



Studies on Antioxidant Properties of Lichen Secondary Metabolites

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Marijana Kosanić and Branislav Ranković

Abstract

At 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 utilize 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 neutralization of free radicals. Lichens are rich in 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 (ROS), such as hydroxyl radical, superoxide anion and hydrogen peroxide, and reactive nitrogen species (RNS), 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

M. Kosanić (✉) · B. Ranković

Department of Biology, Faculty of Science, University of Kragujevac, Kragujevac, Serbia

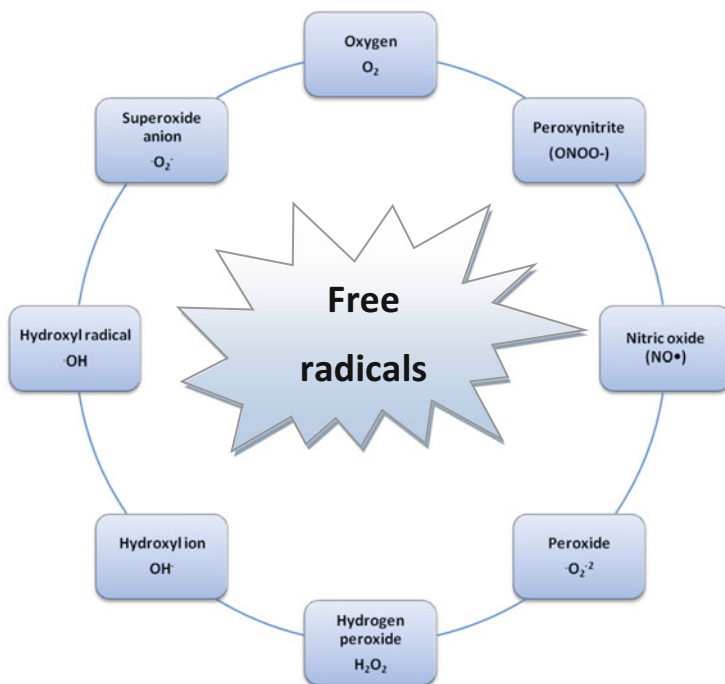


Fig. 4.1 Free radicals (reactive oxygen species and reactive nitrogen species)

of cellular structures and can act as weapons for the host defence system (Halliwell 1996; 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 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. 2009; 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



Fig. 4.2 Diseases induced by oxidative stress

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).

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 Shajyothi and Padmalatha 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. 2002; Naveena et al. 2008).

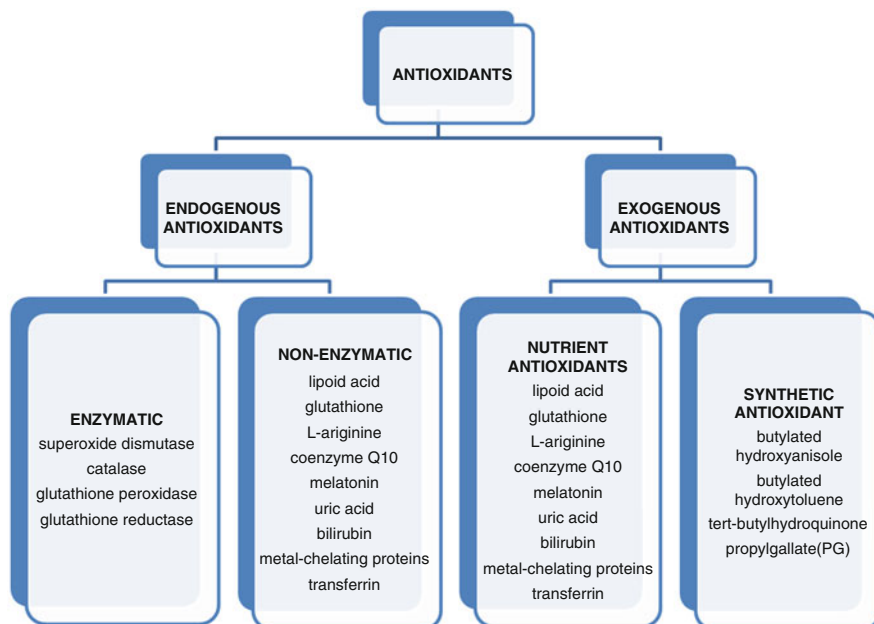


Fig. 4.3 Endogenous antioxidants and exogenous antioxidants for protecting the body from damage caused by oxidative stress

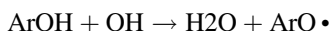
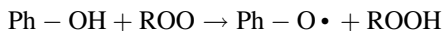
4.2 Antioxidant Properties of Lichens

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. 2008).

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 phenolics are generally secreted by the fungal partner and deposited as crystals on the surface of the cell wall of the fungal hyphae. Lichen phenolics are primarily acetate-polymalonate-derived with

the exception of pulvinic acid derivatives which are synthesized 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 furane heterocycle bond as found in dibenzofurans, such as usnic acid (Ross 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).



It is known 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 stabilization 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.

Phenolics 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:

1. The *ortho* 3',4'-dihydroxy structure in the B ring
2. The 2,3-double bond in conjunction with the 4-oxo group in the C ring (which allows conjugation between A and B rings or electron delocalization)
3. 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 phenolics 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 phenolics 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, b). Kosanić and Ranković (2011a, b) found that total phenolic content of *Cetraria islandica*, *Lecanora atra*, *Parmelia pertusa*, *Pseudevernia furfuracea* and *Umbilicaria cylindrica* extracts 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 phenolic 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 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 phenolic 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 IM (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).

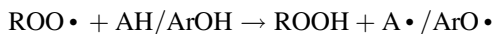
These results suggest that the antioxidant activity of some tested extracts might be attributed to the presence of non-phenolic compounds. Nevertheless, it should be taken into consideration that individual phenolics may have distinct antioxidant activities; there may be antagonistic or synergistic interactions 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, peroxy 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 (ArOH) is transferred to a ROO• radical can be summarized by the reaction



where the aryloxy radical (ArO•) formed from the reaction of antioxidant phenol with peroxy radical is stabilized by resonance. The AH and ArOH 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•, 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) dihydrochloride (AAPH) as the radical generator and β -carotene bleaching assay, although the latter bleaches not only by peroxy 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 (oxidizing agent) instead of peroxy 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).

Except for the most commonly used spectrophotometric methods, the antioxidant capacity of various compounds is also frequently evaluated using electrochemical

methods. Commonly used electrochemical methods are cyclic voltammetric methods, amperometric technique and bioamperometric and biosensor technique.

Cyclic voltammetric technique: This method is based on the potentiodynamic electrochemical evaluation. In this process the working electrode is ramped linearly to the time. The working electrode potential is measured at different intervals in order to calculate the current intensity at respective electrode. On attaining the preset potential, the potential ramp of working electrode is reversed. In a single experiment, this reversal can take place many times. Then the cyclic voltammogram is made by plating working electrode's current to the applied voltage. A cyclic voltammogram gives the following significant parameters such as cathodic oxidation potential, anodic oxidation potential, anodic peaks and cathodic peaks (Chevion et al. 2000; Ahmad et al. 2014).

Amperometric technique: It is based on the principle that the intensity of the current flowing between a reference and a working electrode is measured at a fixed potential. A current is produced by the oxidation/reduction reaction of an analyte whose value is fixed at a preset value as compared to the reference electrode. The measurement of the antioxidant activity by an amperometric method is based on the reduction of the DPPH using carbon electrode (Milardovic et al. 2006; Ahmad et al. 2014).

Biamperometric technique: In biamperometry, between two same working electrodes that is polarized at the minute difference in potential and immersed in a solution that contains a reversible redox couple. The current flows in this process are measured. Commonly used redox couple in biamperometric measurement is $\text{Fe}^{3+}/\text{Fe}^{2+}$, I^2/I^- , $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$. Controlled parameters in biamperometry is potential difference between two same kind of working electrodes while the potential value of reference electrode is not controlled with respect to two electrodes (Tougas et al. 1985; Ahmad et al. 2014).

Biosensor technique: Because of their electron giving nature, oxidoreductases are very commonly used biosensors. These are very stable in nature and do not require any other cofactor or chemical for their working. They have been broadly studied and discussed in the literature. In broader sense most of the antioxidant properties of the extract is due to their polyphenolic constituents. These polyphenols are detected by the enzyme-based biosensors. These enzymes include tyrosinase and laccase (Barroso et al. 2011; Ahmad et al. 2014).

Chromatographic techniques are also employed for the assessment of the antioxidant capacity of the extracts. Of the chromatographic techniques, gas chromatography is used for these purposes. Gas chromatography is one of the primary types of chromatography that is extensively applied for the separation and analysis of stable but vaporizable compounds. The process is carried out by passing the mixture through the stationary phase which is liquid, and the mobile phase in this type of chromatography is gas. Gases used as mobile phase are helium and nitrogen that are considered to be inert, whereas the stationary phase is a microfilm of a liquid incorporated on to a non-reactive solid support. The retention time comparison gives its value. Usually thermal conductivity and flame ionization detectors are used in gas chromatography (Ahmad et al. 2014).

4.4 Antioxidant Activities of Lichen Extracts

Lichens are proven to be a 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 anti-oxidation 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 the antioxidant activity of methanol extract of *Evernia divaricata*, *Evernia prunastri*, *Cladonia foliacea*, *Dermatocarpon miniatum* and *Neofuscella pulla* by scavenging 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 that they exhibited DPPH radical scavenging activity.

The antioxidant activity of ethanol extract of *Sanionia uncinata* was evaluated by Bhattarai et al. (2008a, b) by analysing its reducing power, superoxide scavenging activity, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic 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. (2010a, b) 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*. A strong antioxidant effect was also found in the methanol extract of *Usnea ghattensis* (Verma et al. 2008a), 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 extracts of *Ramalina roesleri* species and found that the DPPH radical scavenging activity of extracts ranged from 29.42 to 87.90%. Sharma and Kalikotay (2012) examined the antioxidant activity of two common lichens, namely, *Parmotrema reticulatum* and *Usnea* sp., from Darjeeling hills. The antioxidant assay of different concentrations 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 *Parmotermia* 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. 2008).

In the study described by Mastan et al. (2014), the lichens *Cladonia fimbriata*, *Permilopsis ambigua*, *Punctelia subrudecta* and *Evernia mesomorpha* secondary metabolites 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 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 radical and strong lipid peroxidation inhibition. Kekuda et al. (2009) found 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 with 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 activity.

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 a 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 lyophilisate), 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 crinita* 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 lichen *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).

The antioxidant effect of methanol, chloroform and petrol ether extracts from the lichen *Toninia candida* was assayed 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, 2014a, b; 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 phenolic 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), 2,2'-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 highest FRAP value. Methanolic extract of *Xanthoparmelia stenophylla* showed the highest ABTS radical scavenging capacity. The n-hexane extract of *Rhizoplaca chrysoleuca* exhibited highest DPPH radical scavenging capacity. 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 highest NO scavenging capacity. Among n-hexane, methanol and water extracts of all lichens, the methanolic extract of *Xanthoparmelia mexicana* showed highest total proanthocyanidin, flavonoid and polyphenol content.

In the study by Vivek et al. (2014), the radical scavenging potential of three *Parmotrema* species, viz. *Parmotrema tinctorum*, *Parmotrema grayanum* and *Parmotrema praesorediosum*, from Maragalale and Guli Guli Shankara, Western Ghats of Karnataka, India, was reported. 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, *Parmoterma grayanum* showed higher scavenging potential as indicated by lower IC50 value. Total phenolic content was also high in *Parmoterma grayanum*.

Kosanić et al. (2014c) investigated antioxidant properties of acetone extracts of the lichens *Parmelia arseneana* and *Acarospora fuscata*. Antioxidant activity was evaluated by measuring the scavenging capacity of tested samples on DPPH and superoxide anion radicals and reducing the power of extracts. As a result of the study, *Parmelia arseneana* extract was found to have the larger DPPH radical scavenging activity, superoxide anion radical scavenging and reducing power than *Acarospora fuscata* extract.

In a study described by Ristić et al. (2016a), DPPH radical scavenging activity and reducing power of the acetone extracts of *Melanelia subaurifera* and *Melanelia fuliginosa* lichens were determined. The tested extracts showed a dose-dependent

activity. The results of this assay present that acetone extract of *M. subaurifera* exhibits larger DPPH radical scavenging activity ($IC_{50} = 165.13 \mu\text{g/ml}$) than extract of *M. fuliginosa* ($IC_{50} = 266.32 \mu\text{g/ml}$) as well as showed higher reducing power. The same authors explored two *Ramalina* lichens for their antioxidant capacity. Extracts of the studied lichens showed a moderate DPPH radical scavenging activity. The IC_{50} values for the studied extracts were 285.45 and 423.51 $\mu\text{g/mL}$ for *R. fraxinea* and *R. fastigiata*, respectively. Acetone extract of lichen *R. fraxinea* also showed the higher reducing power (Ristić et al. 2016b). Kosanić et al. (2016) investigated antioxidant activity of the *Lasallia pustulata* methanol extract by measuring free radical scavenging activity, superoxide anion scavenging activity and reducing power. Their results revealed that the methanol extract had moderate free radical scavenging activity with IC_{50} values of 395.56 $\mu\text{g/mL}$. Moreover, the extract tested had effective reducing power and superoxide anion radical scavenging.

Similarly, the antioxidant potential of the lichen *Pleurosticta acetabulum* was evaluated by Tomović et al. (2017). The antioxidant capacity of the acetone lichen extract was evaluated by determining the radical scavenging capacity on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and reducing power. They found that *P. acetabulum* extract exhibited moderate free radical scavenging activity (IC_{50} of 151.7301 $\mu\text{g/mL}$), while spectrophotometric absorbance for reducing power varied from 0.035 to 0.127.

The study performed by Aoussar et al. (2017) focused on screening of antioxidant activity of dichloromethane, acetone and methanol extracts of *Evernia prunastri* and *Pseudevernia furfuracea* growing in Morocco. The antioxidant activity was performed in vitro by DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging method and ferric reducing antioxidant power assay. The highest DPPH radical scavenging activity was obtained from the acetone extract of *P. furfuracea*. Also, for *P. furfuracea*, the absorbance of the acetone extract was higher than those of other extracts and increased from 0.043 ± 0.005 to 0.890 ± 0.036 .

Five lichen species of *Cladonia* genus (*C. fimbriata*, *C. furcata*, *C. subulata*, *C. foliacea* and *C. rangiferina*) were examined for their antioxidant effect by Kosanić et al. (2018). They found that all tested extracts showed relatively high DPPH radical scavenging activity (IC_{50} values of lichen extracts range from 183.41 to 312.56 $\mu\text{g/mL}$). The extract of *C. furcata* showed the highest DPPH radical scavenging activity. Measured values of absorbances for reducing power varied in range from 0.010 to 0.1178. The extract of *C. furcata* showed the highest reducing power, followed by *C. foliacea*, *C. fimbriata*, *C. rangiferina* and *C. subulata*. The extract of *C. fimbriata* contains hypoprotocetraric acid, fumarprotocetraric acid, usnic acid (USN) and a small amount of the atranorin for which is a proven antioxidant activity, so that such greater activity was expected.

Ganesan et al. (2017) evaluated the antioxidant activities of *Parmotrema reticulatum* obtained from Yercaud hills of southern India. *P. reticulatum* was extracted with different solvents such as petroleum ether, ethyl acetate, ethanol, methanol, acetone and water based on their polarity. Then, it was subjected to antioxidant assays such as 1,1-diphenyl-2-picrylhydrazyl and ferric reduction activity potential. The DPPH free radical scavenging activity of lichen extracts using

different solvents was found to increase while increasing the concentration of extracts. Methanol extracts of *P. reticulatum* showed highest activity of capturing hydrogen-free radicals than the other extracts. The IC₅₀ value was found to be 19 µg/mL indicating the presence of bioactive molecules. The free radical capturing activity was found to be increased in the order of methanol > petroleum ether > water > acetone > ethanol > ethyl acetate extracts of *P. reticulatum*. The result revealed that methanol extract has the similar potential as that of standard ascorbic acid. In contrast, very less antioxidant activity was observed in the extract of ethyl acetate, and it was measured with an inhibition of 32.8% at 100 µg/ml. For ferric reduction activity potential, the absorbance was found to be increasing with an increase in the concentration of various extracts of lichen, and the activity was higher in the ethanol extracts among all other extracts. The order of activity was methanol > ethyl acetate > petroleum ether > ethanol > acetone > water. The same authors studied lichen species such as *Parmotrema austrosinense*, *P. hababianum* and *P. tinctorum* collected from the Eastern Ghats of India. From this species, the extracts were obtained by using various solvents; they were then subjected to various antioxidants assays, such as 2,2-diphenyl-1-picrylhydrazyl, ferric reducing antioxidant power and hydrogen peroxide. *P. tinctorum* showed very good antioxidant activity followed by *P. hababianum* and *P. austrosinense*. With respect to IC₅₀, acetone extracts of *P. tinctorum* were found to be superior (18.41 µg/mL) followed by methanol (60.3 µg/mL) and petroleum ether (74.5 µg/mL) extracts. The obtained results in this study indicated that selected lichen species showed moderate levels of reducing the ferric ions. In this regard, acetone extracts of *P. tinctorum* showed the highest reducing power as 3.77 at 1000 µg of concentration followed by petroleum ether extracts (1.46), whereas the least activity was measured for methanol extracts (0.125). In case of *P. hababianum*, methanol extracts showed highest reducing power (0.687) followed by water extracts (0.45). In contrast, water extracts of *P. austrosinense* showed better activity (0.48). Similar effects were seen for both methanol and petroleum ether extracts of *P. austrosinense*. For hydrogen peroxide scavenging assays, methanol and benzene extracts of *P. hababianum* showed the highest potential of scavenging, whereas other extracts did not show any activity. Benzene extract of *P. tinctorum* showed highest scavenging power as 26.5 followed by acetone extracts (35.32). Similar effects were observed in both methanol and benzene extracts of *P. austrosinense* for scavenging power of 80.99 and 89.85, respectively (Ganesan et al. 2015).

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

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. (2008b), Buçukoglu et al. (2013), Ristić et al. (2016a)
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, c)
2,4-Dihydroxy-6-propyl	Sisodia et al. (2013)
Ramalin	Paudel et al. (2008)
Barbatic acid	Verma et al. (2008b)
Divaricatic acid	Hidalgo et al. (1994)
Pannarin	Hidalgo et al. (1994), Russo et al. (2008)
Zeorin	Verma et al. (2008b)
Gyrophoric acid	Buçukoglu et al. (2013), Kosanić et al. (2014c)
Umbilicatic acid	Buçukoglu et al. (2013)
Protocetraric acid	Manojlović et al. (2012)
Fumarprotocetraric acid	de Barros Alves et al. (2014), Kosanić et al. (2014a, b, c)
Stictic acid	Lohézic-Le Dévéhat et al. (2007)
Salazinic acid	Verma et al. (2012), Manojlović et al. (2012), Selvaraj et al. (2015)
Usnic acid	Odabasoglu et al. (2006), Manojlović et al. (2012), Ranković et al. (2012, 2014a, b), Verma et al. (2012), Sisodia et al. (2013), Ananthi et al. (2015)
Variolaric acid	Brisdelli et al. (2013)
2-Hydroxy-4-methoxy-6-propylbenzoic acid	Sisodia et al. (2013)
Evernic acid	Kosanić et al. (2013a, b)
Erythrin	Choudhary et al. (2009)
2,4-Dihydroxy-3,6-dimethylbenzoate	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)
Vicanic	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)

(continued)

Table 4.1 (continued)

Lichen substances	References
Pulmonarianin	Atalay et al. (2011)
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)
2'-O-methyl anziaic acid	Ristić et al. (2016a)
Obtusatic acid	Ristić et al. (2016b)
Methyl evermate	Ristić et al. (2016b)

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 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, pannarin being more efficient, confirming Hidalgo et al. (1994). Similarly, de Barros Alves et al. (2014) found high 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) assessed the antioxidant activity of several classes of lichen metabolites in the in vitro superoxide radical (SOR), nitric oxide radical and 2,2-diphenyl-1-picrylhydrazyl radical scavenging assays. The depsides sekikaic acid and lecanoric acid showed promising antioxidant activity in SOR assay with IC_{50} values of $82.0 \pm 0.3 \mu\text{mol}$ and $91.5 \pm 2.1 \mu\text{mol}$, respectively, while the depsidone lobaric acid exhibited an IC_{50} value of $97.9 \pm 1.6 \mu\text{mol}$, all relative to the standard, propyl gallate ($IC_{50} = 106.0 \pm 1.7 \mu\text{mol}$). One of the most abundant mononuclear phenolic compounds, methyl- β -orcinol carboxylate, was found to be a potent NO scavenger ($IC_{50} = 84.7 \pm 0.1 \mu\text{mol}$), compared to the standard rutin ($IC_{50} = 86.8 \pm 1.9 \mu\text{mol}$).

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 and 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 structural feature in all of the above compounds is having two aromatic rings connected by an ester linkage and ortho to the carbonyl bearing carbon of ring A, 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 stabilized due to extended conjugation available in such compounds. In the case of depsidone lobaric acid, the electron accepted by C2O could be stabilized by both aromatic rings. The SOR activity of both the depsides lecanoric acid and erythrin was lost on permethylation suggesting that when C2O is methylated, the molecule loses its ability to accept electrons. Importantly, the IC₅₀ 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 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. All tested doses of usnic acid 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 derivatives, 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 umbilicic 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, umbilicic 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, orsenillic 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 acid and 8'-methylconstictic acid along with the metabolites 8'-methylstictic acid, 8'-methylmenegazziaic acid, stictic acid, 8'-ethylstictic acid and atranorin, which 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 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 possess 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.

Cuculloquinone, a bisnaphthoquinone of *Flavocetraria cucullata* inactivated DPPH to a 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.

Various antioxidant assays were used by Paudel et al. (2008) 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 1-diphenyl-2-picrylhydrazyl (DPPH) free radicals, 27 times more potent in scavenging 2,2'-azino-bis(3-ethylbenzothiazoline-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 μ g/ml ramalin reduced the produced hydrogen peroxide in LPS (lipopolysaccharide)-stimulated murine macrophage Raw 264.7 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 peroxy 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 free radical scavenging activities of salazinic acid, sekikaic acid and usnic acid isolated from three terrestrial natural lichen species *Ramalina celastri*, *Ramalina nervulosa* and *Ramalina pacifica*. 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 DPPH 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, b) who found varying antioxidant success in free radical scavenging, superoxide anion radical scavenging and reducing power, with physodic acid being 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. (2014a, b). 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, b, c) investigated the 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 largest free radical scavenging activity with IC_{50} values 131.48 $\mu\text{g/mL}$. Moreover, atranorin had the most effective reducing power and superoxide anion radical scavenging.

Kosanić et al. (2014c) studied antioxidant capacity for gyrophoric acid from *Acarospora fuscata* lichen. The isolated lichen component demonstrated strong DPPH radical scavenging activity. The IC_{50} value for gyrophoric acid was 105.75 $\mu\text{g/mL}$. The isolated component also expressed the high superoxide anion radical scavenging activity (the IC_{50} was 196.62 $\mu\text{g/mL}$) and reducing power. Ristić et al. (2016a) tested antioxidant properties for 2'-O-methyl anziaic acid from lichen *M. fuliginosa* and lecanoric acid from lichen *M. subaurifera*. Both tested compounds showed a dose-dependent activity. For DPPH assay, the IC_{50} value of lecanoric acid was higher ($IC_{50} = 424.51 \mu\text{g/mL}$) compared to another compound 2'-O-methyl anziaic acid ($IC_{50} = 121.52 \mu\text{g/mL}$). Compound 2'-O-methyl anziaic acid also showed higher reducing power than lecanoric acid. The same group of authors have done similar research with two *Ramalina* lichens from which isolated obtusatic acid (from *R. fraxinea*) and methyl evernate (from *R. fastigiata*). Both compounds exhibited lower DPPH radical scavenging activity. For reducing power it is interesting that the isolated components showed higher effect than lichen extracts from which they are isolated (Ristić et al. 2016b).

Study performed by Selvaraj et al. (2015) was to estimate the antioxidant activity of salazinic acid and its derivate hexaacetyl salazinic acid isolated from *Parmotrema reticulatum*. The antioxidant properties were studied under the categories DPPH, FRAP, metal chelating activity, hydroxyl scavenging activity, lipid peroxidation activity, phosphomolybdenum activity and superoxide dismutase activity. But among the two test compounds, hexaacetyl salazinic acid gives lower IC_{50} value and shows higher inhibition activity than salazinic acid for DPPH, superoxide dismutase activity and hydroxyl radical scavenging activity. It was already known that the lower the IC_{50} value, the higher is the inhibition activity. In lipid peroxidation, both the test compounds show nearly equivalent activity. In phosphomolybdenum assay, salazinic acid shows better activity than hexaacetyl salazinic acid. In FRAP assay, hexaacetyl salazinic acid shows better activity than salazinic acid. The metal chelating capacity of both the test compounds was found to be less.

Ananthi et al. (2015) repeated the same experiment with usnic acid and its derivative usnic acid diacetate isolated from *Usnea luridorufa*. They concluded that the lichen secondary metabolite usnic acid and its derivative usnic acid diacetate have possessed efficient antioxidant properties and are reported to be dose dependent. Among these two test compounds usnic acid gives lower IC_{50} value for hydroxyl scavenging activity, superoxide dismutase scavenging activity and shows better activity than usnic acid diacetate, for DPPH radical scavenging while usnic acid diacetate gives lower IC_{50} value and possesses better activity than usnic acid for lipid peroxidation inhibiting activity both the test compounds shows lesser activity. Also usnic acid diacetate possesses higher total antioxidant activity via phosphomolybdenum assay than usnic acid. In FRAP assay usnic acid diacetate shows higher activity than usnic acid. On the other hand, metal chelating activity of both usnic acid and usnic acid diacetate was found to be very less.

This work reveals that the lichens can be an interesting source of new anti-oxidative 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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