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Smitha KC
Department of Studies in Botany, University of Mysore, Manasagangotri, Mysore – 570 006, Karnataka State, India.

***Rajkumar H Garampalli**
Department of Studies in Botany, University of Mysore, Manasagangotri, Mysore – 570 006, Karnataka State, India.

Correspondence
Rajkumar H Garampalli
Department of Studies in Botany, University of Mysore, Manasagangotri, Mysore – 570 006, Karnataka State, India.

Evaluation of phytochemicals and *in vitro* antioxidant activity of *Ramalina pacifica* and *Roccella montagnei*

Smitha KC, Rajkumar H Garampalli*

Abstract
The experiment conducted to record the phytochemicals present and antioxidant activities of two medical medicinal lichens *Ramalina pacifica* and *Roccella montagnei*. The extracts were obtained in different solvents of chloroform, ethyl acetate, ethanol and methanol. Phytochemical analysis revealed the presence of secondary metabolites namely, alkaloids, carbohydrates, saponins, phytosterols, steroids, phenols, tannins, flavanoids, and proteins. Phytosterols were present in high concentration in all the extracts compare to other secondary metabolites. The antioxidant activity was determined by DPPH radical scavenging activity, reducing power assay (FRAP) and the total phenolic content of all extracts. The ethanol extract of *Ramalina pacifica* was found to be more effective in free radical scavenging activity which showed 76.72±0.19 and the reducing power which showed 72.38±0.13. *Roccella montagnei* did not show any significant activity when compare to the *Ramalina pacifica*. The *in vitro* studies clearly showed that the ethanol extract of the *Ramalina pacifica* may be a good source of natural antioxidants.

Keywords: Lichen, Antioxidant, DPPH, Reducing power, *Ramalina pacifica*.

Introduction
Oxidation is an essential metabolic process of living organisms for the production of energy needed for biological processes. The chemical reaction involves the transfer of electrons or hydrogens from a substance to an oxidizing agents like nicotinamide adenine dinucleotide (NAD+), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) [1, 2]. Excessive amount of accumulation of reactive oxygen species in the body causes oxidative stress which in turn enhance the risk of several diseases in humans like, cancer, diabetes, cardiovascular diseases, neurodegenerative diseases, pulmonary diseases, aging, atherosclerosis, chronic inflammation [3-6].

Antioxidants are the substance that can be effective by preventing the formation of free radicals by scavenging reactive oxygen species. These antioxidants can be categorized into synthetic and natural. Synthetic antioxidants are widely used as protectants for oxidative stress in biological system, but continuous and uncontrolled use of these can led to toxicity, carcinogenic side effects and sometimes resistance. Thus, restriction is being imposed on the use of synthetic antioxidants [7-9]. The increased concern regarding safety issues of using synthetic antioxidants, the trend of research is being increased greatly towards finding antioxidants from natural sources without undesirable side effects [10-15]. Primarily, natural antioxidants are from whole grains, fruits and vegetables. Antioxidants such as carotenes, phenolic acids, phytate, vitamin C, vitamin E and phytoestrogens from plant food source have the potential to reduce disease risk [2]. *In vitro* and epidemiological studies of natural sources like medicinal plants and vegetables proved to be effective against oxidative stress in biological [16-18]. These studies have strongly supported the idea of developing antioxidants from natural sources.

Lichens are the symbiotic association of two living organisms one being, fungal partner (Mycobiont) and other algal/cyanobacterial (Photobiont) partner and they can survive in extreme environments [19-21]. Lichens produces various number of secondary metabolites which are known as 'lichen substance' and most of these are from the fungal partner [22, 23], exerts a wide variety of biological actions namely, antibiotic, antimycotic, antiviral, antiinflammatory, analgesic and antipyretic, antiproliferative and cytotoxic effects [24-26]. Aromatic polyketides are the most common compounds of lichen particularly, depsides, depsidones, diphenyl ethers and dibenzofurans, makes the lichens as rich source of phenolic compounds [27]. There were many researchers who paid attention to find antioxidant and antimicrobial activities of lichens

like 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 [28-32]. The antioxidant properties of lichens are poorly known and there have also been limited reports on the relationship between the antioxidant activity of lichen samples and their pure phenolic compounds. Therefore, the aim of this study was to investigate total phenolic content and to test the antioxidant activity of two lichen species *Ramalina pacifica* and *Roccella montagnei*.

Materials and Methods

Collection and identification of lichen

Lichens specimens were collected from the Chamundi hill, Mysuru district, Karnataka falls between 11°36'N latitude and 76°55'N E longitude, is a small mountain with scrubby forest with an altitude of 1000 m from the base of the hill. The relative humidity varies from 19 to 75% with temperature ranges from 17 to 35°C [33]. The samples were collected with the help of chisel and hammer along with ecological notes for identification purpose, from different locations in different habitat types. The lichens were identified based on the morphology and anatomy of thallus structure and their mode of reproduction. External morphology was studied under stereo-binocular microscope. The anatomy of the thallus and apothecia was studied under compound microscope and also identified by the color spot test. Color reactions were performed on cortex and medulla. The identifications were confirmed with the help of experts at National Botanical Research Institute (NBRI), Lucknow, India.

Extraction of lichen samples

Lichens samples were cleaned, shade dried and ground into fine powder by mortor and pestle. The lichen powder samples were subjected to successive solvent extractions in soxhlet apparatus. 10 g of lichen powder was run in series of 100 ml solvents viz. chloroform, ethyl acetate, ethanol and methanol. Extraction power of solvents varied from 2-48 hrs. The solvent extracts were collected, dried and stored at 4 °C for further experimental use.

Qualitative phytochemical analysis of lichens

Phytochemical tests were carried out to detect the presence of metabolites using standard procedure [34].

Total Phenolic content of lichen extracts

Total phenolic content of lichen solvent extracts were determined by the method of Singleton *et al*. [35]. 50 µl of extract (5 mg/ ml) was mixed with 0.75 ml of 20% sodium carbonate solution and 0.25 ml of Folin Ciocalteu reagent. The reaction mixture was allowed to stand in light for 3 min and incubated for 2 h in dark. The absorbance was measured at 765 nm using UV-Visible Spectrophotometer. Total phenolics were quantified by calibration curve obtain from measuring the absorbance of known concentrations of gallic acid standard (0-100 µg/ ml). Total phenolic content expressed as gallic acid equivalent to mg of gallic acid/g dry extract used.

Determination of Free Radical Scavenging Activity

Estimation of antioxidant activity

Antioxidant activity of was determined using 2,2-Diphenyl-1-picryl hydrazyl radical (DPPH) [36, 37]. Briefly, 100µg of Lichen solvent extracts were mixed with 5 ml of 0.1mM methanolic solution of DPPH and incubated at 20°C for 20 minutes in complete dark. The DPPH alone served as control and methanol was used for the base line correction. The absorbance of the samples was measured at 517 nm and radical scavenging activity was expressed as percentage activity using the following formula.

$$\% \text{ Inhibition} = [(A\text{DPPH} - A\text{Extr})/A\text{DPPH}] \times 100$$

Reducing Power Assay

The reducing power ability of lichen extracts were determined by the FRAP method [38]. The reaction mixture contains, extract (100µg/ml) with equal volume of 0.2 M phosphate buffer pH 6.6 and potassium ferric cyanide was incubated at 50 °C for 20 min. centrifuged at 3000 rpm for 10min by adding equal volume of 10% TCA to the mixture. To the supernatant, distilled water and 0.1% ferric chloride was added in the ratio of 1:2 (v/v). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture compare to blank indicates increased reducing power activity. BHA is used as standard.

Results and Discussion

Phytochemical analysis of lichens

In the present study, the presence of phytochemicals namely, alkaloids, phenols, tannins and flavanoids were recorded in the chloroform extract of the both *Ramalina pacifica* and *Roccella montagnei* lichen species. phenols are present in all extracts of the both species when tested with FC reagent test, but phenols absent in *Ramalina pacifica* and chloroform and methanol extract of *Roccella montagnei* when tested with Ferric chloride test. Presence of phytosterols was also recorded in all extract except in ethyl acetate extract of *Ramalina pacifica.* Proteins were not recorded in any of the extract tested from both the lichens studied. Carbohydrates were found only in chloroform extract of the *Ramalina pacific* (Table-1).

Table 1: Phyto-chemical analysis of lichen extracts

	Ramalina pacifica				***Roccella montagnei***			
	Chl	**E.A**	**Et**	**Me**	**Chl**	**E.A**	**Et**	**Me**
Detection of Alkaloid								
Mayer test	+	-	-	-	+	+	-	-
Draggendorffs test	+	-	-	-	+	-	-	-
Detection of Carbohydrates								
Benedicts test	-	-	-	-	-	-	-	-
Fehlings test	+	-	-	-	-	-	-	-

Detection of Saponins								
Foam test	-	-	+	+	-	-	-	+
Detection of Phytosterols								
Salkowski	+	-	-	-	+	-	+	-
Liebermann burchard	+	-	+	+	+	+	+	+
Detection of Phenols								
Ferric chloride	-	-	-	-	-	+	+	-
FC reagent test	+	+	+	+	+	+	+	+
Detection of Tannins								
Gelatin test	+	+	-	-	+	+	-	-
Detection of Flavanoids								
Lead acetate	+	-	-	-	+	-	-	+
Detection of Protein								
Ninhydrin test	-	-	-	-	-	-	-	-

'+' - presence of metabolites; ' ' - absence of metabolites

Total phenolic content

Total phenolic content of *Ramalina pacifica* and *Roccella montagnei* was determined by using the Folin-ciocalteu reagent and the determined as the gallic acid equivalent using an equation obtained from gallic acid curve (Y=0.115x-0.057, R^2=0.996) (Fig-1). The highest percentage total phenolic constituent was recorded in ethyl acetate and ethanol extract of *Roccella montagnei*, the quantity of which was 77.80 mg/ml and in chloroform extract of *Ramalina pacifica*, it was 67.33mg/ml. The chloroform extract of the *Roccella montagnei* gave less total phenolic content if 8.66mg/ml (Table-2).

Table 2: Total phenolic content measured

lichens / Extracts	*Ramalina pacifica**#*	*Roccella montagnei**#*
Chloroform	67.33± 4.0	8.66± 0.32
Ethyl acetate	27.03±1.21	77.80±4.77
Ethanol	18.70±0.55	77.80±4.77
Methanol	24.63±0.9	24.73±1.27

* Total phenolic content expressed as gallic acid equivalent to mg gallic acid/g dry extract

The values are presented as mean ± SE

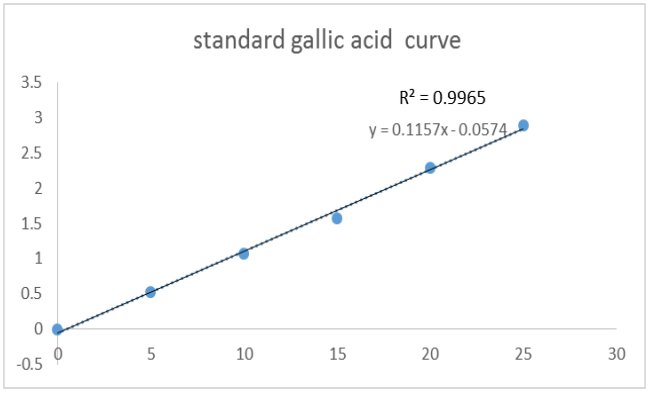

Fig 1: Standard gallic acid curve for total phenolic content

Antioxidant activity

The estimation of DPPH radical scavenging activity lichen extracts as in the Fig-2. The determination of DPPH radical scavenging activity of chloroform, Ethyl acetate, Ethanol and methanol extract of *Ramalina pacifica* showed relatively good scavenging activity and there observed a statistically significant difference when compare to the chloroform and ethyl acetate extracts. In *Roccella montagnei*, though some DPPH radical scavenging activity was recorded but there was no any significant difference when compare to the ascorbic acid standard (Fig-2).

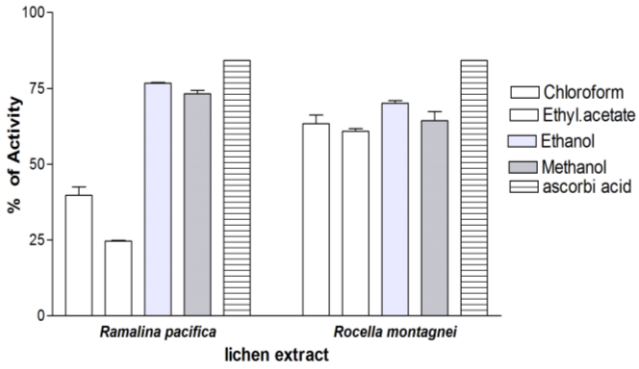

Fig 2: Antioxidant activity of the lichen extracts (100µg/ml)

Reducing power

The high absorbance of reducing power is obtained from the ethanol and methanol extracts of the *Ramalina pacifica* which was less when compared to *Roccella montagnei* (Fig-3). Amongst the four solvent namely, chloroform, ethyl acetate, ethanol, methanol extracts of *Roccella montagnei*, methanol and ethyl acetate extract showed more reducing power when compared to the chloroform and ethanol extract. In the tested lichen extract, the lowest absorbance of reducing power was recorded in ethyl acetate and chloroform extract of *Ramalina pacifica* (Fig-3).

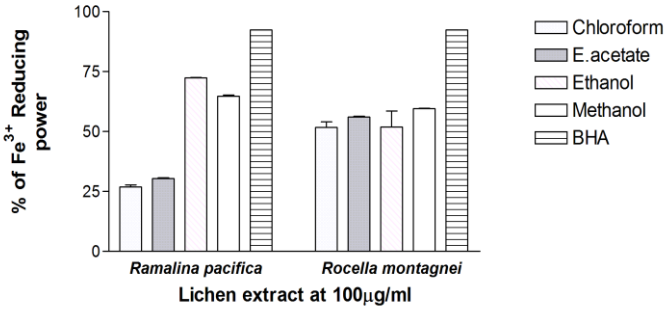

Fig 3: Reducing power of the lichen extracts

The experiment was conducted to study the phytochemical and antioxidant activity of two medicinal lichens *Ramalina pacifica* and *Roccella montagnei* which were collected from the Chamundi hills, Mysore. The analysis of the lichens extracts showed presence of phytochemicals like alkaloids, carbohydrates, saponins, phytosterols, phenols, tannins, flavanoids. Many of the research studies showed that lichens produce more than thousands of the secondary metabolites [39]. Among them most of the secondary metabolites of lichens are kwon to play an important role in biological activities such as antioxidant, antimicrobial, anti-insecticidal, anti-herbivore, anti-tumour, anti-allergic [40]. In the present study also *Ramalina pacifica* and *Roccella montagnei* good antioxidant activity and free radical scavenging activities. Many of the reports suggest that, the total phenolic content of the lichen can be directly correlated with the antioxidant activity [41-46] which corroborate our results. The results of the ferrous reducing power also proved the DPPH radical scavenging activity of the lichens extracts tested in the present study. Hence, *Ramalina pacifica* and *Roccella montagnei* may be used as a potential antioxidant source after thorough clinical investigation.

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