (2008b, c)
Peltigera aphthosa *Peltigera leucophlebia*	Tenuiorin	Depsides	APP	Inhibition of 5-lipoxygenase activity	Ingolfsdottir and Gudmundsdottir (2002)
Protousnea poeppigii	Divaricatinic acid Isodivaricatic acid Usnic acid	Depsides Cleavage product of depsides and depsidones Dibenzofuranes	APP APP APP	Antiprotozoal activity Antifungal activity Cytotoxic activity	Schmeda-Hirschmann et al. (2008) Bezivin et al. (2004)

Psoroma pallidum *Psoroma pulchrum* *Psoroma pholidotoides* (*Psoroma reticulatum*)	Pannarin	Depsidones	APP	Cytotoxic activity	Russo et al. (2006, 2008)
Pseudocyphellaria crocata	Tenuiorin	Depsides	APP	Inhibition of 5-lipoxygenase activity	Ingolfsdottir and Gudmundsdottir (2002)
Ramalina almquistii	Protolichesterinic acid Nephrosterinic acid	Aliphatic and cycloaliphatic	APP APP	Antitumour activity	Hirayama et al. (1980)
Ramalina celastri	Usnic acid	Dibenzofuranes	APP	Radical scavenging activity Glucosidase inhibitory activity	Verma et al. (2012a)
Ramalina farinacea	Evernic acid Norstictic acid Protocetraric acid Obtusatic acid Usnic acid	Depsides Depsidones Depsidones Depsides Dibenzofuranes	APP APP APP APP APP	Antimicrobial activity	Rastogi and Mehrotra (1993) Tay et al. (2004)
Ramalina nervulosa	Sekikaic acid Usnic acid	Depsides Dibenzofuranes	APP APP	Radical scavenging activity Glucosidase inhibitory activity	Verma et al. (2012a, b)
Ramalina pacifica	Salazinic acid Usnic acid	Depsidones Dibenzofuranes	APP APP	Radical scavenging activity Glucosidase inhibitory activity	Verma et al. (2012a, b)
Sphaerophorus globosus	Sphaerophorin	Depsides	APP	Cytotoxic activity Induction of apoptosis	Russo et al. (2006, 2008)
Stereocaulon alpinum *Stereocaulon azoreum* *Stereocaulon ramulosum* *Stereocaulon sasakii* *Stereocaulon tomentosum*	Atranorin Lobaric acid Methyl haematommate Stereocalpin A	Depsides Depsidones Depsides Didepsipeptide	APP APP APP APP	Antiproliferative activity Inhibition of 5-lipoxygenase activity Inhibition of platelet-type 12(S)-lipoxygenase Inhibition of arachidonate	Ingolfsdottir et al. (1996) Haraldsdottir et al. (2004) Bucar et al. (2004) Hickey et al. (1990)

(continued)

Table 8.1 (continued)

Lichen species	Metabolite	Class of compound	Production pathways[a]	Category of use	References
				5-lipoxygenase Inhibition of cyclooxygenase Antifungal activity Antimycobacterial activity Inhibition of tyrosine phosphatase Inhibition of cysteinyl leukotrienes Cytotoxicity	Ingolfsdottir et al. (1998) Seo et al. (2009) Gissurarson et al. (1997) Seo et al. (2008)
Thamnolia vermicularis	Baeomycesic acid	Depsides	APP	Inhibition of 5-lipoxygenase activity	Ingolfsdottir et al. (1997b)
Usnea complanata	Psoromic acid Usnic acid	Depsidones Dibenzofuranes	APP APP	Antioxidant activity Antimicrobial activity Cardiovascular protective activity	Mahadik et al. (2011) Behera et al. (2012)
Usnea ghattensis	Norstictic acid Usnic acid	Depsidones Dibenzofuranes	APP APP	Antioxidant activity Hepatoprotective activity Antibacterial activity Antityrosinase activity	Verma et al. (2008a) Behera et al. (2004b, 2005a, b, 2006d, 2009a)
Umbilicaria sp.	Gyrophoric acid	Depsides	APP	Cytotoxicity activity Antitumour activity	Burlando et al. (2009)
Umbilicaria esculenta	Orcinol	Aromatic	APP	Anti-inflammatory activity	Kim et al. (1996)
Xanthoparmelia scabrosa	Scabrosin esters	Epipolythiodioxopiperazine	APP	Cytotoxic activity	Ernst-Russell et al. (1999b)

Xanthoria parietina	Emodin Fallacinal Parietin	Anthraquinones Anthraquinones Anthraquinones	APP APP APP	Antibacterial activity Antifungal activity Cytotoxic activity Virucidal activity	Manojlovic et al. (1998) Fazio et al. (2007) Ivanova et al. (2000) Manojlovic et al. (1998) Ivanova et al. (2000)

APP acetyl polymalonyl pathway, *SAP* shikimic acid pathway, *MAP* mevalonic acid pathway
[a]Production pathways of lichen secondary metabolites

Jaganmohan (2000) examined antioxidant capacities of methyl orsellinate, atranorin, orsellinic acid and lecanoric acid. Behera and Makhija (2002) demonstrated the potential of biological activities like inhibition of tyrosinase and xanthine oxidase in *Bulbothrix setschwanensis*. In a further study, extracts of dried herbarium specimens of as many as 77 species belonging to the lichen family *Graphidaceae* have been screened for the bioactivities like inhibition of tyrosinase, xanthine oxidase and nitro blue tetrazolium (NBT) and scavenging of superoxide, and many of them have shown potential for these activities (Behera et al. 2003, 2004a, 2006a). Further they have also studied on lichen species *Usnea ghattensis*, *Arthothelium awasthii*, *Heterodermia podocarpa* and *Parmotrema tinctorum* as well as their secondary metabolites, atranorin, barbatic acid, lecanoric acid, norstictic acid, salazinic acid, usnic acid and zeorin, for the antityrosinase and antioxidant activities (Behera et al. 2004b; Verma 2011). Several clinical studies have shown that ethanol ingestion alters the prooxidant–antioxidant balance in the liver by the production of free radicals during its metabolism (Nordmann et al. 1992; Ishii et al. 1997; Aleynik et al. 1998; Mutlu-Turkoglu et al. 2000; Bailey et al. 2001; Balkan et al. 2002). Therefore, hepatoprotective activity in cultured lichen *Usnea ghattensis* has been screened and reported for the first time (Verma et al. 2008a). Bhattarai et al. (2008) reported stronger antioxidant activities in lichens from Antarctica. Mitrovic et al. (2011b) compared the chemical content of lichen extracts (*Flavoparmelia caperata*, *Evernia prunastri*, *Hypogymnia physodes*, *Cladonia foliacea*) and their free radical scavenging ability. Mahadik et al. (2011) reported the antioxidant activity of psoromic acid and usnic acid isolated from a lichen *Usnea complanata*. Later on, radical scavenging and glucosidase activity of lichen metabolites usnic acid, sekikaic acid and salazinic acid isolated from *Ramalina celastri*, *R. nervulosa* and *R. pacifica* have been reported (Verma et al. 2012a).

8.3.3 Antifungal Activity

Lichen species have also been reported for the antifungal activity. Recently, Manojlovic et al. (2005) reported antifungal activity of the anthraquinone parietin isolated from *Caloplaca cerina*. After that, Schmeda-Hirschmann et al. (2008) demonstrated the antifungal properties of Andean lichens *Protousnea poeppigii* and *Usnea rigida*, which contain divaricatinic acid, isodivaricatinic acid, usnic acid and 5-resorcinol. Later on Mitrovic et al. (2011b) reported strong antifungal effect of *Evernia prunastri* and *Hypogymnia physodes*. In the same year Mahadik et al. (2011) reported the antifungal activity of lichen species *Usnea complanata* against 14 fungal strains, *Alternaria alternata*, *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. oryzae*, *A. terreus*, *Candida albicans*, *Cryptococcus albidus*, *Curvularia* sp., *Fusarium moniliformae*, *F. oxysporum*, *Rhizopus stolonifer*, *Trichoderma harzianum* and *T. viride*. Usnic acid and psoromic acid were found to be the main component for the activity.

8.3.4 Antiviral Activity

As far as antiviral properties of lichen secondary metabolites are concerned, Yamamoto et al. (1995) reported the inhibition of Epstein–Barr virus activation by lichesterinic acid and usnic acid from the lichen *Dolichousnea longissima* (*Usnea longissima*). Later on Perry et al. (1999) showed antiviral activity of usnic acid against herpes simplex type 1 and polio type 1 viruses. Lichenan, a widely distributed lichen compound, is reported for the inhibition of tobacco mosaic virus (Stubler and Buchenauer 1996). Anthraquinones are also important constituents of lichens and are of interest as antiviral agents against HIV (Schinazi et al. 1990). In particular, hypericin is of pharmaceutical relevance because of its dramatic antiretroviral activity (Lavie et al. 1989). The derivatives 7,7′-dichlorohypericin and 5,7-dichloroemodin isolated from the lichen *Heterodermia obscurata* (Cohen and Towers 1995) were shown to exhibit strong inhibitory activity against herpes simplex virus type 1 (Cohen et al. 1996). Parietin extracted from *Teloschistes chrysophthalmus* showed activity against Junin and Tacaribe arenaviruses (Fazio et al. 2007).

8.3.5 Antitumour Activity

Antitumour activities of lichen metabolites are of great interest for pharmaceutical industries. The most extensively studied lichen secondary metabolite is a dibenzofurane compound usnic acid, abundantly found in many lichen species and reported for the antitumour activity by many researchers (Cardarelli et al. 1997; Ingolfsdottir 2002; Kristmundsdottir et al. 2002) from the time of its first isolation in 1844 (Mitrovic et al. 2011a). Other compounds like pannarin isolated from *Psoroma pholidotoides* (*Psoroma reticulatum*), *P. pulchrum* and *P. pallidum* are reported to inhibit the cell growth and induce apoptosis in human prostate carcinoma and human melanoma cells (Russo et al. 2006, 2008). Euplectin from *Flavoparmelia euplecta* and naphthazarin, an anthraquinone from lichen *Lecanora hybocarpa*, have been reported for the cytotoxicity activity (Ernst-Russell et al. 1999a, 2000). Protolichesterinic acid of *Cetraria islandica* showed inhibition against the growth of breast cancer cell lines (Ogmundsdottir et al. 1998). A depside gyrophoric acid isolated from *Umbilicaria* sp. demonstrated the cytotoxic and antitumour activity (Burlando et al. 2009).

8.3.6 Antipyretic, Analgesic and Anti-inflammatory Activity

In recent studies, lichen compounds are proved to be a good source of antipyretic, analgesic and anti-inflammatory compounds. Diffractaic acid, usnic acid and

decarboxy stenosporic acid isolated from the lichen *Dolichousnea diffracta* (*Usnea diffracta*) are reported for antipyretic, analgesic and anti-inflammatory activities (Otsuka et al. 1972; Okuyama et al. 1995). Kim et al. (1996) reported the anti-inflammatory activity of orcinol isolated from *Umbilicaria esculenta*. Longissiminone A isolated from *Dolichousnea longissima* (*Usnea longissima*) has shown anti-inflammatory activity (Choudhary et al. 2005).

8.3.7 Herbicidal Activity

Lichens are also a potential source of herbicidal compounds. The herbicidal effects of some lichen secondary metabolites have been reviewed by many workers. Barbatic acid, gyrophoric acid, haemathamnolic acid and lecanoric acid are reported as photosystem II-inhibiting herbicides (Lawrey 1995; Dayan and Romagni 2001, 2002). Evernic acid and protolichesterinic acid isolated from *Dolichousnea longissima* (*Usnea longissima*) are reported as plant growth and photosystem II inhibitor (Nishitoba et al. 1987; Endo et al. 1998). Several analogues of lichen-derived anthraquinones have strong herbicidal activity. Emodin and its analogues are highly active on grasses, causing malformation and bleaching in early seedlings. Usnic acid proved to be a strong herbicidal which has the potential to inhibit carotenoid biosynthesis through the enzyme 4-hydroxyphenylpyruvate dioxygenase (Dayan and Romagni 2001).

8.3.8 Insecticidal Activity

The unique secondary compounds present in lichens can also be used as novel pest control agents. Dayan and Romagni (2001) reviewed that the lichen feeding slug, *Pallifera varia*, when offered to eat some potential lichens, avoided those having wide variety of compounds and preferred those with the lowest number of lichen compounds. The slug-repelling activity of vulpinic acid is being investigated as a seed treatment for protecting higher plants. Likewise, the insect *Spodoptera littoralis* was strongly deterred by usnic acid and vulpinic acid (Dayan and Romagni 2001).

8.4 Harnessing of Pharmaceutically Important Metabolites from Lichen

Lichen species have shown a wide variety of biological activities, but they have been long neglected by mycologists and overlooked by pharmaceutical industries because of their slow growing nature and difficulties in their artificial cultivation

(Crittenden and Porter 1991; Yamamoto et al. 1998; Behera et al. 2004a, b). Hale (1973) made the following generalization about growth rates of lichen species: foliose (0.5–4 mm $year^{-1}$), fruticose (1.5–5 mm $year^{-1}$) and crustose (0.5–2 mm $year^{-1}$). Therefore, industrial-scale harvesting of lichens is neither ecologically sensible nor sustainable and for many species is not feasible (Miao et al. 2001). To prevent the decline of lichen population in protected areas and harnessing of novel biologically active compounds, the only conceivable alternative is to in vitro culture the lichens in bulk quantities (Yamamoto et al. 1995; Brunauer and Stocker-Worgotter 2005).

Ahmadjian (1966) was the first lichenologist who succeeded in artificial reestablishment of the lichen *Cladonia cristatella*. Ahmadjian and Heikkila (1970) reported the successful culture of *Endocarpon pusillum* and *Staurothele clopima*. In the year 1985, Yamamoto et al. (1985) successfully cultured *Usnea rubescens* and *Ramalina yasudae* in vitro by thallus fragments and demonstrated the production of usnic acid in the cultures, and subsequently they patented the method. Yoshimura et al. (1987) reported the culture of *Cladonia vulcani*. Stocker-Worgotter and Turk (1988) cultured the cyanobacterial lichen species, *Peltigera didactyla*, from soredia. Yoshimura et al. (1989) again reported the successful culture of some *Umbilicaria* species of lichenized fungi and the production of secondary metabolites by cultured tissue of *Usnea flexilis*. In the year 1990, Yoshimura et al. (1990a, b) reported the tissue culture of some Antarctic lichens and also cultured *Usnea rubescens* and *Peltigera praetextata* to the vegetative thallus stage.

After resolving various difficulties in establishing lichen tissue culture technique, many researchers have started to screen biological activities of cultured lichens and their metabolites produced in vitro. In this direction, Higuchi et al. (1993) screened as many as 46 cultured lichen species for tyrosinase inhibitory activity and some of them (*Hypogymnia physodes*, *Letharia vulpina* and *Cetraria juniperina*) showed strong activity. Antibacterial, antifungal, superoxide scavenging and tyrosinase inhibitory activity of cultured lichens of families *Cladoniaceae*, *Graphidaceae*, *Parmeliaceae*, *Umbilicariaceae* and *Usneaceae* has been reported by Yamamoto et al. (1993). Behera and Makhija (2002) reported the tyrosinase and xanthine oxidase inhibition activity of cultured thallus of foliose lichen *Bulbothrix setschwanensis*. Further, inhibition of tyrosinase and xanthine oxidase and scavenging of superoxide anions of three cultured species of crustose lichen genus *Graphis*, *G. guimarana*, *G. nakanishiana* and *G. schizograpta*, producing secondary metabolites norstictic acid, constictic acid and stictic acid, have been reported (Behera et al. 2006b). In continuation to this the same workers cultured lichen species *Usnea ghattensis*, *Arthothelium awasthii*, *Heterodermia podocarpa* and *Parmotrema tinctorum*. Secondary metabolites usnic acid, norstictic acid, atranorin, zeorin, lecanoric acid and barbatic acid produced by the derived culture were studied for the antioxidant, antimicrobial, antityrosinase and hepatoprotective properties (Behera et al. 2005a, b, 2006c, d, 2009a; Verma et al. 2008a–c).

In laboratory conditions, lichen tissue grows much faster than natural thalli but more slowly than many other microorganisms (Yamamoto et al. 1993).

To improvise the growth rate of lichen culture tissue for the production of lichen substances in high quantity, very few studies have been carried out so far. In a very recent study, Chooi et al. (2008) cloned and characterized the sequence of nonreducing polyketide synthase (PKS) gene from lichen *Xanthoparmelia semiviridis* and explored the suitability of *Aspergillus nidulans* for heterologous expression of the *X. semiviridis* PKS gene. However, further work is needed to understand the expression of PKS gene in surrogate host organism for desired lichen substances. Behera et al. (2009b) demonstrated the fusion of protoplast of isolated mycobiont of lichen *Usnea ghattensis* with the protoplast of fast-growing fungi *Aspergillus nidulans*. The result showed successful regeneration of fusant with the production of lichen secondary metabolite usnic acid. Further, three lichen species *Ramalina nervulosa*, *R. pacifica* and *Usnea complanata* were successfully cultured in bioreactor for the first time for the production of their secondary metabolites (psoromic acid, sekikaic acid, salazinic acid and usnic acid) in higher quantity (Verma et al. 2012b; Behera et al. 2012).

8.5 Future Prospects

Although the pace of work on lichens in recent days has been increased, it is still far behind with respect to their bioprospection. As of today with the established culturing methods, many lichens have been cultured. However, many more are not responded to the nutrient media and physiological parameters (temperature, pH); hence, they could not be brought under culture. In order to prospect the lichens or their secondary metabolites, we have to improve the conventional lichen tissue culture technique established with formulation of more appropriate nutrient media along with physiological parameter, which can support the symbiont growth with the production of secondary metabolites in larger quantity within a short span of time. Very recently it has been reported that the growth rate and the metabolite production at flask level are not sufficient to solve the problem of commercial exploitation of lichen secondary metabolites. For industrial-scale production of compounds salazinic acid, sekikaic acid and usnic acid found in lichen species *Ramalina nervulosa* and *Ramalina pacifica*, the optimized culture conditions (nutrient media, temperature, pH, stirrer speed and pO_2 level) are different even though they are from the same genus. In bioreactor, culture biomass of *R. nervulosa* and *R. pacifica* obtained 10.3–17.7 g along with the production of sekikaic acid 122.8 mg, salazinic acid 200 mg and usnic acid from 75.4 to 136.8 mg after 100 h batch running for each species (Verma et al. 2012b).

Bioreactor refers to an engineered device that supports a biologically active environment to carry out chemical process by which organisms enable to synthesize biochemically active substances at higher quantity in short duration of time (Mc Naught and Wilkinson 1997). Lichen is a dual organism consisting of photosynthetic partner (algae) and a mycobiont (fungi). The optimal physiological conditions for both the bionts in symbiosis is still unknown. Therefore, the culturing

of lichens in bioreactor is very less. However, large-scale production of lichen culture biomass using bioreactors is a promising approach for industrial-scale utilization of lichen secondary compounds.

In the future, metabolic engineering and biotechnological approaches can be used as an alternative production system to overcome the limited availability of biologically active, commercially valuable and medicinally important secondary metabolite compounds. Advancement in culture techniques may provide new means for the culturing of non-cultured/exotic lichens and production of their secondary compounds as they produce in nature. This may create an ultimate advantage to provide a continuous, reliable source of natural products from this complex organism.

The increasing use of genetic tools in regulation of pathways in secondary metabolism will provide the basis for the production of commercially viable lichen substances. The knowledge of using precursor to activate biosynthetic pathways of desired lichen phytochemical in cultures is still in its infancy; hence, strategies are needed to develop an information on the requirement of precursor for lichen-derived compounds. The introduction of newer techniques of molecular biology, so as to produce transgenic cultures and to effect the expression and regulation of biosynthetic pathways, is also likely to be a significant step toward making cell cultures more generally applicable for the commercial production of secondary metabolites (Hussain et al. 2012). This probably has been the main impetus for understanding and manipulating for biosynthesis of various chemical, physiological and biotechnological pathways.

Recently, many devised techniques are being used for culturing of lichens to enhance their biomass and their unique secondary medicinally important compounds. These techniques include genetic engineering to find out the genes responsible for the production of lichen secondary compounds along with the expression of the gene in fast-growing fungi to reduce its slow growth rate. Furthermore chemistry along with computer-aided drug designing together may be able to develop novel synthetic analogues of lichen substances which can enlarge the access for new drug discovery by the pharmaceutical industries.

Acknowledgements We gratefully acknowledge the various financial supports by the Department of Biotechnology (DBT), Govt. of India, New Delhi [grant nos. BT/PR3133/BCE/08/237/2002; BT/PR8551/NDB/52/15/2006]; Council of Scientific and Industrial Research (CSIR), Govt. of India, New Delhi [grant nos. 09/670(034)/2006-EMR-I; 09/670(0046)2010/EMR-I]; and Science and Engineering Research Board (SERB), Govt. of India, New Delhi [grant no. SR/FT/LS-170/2009]. We are also thankful to the director of Agharkar Research Institute, Pune, for research facilities provided.

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