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Assessment of Antioxidant Potential and Related Structural Diversity of Polyphenols in Indian Foliose Lichens

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ABSTRACT

Lichens have been used since ancient times to cure various ailments. To study the variation in phytochemical composition and antioxidant activity of n-hexane, acetone and methanol extracts in seven lichen species. Phytochemical study includes total phenolic and flavonoid content and quantitative study by HPLC technique. Furthermore the *in vitro* antioxidant activities were determined by DPPH and ABTS methods in seven lichen species. The findings revealed the significant ($p < 0.05$) variations in phytochemical profiles and antioxidant activity for all the analyzed samples. The *Flavoparmelia caperata* extracts showed highest antioxidant activity followed by *Sticta nylanderiana*. The amount of total phenolic and flavonoid contents were highest in methanol extracts of *Everniastrum nepalense* (95.9±2.62 mg GAE/gm dw) and *F. caperata* (78.04 ± 2.31 mg QAE/gm dw) respectively. The phenolic acids and flavonoids contents of lichen samples were quantified by RP- HPLC and compared with standard markers, which varied between 0.03- 970.01 and 0.16- 1316.54 µg/gm dry weight, respectively. Gallic acid was present in all the tested extracts of which, highest concentration was found in acetone (970.01± 2.48 µg/gm dw) and methanol (846.86±1.86 µg/gm dw) extracts of *F. caperata*. Maximum concentration of rutin (1316.54 ± 3.23 µg/gm) was present in acetone extract of *Lobaria retigera.* The scavenging capacity of methanol fraction of lichens were also compared with individual scavenging capacity of the phenolic compounds. The structure–activity relationship was elucidated and discussed within each class of phenolic substances. The variation in radical scavenging activity of the tested phenolic compounds were attributed to structural differences mainly on number and position of hydroxyl groups.

INTRODUCTION

Lichens are the symbiotic association of the mycobiont (fungal partner) and photobiont (algal partner), produce a varied range of secondary metabolites which have attracted much attention in investigations because of their potent biological activities. Throughout the ages, lichens have b een used for various purposes, in particular as dyes, perfumes and remedies in folk medicines (Upreti *et al*., 2005; Singh *et al*., 2015). Use of lichens as traditional medicine has a long history which includes broad spectrum of chemical and biological activities in cluding protective effects against oxidative stress owing to their radical scavenging properties (Tobwala *et al*., 2014). Pharmacological properties of lichens have attracted much attention because of their potent bioactivities such as antioxidant, antimicrobial, antiprotozoal, insecticidal, antiinflammatory, antipyrectic, analgesic, antiproliferative, cytotoxic, neuroactive, enzyme inhibitory and immunomodulator activities (White *et al*., 2014; Thadhani *et al*., 2015; Reddy *et al*., 2016; Aadesariya *et al*., 2017)

Antioxidants, either synthetic or natural, can be effective to help in reducing oxidative damage. Oxidative stress is initiated by reactive oxygen species (ROS), such as superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH) and singlet oxygen ($_1O^2$). ROS are required for cellular activities at normal physiological concentrations as they perform a positive role in energy production, phagocytosis, regulation of cell growth intercellular signaling, and synthesis of biologically important compounds (Gulcin *et al*., 2004b). However, at elevated concentrations, ROS can trigger oxidative damage to proteins, lipids, enzymes, and DNA molecules (Md Obaidul Islam *et al*., 2019). The most effective way to eliminate ROS which cause the oxidative stress is with the help of antioxidants (Gaschler and Stockwell, 2017; Gholamian-Dehkordi *et al*., 2017).

Plant phenolics are important group of low molecular mass secondary metabolites, which are synthesized by plants as a result of plant adaptation to biotic and abiotic stress conditions (Kasote *et al*., 2015).The physiological

and pharmacological functions of plants may originate from antioxidant properties of lichen phenolic compounds (Dandapat and Paul, 2019).

Many plant phenolics exhibited antioxidative activity *in vitro* and *in vivo*. The intensity of antiradical activity of phenols depends on many factors such as number of hydroxyl groups bound to the aromatic ring, number and places of double bonds in the molecule (Gu *et al*., 2019). Also, there have been a few studies on the structure–antioxidant activity relationships of certain natural coumarins, lignans, tannins, and quinines and some synthesized curcuminoids and stilbenes are available (Cai *et al*., 2004). Though little has been discussed on the structure -activity relationship in lichens.

Lichens through its metabolic pathway also produce a large number of phenolic compounds. The complex phenolic antioxidants in the traditional lichen plants may play a significant role in the prevention and treatment of many diseases. The present study is aimed (i) to determine the contents of total phenolic and flavonoids of three organic extracts; (ii) profiling of eight phenolic compounds; and (iii) to estimate the *in vitro* antioxidant capacities and (iv) to investigate and elucidate the structure–radical scavenging activity relationships of representative natural phenolic compounds identified in the traditional seven medicinally potent lichens. The investigation of antioxidant activity of natural phenolic compounds and their structures– activity relationships will contribute towards scientific validation of the lichen species, well known for their traditional/folklore or other ethnic uses.

MATERIAL AND METHODS

Chemicals and reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), aluminium chloride ($AlCl_3$), potassium persulfate ($K_2S_2O_8$), ascorbic acid, gallic acid, protocatechuic, chlorogenic, caffeic, ferulic, rutin, quercetin, Kaempferol, electronic-grade methanol and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin Ciocalteu's phenol reagent, silica gel precoated aluminium TLC plates (20×20 cm), sulphuric acid, methanol, n-hexane, ethanol, toluene, acetone, dioxane, acetic acid, diethyl ether, formic acid, Sodium hydroxide (NaOH), Sodium nitrite ($NaNO_2$) and sodium carbonate (Na_2CO_3), were procured from Merck Chemical Supplies (Merck KGaA, Darmstadt, Germany). All the chemicals and solvents were of analytical grade.

Sample Collection

Lichen samples *viz. Heterodermia diademata* (Taylor) D.D. Awasthi, *Everniastrum nepalense* (Taylor) Hale ex Sipman, *Parmotrema nilgherrense* (Nyl.) Hale, *Flavoparmelia caperata* (L.) Hale, *Nephromopsis laii* (A. Thell & Randlane) Saag & A. Thell, *Lobaria retigera* (Bory) Trevis and *Sticta nylanderiana* Zahlbr. were collected from different localities of Govind Wild Life Sancturay (GWLS), Uttarkashi district, Uttarakhand. The voucher specimens are preserved in Lichen herbarium (LWG), CSIR- National Botanical Research Institute (NBRI), Lucknow, India.

Determination of morpho-anatomical and colorimetric characterization of lichen species

The morphological characters were studied by Leica S8APO stereo-zoom microscope while the anatomical structures of lichen samples were analyzed with the help of Leica DM500 compound microscope. The chemical analysis is carried out by colour test, UV and standardized thin-layer chromatography (TLC). The colour tests were achieved by using frequently used reagents; aqueous solution of KOH (K), calcium hypochlorite (C) and *para*-phenylene-diamine (Pd). Solvent system A (toluene: dioxane: acetic acid glacial; 180: 60: 8) as a mobile phase while silica gel as stationary phase have been used for thin layer chromatography, the technique provided by Walker and James, 1980.

Sample extraction and preparation

Samples were sorted, cleaned of substratum and dried for extraction. The air dried sample (was then powdered and extracted by using three different solvents (viz. n-hexane, acetone and methanol) in a soxhlet extractor. The extract was filtered and then concentrated under reduced pressure in a rotary evaporator at 35-40°C. The dry extract was stored at -20°C until it was used for further phytochemical and antioxidant screening. For further analysis lichen dry extract was dissolved at a concentration of 1 mg/ml in methanol.

ESTIMATION OF ANTIOXIDANT CAPACITIES

DPPH radical scavenging assay

Quantitative measurement of radical scavenging properties of lichen extracts were carried out according to the procedure described by Shimada *et al*. with some modifications (Shimada *et al*., 1992). Briefly, a 0.1 mM solution of 2, 2- diphenyl-1-picryl-hydrazyl (DPPH) in methanol was prepared and 1 ml of this solution was added to 3 ml of

Table 1: Description of Lichen species selected for the study.

S.N.	*Lichen Species*	*Family*	*Habitat*	*Thallus*	*Colour test*	*TLC*
1	*H. diademata*	Physciaceae	Corticolous	Thallus corticolous, terricolous and saxicolous, foliose; lobes linear, to 2.5 mm wide, rarely secondry lobules in central part, corticated on both sides; upper side grey to grey- white, lacking isidia and soredia; lower side pale brown with concolorous, sparse rhizines.	Medulla K+ yellow, Pd+ pale yellow or Pd-, C-.	Zeorin present.
2	*Everniastrum nepalense*	Parmeliaceae	Corticolous	Thallus corticolous, foliose, suberect to pendulous; lobes to 4 mm wide; upper side grey to dark grey, rarely brownish, lacking soredia and isidia; lower side black- brown with simple or branched rhizines.	Medulla K+ yellow turning red, Pd+ orange- red, C-.	Salazinic and protolichesterinic acids
3	*Parmotrema nilgherrrense*	Parmeliaceae	Corticolous	Thallus corticolous, foliose, coriaceous; lobes 10- 20 (-30) mm wide, convolute, ciliate; upper side pale grey or darker, densely white- maculate, lacking isidia and soredia; lower side centrally black, wide marginal zone brown, nude; medulla white.	Medulla K-, Pd-, C-, KC+ pink or red.	Alectoronic and a-collatolic acids
4	*Flavoparmelia caperata*	Parmeliaceae	Corticolous	Thallus corticolous, occasionally saxicolous, foliose, closely adnate; lobes to 10 mm wide; upper side plicate, pustules or ridges developing into descrete or confluent soralia; soredia; soredia; soredia granular; lower side black, narrow marginal zone brownish and shiny ; rhizines short, simple; medulla white.	Upper cortex K-; medulla K-, Pd+ orange- red C-, KC-.	Usnic acid, caperatic and protocetraric acids in medulla.
5	*Nephromopsis laii*	Parmeliaceae	Corticolous	Thallus corticolous, foliose; upper side greenish yellow; lower brownish, pseudocyphellate on lamellae; rhizines short.	Medulla K-, C-, KC-, PD-.	Usnic, lichesterinic, protolichesterinic and caperatic acids
6	*Lobaria retigera*	Lobariaceae	Corticolous	Thallus corticolous, terricolous or saxicolous, foliose, loosely adnate; lobes 10- 30 mm wide, upper side pale brown to darker at margins, scorbiculate, reticulately ridged; isidia usually on ridges, granular, cylindrical, simple to coralloid, rarely minutely lobulate; lower side dark brown to black, tomentose sparsely rhizinate in grooves; convexities, nude, pale brown; photobiont a Nostoc.	Upper cortex K-; medulla K-, Pd-, C-.	Triterpenoids and thelephoric acid
7	*Sticta nylanderiana*	Lobariaceae	Corticolous	Thallus corticolous, fruticose, horizontal; upper side pale grey to darker, lacking isidia and soredia; lower side pale brown to brown, later 0.4-2 mm in diameter.	Medula K-, C-, KC-, PD+ pink to reddish.	Atranorin and gyrophoric acid.

the crude extracts at different concentration (0.05-0.2 mg/ml). Ascorbic acid was used as positive control along with detected phenolic compounds. Discoloration was measured at 517 nm after incubation for 30 min in the dark. Measurements were taken in triplicate. Ascorbic acid was used as a control. The capacity to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = $[A_0 - A_S/A_0] \times 100$

Where, A_0 was absorption of control, A_S was absorption of tested extracts and standards. The half maximal inhibitory concentration (IC_{50}) for scavengers was calculated (Teke *et al*., 2011). The IC_{50} was determined by extrapolation from concentration regression lines obtained from four different concentrations (0.05 - 0.2 mg/ml).

Trolox-Equivalent antioxidant capacity (TEAC) assay

The total antioxidant values were estimated by the Trolox-Equivalent antioxidant capacity assay (Re *et al*., 1999). The method used was modified by Cai *et al.*, 2004. The TEAC value is based on the ability of the antioxidant to scavenge the blue green 2,2'-azinobis (3-ethylbenzothiazolin-6-sulfonate) ($ABTS^{\cdot+}$) radical cation relative to the $ABTS^{\cdot+}$ scavenging ability of the water soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid (Trolox). The dark colored solution containing stable ABTS radical monocation ($ABTS^{\cdot+}$) was generated by the incubation of equal volumes of 7mM ABTS with 2.5 mM potassium per sulfate in the dark at room temperature for 12-16 hr. The prepared ABTS solution was diluted immediately to 50% methanol (1:89 v/v) prior to an assay to an absorbance of 0.70±0.002 at 734 nm. The activity was assessed by mixing 50 µl of different fraction of standards or extract with 300 µl of ABTS solution. Trolox and detected 8 standard phenolic compounds were used as positive control. The reaction mixture was allowed to stand at 30° C for 6 min and the absorbance at 734 nm was immediately recorded. Measurements were taken in triplicate. Ascorbic acid was used as a control. The scavenging activity was estimated based on the percentage of ABTS radicals scavenged by the following formula

% Scavenging = [(Ao-As)/ Ao] ×100

Where Ao is the absorption of control and As is the absorption of extracts and standards. The IC_{50} values were also calculated as described in previous section. The results were expressed in terms of Trolox equivalent antioxidant capacity (TEAC).

ESTIMATION OF PHYTOCHEMICAL CONTENTS

Determination of total polyphenol content (TPC)

Total phenols was determined using the Folin–Ciocalteu reagent as described by Roby *et al.,*(2013) with slight modifications. Samples of 200 µL of extract at different concentrations, in triplicate, were mixed with 1 mL of 1N Folin–Ciocalteu reagent and allowed to stand for 5 min at room temperature. Then, 2 mL of 20 % sodium bicarbonate solution were added to the mixture and incubated for 90 min at room temperature. Absorbance was measured at 750 nm and total phenols were quantified using a standard curve. The standard curve was prepared with the absorbance readings of various concentrations of gallic acid solutions (5µl, 10µl, 25 µl, 50 µl and 100 µl in methanol). The equation $y = 0.010x + 0.036$ ($R^2 = 0.997$) was obtained and used to determine the total phenols in the extract in gallic acid equivalents (GAE), mg/g dry extract. All determinations were carried out in triplicate and the results were reported as the mean ± SD.

Determination of total flavonoid content (TFC)

Total flavonoid content of lichen crude extracts was determined using the procedure as explained by Chang *et al*. (2002) with slight modification. In brief, 50 µl of crude extracts (1 mg/ml in methanol) were then mixed with 4 ml of distilled water and subsequently with 0.3 ml of 5% $NaNO_2$ solution. After 5 min of incubation, 0.3 ml of 10% AlCl3 solution was added and then allowed to stand for 6 min, followed by adding 2 ml of 1 M NaOH solution to the mixture. Then water was added to the mixture to bring the final volume to 10 ml and the mixture was allowed to stand for 15 min. The absorbance was measured at 510 nm.

Total flavonoid content was calculated as quercetin from a calibration curve. The calibration curve was prepared by preparing quercetin solutions at concentrations 5µl, 10µl, 25 µl, 50 µl and 100 µl in methanol. The equation $y = 0.009x + 0.029$ ($R^2 = 0.997$) was obtained and used to determine the total flavonoid in the extract and expressed as mg quercetin (QU)/g of dry weight.

IDENTIFICATION OF LICHEN SUBSTANCES BY HPLC

Standardization of Lichen Extract

The analysis of resident polyphenols like gallic acid, protocatechuic, chlorogenic, caffeic, ferulic acid, rutin, quercetin, and kaempferol in the extract was carried out by using standard qualitative and quantitative methods as

described previously (Niranjan *et al*., 2009). All solvents used for extraction were distilled before use.

HPLC Standardization of Lichen Extract

For HPLC analysis, Stock solutions (20 µg/ml) were prepared in mobile phase for the sample (lichen crude extracts) and gallic acid, protocatechuic, chlorogenic, caffeic, and ferulic acids, rutin, quercetin, and kaempferol as standards. Samples were then filtered through 0.45 µm polytetrafluoroethylene (PTFE) filter (Millipore) to remove any particulate matter. Analysis was performed by utilizing a HPLC–UV with a Shimadzu LC-10A system from Japan comprising an LC-10AT dual-pump system, an SPD-10A UV detector (operated at 254 nm), and Rheodyne injection valve with 20-µL sample loop. Compounds were separated on a 4.6 mm × 250 mm, i.d., 5-µm pore size Phenomenex Luna RP-C18 column protected by a guard column containing the similar packing. The mobile phase was a gradient prepared from 1% (*v*/*v*) acetic acid in HPLC-grade water (component A) and acetonitrile (component B). The gradient was from 20 to 35% B in 0–14 min then from 35–50% B in 14-40 min. The flow rate was 0.6 mL min^{-1}. Data were integrated by Shimadzu class VP series software and results were obtained by comparison with standards. Data were integrated by Shimadzu class VP series software and results were obtained by comparison with standards.

The whole HPLC profiles of all identified phenolics were obtained within 80 min. The retention times of various phenolics identified in this study were approximately in the following ranges: 6.38– 17.38 min for phenolic acids; 13.82–34.71 min for flavonols. Fig. 1 displays typical HPLC chromatogram of parts of phenolic standards.

STATISTICAL ANALYSIS

Experimental results are demonstrated as mean ± standard deviation (SD). Statistical analysis was performed using SPSS software, version 21.0. One-way analysis of variance (ANOVA) and Duncan's multiple range tests were applied to determine the significance of the difference among tested samples, with a significance level of $P < 0.05$. Tests for the correlation (between the content of phenolic compounds and the antioxidant activities) was done by employing standard Pearson correlation. IC_{50} values were calculated and determined by linear regression analysis.

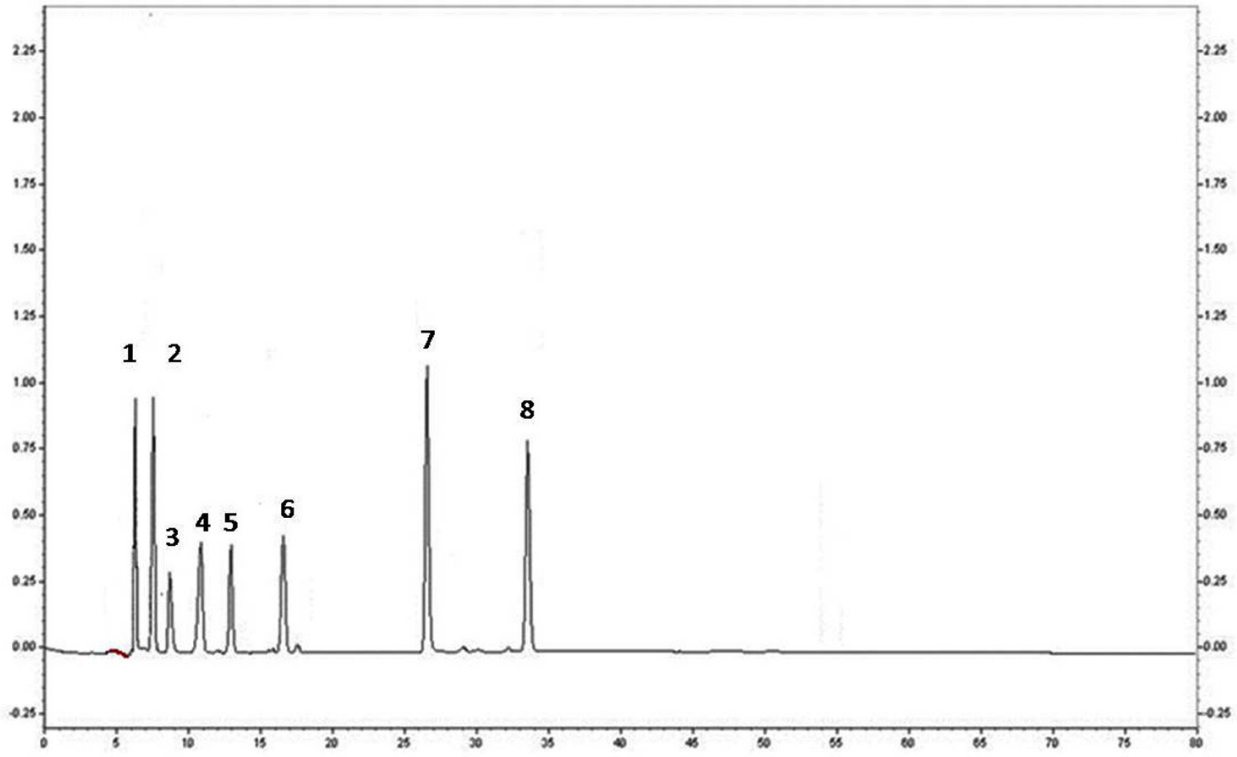

Fig 1: HPLC chromatogram of polyphenols (standard). (1- Gallic acid, 2- Protocatechuic acid, 3- Chlorogenic acid, 4- Caffeic acid, 5- Rutin, 6- Ferulic acid, 7- Quercetin, 8- Kaempeferol).

RESULT AND DISCUSSION

Lichens grow in excessive environmental surroundings which could result in up regulated pathways of secondary metabolites synthesis and enhance the production of polyphenolics (Kumar *et al*., 2014). The key function of phenolic compounds as scavengers of free radicals such as singlet oxygen, superoxide and hydroxyl radicals is highlighted in previous reports (Atalay *et al*., 2011; Kosanic´ *et al*., 2012). The present study is aimed to observe the antioxidant activities that are coupled with the phenolic content and the contribution of each phenolic compound to the activity has been evaluated.

Antioxidant activity

In this study, the antio xidant potential was validated using DPPH and ABTS activity. The pearson correlation (r = 0.95) shows strong positive correlation between both the assays of all examined extracts and phenolic compounds signifying that the assay results of ABTS+ and DPPH methods are consistent. The tested lichen extracts have an excellent antioxidant activity against both the oxidative systems *in vitro*. The variation in antioxidant activity depends on lichen species and preferential fractionation of the secondary metabolites in different solvent based on the polarity (Ngo *et al*., 2017). The half maximal inhibitory concentration (IC_{50}) values were calculated for both the activities. Lower value of IC_{50} indicates higher radical scavenging capacity (Matuszewska *et al*., 2018).The methanol and acetone extracts of all lichen species were found to contain significantly higher ($p<0.05$) activity in comparison with the corresponding n-hexane extracts.

DPPH free radical scavenging assay is a widely employed rapid method to evaluate the antioxidant activities of plant extracts (Mileva *et al*., 2014). The IC_{50} results for DPPH activity ranged from 0.81 to 3.71 mg/ml, 0.23 to 1.45 mg/ml and 0.15 to 1.17 mg/ml in n-hexane, acetone and methanol extracts respectively. The *F. caperata* displayed the highest DPPH radical scavenging capacity in methanol (IC_{50}, 0.15 ± 0.04 mg/ml), acetone (IC_{50}, 0.23 ± 0.01) and n-hexane (IC_{50}, 0.63 ± 0.03 mg/ml) fractions amongst all lichen extracts under the study. Furthermore, the methanol (IC_{50},0.19±0.02 mg/ml), acetone (IC_{50},0.33±0.01 mg/ml) and hexane (IC_{50}, 0.81±0.01 mg/ml) extracts of *S. nylanderiana* also demonstrated significantly higher activity. In addition methanol extracts of *H. diademata* (IC_{50}, 0.92 ± 0.03 mg/ml), *E. nepalense* (IC_{50}, 0.76 ± 0.09 mg/ml) and *P. nilgherrense* (IC_{50}, 0.44 ± 0.03 mg/ml) encountered significantly higher radical scavenging capacity compared to acetone and n-hexane extracts. Vivek and his coworkers found relatively similar IC_{50} value (0.44 mg/ml) for metanol extract of *Parmotrema tinctorum* in connection with our results for *P. nilgherrense* (Vivek *et al*. 2014). Previously published result for *E. nepalense* showed comparatively much lower antioxidant potential than our results with higher IC_{50} value, 64.7 mg/ml (Maharjan and Baral, 2013). Furthermore, the acetone extracts of *L. retigera* (IC_{50}, 0.50 ± 0.01 mg/ml) and *N .laii* (IC_{50}, 0.68 ± 0.07 mg/ml) exhibited higher antioxidant activity than the corresponding methanol and n-hexane extracts. The remaining tested species comparatively showed a significantly lower range of DPPH radical scavenging activity. Ascorbic acid (IC_{50}, 0.14 ± 0.02 mg/ml) was found to produce significantly higher radical scavenging capacity in comparison with lichen extracts (Table 2).

The ABTS assay has been calibrated with the water soluble α-tocopherol analogue, (Trolox) and corresponding ABTS value have been calculated as trolox equivalent antioxidant capacity (TEAC). Tested lichen extracts exhibited excellent TEAC values proving their capacity to scavenge the ABTS radical cation. The extracts scavenged the ABTS radical in a dose dependent manner along with positive controls viz. quercetin and ascorbic acid at concentration of 0.05–0.2 mg/ml. As far as the antioxidant activity is concerned, the maximum ABTS radical scavenging capacity in all three extracts viz methanol (IC_{50}, 0.11 ± 0.03 mg/ml), acetone (IC_{50}, 0.22 ± 0.01 mg/ml) and hexane extracts (IC_{50}, 0.54 ± 0.04 mg/ml) was rendered by *F. caperata*. In addition the methanol extracts of *H. diademata* (IC_{50},0.86 ± 0.02 mg/ml), *E. nepalense* (IC_{50}, 0.66 ± 0.04 mg/ml) and *S. nylanderiana* (IC_{50}, 0.16 ± 0.01 mg/ml) were delineated with significantly higher antioxidant capacity compared to the corresponding n-hexane and acetone extracts. Studies on TEAC activity of several species of *Heterodermia* was also conducted by Behera *et al* (2016). Furthermore, the acetone extracts of *P. nilgherrense* (IC_{50}, 0.31 ± 0.02 mg/ml) and *L. retigera* (IC_{50}, 0.44 ± 0.01 mg/ml) manifested significantly higher antioxidant activity to the corresponding methanol and n-hexane extracts. Relatively simlailar IC_{50} values of ABTS.+ radical scavenging activity was found in ethanol extract of *Diploschistes scruposus* with the value 0.46 mg/ml (Sökmen *et al*., 2012). However acetone (IC_{50}, 0.62 ± 0.06 mg/ml) and methanol (IC_{50}, 0.62 ± 0.08 mg/ml) extracts of *N. laii* demonstrated similar TEAC values. The remaining studied lichen extracts exhibited significant lower values ($p<0.005$). Ascorbic acid (IC_{50}, 0.50 ± 0.01 mg/ml) was found to produce significantly higher radical scavenging capacity compared to various lichen extracts (Table 2).

Kumar *et al*. reported comparatively higher range of IC_{50} values with lower antioxidant potential for DPPH and ABTS activities in some saxicolous lichens than results obtained in tested samples of current study (Kumar *et al*., 2014).

Estimation of total polyphenol content

The quantity of total phenolics, analyzed by Folin–Ciocalteu method, varied significantly amongst different lichen species and its solvents viz. n-hexane, acetone and methanol (Table-3). The methanol and acetone extracts of selected lichen species were found to contain significantly higher ($P<0.005$) total phenolic and flavonoid content in comparison with corresponding hexane extracts, in concurrence with the findings of earlier reports which demonstrated that variation in the polarity of the extracting solvents could result in a ample differences in

Table 2: Radical scavenging capacities (DPPH & ABTS) of lichen extracts

	DPPH (IC_{50}), mg/ml			*ABTS (IC_{50}), mg/ml*		
Lichen species	*Hexane*	*Acetone*	*Methanol*	*Hexane*	*Acetone*	*Methanol*
H. diademata	2.60 ± 0.15^{d3}	1.45 ± 0.17^{e2}	0.92 ± 0.03^{d1}	2.15 ± 0.09^{d3}	1.28 ± 0.07^{f2}	0.86 ± 0.02^{d1}
E. nepalense	1.02 ± 0.09^{b2}	0.82 ± 0.01^{d1}	0.76 ± 0.09^{c1}	0.95 ± 0.11^{bc1}	0.70 ± 0.04^{e1}	0.66 ± 0.04^{c1}
Parmotrema nilgherrrense	0.87 ± 0.04^{ab2}	0.46 ± 0.02^{b1}	0.44 ± 0.03^{b1}	0.81 ± 0.01^{ab3}	0.31 ± 0.02^{b1}	0.40 ± 0.03^{b2}
Flavoparmelia caperata	0.63 ± 0.03^{a3}	0.23 ± 0.01^{a2}	0.15 ± 0.04^{a1}	0.54 ± 0.04^{a3}	0.22 ± 0.01^{a2}	0.11 ± 0.03^{a1}
Nephromopsis laii	1.36 ± 0.21^{c2}	0.68 ± 0.07^{c1}	0.81 ± 0.10^{c1}	1.18 ± 0.26^{c2}	0.62 ± 0.06^{d1}	0.62 ± 0.08^{c1}
Lobaria retigera	3.71 ± 0.40^{e3}	0.50 ± 0.01^{b1}	1.17 ± 0.12^{e2}	2.88 ± 0.27^{e3}	0.44 ± 0.01^{e1}	1.10 ± 0.03^{e2}
Sticta nylanderiana	0.81 ± 0.01^{ab3}	0.33 ± 0.01^{a2}	0.19 ± 1.02^{a1}	0.76 ± 0.02^{ab3}	0.31 ± 0.02^{b2}	0.16 ± 0.01^{a1}
Standards						
Gallic acid	-	-	0.18±0.01			0.22±0.04
Protocatechuic acid	-	-	0.63±0.03			0.89±0.01
Chlorogenic	-	-	0.49±0.01			0.64±0.03
Caffeic acid	-	-	0.52±0.01			0.67±0.05
Rutin	-	-	0.72±0.08			1.21±0.02
Ferulic	-	-	0.37±0.01			0.47±0.02
Quercetin	-	-	0.23±0.01			0.37±0.02
Kaempferol	-	-	0.40±0.02			0.82±0.03
Ascorbic acid	-	-	0.35±0.01			0.45±0.01

Mean + SD of three replicates. Antioxidant activity expressed as IC_{50} values of ABTS scavenging activity of lichen extracts (mg/mL)

Table 3: Total phenolic and flavonoid content in fourteen different lichen extracts determined by biochemical analysis

	Phenolics content mg GAE/gm dw			*Flavonoid content mg QUE/ gm dw*		
Lichen species	*H*	*A*	*M*	*H*	*A*	*M*
H. diademata	23.97 ± 2.36^{b1}	57.83 ± 1.31^{d2}	82.2 ± 1.01^{d3}	16.18 ± 1.45^{c1}	39.56 ± 1.64^{c2}	61.96 ± 0.89^{d3}
E. nepalense	42.7 ± 3.72^{c1}	89.87 ± 0.70^{f2}	95.9 ± 2.62^{f3}	16.74 ± 1.81^{c1}	61.37 ± 2.02^{d2}	67.48 ± 1.26^{e3}
P. nilgherrrense	39.37 ± 2.96^{c1}	85.43 ± 2.08^{e2}	89.2 ± 6.75^{e2}	11.70 ± 1.07^{b1}	66.81 ± 1.62^{e3}	62.85 ± 0.97^{d2}
F.caperata	38.53 ± 2.21^{c1}	94.9 ± 5.05	88.73 ± 1.46^{e2}	30.89 ± 2.08^{e1}	61.59 ± 1.18^{d2}	78.04 ± 2.31^{f3}
N. laii	11.8 ± 3.16^{a1}	48.1 ± 0.66^{c2}	54.97 ± 1.31^{b3}	16.11 ± 2.22^{c1}	27.59 ± 1.34^{a2}	33.33 ± 1.39^{a3}
L. retigera	7.6 ± 1.40^{a1}	28.93 ± 1.46^{b2}	44.77 ± 1.02^{a3}	4.78 ± 0.44^{a1}	38.96 ± 0.68^{c2}	48.33 ± 1.39^{b3}
S. nylanderiana	10.97 ± 1.20^{a}	40.07 ± 1.15^{a2}	67.53 ± 0.75^{c3}	23.85 ± 1.39^{d1}	31.07 ± 1.77^{b2}	54.26 ± 1.33^{c3}

Mean ± SD of three replicates.Total phenolic content expressed as gallic acid equivalent (mg GAE/10 g of dw);Total flavonoid content expressed as quercetin equivalent (mg QAE/10 g of dw);.H- hexane; A- acetone; M- methanol

the polyphenolic contents of extracts owing to the polar nature of phenolic compounds (Truong *et al*., 2019). Total phenolic content of the lichen extracts ranges from 7.60 ± 1.40 mg GAE /gm dw to 42.70 ± 3.72 mg GAE / gm dw in hexane, 28.93 ± 1.46 mg GAE /gm dw to 94.9 ± 5.05 mg GAE/gm in acetone and 44.767 ± 1.02 mg GAE/ gm to 95.9 ± 2.62 mg GAE/ gm in methanol extracts. Among the lichen extracts the methanol fraction of *E. nepalense* exhibited highest TPC (95.9 ± 2.62 mg GAE/gm dw). Furthermore the maximum TPC in n-hexane and acetone extracts were determined in *E. nepalense* (42.7 ± 3.72 mg GAE/gm dw) *F. caperata* (94.9 ± 5.05 mg GAE/gm dw) respectively. Relatively similar TPC result (90.83 ± 0.98 mg GAE/g) for *F. caperata* was acquired by Mitrovic *et al*. 2011. The acetone extracts of *P. nilgherrense* (85.43 ± 2.08 mg GAE/gm) and methanol extracts of *S. nylanderiana* (67.53 ± 0.75 mg GAE/gm), *H. diademata* (82.2 ± 1.01 mg GAE/gm) and *P. nilgherrense* (89.2 ± 6.75 mg GAE/gm) were also found to have significant higher amount of TPC (Table 3). Odabasoglu *et al*. determined the total phenolic content for methanol extracts of *L. pulmonaria* (87.9 mg/g lyophylisate) and *U. longissima* (38.6 mg/g lyophylisate) (Odabasoglu *et al*., 2004).

The total flavonoid content of different lichens extracts varied greatly and ranges from 4.78 ± 0.44 mg QUE/gm to 30.89 ± 2.08 mg QUE/gm in hexane; 27.59 ± 1.34 mg QUE/gm to 66.81 ± 1.62 mg QUE/gm in acetone and 33.33 ± 1.39 mg QUE/gm to 78.04 ± 2.31 mg QUE/gm in methanol extract. The acetone and methanol extracts exhibited the comparatively higher concentrations of TFC to those reported by Mitrovic et al. (2011) in some lichen extracts with the range of 20.14 ± 0.81 mg RuE/g to 44.43 ± 1.22 mg RuE/g, whereas the hexane extracts contain comparatively lower values. The highest TFC concentration was determined in the methanol extract of F. caperata (78.037 ± 2.31 mg QUE/gm), which is relatively higher amount found in a study conducted by Mitrovic et al. (2011) with value of 33.55 ± 0.93 mg QUE/g. Furthermore, the maximum TFC concentration of acetone and hexane extracts were depicted in of P. nilgherrense (66.82 ± 1.62 mg QUE/gm) and F. caperata (30.89±2.08 mg QUE/gm) respectively. Extracts of E. nepalense (Acetone,61.37±2.02 mg QUE/gm; Methanol, 67.48±1.26 mg QUE/gm), H. diademata (Methanol, 61.96±0.89 mg QUE/gm) and S. nylanderiana (Methanol, 54.26±1.33 mg QUE/gm) also exhibited comparatively good content of flavonoid. than the remaining lichen extracts (Table 3).

The results acquired here for TPC and TFC are also similar to *Parmelia sulcata* (88.25 ± 1.02 mg GAE/g TPC, 44.43 ± 1.22 mg RuE/g TFC); *Evernia prunastri* (80.73 ± 1.25 mg GAE/g TPC; 27.46 ± 0.78 mg RuE/g TFC); *Hypogymnia physodes* (141.59 ± 1.12 mg GAE/g TPC; 20.14 ± 0.81 mg RuE/g TFC); *Cladonia foliacea* (78.12 ± 1.31 mg GAE/g; 28.22 ± 0.59 mg RuE/g) (Mitrović *et al*., 2011).

Proportional relation of phenolic acids to flavonoids

Proportional relation of phenolic acids to flavonoids depicts predominating TPC values over TFC (Fig. 2). Polyphenols were found in higher concentrations in green algae containing lichens (*F. caperata, E. nepalense, P. nilgherrense* and *H. diademata*) and are known to serve as filtration mechanism against UV-B radiation except *N. laii* (Harborne and Williams, 2000), Whereas cyanobacteria containing lichens (*L. retigera* and *S. nylanderiana*) exhibited lower values indicating the different biosynthetic pathways are followed by green alga and cyanobacteria for synthesis of their protective compounds.

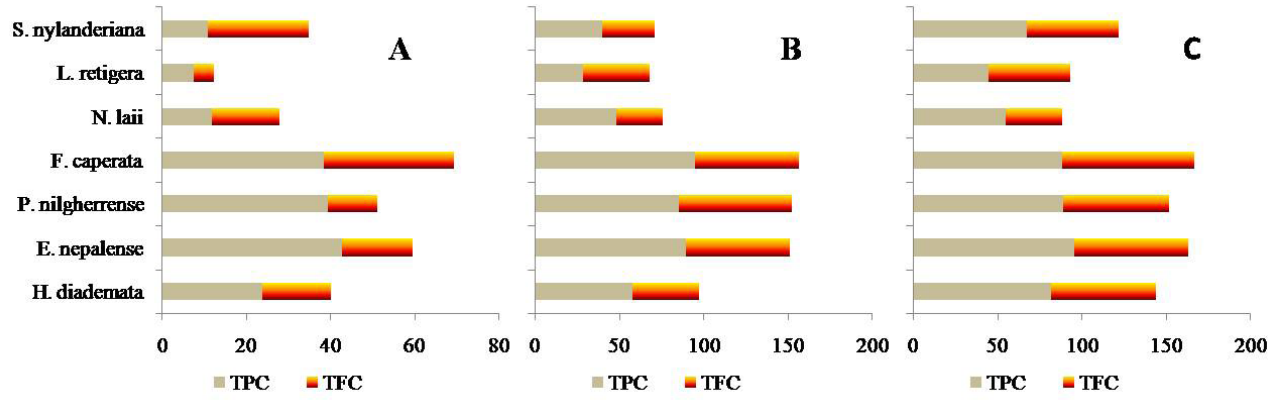

Fig 2: Proportional relation of flavonoids content to phenolic acid in selected lichen species. (A- Hexane extract; B- Acetone extract; C- Methanol extract).

Correlation between total phenolic, flavonoid content and antioxidant capacities (DPPH & ABTS) in various solvent extracts of seven lichen species

The tested lichen extracts exhibited strong DPPH and ABTS radical scavenging activity which is significantly correlated with each other(r = 0.978). Total phenolic content strongly correlated with total flavonoid content (r = 0.905). Since IC_{50} value is minimum inhibitory concentration to scavenge radicals therefore IC_{50} of DPPH and ABTS exhibited moderate negative correlation with the increasing TPC (DPPH r = –0.563; ABTS r = –0.524) and TFC (DPPH r = –0.604; ABTS r = –0.576) values (Table 4). Sample which had the lower IC_{50} of DPPH scavenging capacity gave the higher antioxidant activity (Fig.3). So the negative correlation was found between TPC, TFC with the IC_{50} of DPPH and ABTS activities. It is also shown from the results that antioxidant capacity of extracts do not rely only on amounts of phenolics, but also on the structure and interactions among phenolic constituents, from phenolic acids to flavonoids and the derrivatives (Huyut *et al*., 2017). Furthermore the antioxidant activity may also be attributed to the other non-phenol components such as carotenoids, vitamins and minerals (Odabasoglu *et al*., 2004). Thus, the individual phenolic quantification and its structural correlation can be used to predict their antioxidant activity with reasonable accuracy.

HPLC profile of phenolic acids

Total phenolic and flavonoid content measured in the extracts, does not give a full picture of contribution of each phenolic constituent to the scavenging activity (Katsube *et al*., 2004; Wu *et al*., 2004). Therefore, the chemical analysis by HPLC compared with standard markers revealed the concentration of major types of representative phenolic components identified in the present study mainly included hydroxycinnamic acids - caffeic acid (R_t = 11.93), chlorogenic acid (R_t = 9.19) and ferulic acid (R_t = 17.38); hydroxybenzoic acid- Gallic acid (R_t = 6.38) and protocatechuic acid (R_t = 8.04) and flavonols- rutin (R_t = 13.82), quercetin (R_t = 27.59) and kaempferol (R_t = 34.71) are depicted in Table 5. Considerable variation was found for concentration of phenolic compounds in hexane, acetone and methanol extracts of seven different lichen species (Fig. 4). Similarly gallic acid, protocatechuic, caffeic, ferulic, chlorogenic, quercetin and rutin have also been well separated and quantified by several researchers (Seal, 2016; Gammoh *et al*., 2017).

Acetone extract of *H. diademata* had the highest concentration of chlorogenic acid (106.25±2.76 µg/gm) and caffeic acid (47.77±1.85 µg/gm), while *L. retigera* contains maximum concentration of ferulic acid (33.04±0.85 µg/gm). The content of caffeic acid is much higher than the

Table 4: Correlation among TPC, TFC and antioxidant parameters (DPPH and ABTS) of seven selected lichen species.

Correlation coeffient	*TPC*	*TFC*	*IC_{50}(DPPH)*
TFC	0.9045	1	
IC_{50}(DPPH)	-0.5629	-0.6037	1
IC_{50}(ABTS)	-0.5241	-0.5763	0.9779

r, Correlation coefficient. Significance level at P <0.001

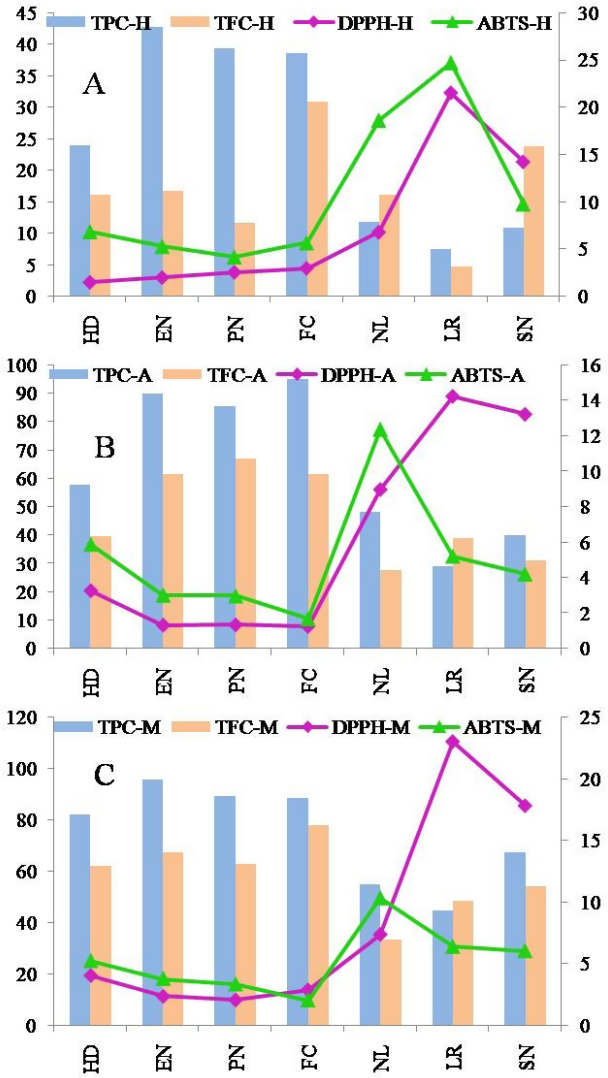

Fig 3: Variation in TPC, TFC and their corresponding antioxidant activities (DPPH and ABTS). (A - hexane extract, B -acetone extract, C -methanol extract; HD- *H. diademata*, EN- *E. nepalense*, PN- *P. nilgherrense*, FC- *F. caperata*, NL- *N. laii*, LR- *L. retigera*, SN- *S. nylanderiana*

Table 5: Concentration of detected phytochemicals in lichen extracts by HPLC analysis

		Phenolic Compounds (µg/gm dw)							
Lichen Species	*Extract*	*Gallic acid*	*Protocatechuic acid*	*Chlorogenic*	*Caffeic acid*	*Rutin*	*Ferulic acid*	*Quercetin*	*Kaempferol*
Heterodermia Diademata	H	41.99±1.84	0.08±0.01	0.03±0.01	ND	ND	ND	1.07±0.04	ND
	A	72.73±1.47	18.17±1.71	106.25±2.76	47.77±1.85	ND	ND	ND	37.12±2
	M	110.29±2.66	ND	ND	0.56±0.05	ND	ND	ND	58.50±5.56
Everniastrum nepalense	H	34.90±3.79	ND	ND	ND	ND	ND	0.28±0.01	ND
	A	48.67±1.42	ND	7.12±1.04	ND	127.54±3.8	0.17±0.01	188.75±6.36	ND
	M	6.64±0.42	ND	ND	ND	0.65±0.03	ND	4.96±0.95	ND
Parmotrema nilgherrense	H	37.56±1.08	0.56±0.03	ND	0.11±0.01	5.64±0.29	ND	22.40±0.90	0.57±0.02
	A	879.10±21.07	ND	ND	23.10±0.22	ND	ND	ND	13.07±0.89
	M	20.97±1.95	15.19±1.03	27.02±0.70	18.51±0.88	ND	9.84±0.97	1.05±0.08	0.89±0.08
Flavoparmelia caperata	H	143.62±0.94	18.10±1.08	24.54±0.78	0.48±0.04	ND	5.30±0.58	19.03±0.99	6.31±0.27
	A	970.01±2.48	ND	2.54±0.14	15.30±0.31	ND	18.53±1.28	ND	12.90±1.08
	M	846.86±1.86	ND	21.01±1.74	0.68±0.07	0.29±0.02	3.46±0.55	1.34±0.17	ND
Nephromopsis laii	H	33.09±0.81	ND	25.66±1.38	9.09±0.45	ND	ND	0.32±0.04	0.17±0.01
	A	48.10±1.74	3.75±0.17	12.73±0.56	0.08±0.01	ND	3.55±0.29	ND	ND
	M	83.42±0.60	24.62±1.23	18.15±0.73	18.60±1.08	10.27±0.72	15.76±1.50	6.97±1.30	1.06±0.06
Lobaria retigera	H	12.25±0.24	2.23±0.35	ND	ND	6.86±2.34	4.53±1.11	3.12±0.10	4.78±0.01
	A	14.26±0.27	9.61±0.35	ND	ND	1316.54±3.23	33.04±0.85	1.02±0.03	ND
	M	96 ±1.60	27.21±0.85	9.52±0.37	12.18±0.40	17.31±0.41	8.34±0.21	0.97±0.02	ND
Sticta nylanderiana	H	45.52±1.11	ND	0.06±0.01	ND	ND	0.76±0.14	ND	0.52±0.01
	A	9.39±0.23	9.60±0.14	28.19±0.59	2.12±0.21	ND	3.14±0.34	0.16±0.02	ND
	M	45.44±1.65	10.55±0.57	8.20±0.24	2.41±0.24	ND	0.17±0.02	1.89±0.20	ND

H - hexane extract; A - acetone extract; M - methanol extract. All the values between the row and column are highly significant $P<0.001$ ($F=9863.9469$ in columns and $F= 22513.668$ in rows as obtained by two-way ANOVA with replication.

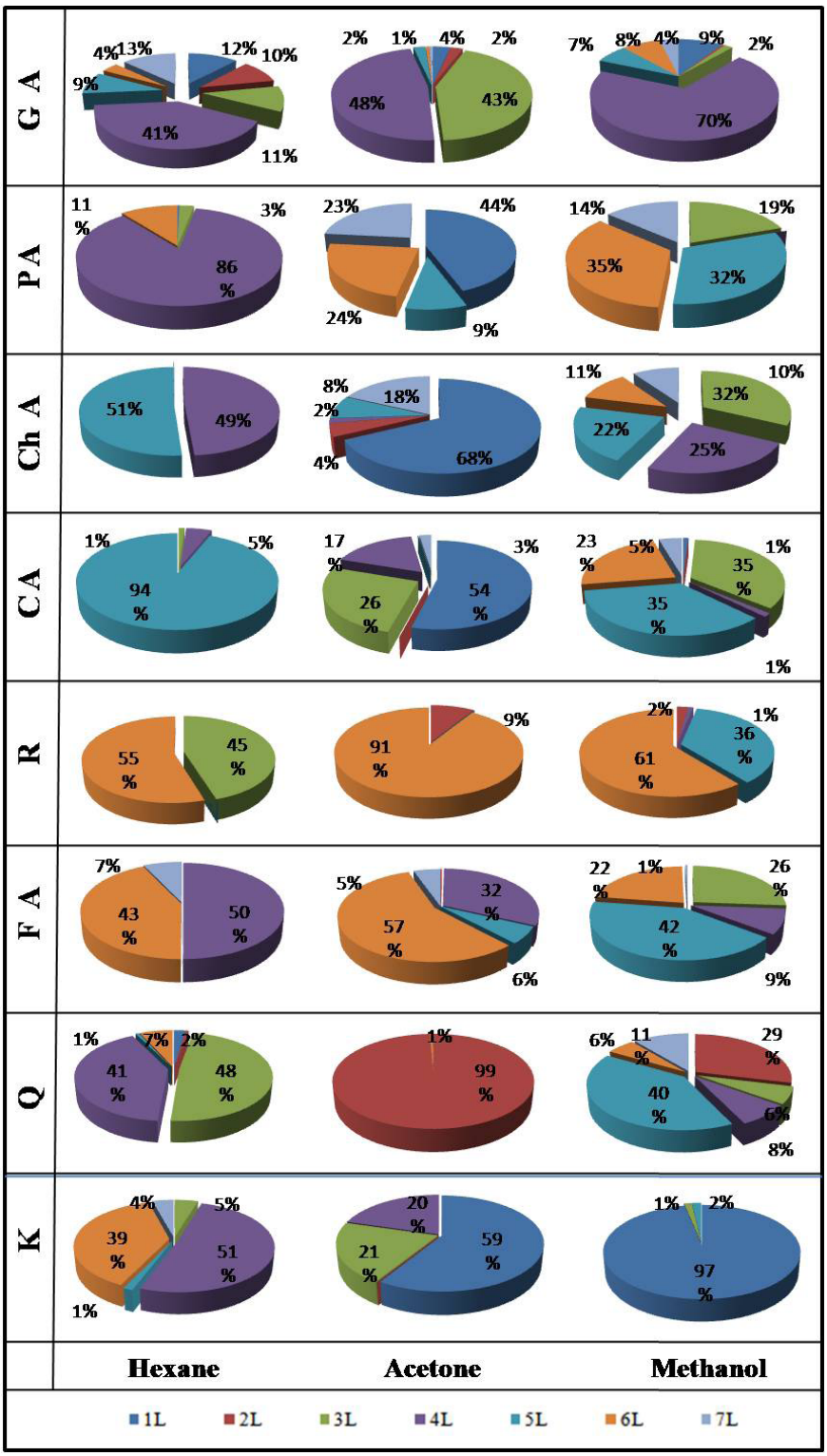

Fig 4: Percentage distribution of individual polyphenols in different lichen extracts. (H - hexane extract, A -acetone extract, M -methanol extract; 1L- *H. diademata*, 2L- *E. nepalense*, 3L- *P. nilgherrense*, 4L- *F. caperata*, 5L- *N. laii*, 6L- *L. retigera*, 7L- *S. nylanderiana*; GA- Gallic acid, PA- Protocatechuic acid, ChA- Chlorogenic acid, CA- Caffeic acid, R- Rutin, FA- Ferulic acid, Q- Quercetin, K- Kaempeferol).

values obtained by Nowacka *et al*. (2015) who obtained maximum value of 2.86+0.02 µg/gm. Ferulic acid and caffeic acid were not detected in all three extracts of *H. diademata* and *E. nepalense* respectively. Nowacka *et al*. (2015) also identified ferulic acid in *L. vellereus, Lactarius aurantiacus* (0.75±0.01 µg/gm) and *Lenzites betulinus* (0.16±0.0 µg/gm); caffeic acid in *Gymnopilus penetrans* (2.86±0.02 µg/gm) *Hyphodontia paradoxa* (1.14±0.02 µg/gm) *Fomes fomentarius* (1.35 ±0.03 µg/gm).

Among the detected hydroxybenzoic acids, gallic acid is only compound which is detected as predominant compound in all lichen extracts, with the highest value in acetone extract of *F. caperata* (970±22.48 µg/gm). Our results are in concordance with several studies in which gallic acid showed the highest level, and potential antioxidant activity was found for gallic and caffeic acids (Sato *et al*., 2011; Cho *et al*., 2001). Protocatechuic acid was not detected in most of the lichen extracts. Maximum amount of protocatechuic acid is detected in methanol extract of *L. retigera* (27.21±0.85 µg/gm) while lowest amount was quantified in hexane extract of *H. diademata* (0.08±0.01 µg/gm). Reports are also available for the presence of p- hydroxybenzoic acid derivatives ; vanilic and protocatechuic in lichen species *Cetraria islandica* and *Cladonia arbuscula* respectively (Zagoskina *et al*., 2013).

Amongst detected flavonoid compounds viz. quercetin, kaempferol and rutin were significantly present. The compounds, quercetin and kaempferol encountered maximum values in acetone extract of *E. nepalense* (188.75±6.36 µg/gm) and methanol extract of *H. diademata* (58.50±5.56 µg/gm) respectively. Rutin was not detected in all the three extracts of *H. diademata* and *S. nylanderiana*. The rutin content in acetone extract of *L. retigera* represents highest value (1316.54±3.23 µg/gm) amongst all the detected polyphenolic compounds of tested samples. This observation is in agreement with Kim *et al*. who found the rutin as dominant flavonol in green tea infusions (Kim *et al*., 2011). other lichen extracts represent very low concentration comparatively. The flavonoids (quercetin, kaempferol and rutin) identified here in lichen extracts have been previously depicted as having antioxidant properties (Singh *et al*., 2017)

Structural criteria of phenolic compounds to antioxidant activity

In the present study the free radical scavenging capacity was analyzed by comparing its results with eight tested phenolic compounds to thoroughly investigate the structure-activity relationships of phenolics and flavonoids in tested lichen extracts. The correlation between phenolic compounds and antioxidant activity has been verified and confirmed by several authors (Gan *et al*., 2017; Fidrianny *et al*.,2018). The intensity of antiradical activity of detected phenolic standards and lichen extracts depends on many factors such as their varying levels, types of natural polyphenols and the different chemical structures (Dai and Mumper, 2010).

The activities of phenol derivatives are connected with (i) the effect of -CH = CH-COOH group, (ii) The relationship between the number and position of hydroxyl (-OH) groups in aromatic ring, (Sroka and Cisowski, 2003) (iii) Number and position of double bonds in molecule, (Burda and Oleszek, 2001) (iv) methoxy (-OCH3) substituents in the molecule (Cai *et al*., 2006). Major representative compounds of polyphenols quantified in tested lichen species and their structural characteristics such as the number, position of hydroxyl groups and other substituents or glycosylation are shown in Table 6 and
. 5.

Amongst tested standard phenolic compounds and positive controls, gallic acid exhibited highest DPPH ($IC_{50,}$ 0.17±0.017 mg/ml) and ABTS ($IC_{50,}$ 0.22±0.036 mg/ml) radical scavenging capacity. This result is in support by the studies of Cai *et al*. (2006), which revealed that amongst all the tested phenolic acids, gallic acid (3, 4, 5- trihydroxybenzoic acid) with most hydroxyl groups, had the strongest radical scavenging activity, because of the pyrogallol structural advantage and potent H-donating ability (three free hydroxyl groups).

In our studies for the tested hydroxycinnamic acids, it has been observed that dihydroxycinnamates, caffeic (DPPH $IC_{50,}$ 0.52±0.036 mg/ml ; ABTS $IC_{50,}$ 0.67±0.026 mg/ml) and chlorogenic (DPPH $IC_{50,}$ 0.49±0.017 mg/ml ; ABTS $IC_{50,}$ 0.64±0.042 mg/ml) were less effective than other monohydroxycinnamate, ferulic acid (DPPH $IC_{50,}$ 0.38±0.026 mg/ml ; ABTS $IC_{50,}$ 0.47±0.026 mg/ml). This is perhaps due to the effect of substitution of the 3- or 4- hydroxyl group of caffeic acid by a methoxy groups as in ferulic acid which raised the scavenging activity as demonstrated in several studies (Cai *et al*., 2006). This indicated that methoxy groups could noticeably enhance the radical scavenging activity of the hydroxycinnamic acids. Observations were in conformity with the results described by Rice Evans *et al*. (1996) and Natella *et al*. (1999). In addition, hydroxyl group present in *para*-position (4-OH) also improve the radical scavenging activity of ferulic acid.

Albeit with the equivalent number of hydroxyl and methoxy groups, hydroxycinnamic acids tends to be more effective (value) than hydroxybenzoic acid

Table 6: Basic structural skeleton of representative phenolic compounds identified in tested lichen species (After Cai et al., 2006)

S.N.	*Representative phenolic*	*Hydroxyl (OH) substituents*		*Other substituents and glycosylated position*
		-OH position	Number of -OH	
	Phenolic acids (Hydroxycinnamic acids)			
1.	Caffeic acid	3,4-OH	2	
2.	Chlorogenic acid	3,4-OH	2	R=tetrahydroxycyclohexanecarboxylic group
3.	Ferulic acid			
	Phenolic acids (Hydroxybenzoic acids)			
4.	Gallic acid	3,4,5-OH	3	
5.	Protocatechuic acid	3,4-OH	2	
	Flavonoids (Flavonols)			
6.	Quercetin	3,5,7,3',4'-OH	5	
7.	Kaempferol	3,5,7,4'-OH	4	
8.	Rutin (Quercetin-3-rutinoside)	5,7,3',4'-OH	4	3-O-Rutinose

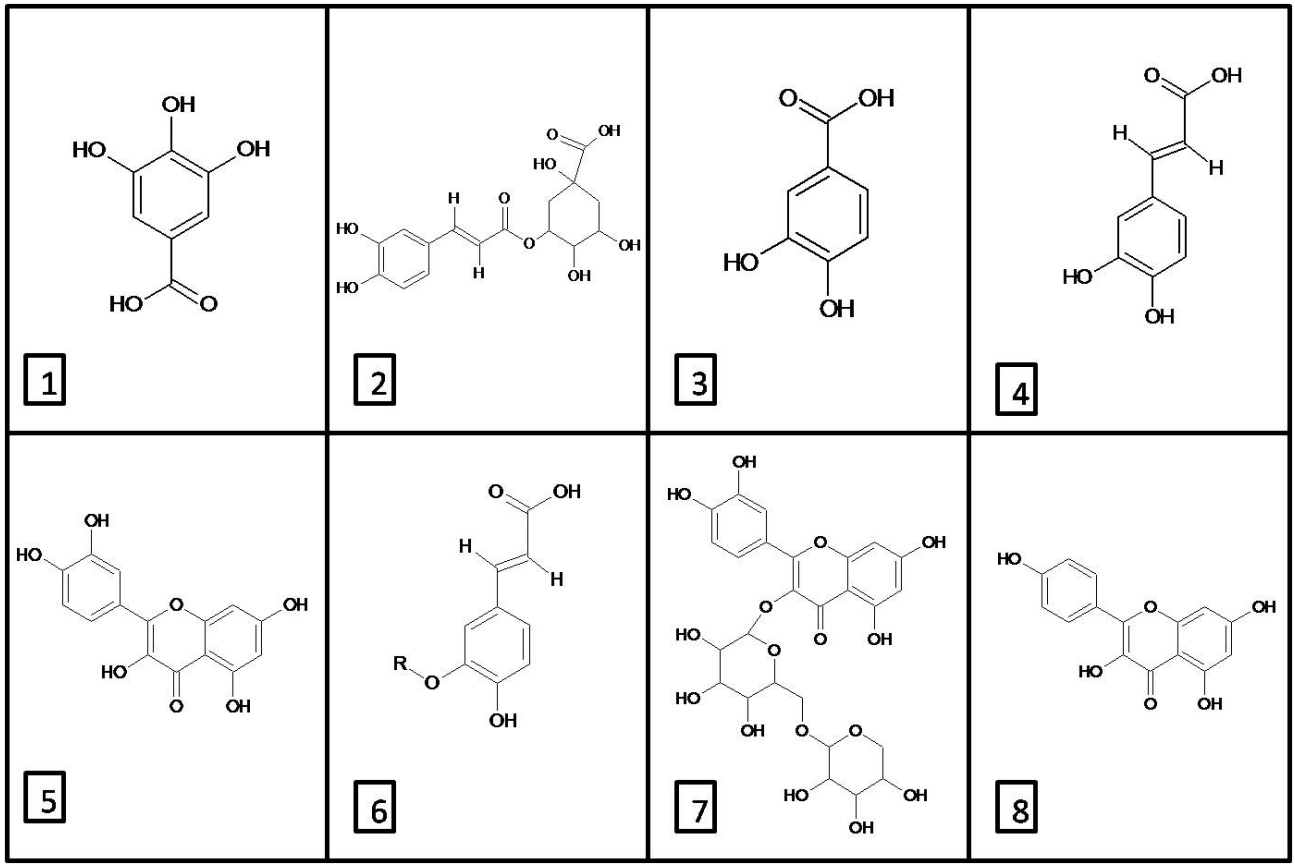

Fig 5: Basic structural skeleton of representative phenolic compound quantified in tested lichen species. (1- Gallic acid, 2- Protocatechuic acid, 3- Chlorogenic acid, 4- Caffeic acid, 5- Rutin, 6- Ferulic acid, 7- Quercetin, 8- Kaempeferol).

(protocatechuic acid, DPPH $IC_{50,}$ 0.63±0.02 mg/ml ; ABTS $IC_{50,}$ 0.89±0.056 mg/ml) except gallic acid. It could be due to the –CH = CH-COOH group coupled to phenyl ring of the hydroxycinnamic acid plays a role in stabilizing the radical by resonance. The presence of the –CH = CH-COOH group in hydroxycinnamic acid ensures greater H-donating ability and subsequent radical stabilization than the carboxylate (CH_2-COOH) group in hydroxy benzoic acid (Natella *et al*., 1999).

The relationship between the chemical structure of flavonoids and their radical scavenging activities was analyzed by (Li *et al*., 2018). The main structural features of flavonoids necessary for efficient radical scavenging are: a) *ortho*-dihydroxy structure in the B-ring because it makes possible electron delocalization; b) 2,3-double bond in the C-ring in conjugation with a 4-keto group provides electron delocalization from the B-ring; c) free hydroxy groups at 3 (ring C) and 5 (ring A) positions provide hydrogen bonding to the 4-keto group (Croft, 1998; Kim and Lee, 2004). It has been revealed that flavonols with a free 3-hydroxy group such as Kaempferol (DPPH $IC_{50,}$ 0.40±0.026 mg/ml ; ABTS $IC_{50,}$ 0.82±0.036 mg/ml) and quercetin (DPPH $IC_{50,}$ 0.24±0.044 mg/ml ; ABTS $IC_{50,}$ 0.38±0.061 mg/ml) were stronger antioxidants than flavonoids with blocked 3- hydroxy group at 3 position in C ring by glycosylation such as rutin (DPPH $IC_{50,}$ 0.73±0.017 mg/ml ; ABTS $IC_{50,}$ 1.21±0.053 mg/ml) (Sroka, 2005). Rutin with its high concentration had a little correlation with the radical scavenging capacities measured by the two methods in this study, which is in concurrence with other reports (Fei *et al*., 2013).

Structure of phenolic compounds may contain other constituents such as -COOH, -CHO, -CH_2OH, -CH_3 and other alkyl groups. -COOH groups have electron-withdrawing properties detrimental to the H- donating ability of hydroxyl acids. In contrast, carboxylate groups (-COO-), which are electron-donating, favor H-atom transfer and electron donation based on radical scavenging (Ji *et al*., 2006)

In conjunction with structural features of phenolic compounds, the effect of solvent on antioxidant activity should also be taken into account. Hydrogen transfer rely on the polarilty of the solvent utilized, which is regulate by two factors : strength of the phenolic O-H bond and magnitude of the kinetic solvent effect. The stronger these affinities and their number, the larger the number of solvent molecules to be displaced before hydrogen can be removed by the free radical. Solvent interaction with hydroxyls reduces the reaction rate, which is reflected as lower antioxidant activity (Litiwinienko and Ingold, 2007).

Lichen species *F. caperata* were found to contain significantly higher amount of gallic acid and exhibited significantly higher radical scavenging activity. Results revealed that gallic acid is a major bioactive component in *F. caperata* having highest IC_{50} values, which could potentially inhibit the ROS levels due to its pyrogallol structural activity. Other promising inhibition activities of gallic acid were also discussed by Cai *et al*. (2006) and Chan *et al*. (2015). Similar potential activities were also observed in *S. nylanderiana* which not only contain substantial amount of polyphenols, but instead is known to contain atranorin and gyrophoric acid which are known antioxidants (Buçukoğlu *et al*. 2013; Sisodia *et al*. 2013; Ranković *et al*. 2014 a &b; Kosanić *et al*. 2014 a &b).

The present findings dete rmined that lichens are characterized by production of not only lichen acids, but also of phenol carboxylic acid derivatives widespread in the plant kingdom which are formed at the earlier stages of phenolic compound biogenesis and participate in many biological and physiological processess (Zavarzina and Zavarzin, 2006; Laufer *et al*. 2006; Laufer *et al*. 2009). According to some researchers lichen biosynthesize a unique class of substances depsides and depsidones. Depsides, tridepsides and tetradepsides also consist of two, three and four hydroxybenzoic acid residues linked by ester groups while Depsidones in lichen are believed to arise by oxidative cyclization of depsides which recommend the structural relation of lichen substances with known phenolics (Manojlović *et al*., 2012). Therefore the study presents in open question for extensive investigation on the structure-radical scavenging activity relationships of representative phenolic substances and potential bioactive lichen substances which will be beneficial for commercial application.

CONCLUSION

Based on the results, it has been observed that among the tested lichen extracts, methanol and acetone extracts exhibited potential radical scavenging activity. The increased concentration of gallic acid probably enhance the antioxidant activity of these lichen species while higher concentration of other phenolic compounds do not have much noticeable effect on detected antioxidant potential lichen extracts. A high correlation was found between TPC and TFC, as well as the antioxidant activity. *F. caperata* and *S.nylanderiana* demonstrated strongest antioxidant potential followed by *P. nilgherrense*, *E,.nepalense* and *H. diademata*. Chemical structure of most of the lichen substances is simple which facilitates its synthesis at large scale without affecting the ecosystem. Therefore this study

contributes to the improvement of scientific understanding of structure-activity relationships and functionality of phenolic constituents in regulating the bioactive potential which will be beneficial for the commercial production of bioactive components by functional group modulation through synthetic chemistry. The elevated antioxidant activity of *F. caperata* and *S. nylanderiana* may be considered as potential source of natural antioxidants for medicinal and commercial uses.

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