Antioxidative potential of natural lichens

For the study of antioxidative potential four species of lichens *Arthothelium awasthii*, *Heterodermia podocarpa*, *Parmotrema tinctorum* and *Usnea ghattensis* have been selected. The natural thalli of the lichen species namely *Arthothelium awasthii* (voucher no. 03.379 AMH) produces barbatic acid and two unknown substances, *Heterodermia podocarpa* (voucher no. 04.59 AMH) produces atranorin, norstictic acid, salazinic acid and zeorin, *Parmotrema tinctorum* (voucher no. 04.101 AMH) produces atranorin and lecanoric acid and *Usnea ghattensis* (voucher no. 03.121 AMH) produces usnic acid and norstictic acid in nature.

Materials and methods

Collection of natural lichen species

The lichen species were collected at various localities from Maharashtra State and authenticated by Dr. Urmila Makhija, a lichen taxonomist at Agharkar Research Institute. A part of the material of each species has been preserved as dried herbarium specimen in the Ajrekar Mycological Herbarium (AMH), at Agharkar Research Institute, Pune, India, as:


*Heterodermia podocarpa*, Maharashtra, Satara District, Lodwick Point, 4 km from Mahabaleshwar, 21.1.2004, B. C. Behera & G. S. Chitale, 04.59-AMH.

*Parmotrema tinctorum*, Maharashtra, Satara District, Kas Lake, 22 km from Satara, 2.8.2004, B. A. Adawadkar, 04.101-AMH.

*Usnea ghattensis*, Maharashtra, Satara District, Gureghar, 7 km from Mahabaleshwar, 15.7.2003, B. C. Behera & G. S. Chitale, 03.121-AMH.
Determination of lichen substances

Lichen substances were determined with standardized thin layer chromatography (TLC) method of Culberson & all (1972) and high-performance liquid chromatography (HPLC) (Feige & all, 1993). The natural thalli of the lichen species A. awasthii, H. podocarpa, P. tinctorum and U. ghattensis (1 gm each) were extracted in acetone/methanol by the soxhlet extractor.

For TLC analysis commercially available silica coated aluminum TLC plates (Silica gel 60 F_{254}, Merck) were used. The acetone residue was extracted from the lichen thallus and spotted on the TLC plates 2 cm from the base. The loaded plates were run in two standard solvent systems BDA (benzene : dioxane : acetic acid; 180 ml : 45 ml : 5 ml, total volume 230 ml) and HEF (hexane : ethylether : formic acid, 130 ml : 80 ml : 20 ml, total volume 230 ml). The plates were dried and sprayed with 10% sulfuric acid (H$_2$SO$_4$) and heated in an oven at 110°C, till the development of the spots. Identification of lichen substances was done using Rf values and colours of standards substances by loading and running the two simultaneously, for direct comparison.

The HPLC analysis was carried out on Agilent 1100 system with a 360 autosampler, C8 (Zorbax) column (Eclipse × DB-C8, 4.5 × 150 mm, 5 µm) and UV spectrophotometric detector, at 28°C with solvent: methanol-water-phosphoric acid (80 : 20 : 0.9, v/v/v). The detection wavelength was 254 nm and the injection volume was 5 µl, with a flow rate of 1 ml/min. Lichen substances were identified by their peck symmetry and their retention time, by comparison with authentic substances made to standard concentration. The retention times were 2.21 min for atranorin, 2.45 min for barbatic acid, 7.49 min for lecanoric acid, 6.0 min for norstictic acid, 1.7 min for salazinic acid, 7.21 min for usnic acid, and 2.35 min for zeorin respectively.

Extraction

Lichen substances are poorly soluble in water (Kristmundsdóttir & all, 2005), so various solvents viz. methanol, acetone, ethanol, dimethylsulphoxide (DMSO),
hexane have been used for the extraction and determination of suitable solvent with maximum antioxidant activity. For extraction, 1 gm thallus of each lichen species was extracted using soxhlet apparatus in 20 ml of 10% aqueous solution of each solvent. The extracts were then used to study for their antioxidative properties following various assays.

**Free radical-scavenging activity**

The free radical-scavenging activity (FRS) of lichen extracts was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) according to the method described by Blois (1958). DPPH solution of 0.1 mM in ethanol prepared and 1 ml of this solution was added to 3 ml of lichen extract (20 µg/ml) and was allowed to stand at room temperature for 30 min, and then absorbance was read at 517 nm against blank samples, using UV-Vis spectrophotometer (Shimadzu-1601, Japan). The standard antioxidants (20 µg/ml) of each; butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Trolox, a water-soluble vitamin E analogue, quercetin, melatonin and ascorbic acid were used as positive control. A lower absorbance of the reaction mixture indicated a higher free radical scavenging activity. Radical scavenging was expressed as the inhibition percentage and was calculated using the following formula (Yen & Duh, 1994):

\[
\text{% Inhibition} = \left(\frac{A_{DPPH} - A_{Extr}}{A_{DPPH}}\right) \times 100
\]

Where \(A_{DPPH}\) is the absorbance value of the DPPH blank sample, and \(A_{Extr}\) is the absorbance value of the test solution. \(A_{Extr}\) was evaluated as the difference between the absorbance value of the test solution and the absorbance value of its blank.

**Superoxide anion-scavenging activity**

The superoxide anion-scavenging activity (SAS) of lichen extracts was measured according to the method described by Nishimiki & all (1972), with slight modification. 1 ml of nitroblue tetrazolium (NBT) solution (150 µM NBT in 100 mM phosphate buffer, pH 7.4), 1 ml nicotinamide adenine dinucleotide (NADH) solution (468 µM in 100 mM phosphate buffer, pH 7.4), and 0.1 ml of extract (20 µg/ml) were
mixed. The reaction started by adding 100 µl of phenazine methosulphate (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance was measured at 560 nm against blank samples. Decreased absorbance indicated the increased superoxide anion-scavenging activity. Same concentrations of standard antioxidants, melatonin, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), quercetin, Trolox, a water-soluble vitamin E analogue and ascorbic acid were used as positive control.

Nitric oxide-scavenging activity

The nitric oxide-scavenging (NOS) ability of lichen extracts were measured according to the method of Marcocci & all (1994). Aliquots of 4 ml of extract solution (20 µg/ml) were added to 1 ml of sodium nitroprusside solution (25 mM) in a test tube and then incubated at 37°C for 1 h. A volume of 0.5 ml of the incubation solution was removed and diluted with 0.3 ml of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotination of nitrite with sulfanilamide and subsequent coupling with napthylethylenediamine dihydrochloride was immediately read at 570 nm and referred to the absorbance of standard solutions of sodium nitrite salt treated in the same way with Griess reagent.

Hydroxyl radical-scavenging activity: Deoxyribose assay

As a major intermediate in the metabolism of ROS, hydrogen peroxide can be formed from superoxide anions by the action of superoxide dismutase (SOD), and it is metabolized by Fe (II) to hydroxyl radicals or by the enzyme catalase (CAT) to water and molecular oxygen, according to the Haber-Weiss reactions (Benzie & all, 1996):

\[
Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2
\]
\[
Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdotOH + OH^-\]
The ability of the extract to inhibit the iron-induced decomposition of deoxyribose was assessed following the procedure described by Halliwell & Gutteridge (1981). The reaction mixture contained, in a final volume of 1.2 ml, the following reagents at the final concentrations stated: deoxyribose (2.8 mM), ferric chloride (FeCl$_3$; 25 μM), and nitrilotriacetic acid (NTA; 100 μM). NTA and Fe$^{3+}$ ions are premixed at the ratio given prior to the addition of deoxyribose, phosphate buffer, pH 7.4 (10 mM), different concentrations of the extract (10-200 μg/ml), and hydrogen peroxide (H$_2$O$_2$; 2.8 mM). The test tubes were incubated at 37°C for 1 h. Products of 'OH attack upon deoxyribose was assessed by reaction with thiobarbituric acid (TBA; 1%) in acid solution (2.8% trichloroacetic acid). After 15 min incubation at 100°C and then cooling over ice, the absorbance of the pink chromophore was measured at 532 nm.

**Trolox-equivalent antioxidant capacity assay**

Trolox-equivalent antioxidant capacity (TEAC) of lichen extracts were carried out by the procedure described by Miller & all (1995) with minor modifications. The TEAC value is based on the ability of the antioxidant to scavenge the blue-green 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS$^{•+}$) radical cation relative to the ABTS$^{•+}$ scavenging ability of the water soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). ABTS$^{•+}$ radical cation can be generated by the interaction of ABTS$^{•+}$ (100 μM), H$_2$O$_2$ (50 μM) and peroxidase enzyme (4.4 unit/ml). To measure antioxidant capacity, 200 μl of lichen extract (20 μg/ml) was mixed with an equal volume of ABTS$^{•+}$, H$_2$O$_2$, peroxidase enzyme and deionized water. The absorbance was monitored at 734 nm for 10 min. The decrease in absorption at 734 nm after the addition of reactant was used to calculate the TEAC value. The TEAC value was expressed as mM of Trolox solution having the antioxidant equivalent to a 0.1% (w/v) extract solution. The higher the TEAC value of the sample indicating a stronger the antioxidant ability.
Inhibition of lipid peroxidation

The inhibition of lipid peroxidation (ILP) of lichen extracts was determined according to the method of Liegeois & al (2000). An aqueous solution of linoleic acid and 2,2′-azobis,2-amidineopropane dihydrochloride (AAPH) solution was prepared as described by Liegeois & al (2000). A 30 µl aliquot of 16 mM linoleic acid dispersion was added to a UV cuvette containing 2.81 ml of 0.05 M phosphate buffer, pH 7.4, pre-thermo stated at 40°C. The oxidation reaction was initiated at 37°C under air by adding 150 µl of 40 mM AAPH solution. Oxidation was carried out in the presence of aliquots (20 µl) of the lichen extract (20µg/ml). The rate of peroxidation at 37°C was monitored by recording the increase in absorbance at 234 nm caused by conjugated diene hydroperoxides. The same concentration (20 µg/ml) of standard antioxidants; butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Trolox, quercetin, melatonin, and ascorbic acid were used as positive control. The percentage inhibition of lipid peroxidation was calculated by the following equation:

\[
\% \text{ Inhibition} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

Where \( A_0 \) is the absorbance of the control reaction and \( A_1 \) is the absorbance in the presence of the extract.

Polysaccharide assay

The polysaccharide content in lichen extracts was determined, using the phenol-sulfuric acid method described by Dubois & al (1956). 1 ml of extract solution (20 µg/ml) was added with 25 µl of 80% phenol and 1 ml sulfuric acid (H₂SO₄). Mixture was shaken and allowed to stand at 30°C for 30 min. The absorbance was measured by UV-Vis spectrophotometer at 490 nm. Polysaccharide content was estimated by a standard curve using known amounts of standard polysaccharide solution (Himedia Chemicals, India).
Protein assay

Protein content in lichen extracts was determined by the Coomassie-dye binding method described by Bradford (1976). 1 gm of lichen thallus was homogenized in 2 ml of 0.1 M potassium phosphate buffer, pH-7.5 and centrifuged at 7000 rpm in a micro-centrifuge. Protein was precipitated by the addition of 20% Tri-carboxylic acid (TCA) at 4°C. After over night incubation the precipitate was separated from the remaining solution by centrifugation at 12000 rpm in micro-centrifuge for 10 min. The pellet of protein was added to cuvette containing 5 ml Bradford reagent (0.01% Coomassie brilliant blue G - 250, 4.7% ethanol and 8.5% phosphoric acid) and the mixture was allowed to stand for 30 min at room temperature. The absorbance was measured at 595 nm. The protein content was estimated by a standard curve using known amount of bovine serum albumin (BSA).

Total soluble phenolic content

Total soluble phenolics in lichen extracts were determined with Folin-Ciocalteu reagent according to the method described by Slinkard & Singleton (1977), using pyrocatechol as a standard. An extract of 0.1 ml and 2 ml of 2% sodium carbonate (Na$_2$CO$_3$) was added and mixed thoroughly. After 5 min of incubation, 0.1 ml of 50% Folin-Ciocalteu reagent was added and allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm. The concentration of total phenolic compounds in the extract was determined as micrograms of pyrocatechol, equivalent by using an equation that was obtained from the standard pyrocatechol graph given below:

$$\text{Absorbance} = 0.001 \times \text{Pyrocatechol (µg)} + 0.0033$$

Statistical analysis

Experimental results are the mean (± SD) of five parallel measurements of the individual activity. Values of $p < 0.05$ were regarded as significant and $p < 0.01$ as very significant.
Chapter 1

Results

Antioxidative potential of lichens

The antioxidant activity of various solvent extracts of lichens; Arthothelium awasthii, Heterodermia podocarpa, Parmotrema tinctorum and Usnea ghattensis was measured in terms of lipid peroxidation inhibition, scavenging of free radical, superoxide anion, nitric oxide, hydroxyl radical, and Trolox-equivalent antioxidant capacity using various in vitro assay systems.

Free radical-scavenging activity (FRS)

The free radical-scavenging activity of various solvent extracts of lichen thalli is presented in table 1.1. The FRS activity of all the solvent extracts (methanol, acetone, ethanol, DMSO, hexane) was found ranging from 9.3% to 75%. The methanol extract of U. ghattensis showed 75% FRS activity, which was found to be higher than the activity of other lichens A. awasthii, H. podocarpa, P. tinctorum and standard antioxidants quercetin, melatonin, ascorbic acid, but found lower than the BHA and BHT. The methanol extract of A. awasthii has shown 58% FRS activity which was more or less equivalent to the activity of standard antioxidants quercetin, melatonin and ascorbic acid. However the FRS activity in H. podocarpa and P. tinctorum (9.3% to 48.3%) were found to be lower than the FRS activity of U. ghattensis and A. awasthii.

Superoxide anion-scavenging activity (SAS)

The superoxide anion-scavenging activity of various solvent extracts of lichen thalli is presented in table 1.1. The SAS activity of all the solvent extracts (methanol, acetone, ethanol, DMSO, hexane) was found ranging from 10.2% to 56.3%. The methanol extract showed 19.8% to 56.3% SAS activity, which was higher than the activity of other solvent extracts acetone, ethanol, DMSO, hexane. The methanol extract of U. ghattensis showed highest SAS activity (56.3%) and found to be higher than the activity of other lichens A. awasthii, H. podocarpa, P. tinctorum and standard
antioxidants (quercetin, melatonin, ascorbic acid), but it was lower than the BHA and BHT. No SAS activity was found by hexane extract.

**Nitric oxide-scavenging activity (NOS)**

The nitric oxide-scavenging activity of various solvent extracts of lichen thalli is presented in table 1.1. The NOS activity of all solvent extracts (methanol, acetone, ethanol, DMSO, hexane) was found ranging from 12.2% to 82.6%. The methanol extract of *U. ghattensis, A. awasthii, H. podocarpa, P. tinctorum* showed 82.6%, 47.3%, 51.8% and 45.4% NOS activity, which were higher than the activity of other solvent extracts (acetone, ethanol, DMSO, hexane) and the standard antioxidants (BHA, quercetin, melatonin, ascorbic acid).

**Hydroxyl radical-scavenging activity (HRS)**

The hydroxyl radical-scavenging activity of various solvent extracts of lichen thalli was found ranging from 8.6% to 89.1%, presented in table 1.1. The methanol extract of *U. ghattensis* and *A. awasthii* showed 89.1% and 68.1% HRS activity, which were found to be higher than the activity shown by *H. podocarpa, P. tinctorum* and standard antioxidants. However, no HRS activity was found by the hexane extract of *A. awasthii, H. podocarpa* and *P. tinctorum*.

**Trolox-equivalent antioxidant capacity (TEAC)**

As far as the Trolox-equivalent antioxidant capacity of various solvent extracts of lichen thalli is concerned, the methanol extract of *U. ghattensis* showed highest TEAC 4.0 mM, which was found to be higher than the other solvent extracts (acetone, ethanol, DMSO, hexane) of lichens (*A. awasthii, H. podocarpa, P. tinctorum*) and the standard Trolox, a water soluble vitamin-E analogue (Table 1.1).

**Inhibition of lipid peroxidation (ILP)**

The various solvent extracts of lichen thalli showed inhibition of lipid peroxidation activity ranging from 15.3% to 86%, presented in table 1.1. The methanol extract of
U. ghattensis, A. awasthii, P. tinctorum and H. podocarpa were showed ILP activity in the order (86% > 56% > 46.8% > 36.8%) in comparison to other solvent extracts (acetone, ethanol, DMSO, hexane). The ILP activity of U. ghattensis and A. awasthii (> 50%) found to be higher than the activity (36% to 55.3%) shown by standard antioxidants (BHA, BHT, Trolox, quercetin, melatonin and ascorbic acid). However, H. podocarpa and P. tinctorum showed < 50% lipid peroxidation activity than the standard antioxidants.

Protein, polysaccharide and polyphenol content of lichens

Protein, polysaccharide and polyphenol content in lichen, U. ghattensis, A. awasthii, H. podocarpa and P. tinctorum are presented in table 1.1. The ratio of protein/polysaccharide was found 0.2 to 5.9. The polysaccharide content and polyphenol content, obtained from the thallus of lichens (U. ghattensis, A. awasthii, H. podocarpa, P. tinctorum) were ranging from 0.1 mg to 5 mg/gm dry wt and 0.04 mg to 17.7 mg/gm dry wt respectively. A high (9.5 mg to 17.7 mg/gm dry wt) polyphenolic content was found in methanol extract.

Half inhibiting concentration (IC$_{50}$)

The half inhibiting concentration (IC$_{50}$) value is a half maximal (50%) inhibitory concentration (IC) of a substance which is needed to inhibit a given biological process by half. The IC$_{50}$ value for the antioxidant activities of the lichen extracts and the commercial standards were calculated by extrapolation from concentration/effect regression lines obtained from 3 to 4 different concentrations (2, 5, 10 or 20 µg/ml) and results are presented in table 1.2.

The IC$_{50}$ value of methanol extract of natural lichen U. ghattensis was found to be lower (11.2 µg to 17.8 µg/ml) for 50%, lipid peroxidation inhibition, scavenging of free radicals, superoxide anion, nitric oxide, and hydroxyl radical in the assay system. However, the IC$_{50}$ values of other lichen species A. awasthii, H. podocarpa and P. tinctorum were found (14.6 µg to 50.5 µg/ml) to be higher than the standard antioxidants.
Antioxidant activities in relation to protein, polysaccharide and polyphenol content

Several components, such as phenols and polysaccharides, have been isolated from lichens. Recently Liu & all (1997) reported that polysaccharide in the extracts of mushrooms had scavenging effects on superoxide that appeared to be dependent on the amount of protein present in the protein-polysaccharide complexes. Keeping in view, protein/polysaccharide ratio in the lichen extracts have also been determined and correlated with the antioxidant activities. The correlation between total phenol content and free radical scavenging activity showed correlation $R^2 = 0.886$, $p < 0.01$ (Fig. 1.1); superoxide anion-scavenging activity $R^2 = 0.544$, $p < 0.05$ (Fig. 1.2); nitric oxide-scavenging activity $R^2 = 0.699$, $p < 0.05$ (Fig. 1.3); hydroxyl radical-scavenging activity $R^2 = 0.641$, $p < 0.05$ (Fig. 1.4); Trolox-equivalent antioxidant capacity $R^2 = 0.636$, $p < 0.01$ (Fig. 1.5) and with inhibition of lipid peroxidation $R^2 = 0.717$, $p < 0.05$ (Fig. 1.6). However, the correlation between protein/polysaccharide ratio and the scavenging of free radical, superoxide; nitric oxide, hydroxyl radical, Trolox-equivalent antioxidant capacity and inhibition of lipid peroxidation showed insignificant correlation as $R^2 = 0.363$, 0.226, 0.115, 0.161, 0.189 and 0.126 respectively.

Discussion

The methanol extract of lichen species have showed antioxidative potential in the order of *Usnea ghattensis > Arthothelium awasthii > Heterodermia podocarpa > Parmotrema tinctorum* (Fig. 1.7). Lichens produce a number of secondary metabolites many of which are phenolic compounds (Nash, 1996). Since a significant correlation was found between the total phenols in the extracts and scavenging of free radical, superoxide, nitric oxide, hydroxyl radical, Trolox-equivalent antioxidant capacity and lipid peroxidation inhibition activity by the extracts, it is believed that the antioxidant activity shown by the extracts was probably due to the presence of phenolic compounds. These results are in agreement with those that reported the ability of phenolic compounds to scavenge free radicals and active oxygen species (Hatano & all, 1989; Duh & all, 1999).
Crude extracts may contain many compounds along with the various lichen substances. Although the levels of these phytochemicals are influenced by genetics and growth conditions (Marin & all, 2004), we can only conclude that the biological activities reported here are species specific.

On the basis of the results obtained it can be suggested that the extract of natural lichens; *U. ghattensis, A. awasthii, H. podocarpa* and *P. tinctorum* could be of use as an easily accessible source of natural antioxidants for a possible food supplement or in the pharmaceutical industry.