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Screening of antioxidant activity of lichens *Parmotrema reticulatum* and *Usnea* sp. from Darjeeling hills, India

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Abstract—Till date many plants have been screened for their antioxidant activity. Oxidative stress can lead to the production of free radicals which may cause many degenerative diseases. These free radicals can be eliminated with the help of antioxidants which may be of a natural origin. The aim of this study was to examine 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, 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 were high and total flavonoids content was moderate. Tested lichen species were found to possess considerable antioxidant activities and could be evaluated as good natural sources of antioxidants.

Keywords—Antioxidants, Parmotrema reticulatum, Usnea sp., Darjeeling.

I. INTRODUCTION

Free radicals which have one or more unpaired electrons are produced in normal or pathological cell metabolism. Reactive oxygen species (ROS) react easily with free radicals to become radicals themselves. ROS are various forms of activated oxygen, which include free radicals such as superoxide anion radicals (O^{-2}) and hydroxyl radicals (OH^{-1}), as well as non-free radical species (H_2O_2) and the singlet oxygen ($^1O_2$)[1, 2, 3, 4]. Also, excessive generation of ROS, induced by various stimuli and which exceed the antioxidant capacity of the organism, leads to a variety of pathophysiological processes such as inflammation, diabetes, genotoxicity, and cancer [5, 6, 7]. Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents, and pesticides [3, 8, 9, 10]. The most effective way to eliminate free radicals which cause the oxidative stress is with the help of antioxidants.

Antioxidants, both exogenous and endogenous, whether synthetic or natural, can be effective in preventing free radical formation by scavenging and suppressing such disorders [11, 12-14]. Currently, there is a growing interest toward natural antioxidants of herbal resources [10-12]. Epidemiological and in vitro studies on medicinal plants and vegetables strongly supported this idea that plant constituents with antioxidant activity are capable of exerting protective effects against oxidative stress in biological systems [13-16].

Lichens are the symbiotic organisms including a fungal partner and an algal partner and are known to have therapeutic effects on various diseases in folk medicine of many countries. Recently, much attention has been paid to lichens as resources of natural antioxidants. Scientist already investigated the antioxidant activity of some species of lichens, such as *Bryoria fuscescens, Cetraria islandica, Dermatocarpon intestiniformis, Parmelia saxatilis, Peltigera rufescens, Platismatia glauca, Ramalina pollinaria, R. polymorph, Umbilicaria nylanderiana, Usnea ghattenis,* and *U. longissima* and some of them have very good antioxidant activity [17, 18, 19, 20].

India is a rich centre of lichens diversity, contributing nearly 15% of the 13500 species of lichens so far recorded [21]. Darjeeling hills, one of the picturesque hill stations of Eastern Himalayas is situated at coordinates 27◦13′N to 26◦ 27'N and 88◦53'E to 87◦58'E. It has an area of 3,149 sq km. Its annual mean maximum temperature is 14.9◦C and annual mean minimum temperature is 8.9◦C and average annual rainfall is 3092mm.

Lichens occur in abundance in Darjeeling Hills of West Bengal, India. Hence this work was set out in order to screen the antioxidant activity of two common lichen species namely *Parmotrema reticulatum* and *Usnea* sp of Darjeeling hills.

II. MATERIALS AND METHODS

2.1 Collection and identification of lichen materials:

The lichen specimens was collected from the trees growing around Darjeeling town and characterised with the help of their morphology, anatomy, colour reaction, thin layer chromatography and identified from National Botanical Research Institute, Lucknow as *Parmotrema reticulatum* 08-0017193 (LWG) and *Usnea* sp 09-0017196 (LWG). Specimen samples namely SK1 and SK2 are preserved in the Herbarium of Darjeeling Government College, Darjeeling.

2.2 Extract preparation:

Lichen specimens were air dried at room temperature to constant weights. The dried plant materials were ground separately to powder. Ten grams of each powdered lichen material were extracted in methanol and ethanol (50 ml each) by shaking for 48 hours in shaking incubator (Orbital Shaking Incubator MSW232, Macro Scientific Works, New Delhi, INDIA). Each extract was filtered with Tarsons Nylon Membrane Filter 66, Riviera, Kolkata of pore size 450nm. Each filtrate was evaporated and residue obtained stored in refrigerator.

2.3 Chemicals used

1,1-Diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, aluminum nitrate, potassium acetate, quercetin, Folin-Ciocalteus's phenol reagent, sodium carbonate, tannic acid, potassium ferricyanide, phosphate buffer, trichloroacetic acid, ferric chloride, butylated hydroxytoluene (BHT), sodium phosphate, ammonium molybdate, α-tocopherol were obtained HiMedia Laboratories, Mumbai, INDIA. All the chemicals used including the solvents were of analytical grade.

2.4 DPPH radical scavenging assay

The effect of the extracts on DPPH radical was estimated using the method of Liyana- Pathiranan and Shahidi [26]. A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of extract in methanol containing 0.02–0.1 mg of the extract. The reaction mixture left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid was used as reference. The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%) = [(Abs control – Abs sample)]/ (Abs control) x 100 where Abs control is the absorbance of DPPH radical + methanol or ethanol; Abs sample is the absorbance of DPPH radical + sample extract /standard.

2.5 Determination of ferric reducing antioxidant power (FRAP)

Reducing power of both extracts of the lichen specimens were measured by method of Oyaizu [28] with slight modifications. According to this method the reduction of Fe^{3+} to Fe^{2+} was determined by measuring absorbance of the Perl's Prussian blue complex. This method is based on the reduction of (Fe^{3+}) ferricyanide in stoichiometric excess relative to the antioxidants. For this purpose, different concentrations (25,50,100 and 200 µg/mL) of lichen extracts in ethanol and methanol, and standard antioxidants (BHT) was added to the each tube, volumes were adjusted with distilled water to 0.75 mL, separately. Then, they were mixed with 1 ml of 200 mM sodium phosphate buffer (pH 6.6) and 1 ml (1%) of potassium ferricyanide [$K_3Fe(CN)_6$]. After 20 min of incubation at 50°C, the reaction mixtures were acidified with 1 ml of trichloroacetic acid (10%). Finally, 0.25 ml of $FeCl_3$ (0.1%) was added to this solution. Distilled water was used as control. Absorbance of these mixtures was measured at 700 nm using spectrophotometer [29]. Decreased absorbance indicates ferric reducing power capability of sample [30].

2.6 Determination of total phenolics

Using modified Folin-Ciocalteu method [24], total phenolic contents of the extracts were determined. An aliquot of the extract was mixed with 5 ml Folin-Ciocalteu reagent (previously diluted with water at 1:10 v/v) and 4 ml (75 g/l) of sodium carbonate. The tubes were vortexed for 15 sec and allowed to stand for 30 min at 40°C for color development. Absorbance was then measured at 765nm in a spectrophotometer (UV-1700 PharmaSpec UV-VS Spectrophotometer, Shimadzu, Japan). Samples of extracts were evaluated at a final concentration of 0.1 mg/ml. Total phenolic content were expressed as mg/g tannic acid equivalent using the equation based on the calibration curve: y = .007x-0.186, R2= 0.938, where x was the absorbance and y was the tannic acid equivalent (mg/g).

2.7 Determination of total flavonoids

Estimation of the total flavonoids in the plant extracts was carried out using the method of Ordon Ez et al [25]. To 0.5 ml of sample, 0.5 ml of 2% $AlCl_3$ ethanol solution was added. After one hour at room temperature, the absorbance was measured at 420 nm. A yellow colour indicated the presence of flavonoid. Extract samples were evaluated at a final concentration of 0.1 mg/ml. Total flavonoid content was calculated as quercetin (mg/g) using the following equation based on the calibration curve: y = 0.356x-0.461, R2 = 0.697, where x was the absorbance and was the quercetin equivalent (mg/g).

2.8 Determination of total antioxidant capacity

The total antioxidant capacity of lichen extracts, and its different fractions was evaluated by the method of Prieto et al. [27]. An aliquot of 0.1 ml of sample (100 µg) solution was combined with 1 ml of reagent (0.6 M

sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95° C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank in a spectrophotometer (UV-1700 PharmaSpec UV-VS spectrophotometer, Shimadzu, Japan). A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. For samples of unknown composition, antioxidant capacity was expressed as equivalents of α-tocopherol (mg/g).

2.9 Statistical analysis

All analysis was done in triplicates. Data were analyzed in Microsoft EXCEL-2007 by taking triplicates and thus mean and Standard Deviation (SD) obtained.

III. RESULTS AND DISCUSSIONS

3.1 DPPH Radical Scavenging Activity

Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS [26, 29]. The synthetic antioxidants like BHA, BHT, gallic acid esters etc., have been suspected to cause or prompt negative health effects. Strong restrictions have been placed on their application [30, 31]. In recent years much attention has been devoted to natural antioxidant and their association with health benefits [32]. The antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [33]. Numerous antioxidant methods and modifications have been proposed to evaluate antioxidant activity and to explain how antioxidants function. Of these, total antioxidant activity, reducing power, DPPH assay, metal chelating, ROS quenching assays are commonly used for evaluation of antioxidant activities of extracts [34, 35].

Table 1. DPPH radical scavenging of the ethanol and methanol extracts of the lichen *Parmotrema reticulatum*, *Usnea* sp and ascorbic acid.

Ascorbic acid	Concentration (µg/ml)	OD517 nm	Inhibition %
	control	0.750±0.0057	-
	25	0.638±0.0057	16
	50	0.562±0.0057	26
	100	0.481±0.0057	36.7
	200	0.231±0.0057	69.6
***Usnea* sp (M)**	control	0.76±.0.057	-
	25	0.63±0.0057	17
	50	0.61±0.0057	19.4
	100	0.57±0.0057	24.4
	200	0.52±0.0057	31.5
***Usnea* sp (E)**	control	0.82±0.0057	-
	25	0.71±0.001	12.8
	50	0.62±0.0057	24.3
	100	0.51±0.00057	37.1
	200	0.41±0.0057	49.7
***Parmotrema reticulatum* (E)**	control	.43±.00057	
	25	0.363±0.00057	16.5
	50	0.359±0.00015	17
	100	0.356±0.002	18.1
	200	0.351±0.002	19.3
***Parmotrema reticulatum* (M)**	control	0.796±.0005	
	25	.716±.0005	10.1
	50	.667±.0005	15.5
	100	.619±0.321	21.6
	200	.611±.0011	22.6

Data represented as mean±SD of three independent readings

E- Ethanolic extract; M- Methanolic extract

The change in absorbance produced by reduced DPPH was used to evaluate the ability of test compounds to act as free radical scavengers. It was observed (Table:1) that methanolic extracts of *Parmotrema reticulatum* and *Usnea* sp showed potent radical scavenging activity with IC50 value of 4.39 µg/ml and 3.21 µg/ml respectively, the IC50 value of ethanolic extracts of Usnea sp and Parmotrema reticulatum are 4.023µg/ml and 2.95µg/ml respectively. Removal of free radical increased by 10.1 to 49.7 % in accordance with the increase of the concentrations of the extract from 25µg/ml to 200µg/ml, compared to the negative control and moreover, the scavenging ability of the extract was as moderately less as that of ascorbic acid at all the concentrations tested. Significant correlation was found between the free radical scavenging activity and the concentration of lichen extract or the compound used as positive control. The DPPH assay is a widely used method to evaluate antioxidant activities in a relatively short time compared to other methods [36]. The reduction capability on the DPPH radical is determined by the decrease in its absorbance at 517 nm. The decrease in absorbance of DPPH radical caused by antioxidants is due to the reaction between antioxidant molecules and radical, which results in the scavenging of the radical by hydrogen donation. This is visualized as a discoloration from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate antioxidant activity [37, 38]. In this study, a significant decrease in the concentration of DPPH radical due to the scavenging ability of the extract was observed. Though the DPPH radical scavenging abilities of the lichen extracts was less than that of standard, the study showed that the extracts has proton-donating ability and could serve as a natural antioxidant.

3.2 Reducing power assays

Reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Antioxidant compounds are able to donate electrons to reactive radicals, reducing them into more stable and unreactive species [18, 19].The reducing power of the extracts was compared with standard Butylatedhydroxytoluene (BHT) and it increased with increasing concentration of the extracts. The methanolic extracts of *Parmotrema reticulatum* showed high reducing ability with absorbance of 0.491 at concentration of 300µg/ml and absorbance of methanolic extracts of *Usnea* sp was highest as 0 .514 at 300µg/ml. Antioxidant compounds cause the reduction of ferric (Fe^{3+}) form to the ferrous (Fe^{2+}) form because of their reductive capabilities. Prussian blue coloured complex formed by adding $FeCl_3$ to the ferrous (Fe^{2+}) form. Therefore, reduction can be determined by measuring the formation of Perl's Prussian blue at 700 nm [20, 30]. In this assay, yellow colour of the test solution changes to green or blue colour depending on the reducing power of antioxidant samples. A higher absorbance indicates a higher ferric reducing power.

Table 2: Reducing power of extracts of lichen *Parmotrema reticulatum*, *Usnea* sp and BHT.

	Concentration in µg/ml	O.D 700nm
BHT	50	.555±.002
	100	.751±.0005
	200	.815±.001
	300	.893±.004
***Usnea* sp (E)**	50	.413±.001
	100	.421±.001
	200	.425±.002
	300	.434±.002
***Usnea* sp (M)**	50	.402±.001
	100	.411±.001
	200	.502±.003
	300	.514±.003
***Parmotrema reticulatum* (E)**	50	.195±.003
	100	.205±.003
	200	.342±.003
	300	.376±.002
***Parmotrema reticulatum*(M)**	50	.363±.001
	100	.394±.002
	200	.406±.001
	300	.491±.002

Data represented as mean±SD of three independent readings

E- Ethanolic extract; M- Methanolic extract

3.3 Total phenolic and flavonoids contents

Table 3: Total phenolic and flavonoid content of lichen Parmotrema reticulatum and Usnea sp

Lichen extracts	Total phenolic content (µg tannic acid equivalent/mg)	Total flavonoid content (µg quercetin equivalent/mg)
***Parmotrema reticulatum*(E)**	**113 ±1**	**1.42±0.01**
***Parmotrema reticulatum*(M)**	**151 ±0.577**	**1.38±0.0057**
***Usnea* sp(E)**	**110 ±0.577**	**1.498±0.001**
***Usnea* sp(M)**	**148 ±0.577**	**1.543±0.0057**

Data represented as mean±SD of three independent readings

E- Ethanolic extract; M- Methanolic extract

Results obtained in the present study revealed that the level of these phenolic compounds in the ethanol extracts of the *Parmotrema reticulatum* and *Usnea* sp were considerable. The phenolic content of methanolic extracts of *Parmotrema reticulatum* and *Usnea* sp are 151µg and 148µg tannic acid equivalent. Polyphenolic compounds are known to have antioxidant activity and it is likely that the activity of the extracts is due to these compounds [31, 32]. This activity is believed to be mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [33]. In fact, many medicinal plants contain large amounts of antioxidants such as polyphenols. Many of these phytochemicals possess significant antioxidant capacities that are associated with lower occurrence rates of several human diseases [34, 35]. Phenols are very important constituents because of their scavenging ability due to the presence of hydroxyl groups [39].

Flavonoids are wide group of natural compounds and also the most important natural phenolics. These compounds have a large number of biological and chemical activities including radical scavenging properties [40]. The amount of flavonoids in the lichen extracts were very less such as 1.54µg and 1.49µg for ethanolic and methanolic extracts of *Usnea* sp respectively

3.4 Total antioxidant activity

The phosphomolybdenum method has been used to investigate the total antioxidant capacity of the extracts. This method is quantitative, since the total antioxidant capacity is expressed as α- tocopherol equivalents. The ethanolic and methanolic extracts of *Parmotrema reticulatum* contained 0.781 and 1.58 µg vitamin E equivalent /mg and that of *Usnea* sp was 2.025µg and 0.690µg vitamin E equivalent /mg respectively. In fact, many medicinal plants contain large amounts of antioxidants such as polyphenols. Many of these phytochemicals possess significant antioxidant capacities that are associated with lower occurrence rates of several human diseases [34,35].

Table 4. Total antioxidant activity of lichen *Parmotrema reticulatum* and *Usnea* sp.

Lichen extract	Total antioxidant activity(µg vitamin E equivalent /mg)
***Parmotrema reticulatum* (E)**	**0.781±.00057**
***Parmotrema reticulatum* (M)**	**1.58 ±.00577**
***Usnea* sp(E)**	**2.025 ±.001**
***Usnea* sp(M)**	**0 .690 ±.001**

Data represented as mean±SD of three independent readings

E- Ethanolic extract; M- Methanolic extract

IV. CONCLUSION

The investigation for bioactive compounds from natural resources to improve pharmaceutical, cosmetic and agriculture applications is extremely advancing till today. The lichen compounds are also being investigated for its phytochemical properties. Peroxidation (auto-oxidation) of lipids exposed to oxygen is responsible not only for deterioration of foods (rancidity) but also damage to tissue in vivo, where it may be a cause of cancer, inflammatory disease, ageing etc. Thus there is an increase in demand for drugs of natural origin for healing several diseases. On the basis of the results, it can be concluded that tested lichen extracts show a strong antioxidant activity in vitro. The intensity of antioxidant activity depended on the tested lichen species and the solvent that used for extraction. Different antioxidant activities of different solvents depend on their different capabilities to extract bioactive substances [41]. Ethanolic and methanolic extracts of *Parmotrema reticulatum* and *Usnea* sp possessed potent antioxidant activity and DPPH radical scavenging activity. Presence of an appreciable amount of phenol and flavonoid content could suggest for the use of these extracts as natural source of antioxidants. Further, the bioactive substances from these samples are under investigation.

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