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Screening and characterization of bioactive compounds from two epiphytic microlichen and evaluation of their in vitro antioxidant activity

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Abstract

Background Lichens in symbiosis produce a wide range of primary and secondary fine compounds in extreme environmental conditions that have a broad range of biological properties as well as antioxidant potential and can be used in future pharmaceuticals as a natural source of antioxidant molecules.

Results The two microlichen species collected are identified based on morphological and molecular techniques; further studies are carried out by analyzing phytochemicals (FTIR, GC MS), and antioxidant assays are evaluated. The non-enzymatic antioxidant activity is evaluated by DPPH and FRAP assays. The methanol extract of both lichens showed virtuous DPPH scavenge with IC50 of *P. nitida* (125.76 ± 0.023 µg/ml) and *G. scripta* IC50 (176.90 ± 0.058 µg/ml). FRAP activity was prominent in the methalonic extract. The enzymatic antioxidant activity is observed by SOD and catalase activity. The cytosolic (Cu–Zn-SOD and Fe-SOD) and mitochondrial SOD (Mn-SOD) are detected in lichens, though *P. nitida* shows mitochondrial Mn-SOD and cytosolic Cu–Zn-SOD and Fe-SOD, whereas *G. scripta* has a single cytosolic Cu–Zn-SOD; however, two isoforms of catalase were reported. GC–MS analysis screened bioactive metabolites such as phenols, Quinons, heterocyclic compounds, benzofurans, fatty acids, pyrans, carboxylic acid, aliphatic aldehydes, organic alcohol, fluorinated aliphatic substances, ketones, terpenes and fatty alcohols in *P. nitida*, whereas, in *G. scripta* screened fatty acids, alcohols, hydrocarbons, carbonyl compounds, polyols, terpenes, glycosides, phenols, and sugar alcohols detected in the chromatogram peak. FTIR analysis revealed functional groups like Alcohols, Amines, Amides, Alkanes, Aldehydes, Carboxylic acid, Alkynes, Esters, Ketones, Anhydrides, Acid chlorides, Alkenes, Aromatic compounds, Nitro compounds, Alkyl and Aryl Halides in both lichens.

Conclusions The results obtained in the present study proved that *P. nitida* and *G. scripta* have promising antioxidant activity owing to the presence of polyphenols and terpenes, as evidenced by DPPH and FRAP assay along with enzymatic analysis (SOD and CAT). Thus both the lichens may be used as natural sources of new bioactive molecules having pharmaceutical interest.

Keywords 28s rRNA, Antioxidant, FTIR, GC–MS, Lichen, Phytochemicals, SEM

Background

Lichens are poikilohydric symbiotic organisms with non-flowering living structure composed of an alga (phycobiont) and a fungus (mycobiont). They live in their natural habitats and can subsist in extreme environments. The water content of lichen thallus broadly depends upon

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environmental conditions. They have the potential to sustain long durations in a dry ecological condition with high adaptability to slower metabolic activity but can rapidly revive normal physiological activity upon rehydration [1].

Metabolic activities like respiration and photosynthesis are sensible for the generation of ROS (reactive oxygen species) causing damage to cell, thereby enhancing a number of degenerative diseases such as premature aging, inflammation, atherosclerosis, deoxygenation of ischemic tissues and cancer [2]. Free radicals attack unsaturated fatty acids, including PUFAs (polyunsaturated fatty acids), in plasma membranes, resulting in lipid peroxidation, decreased membrane fluidity and dissolution of membrane-associated proteins. Oxidative stress is influenced by environmental factors and aging, but some oxidative diseases and stresses can be treated with antioxidant agents. Antioxidants prevent oxidative chain reactions by eliminating free radical intermediates, which prevent cellular components from getting oxidized [3]. The carcinogenetic effect of synthetic antioxidants implicates the growing interest in natural antioxidants for human sustainability [4]. These are enhanced or triggered stress, such as dietary restriction, xenobiotic exposure, desiccation and/or rehydration. To overcome the latent damaging effects of reactive oxygen species (ROS) and maintain their limited consistency, cells have adopted protection mechanisms, including non-enzymatic antioxidants such as ascorbate, α-tocopherols, β-carotene and other minerals, and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GTX), as well as low molecular weight antioxidants such as glutathione reductase (GRX) and glucose 6-phosphate dehydrogenase (G6PD) [5].

Graphis scripta and *Pyrenula nitida* are two epiphytic crustose lichens predominantly distributed to different phorophytes on the MSCBU campus. The lichenized thallus of both species consists of Ascomycetes fungus with associated *Trentepohlia* alga. This was associated with a burst of cellular ROS generation by both the mycobiont and the photobiont, as well as nitric oxide production that we only found in the fungus. These actions resulted in a transitory decrease in water-soluble low molecular weight antioxidant capacity but did not induce significant membrane damage. Imbibition of some lichens after desiccation stimulated extracellular antioxidant production [6]. Some recent evidence regarding the antioxidant response of lichens to rehydration reveals that this increases with thallus pre-treatment prior to testing species, drying and rehydration procedures, and experimental time periods [7, 8]

Several lichens have reported numbers of unique secondary metabolites such as terpenes, usnic acid, pulvinic acids, chromones, depsons, depsidones and xanthone derivatives that have been broadly labeled in the regulation of lichen growth as well as protection along with series of biological activities [9] and the synthesis of these compounds increases their antioxidant capacities [10]. Recently, lichens have emerged as a valuable source of antioxidants, with implications for the development of pharmaceuticals and cosmetics [11]. Furthermore, this study was carried out to screen and identify the major bioactive constituents of both the lichens, *P. nitida* and *G. scripta*, and to evaluate in vitro, the enzymatic and non-enzymatic antioxidant activity by using different solvent extracts. This is the first report from this region on the isolation and characterization of compounds from both the lichen species having their antioxidant activity.

Methods

Sampling and study area

MSCBU campus positioned at 21.9303° N 86.7636° E, Odisha, India (Fig. 1). Seasonal field visits (Spring, Rainy and Winter) and collection (Dates: 07/03/2021, 22/08/2021 and 21/01/2022) of lichens were performed regularly, and all collected lichens were sampled by random sampling technique. Collected lichens are wrapped in white paper bags with zippers, with its microhabitat data (latitude, longitude, temperature and humidity) of each bisect.

Diversity index of collected samples

This study explored a number of epiphytic lichen species under 12 genera in 8 families from the MSCBU campus, which is situated near the Similipal Biosphere Reserve (SBR). The preceding study was carried out by studying the epiphytic lichen species on twenty dominant trees/phorophytes. The lichen family Graphidaceae has the maximum distribution and is represented by SWI (Shannon-Weiner Index) as $H=2.053$, with a SIV (species index value) of 206.72, and both the lichen species with SIV as in *Pyrenula nitida*- OQ146904 (55.96) and *Graphis scripta*-OP861477 (63.46) were distributed within vegetation.

Morphological identification of lichens

Morphological identification was done using a stereo-zoom microscope (Stemi-305, 40×magnifications) and scanning electron microscope (Model-S3400N, magnification 10X to 300,000 X Max) observation. Morphological identifying characteristics are studied using the lichen identifying key book [12].

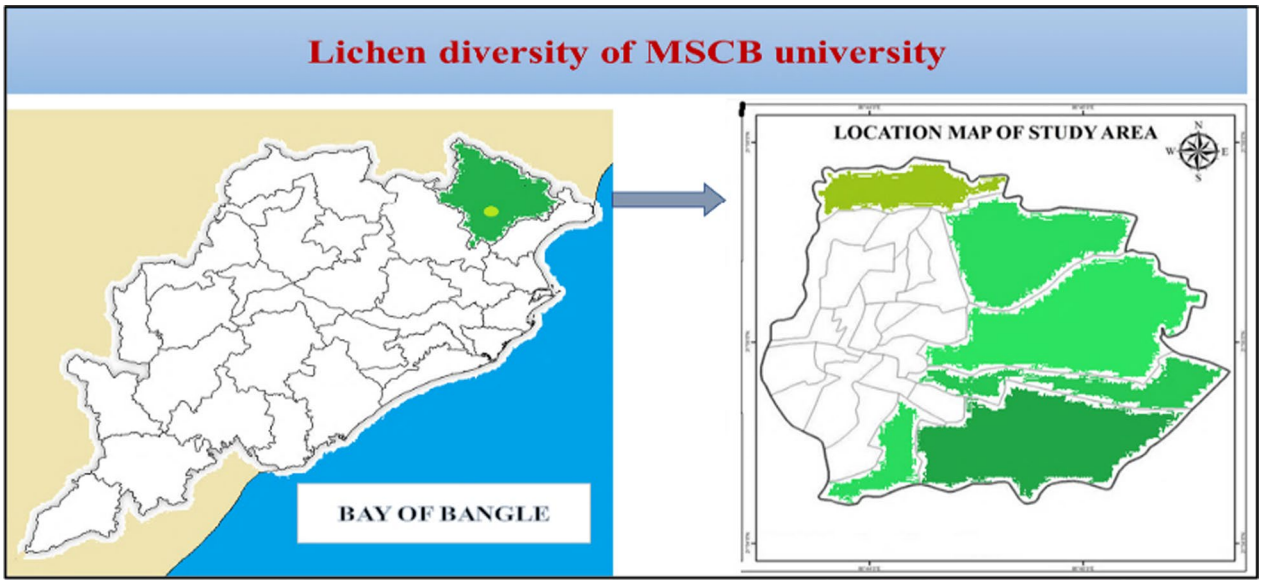


Fig. 1 Study area: MSCBU campus, shadow area showing coverage and distribution of lichens

Molecular identification

DNA isolation from lichens

For the isolation of DNA from Lichen thallus, 10 mg of scraped lichen thallus was used and put into a microvial, deepen in liquid nitrogen with three or four 2.5-mm sterile glass beads for 30 s and disrupted with a Mini-Beadbeater-24. The sample was stirred vertically with 300 µL KCl extraction buffer, 300 µL chloroform and kept reversed. The sample mixture was centrifuged at 12,000 rpm for 1 min. The supernatant was transferred to a microcentrifuge vial with the addition of 180 µL of chilled isopropanol and centrifuge at 12,000 rpm for 1 min; the supernatant was descanted. The remaining pellet was poured out with 300 µL chilled ethanol (70%). The pellet was dried at 55 °C in oven, then 100 µL of TE buffer was added, and purified DNA was stored at 4 °C [13].

PCR and sequencing

A partial sequence of DNA for analysis (28s, and 18s rDNA) was isolated from the upper cortex of both lichens. Amplification of rDNA performed using PCR primers for the 28s larger sub-unit of *P. nitida* strain PNSB04 used forward primer used was PN1- 5′-aacaggggggtgagatgtcaga-3′ (25 nm STD, GC: 54.5%, Tm: 60 °C, ΔG: − 41.7 kcal/mol), and reverse primer PN2-5′-ctagtacgatacattcaaatgt-3′ (25 nm STD, GC: 31.8%, Tm: 47.8 °C, ΔG: -34.33 kcal/mol) for *G. scripta strain* SPB25 rDNA, Forward primer-GS1: 5′-ttgtaatttggagaaggtgttt-3′ (25 nm STD, GC: 31.8%, Tm: 50.4 ΔG: − 38.42 kcal/mol), and reverse primer-GS2: 5′-catcctagcttttgcgcggacc-3′ was used, following the protocol of White et al. [14]. PCR products were visualized on a 1.5% agarose gel analysis for 25 min with content 120 V, to authenticate the presence of amplicons size, then exonuclease-I and recombinant shrimp alkaline phosphatase used for molecular purification and sequenced bidirectionally by genetic analyzer (ABI 3730). Proofreads (forward and reverse) were assembled in MEGA and Sequencher v. 5, with GenBank submission having accession number for *G. scripta* (OP861477) and *P. nitida* (OQ146904).

Antioxidant activity of Lichens

Preparation of the lichen extracts for non-enzymatic antioxidant activity Collected lichen samples were dried in a hot air oven and then crushed to a fine powder, and 10 g of dry powder was poured into a Borosil Soxhlet at 45–50 °C for 24 h using methanol, acetone, benzene and diethyl ether. After 6–8 cycles, the solutions are collected and further evaporated in oven at 42–45 °C. After evaporation, the dry extract of each solvent was taken for further analysis.

DPPH radicals scavenging assay DPPH (1,1-diphenyl-2-picryl-hydrazil) is a complex radical mixture used in an H^+ Ion transformed-based scavenging non-enzymatic antioxidant assay [15]. An earlier prepared 1 ml DPPH solution (0.1 mM) was added to 3 ml of different progressive concentrations (100, 250, 500, 750 and 1000 µg/ml) of lichen extract and instantly incubated in dark conditions for 30 min at room temperature. After incubation, the

absorbance was taken at 517 nm using a Janway-119 spectrophotometer. The radical scavenging activity (RSA) was measured in percentage (%) by using the formula Activity (%) = [(control Absorbance (A_0)—sample Absorbance (A_1))/control Absorbance (A_0)] × 100, where A_0 is the absorbance of the − ve control, and A_1 is the absorbance of the sample added reaction mixture or standards, i.e., butylated hydroxytoluene. Based on the percentage of radical scavenging activity, the IC-50 value was calculated according to the increase in percentage of radical scavenging. For comparative analysis, the natural antioxidant Ascorbate was used as a + ve control against the sample.

Ferric-reducing assay ET (electron transfer)-based antioxidant assay determined the ferric ion-reducing activity of both lichens by the method of Oyaizu [16]. The concentrations of a standard range of lichen extracts (100–1000 µg/ml) were mixed with 2.5 ml of PO_4 buffer of pH 6.6 (0.2 M) with 1% potassium ferricyanide and incubated for 25 min at 50 °C and subsequently mixed with 10% trichloroacetic acid, followed by centrifugation at 3000 rpm for 20 min. The collected supernatant was vigorously mixed with 2.5 ml of distilled water while simultaneously adding 0.5 ml of 0.1% $FeCl_3$, and absorbance was measured at 700 nm. For the comparative observation, BHT (butylated hydroxytoluene) was taken as the positive control.

Determination of antioxidant enzymatic assay

Preparation of cellular extract For analysis of superoxide dismutase and catalase activity, 0.5 g of lichen extract was prepared by using ethylene diamine-tetra acetate (50 mM), sodium phosphate buffer of pH 7.4 (50 mM), polyvinyl pyrrolidone 10% (*w*/*v*) and phenyl methyl sulfonyl fluoride (2 mM) in a frozen condition with a pestle in the dark. The well-gelatinous ground material was centrifuged at 14,000 rpm at 4 °C for 20 min in a collapsing centrifuge. After centrifugation, the supernatant was evaluated for SOD and CAT activities.

Superoxide dismutase (SOD) activity The activity and quantity measures of SOD were evaluated by the standard procedure of Das et al. [17]. This activity measured the superoxide-driven nitrite formation inhibition from hydroxylamine hydrochloride. The reaction cocktail was prepared in dark conditions by adding 1.11 ml of phosphate buffer of 50 mM (pH 7.8), 0.075 ml of 10 mM hydroxylamine hydrochloride, 0.04 ml of 1% Triton X-100 (*v*/*v*), 0.075 ml of L-methionine (20 mM), 0.1 ml of 50 mM EDTA and 80 µl of 50 mM, riboflavin to the mixture. After preparation of the reaction mixture, 10 min of light exposure is required to produce the appearance of white fluorescence and absorbance measured at 543 nm with the addition of Griess reagent. A single unit of enzyme activity represented the amount of SOD, which prevented 50% of nitrite formation. The enzymatic activity was measured using the formula V_0/V_{-1}, where V_0 (control absorbance) and V (sample absorbance). The activity and total enzyme were expressed in units nkat (nanokatal per liter)/mg or U (Unit per liter)/ml.

Catalase (CAT) activities The catalase activity of two tested lichens is evaluated by the method of Aebi [18]. The preparative chemical mixture for catalase analysis contained 2 ml of 0.1, potassium phosphate buffer (pH 6.8), an enzyme extract of 500 µL and H_2O_2 of 500 µL that reached a final volume of 3 ml. The catalase activity was measured over a 3-min time interval at 240 nm against the blank by the rate of H_2O_2 consumption.

Native-PAGE analysis for SOD and CAT enzyme For the analysis and estimation of total SOD and catalase, native-PAGE analysis was performed. The SOD and catalase staining was performed by Beauchamp and Fridovich [19]. Enzymatic analysis through native-PAGE is done by early preparation of 10% resolving and 5% stacking gels and then loaded the sample solution at 4 °C with a constant current of 40 V for 12 h. For gel support, 10% glycerol was added [20]. The gel was stained in dark for 30 min by using solutions (i.e., 50 mM sodium phosphate buffer of pH 7.8), tetramethylethylenediamine (28 mM), riboflavin (0.003 mM), NBT(0.25 mM) and EDTA (0.1 mM), and the gel after 30 min of light exposure, resulting in the visible protein bands. Similarly, the catalase staining was done in a dark condition. The gel was washed with water (ddH_2O) and stained with 0.003% H_2O_2 for 10 min. Thereafter, a mixture of 2% potassium ferric cyanide, 2% ferric chloride and 1% HCl was added for the catalase isoforms to appear dark green in the background of the gel. The activity and total enzyme were expressed in units nkat (nanokatal per liter)/mg or U (Unit per liter)/ml.

Fourier transform infrared spectroscopy (FTIR) In FTIR analysis, the IR spectra are equipped with a Thermo Nicolet iS10 FTIR spectrometer (Thermo Scientific, USA) with the Smart iTR attenuated total refraction (ATR) accessory. Soxhlated dry lichen extract is placed on a horizontal ATR crystal of the spectrometer under constant pressure. The sample is 32 scans across the range of (ν4000 to 400 cm^{-1}) with a resolution of 4.0 cm^{-1}, an absolute threshold of 96.395 and a sensitivity of 50. For analysis of spectral data, essential FTIR and prism software (version 8.0.1) was used, and all sample analysis was carried out in triplicate.

Gas chromatography–mass spectrometry analysis (GC–MS) For GC–MS analysis, methanol is used as a reliable

solvent for extraction. GC–MS analyses were performed on an Agilent Capillary-column 60.0 m × 250 µm × 25 µm. Injection in autosampler was used 1.5 µl of the sample split to 10:1. Oven: Initial temp 60 °C, ramp (temperature regulation) 7 C/min to 200 °C, (hold for 3 min), ramp 10 °C /min to 300 °C (hold 5 min), inject autosampler = 280 °C, Volume = 1.5 µl, split = 10:1, Helium as carrier gas, Solvent delay = 7.00 min, Transfer temp = 160 °C, Source temp = 150 °C, scan = 50 to 600 Da and column 60.0 m × 250 µm. The GC peak regions were used to calculate the percentage of extract composition. PubChem and the NIST Chemistry web-book used for analysis.

Determination of total phenolic The estimation of total phenols includes total aromatic and polyphenols and is expressed against the gallic acid standard calibration curve as GAE mg/g of total DE (dry extract). The Folin–Ciocalteu reagent method is used for estimating total phenol [21]. Lichen extract of 100 µl was taken and mixed with 2 ml of 2% sodium carbonate, and in a 10-min interval, 500 µl of Folin's reagent was added and incubated for 20 min. Absorbance was measured by Jenway-119 spectrometer at 650 nm.

Estimation of total flavonoid Total flavonoid estimation of both lichen species was carried out by method of Zhishen et al. [22] and expressed as quercetin equivalent, i.e., µg QE/g dry extract. One milliliter of Lichen extract was mixed with 10% of aluminum chloride (300 µl), 500 µl of sodium nitrite and 1 ml of 1 M hydroxide, and final volume was made up to 5 ml with dd H_2O. After a period of incubation absorbance was measured at 510 nm.

Estimation of total tannin The total tannin content of two lichens was estimated, expressed as tannic acid equivalent (TAE/mg/g DE) of dry extract and calibrated in a tannic acid calibration curve using the method of Oyaizu [16]. The lichen extract of 200 µg/ml was taken and added to 7 ml of distilled water, followed by 0.5 ml of Folin phenol reagent and 1 ml of a 35% sodium carbonate solution and volume adjusted with added dd H_2O and up to 10 ml. The mixture was shaken and incubated for 30 min, and absorbance was taken at 725 nm.

Estimation of total terpene The quantitative estimation of total terpene in both lichens was carried out by the standard procedure of Ghorai et al. [23], and total terpene was plotted against the linalool equivalent (a monoterpene) calibration curve and expressed as LE mg/g DW. Lichen extract of 1 ml was mixed with conc. 1 ml, conc. H_2SO_4 and 1 ml chloroform and then shaked gently, to take absorbance at 538 nm.

Results

Thallus morphology and identification

Pyrenula nitida PNSB04

Substrate: Mostly on bark, but also on non-calcareous rock, the genus is in its current delimitation characterized by the combination of clypeate ascomata and brown, distoseptate ascospores (brown, ellipsoid to fusiform) and simple paraphyses. Thallus: crustose, submerged but sometimes exposed, areolate, possibly with pseudocyphellae or crystal pockets, ascomata (perithecial) are black masses that are habitually grouped in pseudostromata. Ascomatal wall: black, generally continuous under the hamathecium and frequently partially buried in the thallus with true exciple (brown to pale brown, entire or not developed below the perithecial cavity, colorless or orange) (Fig. 2A, 3A).

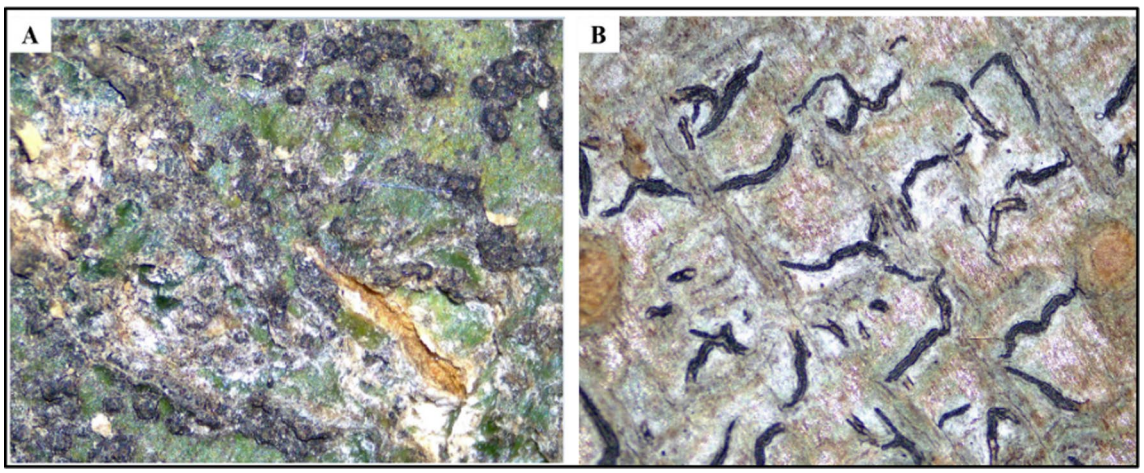


Fig. 2 **A** *Pyrenula nitida* and fig **B** *Graphis scripta* under stereo-zoom microscope (Stemi-305)

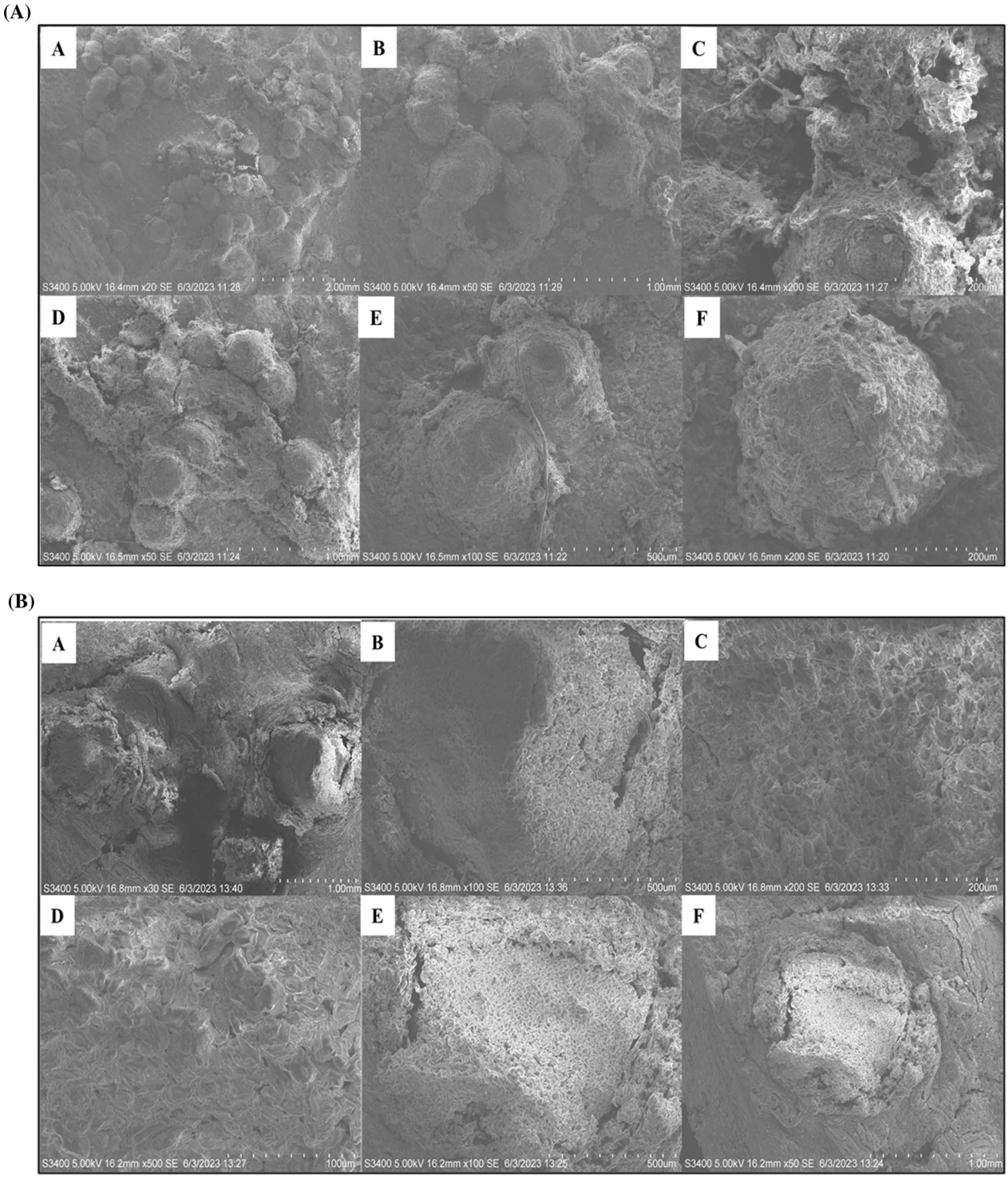


Fig. 3 **A** Scanning electron microscope (Model-S3400N) observation showing: A colonies of perithecium in upper surface of thallus, B–D bunch of ascomata (perithecium), E two perithecium with ostiolar canal opening, F individual perithecia with ascospore and ostiole canal opening of *P. nitida*. **B** Scanning electron microscope (Model-S3400N) observation showing: A and B branching Apothecia on thallus surface with dark gray to whitish pruina margin with black and thin exciple, C–Edistally carbonized excipular lips (base and entire), F individual apothecia with carbonized excipular lips and epihymenium of *G. scripta*

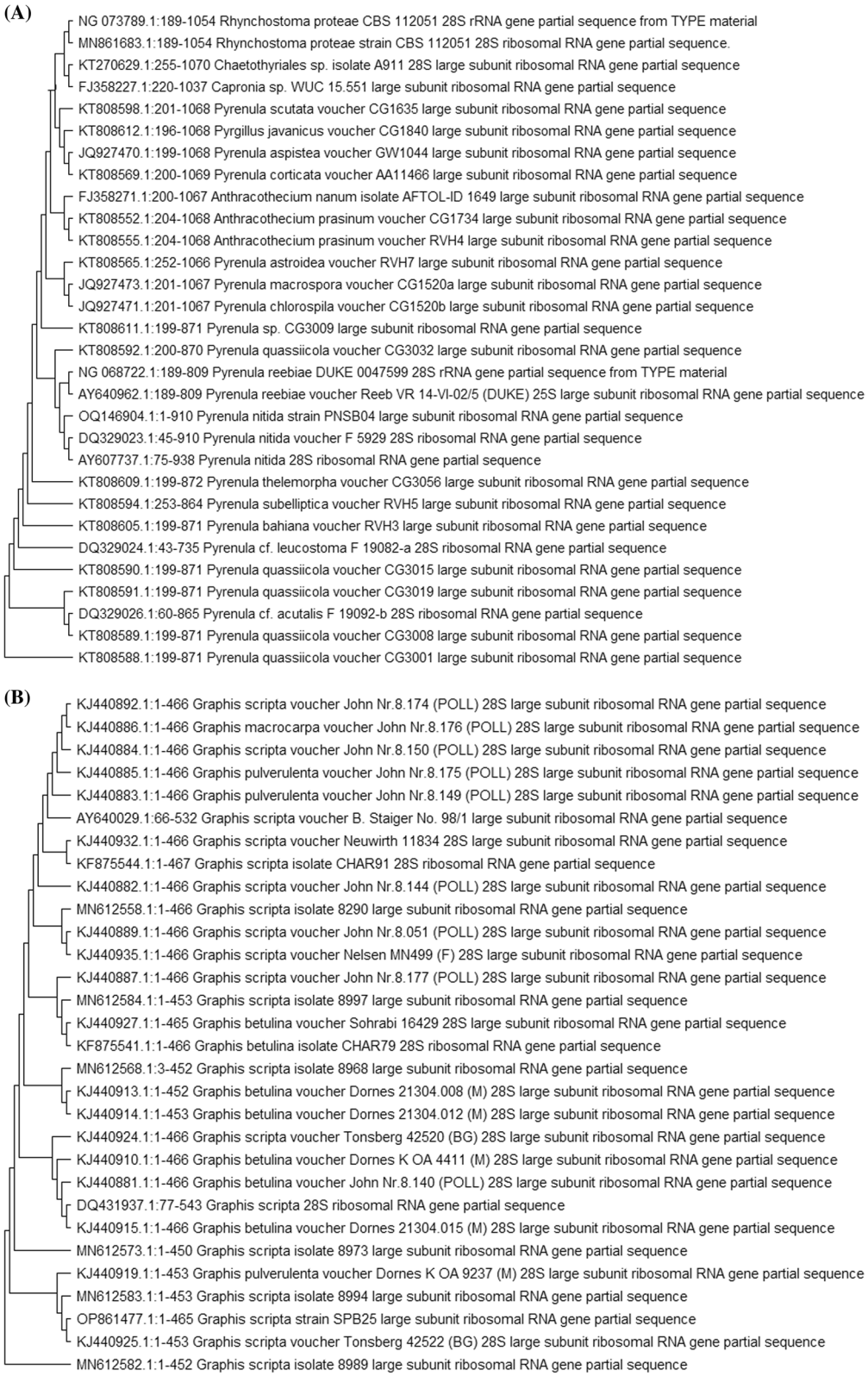


Fig. 4 **A** Phylogenetic analysis of *P. nitida* by using Minimum Evolution method of MEGA 11 software. **B** Phylogenetic analysis of *G. scripta* by using Minimum Evolution method of MEGA 11 software

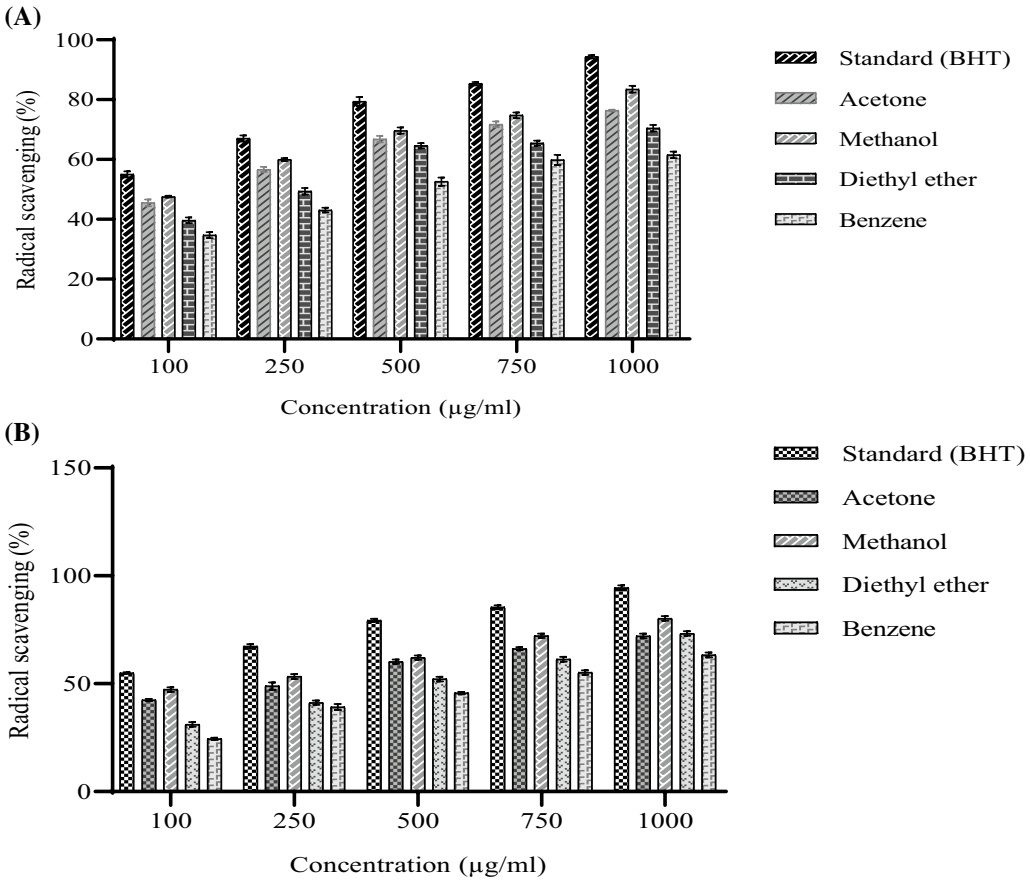


Fig. 5 **A** DPPH radical scavenging activity of *Pyrenula nitida* was carried out by taking 100–1000 µg/ml concentration of lichen extract in X-axis and scavenging activity (% of inhibition) in Y-axis, butylated hydroxy toluene (BHT) represented as standard. **B** DPPH radical scavenging activity of *Graphis scripta* was carried out by taking 100–1000 µg/ml concentration of lichen extract in X-axis and scavenging activity (% of inhibition) in Y-axis, butylated hydroxy toluene (BHT) represented as standard

Graphis scripta SPB25

Substrate: Found on the smooth bark of deciduous trees in humid and submontane zones. Thallus: crustose surface with a continuous to somewhat rugose surface: dull, cream-colored, white, pale gray or grayish green apothecia. Apothecia: oblong, flexuous and branching, 1–3×0.2–0.4 mm. Disc: narrow to broad and open, dark gray to brown with whitish pruina margin: strongly developed, covering the lateral half of the ascocarps excipular lips. Excipular lips: black, whole and occasionally thin Exciple: poorly formed and not carbonized at the base, and carbonized distally, entire excipular lips, the basal slice of which is typically less developed and less carbonized epihymenium. Epihymenium: brown, with distinctive brown or yellowish-brown tips ascospores (Fig. 2B, 3B).

Molecular identification

Evolutionary relationships of taxa

The evolutionary history analysis of both lichens using the Minimum Evolution method [24]. The bootstrap consensus tree inferred from 600 replicates that represent the evolutionary history of the taxa by analyzed Cluster-W preliminarily. Branches with lesser then 50% bootstrap are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of 600 replicates are shown next to the branches [25]. The evolutionary distances were measured by Maximum Composite Likelihood method and the molecular evolutionary tree (MET) tree was searched using the CNI (Close-Neighbor-Interchange) algorithm at a search level of 1. [26, 27] Here, the neighbor-joining algorithm was also used to generate the phyla analysis [28]. This analysis involved 30 closer neighbors with high accuracy of nucleotide similarity. There were a total of 928 positions *for P. nitida* PNSB04and 472 positions for *G. scripta* SPB25

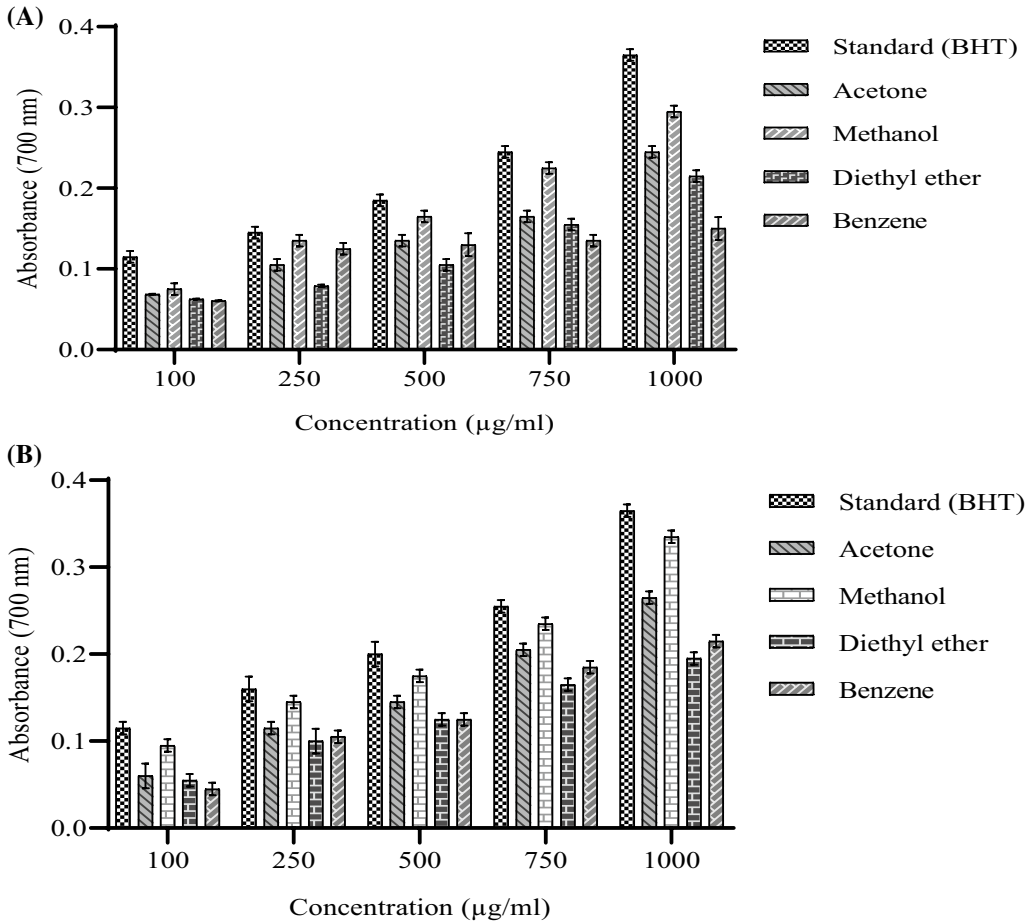


Fig. 6 **A** Ferric-reducing antioxidant power assay of *P. nitida* was carried out taking 100–1000 µg/ml concentration of lichen extract in X-axis and reducing activity (absorbance at 700 nm) in Y-axis. The experiment was carried out in triplicate, while the error bars indicate standard deviation among the replicates. **B** Ferric-reducing antioxidant power assay of *G. scripta* was carried out taking 100–1000 µg/ml concentration of lichen extract in X-axis and reducing activity (absorbance at 700 nm) in Y-axis. The experiment was carried out in triplicate, while the error bars indicate standard deviation among the replicates

in the final dataset (Fig. 4 (A&B)). Evolutionary analyses were conducted in MEGA11 software [29].

Phylogenetic relationship of taxa

Data validation All nucleotide is NCBI (National Centre for Biotechnology Information)/BLAST (http://www.ncbi.nlm.nih.gov/ Basic Local Alignment Search Tool) analysis and submitted to GenBank with accession number, *G. scripta* (OP861477) and *P. nitida* (OQ146904). Proofreads were edited and assembled in MEGA (Molecular Evolutionary Genetics Analysis-Version 11) and Sequencher v. 5.

Non-enzymatic antioxidant activity

DPPH scavenging activity

DPPH scavenging activities were analyzed in both lichens by using different solvent extractions such as acetone, methanol, diethyl ether and benzene in a particular concentration range (100, 250, 500, 750 and 1000 µg/ml). Both species highlighted significant radical scavenging in the methanol extracts i.e., *P. nitida* (83.09 ± 0.89%) (Fig. 5A) and *G. scripta* (80.65 ± 0.96%) (Fig. 5B). The acetone and diethyl ether extracts of both the lichens show moderate radical scavenging activity, whereas the benzene extracts have minimal scavenging activity. However, the methanolic extract of *P. nitida* showed comparatively higher scavenging activity with half the minimal concentration, IC-50 (165.76 ± 0.003 µg/ml) followed by *G. scripta* IC-50 (176.90 ± 0.025 µg/ml) in the final data set.

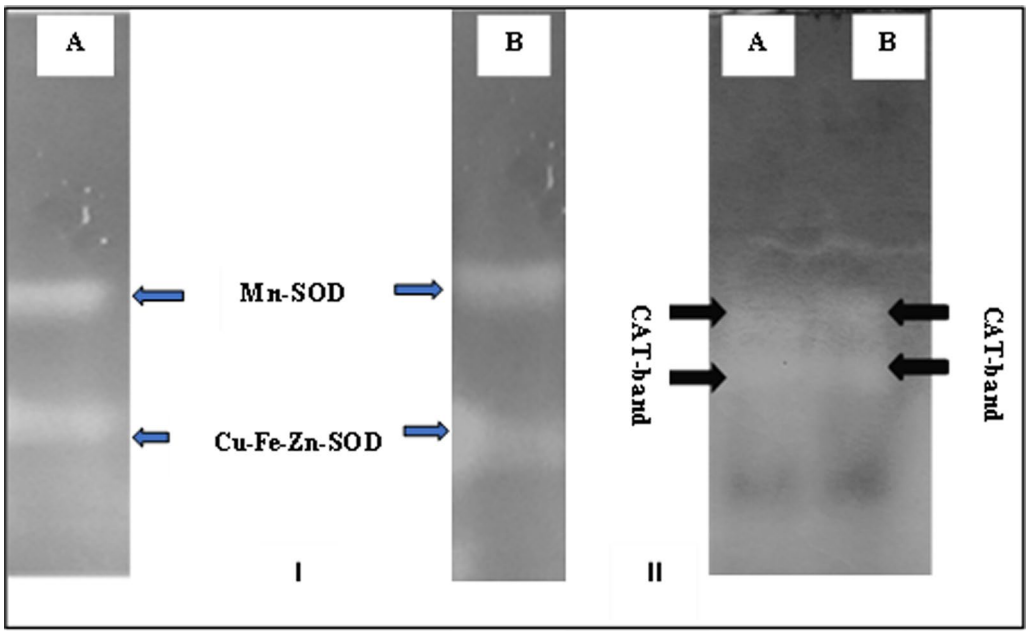


Fig. 7 **i** and **ii** Native-PAGE analysis of SOD and catalase enzyme in two lichens, arrow indicates SOD and catalase isoforms. A- *P. nitida* and B- *G. scripta*

Table 1 Quantitative phytochemical estimation of *P. nitida* and *G. scripta*

Solvents	*Pyrenula nitida*				*Graphis scripta*			
	Phenolic (mg/g) GAE	Flavonoid (mg/g) QE	Tannin (mg/g) TAE	Terpenoid (mg/g) LE	Phenolics (mg/g) GAE	Flavonoid (mg/g) QE	Tannin (mg/g) TAE	Terpenoid (mg/g) LE
Acetone	0.378 ± 0.013	0.031 ± 0.007	0.21 ± 0.0012	0.16 ± 0.041	0.297 ± 0.033	0.029 ± 0.01	0.184 ± 0.002	0.180 ± 0.0018
Methanol	0.511 ± 0.07	0.049 ± 0.002	0.47 ± 0.003	0.192 ± 0.003	0.682 ± 0.008	0.041 ± 0.03	0.32 ± 0.007	0.252 ± 0.0012
Diethyl ether	0.2 ± 0.0014	0.21 ± 0.008	0.134 ± 0.001	0.144 ± 0.003	0.197 ± 0.035	0.018 ± 0.002	0.11 ± 0.003	0.162 ± 0.003
Benzene	0.191 ± 0.04	0.014 ± 0.001	0.114 ± 0.004	0.082 ± 0.002	0.157 ± 0.071	0.011 ± 0.014	0.1 ± 0.006	0.096 ± 0.008

Ferric-reducing activity

The ferric-reducing activity of both the lichens was analyzed using four organic solvents such as acetone, methanol, diethyl ether and benzene. The reducing activity was significant in methanolic extract of test lichen species followed by acetone, diethyl ether and benzene. However, the methanol extract of *P. nitida* and *G. scripta* exhibits comparatively maximal ferric ion reducing activity (0.35 ± 0.051 and 0.33 ± 0.036 nm) (Fig. 6A and B) in the final data set against standard butylated hydroxytoluene (BHT).

Enzymatic antioxidant activities (SOD and CAT)

The cellular extracts of two microlichens were evaluated for SOD and CAT activity. The better SOD and CAT activities were obtained in *P. nitida* (34 U/ml or 566. 667 nkat/mg and 3.120 U/ml or 52 nkat/mg), compared to *G. scripta* (27 U/ml or 450 nkat/mg and 2.280 U/ml or 38 nkat/mg). Further, in 2D native-PAGE analysis, *P. nitida* showed two significant isoforms of SOD, such as mitochondrial Mn-SOD and cytosolic Cu–Zn- Fe-SOD**,** whereas *G. scripta* had purely a single cytosolic Cu–Zn-SOD. On the contrary, both lichens from the same habitat contained two isoforms of CAT in native gel analysis (Fig. 7 i, ii).

Quantitative estimation of total phenol, flavonoid, tannin and terpene

Quantitative phytochemical estimation of lichens was carried out by estimating major phytochemicals, i.e., phenols, flavonoids, tannins and terpenes. Maximum phytochemical content was recorded in methanol extract of both the lichens i.e., *Pyrenula nitida* and *Graphis scripta* (Table 1).

Phytochemicals were estimated in triplicate and expressed as mg/g of dry extract, phenol (GAE—gallic

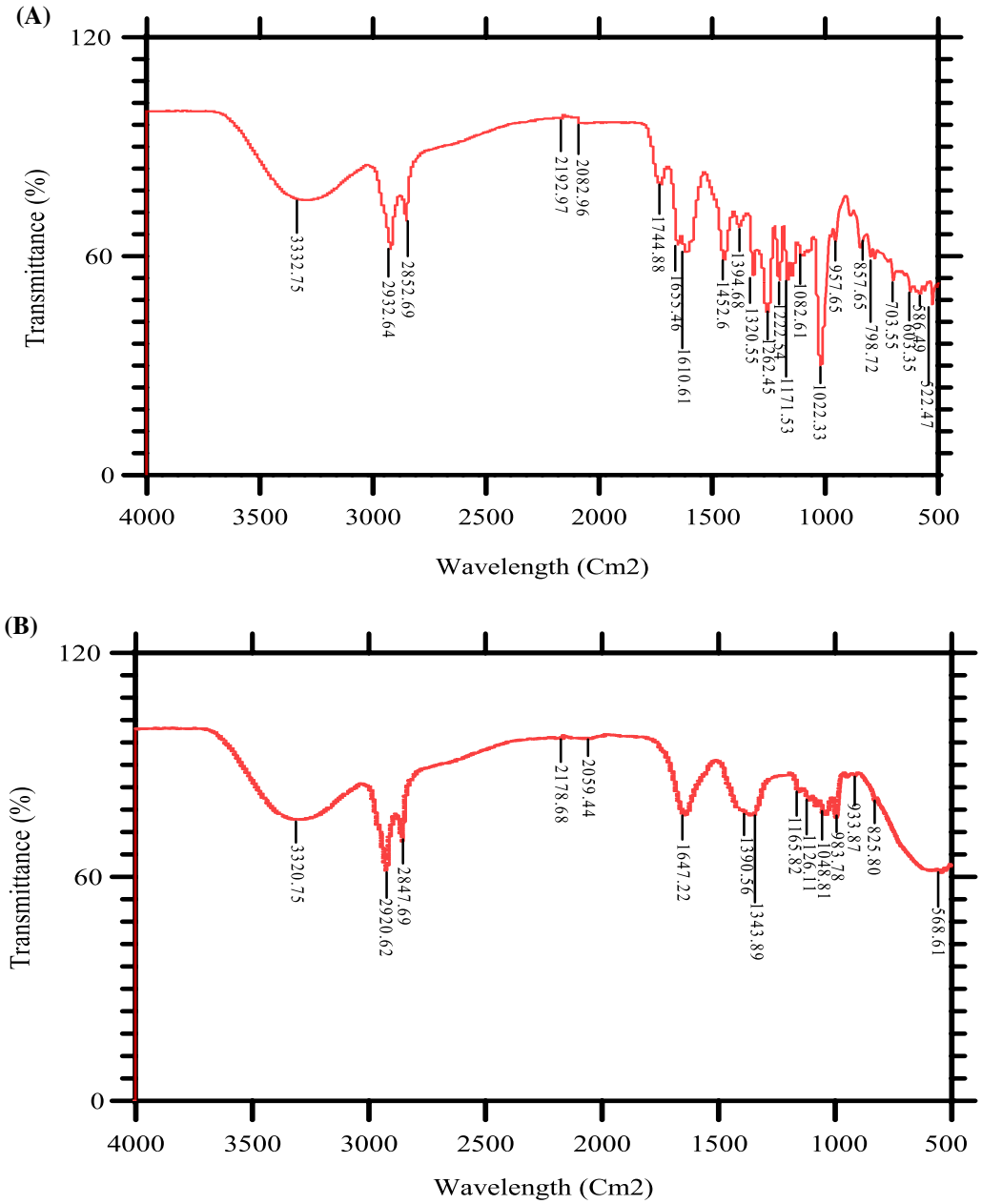


Fig. 8 **A** FTIR analysis *P. nitida*, wavelength in ranges between (500 and 4000 Cm^2), wavelength in plotted in X-axis and transmittance (%) in Y-axis, graph with numerical order showing presence of functional group in IR spectrum range (ν). **B** FTIR analysis *G. scripta*, wavelength in ranges between (500 and 4000 Cm^2), wavelength in plotted in X-axis and transmittance (%) in Y-axis, graph with numerical order showing presence of functional group in IR spectrum range (ν)

acid equivalent), flavonoid (QE—quercetin equivalent), tannin (TAE—tannic acid equivalent) and terpenoid (LE—linalool equivalent), and data were interpreted as mean and standard deviation.

FTIR analysis

For FTIR analysis for *P. nitida* (L_1), here, IR spectrum shows bands with following frequencies (cm − 1) (Fig. 8A), ν Amines, Amides (N–H stretch) and Alcohols (O–H stretch), Alkanes (C–H stretch), (3332–2932 cm^{-1}), νAlkanes, Aldehydes (C–H stretch), and Carboxylic acid (O–H stretch) (2932–2853 cm^{-1}),

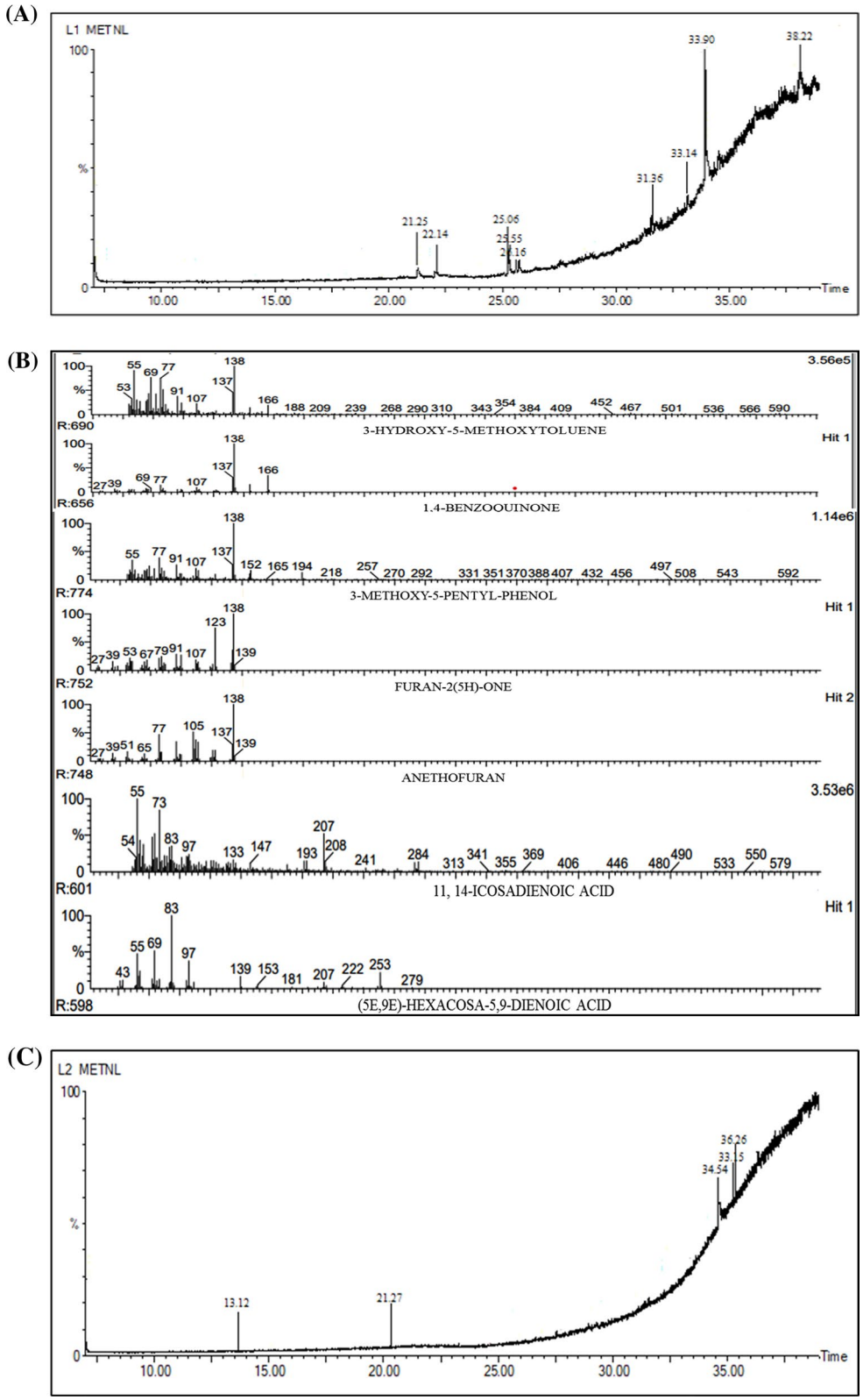


Fig. 9 **A** Gas chromatogram in *P. nitida* showing retention time in the X-axis and % of peak area in the Y-axis, autosampler ejection and running carried out thrice for retention peak conformation. **B** Major compounds in chromatogram of *P. nitida*. **C** GC–MS chromatogram of *G. scripta* showing retention time in the X-axis and % of peak area in the Y-axis, autosampler ejection and running carried out thrice for retention peak conformation. **D** Major compounds in chromatogram of *G. scripta*

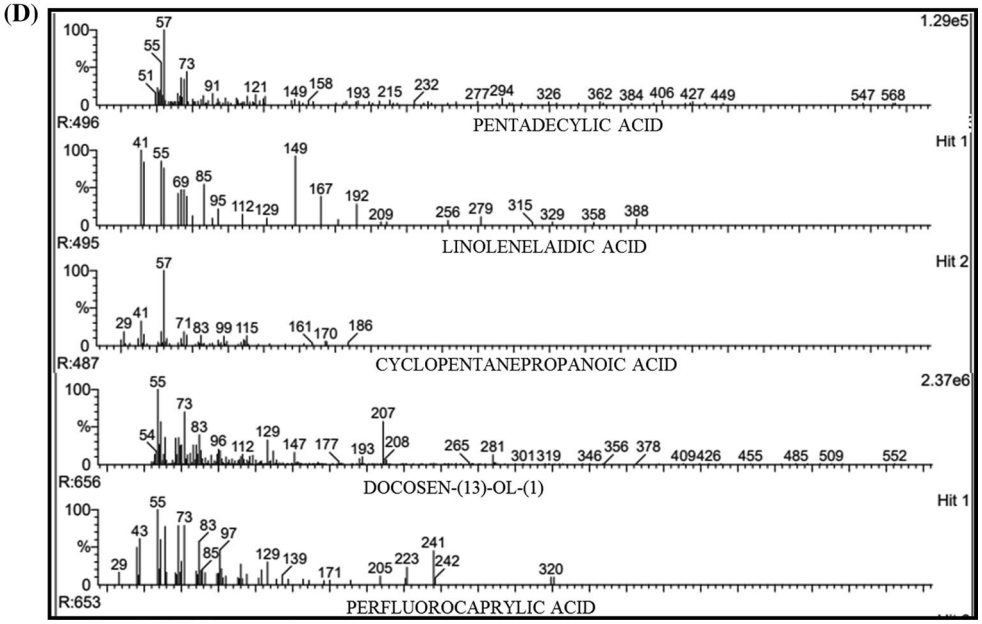


Fig. 9 continued

νAlkanes, Aldehydes (C–H) and Alkynes (C≡C) and (2853–2192 cm^{-1}), νAlkynes (C≡C stretch), Carboxylic acid, Esters, Ketones, Aldehydes, Amides, Anhydrides and Acid chlorides (C=O stretch) (3192–1744 cm^{-1}), νAlkenes (C=C stretch), Aromatic compounds (C=C stretch and C–H bend), Amides (C=O stretch), and Nitro compounds (NO_2 stretch) (1744–1452 cm^{-1}), νAlkyl and Aryl Halides (C–F stretch, C–Cl stretch and C–Br stretch) and (1394–857 cm^{-1}).

For FTIR analysis for *G. scripta* (L2), Here, IR spectrum showing bands with following frequencies (cm^{-1}) (Fig. 8B), νAlkynes (≡C–H stretch), Alcohols (O–H stretch), Amines (N–H stretch), Amides (N–H stretch), Alkanes, Aldehyde (C–H stretch) and Carboxylic acid (O–H stretch) (3320–2920 cm^{-1}), νAlkanes, Aldehydes (C–H stretch), and Carboxylic acid (O–H stretch) (2920–2847 cm^{-1}), νAlkynes (C≡C stretch) (2178–2059 cm^{-1}), νAlkenes, Aromatic compounds (C=C stretch), Amides (C=O stretch), Alkyl & Aryl Halides (C–F stretch), and Nitro compounds (NO2 stretch) (1647–1048 cm^{-1}), νAlkyl and Aryls (C–F stretch), Alkenes (=C-H bend) and Aromatic compounds (C-H bend) (1048–568 cm^{-1}).

GC–MS analysis

Gas chromatography–mass spectrometry screened for lichens and some compounds are analyzed through gas chromatography and other known and unknown class of compounds are analyzed throughout mass spectrometry (Fig. 9A–D). Compounds classes like phenols, Quinons, benzofurans, heterocyclic compounds, pyrans, hydrocarbons, carboxylic acid, aliphatic aldehydes, alkanes and fatty acids characterized through gas chromatography, while mass spectrometry detected some classes of compounds like phenolics, organic alcohol, fatty acids, fluorinated aliphatics, ketones, terpenes and fatty alcohols in *P. nitida* (Table 2). However, in *G. scripta*, GC detected fatty acids, alcohols, hydrocarbons, carbonyl compounds and fatty alcohols, and MS detected terpenes, glycosides, phenols and sugar alcohols in highlighted pick areas of chromatogram (Table 3).

Statistical analysis

Statistical analyses were performed with the EXCEL, IBM-SPSS and GraphPad Prism software version 8.0.1. Data were presented as mean ± standard deviation (SD) of three replicates. The *P*-values less than 0.05 were considered significant.

Discussion

In the current study, both the tested lichens have marked antioxidant properties; through the intensity of antioxidant activity in *in-vitro* oxidative systems depends upon the solvents used for extraction. The variations in the antioxidant potential of solvents could be related to their ability to extract bioactive molecules [30], whereas the aqueous extracts had the least amount of antioxidant activity because lichen metabolites are partially soluble or insoluble in aquas [31]. Thus, organic solvents were

Table 2 Bioactive compounds obtain from *P. nitida* through GC–MS analysis with their molecular formula, compound class, retention time, percentage (%) of area and retention index

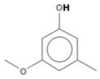
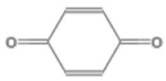
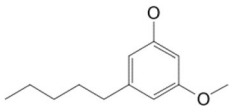
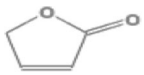
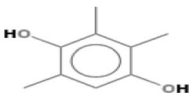
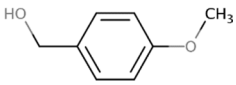
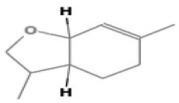
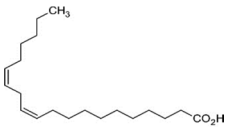
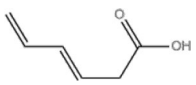
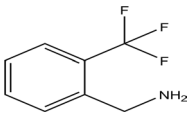
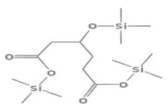
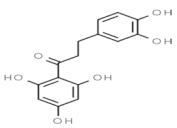
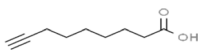
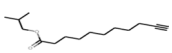
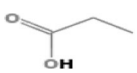
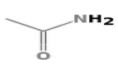
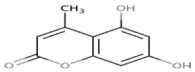
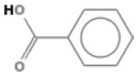
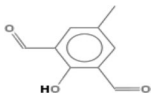
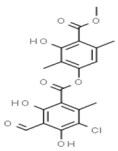
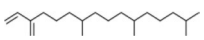
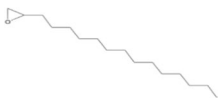
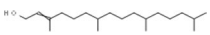
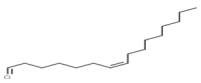
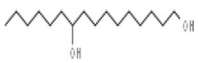
Compound	Compound structure	Compound class	Retention time	% of area	Retention index
3-Hydroxy-5-methoxytoluene ($C_8H_{10}O_2$)		Phenol	21.25	1.27	1342
1,4-BENZOQUINONE ($C_6H_4O_2$)		Quinone	21.25	1.27	143
3-methoxy-5-pentyl-phenol ($C_{12}H_{18}O_2$)		Phenolics	25.06	2.08	–
Furan-2(5H)-one ($C_4H_4O_2$)		Heterocyclic compound	25.06	2.08	915
2,3,5-trimethylbenzene-1,4-diol ($C_9H_{12}O_2$)		Phenols	25.06	2.08	–
[4-(hydroxymethyl) phenyl] methanol ($C_8H_{10}O_2$)		Alcohol	25.06	2.08	–
Anethofuran ($C_{10}H_{16}O$)		Benzofurans	25.06	2.08	1178
11, 14-icosadienoic acid ($C_{20}H_{36}O_2$)		Fatty acid	33.90	11.38	–
(5E,9E)-hexacosa-5,9-dienoic acid ($C_{26}H_{48}O_2$)		Fatty acid	33.90	11.38	–
4-Pentyl-5-propyl-2,2-bis(trifluoromethyl)-1,3-dioxolane ($C_{13}H_{20}F_6O_2$)		Fluorinated substances	38.22	0.77	–
2-trimethylsiloxy-6-hexadecenoic acid ($C_{20}H_{40}O_3Si$)		Fatty acid	38.22	0.77	–
1-(2,4,6-trihydroxyphenyl) propan-1-one ($C_9H_{10}O_4$)		Ketones	22.14	0.62	–
Non-8-ynoic acid ($C_9H_{14}O_2$)		Fatty acids	22.14	0.62	–
Undec-10-ynoic acid ($C_{11}H_{18}O_2$)		Fatty acids	22.14	0.62	–
Ethylformic acid ($C_3H_6O_2$)		Fatty acids	22.14	0.62	745
Methanecarboxamide (C_2H_5NO)		Fatty acids	25.55	0.42	1763

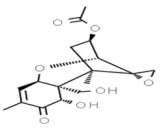
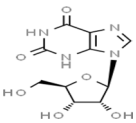
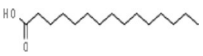
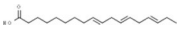
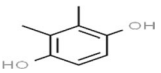
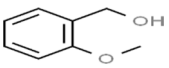
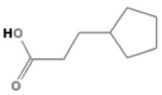
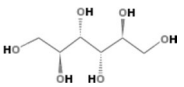
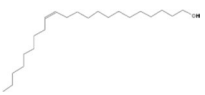
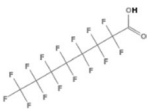
Table 2 (continued)

Compound	Compound structure	Compound class	Retention time	% of area	Retention index
Tetrahydrobenzene (C_6H_{10})		Hydrocarbons, cyclic	25.55	0.42	674
4-Methyl-5,7-dihydroxycoumarin ($C_{10}H_8O_4$)		Pyrans	25.55	0.42	2451
Benzenecarboxylic acid ($C_7H_6O_2$)		Carboxylic acid	26.16	0.76	2380
1,3-Benzenedicarboxaldehyde, 2-hydroxy-5-methyl- ($C_9H_8O_3$)		Phenols	26.16	0.76	–
Chloratranorin ($C_{19}H_{17}ClO_8$)		Phenols	26.16	0.76	–
(Z)-Octadec-9-enoic acid ($C_{18}H_{34}O_2$)		Fatty acid	31.36	0.96	–
7,11,15-trimethyl-3-methylidenehexadec-1-ene ($C_{20}H_{38}$)		Terpene	31.36	0.96	–
2-Tetradecyloxirane ($C_{16}H_{32}O$)		Alkanes	31.36	0.96	1708
2-Hexadecen-1-ol, 3,7,11,15-tetramethyl- ($C_{20}H_{40}O$)		Terpene	31.36	0.96	–
(Z)-7-Hexadecenal ($C_{16}H_{30}O$)		Aliphatic aldehyde	31.36	0.96	2144
1,10-hexadecanediol ($C_{16}H_{34}O_2$)		Fatty alcohols	33.14	0.73	–

used which withstand significant extraction of bioactive metabolites. Several findings proved that environmental factors such as air pollution, desiccation, high temperatures, rehydration and high light have a great influence on the antioxidant activity of lichens, affecting the increasing as well as decreasing and synthesis of antioxidants [32, 33]. Formerly, it was concluded that natural compounds, including tocopherols, phenolics, flavonoids, terpenes and carotenoids, have strong antioxidant activity. Due to their ability to scavenge free radicals such as singlet oxygen, superoxide and hydroxyl radicals, phenolic ingredients such as flavonoids, tannins and lichen phenolics such as depsides, depsidones, diphenyl ethers, polycyclic and monocyclic aromatic classes are classified as high-level antioxidants [34].

The present study was aimed at exploring the antioxidant potential, screening and characterization of the total phytochemicals of *Graphis scripta* and *Pyrenula nitida*. For this purpose, enzymatic (SOD and catalase) and non-enzymatic antioxidant activities (DPPH and FRAP), reducing power, and total phenolic, flavonoid, tannin and terpene content of the extracts of both lichens were determined, in vitro. Tatipamula and Kukavica [35] examined *D. consimilis* with acetone (DA) and methanol (DM) extracts against DPPH assays. From their findings acetone extract of *D. consimilis* showed better IC50 values of 80.90 ± 6.42 mg/mL against DPPH compared to DM with 95.04 ± 6.36 mg/mL. As indicated in our findings, relatively maximal radical scavenging was measured in the methanol extract of *P. nitida* at 83.99% of RSA with an IC-50 62.90 ± 0.003 mg/ml and it was also rich in terms of total phytochemicals. From our previous findings indicated methanol extracts of *D. applanata* and *P. andium* have strong DPPH scavenging activity with IC50 i.e., 471.16 ± 0.85 µg/ml and 534.77 ± 0.75 µg/ml with acetone extract showing comparatively lower scavenging

Table 3 Bioactive compounds obtain from *G. scripta* through GC–MS analysis with their molecular formula, compound class, retention time, percentage (%) of area and retention index

Compound	Compound structure	Compound type	Retention time	% of area	Retention index
Deoxynivalenol 3-acetate ($C_{17}H_{22}O_7$)		Terpenes	21.27	1.27	-
2,3-Dihydroxanthosine ($C_{10}H_{12}N_4O_6$)		Glycosides	13.12	1.18	-
Pentadecylic acid ($C_{15}H_{30}O_2$)		Fatty acids	34.54	4.46	2822
Linolenelaidic acid ($C_{20}H_{34}O_2$)		Fatty acids	34.54	4.46	1671
2,3-dimethylbenzene-1,4-diol ($C_8H_{10}O_2$)		Phenol	34.54	4.46	-
Benzenemethanol, 2-methoxy- ($C_8H_{10}O_2$)		Alcohol	34.54	4.46	2083
4-propan-2-ylcyclohex-3-ene-1-carbaldehyde ($C_{10}H_{16}O$)		Hydrocarbons	34.54	4.46	1195
Cyclopentanepropanoic acid ($C_8H_{14}O_2$)		Carbonyl compound	34.54	4.46	2168
D-mannitol ($C_6H_{14}O_6$)		Sugar alcohols	36.26	4.46	-
Docosen-(13)-ol-(1) ($C_{22}H_{44}O$)		Fatty alcohols	33.15	0.73	2468
Perfluorocaprylic acid ($C_8HF_{15}O_2$)		Fatty acids	33.15	0.73	1046

in both species [36]. Thus, present findings are in agreement with the experimental results of previous workers Manojlović et al.; Hawrył et al. [37, 38].

According to some researchers, complex ion-reducing power is usually a better indicator of the antioxidant capacity of extracts and purified compounds. Thus, the extracts, which had metal ion-reducing power, were considered a potential antioxidant source [15]. While Gordan [39] established antioxidant effect of reaction chain termination of the free radicals by the donation of electrons, the reduction of ferrous ion (Fe-3) to ferric ion (Fe-2) is measured qualitatively by the density of visibility (green–blue outline of solution) that measured by spectroscopically (absorbance at 700 nm). The reducing power of the methanol extract of *Cetraria islandica* may also indicate its potential antioxidant activity [40]. However, the reducing power of the methanol extract of *P. nitida* was higher as compared to other solvents (0.33 ± 0.054 nm). Earlier study reported methalonic extracts of *P. nitida* had strong ferric-reducing activity compared to *G. scripta*, which related to [36]. The reducing features are mainly linked to the presence of ionic reduction reactions. The outcomes provided here suggest that the extract's high ferric-reducing power activity is related to the presence of polyphenols (phenol and its derivatives), which may operate similarly to reductions in that they react with free radicals to convert them into stable products and terminate free radical chain reactions [41]. SOD and catalase estimation by native-PAGE analysis of *Pyrenula nitida* and *Graphis scripta* was used for

the first time to perform qualitative enzymatic antioxidant analysis.

Multiple studies have found a link between lichen antioxidative activity and the quantity of phenolic compounds content [42]. Kosanić and Ranković [43] estimated the total phenolic and flavonoid constituents in *C. furcata* and *U. polyphylla* and *H. physodes* at 52.76 µg/ml of pyrocatechol equivalents (PE); however, higher phenolic contents were also found in methanol extracts of *C. furcata* and *U. polyphylla* with 52.67 and 52.65 µg/ml PE. But in the present study of total phenol estimated against gallic acid equivalent (GAE mg/g) using acetone, methanol, diethyl ether and benzene extract, maximal phenol content was reported in methalonic extracts of both lichens, *P. nitida* (0.511 ± 0.07 mg/g GAE) and *G. scripta* (0.682 ± 0.008 mg/g GAE), whereas benzene extract showed relatively lower content in both lichen species. Total flavonoid content was found in the acetone and methanol extracts of *U. polyphylla* (30.31 µg/ml of Rutin equivalents (RE) for acetone and 47.24 µg/ml for methanol extracts). Good flavonoid content was also found in the acetone and methanol extracts of the lichen *H. physodes* (30.09 µg/ml of RE for acetone and 32 µg/ml of RE for methanol extracts) as reported by [44]. The methalonic extract of both lichens reported a higher content of flavonoid *P. nitida* (0.049 ± 0.002 mg/g QE) and *G. scripta* (0.041 ± 0.03 mg/g QE), among other solvents. However, the quantification of total tannin against tannic acid equivalent (TAE mg/g) and total terpene against linalool equivalent (LE mg/g) was carried out for the first time and the relatively higher content of tannin and terpene recorded was methanolic extract of both lichens, i.e., *P. nitida* (0.47 ± 0.03 mg/g TAE and 0.252 ± 0.012 mg/ml LE) and *G. scripta* (0.32 ± 0.007 mg/g TAE and 0.192 ± 0.006 mg/ml LE); these estimations support the previous findings that methanol extract of *Trypethellium virens* SPTV02 and *Phaeographis dendritica* SPB041 quantified relatively maximum and acetone and diethyl ether contains moderate and benzene extract quantify lowest in all tested lichens [45].

Conclusions

In vitro antioxidant and phytochemical screening of both lichens showed high bioactive potential. GC–MS and FTIR analysis confirm the presence of active metabolites, including primary and secondary metabolites such as phenols, quinons, benzofurans, carboxylic acid, alkanes, aliphatic aldehydes, organic alcohol, fluorinated aliphatic compounds, ketones, terpenes, and fatty alcohols, fatty acids, alcohols, hydrocarbons, fatty alcohols, terpenes, glycosides and sugar alcohols. As a result, this investigation deals with the use of lichen extracts as natural antioxidants. These lichens appear to be promising and alternate supply of synthetic antioxidant chemicals as well as a momentous source of polyphenols. Upcoming research should essence on isolating different bioactive phenolic compounds at the next step to assess their natural qualities in vitro and in vivo having a wider application in pharmaceuticals and cosmeceuticals.

Abbreviations

$AlCl_3 \cdot H_2O$	Aluminum chloride
ANOVA	Analysis of variance
ATR	Attenuated total refraction
BHT	Butylated hydroxyl toluene
CAT	Catalase
OD	Optical density
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-Diphenyl-2-picryl-hydrazil
$FeCl_3$	Ferric chloride
FRAP	Ferric-reducing antioxidant power assay
FTIR	Fourier transform infrared spectroscopy
G. scripta	*Graphis scripta*
GAE	Gallic acid equivalent
GC–MS	Gas chromatography–mass spectrometry
H_2O_2	Hydrogen peroxide
IC_{50}	Half-maximal inhibitory concentration
$K_2S_2O_8$	Potassium persulfate
$K_3Fe\ (CN)_6$	Potassium ferricyanide
KCI	Potassium chloride
LE	Linalool equivalent
Na_2CO_3	Sodium carbonate
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NIST	National Institute of Standards and Technology
P. nitida	*Pyrenula nitida*
PCR	Polymerase chain reaction
PO_4 buffer	Phosphate buffer
PUFAs	Polyunsaturated fatty acids
QE	Quercetin equivalents
ROS	Reactive oxygen species
SD	Standard deviation
SOD	Superoxide dismutase
TAE	Tannic acid equivalent
TCA	Trichloro acetic acid
TE buffer	Tris–EDTA buffer
TFC	Total flavonoid contents
TPC	Total phenolic content

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Declarations

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Consent for publication
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