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Assessment of lichen metabolites as possible natural antioxidant and antimicrobial agents

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ABSTRACT

Background: Lichens are known to produce diverse secondary metabolites with significant pharmacological properties, including antioxidant and antimicrobial activities. However, studies on lichens from the Similipal Biosphere Reserve remain limited. **Objective**: The present study aimed to evaluate the phytochemical profile, antioxidant potential, and antimicrobial activity of four predominant lichen species from the Similipal Biosphere Reserve. **Materials and Methods**: Four lichens *Arthonia polymorpha* (OQ845612), *Harpothellon rubrocincta* (OQ832495), *Parmelia saxatilis* (OQ825976), and *Parmotrema reticulatum* (OQ832494) were collected and identified using both morphological and molecular (18S rRNA, 28S rRNA, 5.8S rRNA, 1.5S rRNA, and ITS regions) approaches. The antioxidant activity was determined using radical scavenging assays (DPPH and ABTS) and enzymatic assays (SOD and CAT). Antimicrobial activity was evaluated using antibacterial assays and determination of minimum inhibitory concentration (MIC) values. GC-MS analysis was performed to identify bioactive secondary metabolites. **Results**: All lichen methanolic extracts exhibited promising radical scavenging activity in both DPPH and ABTS assays. Enzymatic antioxidant analysis revealed multiple isoforms of SOD (cytosolic and mitochondrial) and significant CAT activity. Antibacterial assays demonstrated that methanol and acetone extract of *P. reticulatum* and *P. saxatilis* had strong bactericidal effects, with notable MIC values against pathogenic bacteria. GC-MS profiling identified several bioactive compounds including polyphenols, hydroquinones, coumarins, alkaloids, flavonoids, arenes, meta-divarinol, benzoquinones, terpenes, azaarenes, and aromatic ethers, particularly in *P. saxatilis* and *P. reticulatum*. These compounds are associated with antioxidant, antibacterial, and cytotoxic activities. **Conclusion:** This study highlights the pharmacological significance of secondary metabolites present in lichens from the Similipal Biosphere Reserve. The findings demonstrate their potential as valuable bioresources for future therapeutic applications.

Keywords: Lichens, Secondary metabolites, Antioxidant activity, Antimicrobial activity, GC-MS analysis, Similipal Biosphere Reserve.

INTRODUCTION

The Similipal Biosphere Reserve, spanning 2750 square kilometers in eastern India and commonly known as the Similipal Tiger Reserve, boasts significant flora and fauna. This reserve is home to several species of lichens, indicating that it is an ecologically healthy ecosystem. However, due to habitat loss and changing climatic conditions, there is a need for new lichen assessments, and some lichens may have permanently lost their habitats. Despite these challenges, several lichen families such as Parmeliaceae, Graphidaceae, Arthoniaceae, Caliciaceae, and Trypetheliaceae have maintained their presence throughout the reserve. During extreme conditions, the intrinsic resistance of lichens produces diverse spectrum of secondary metabolites. Lichens produce several more different chemical substances, including aliphatic acids, depsides, depsidones, diterpenes, dibenzofurans, naphthoquinones, anthraquinones, pulvinic acids, usnic acids, and xanthones [1-4], which has prominent antimicrobial, antioxidant, and cytotoxic activities [5]. Lichen genera like Parmelia and Parmotrema have traditionally been used as condiments, therapeutic drugs, and spices in Unani medicines [6-8]. Accordingly, recent study has been designed to evaluate bioactivity of four lichen species, *Arthonia polymorpha*, *Harpothellon rubrocincta*, *Parmotrema reticulatum*, and *Parmelia saxatilis* isolated from Similipal, for their *in vitro* antioxidant (enzymatic and non-enzymatic) and antimicrobial properties. Additionally, a GC-MS-based phytochemical screening analysis has been conducted. These studies suggest that lichens are a potential source of pharmaceutical compounds with several therapeutic applications.

MATERIAL AND METHODS

Study site and sample collection

The Similipal Biosphere Reserve, located between 85°40′ E and 87°10′ E in the Mayurbhanj district of Odisha State, India, features dry deciduous vegetation and is known for its rich biodiversity. Frequent field visits were conducted, and documentation was carried out using a random sampling technique. Samples were collected, wrapped in white paper bags, packed in polythene bags, and transported to the research laboratory for analysis. During the collection process, the altitude of each sample site was recorded using Garmin Etrex GPS device. The temperature and humidity at each site were measured with a hygrometer (HTC), and detailed microhabitat data were recorded for each transect. This comprehensive approach ensured accurate documentation of the environmental conditions and facilitated a thorough analysis of the collected samples.

Morphological Identification

Several samples were collected, separated, and identified based on their morphology using the research microscope (compound microscope, Carl Zeiss Stemi 304) and lichen identification manual [9-14]. Various lichen species from SBR were explored and were identified based upon their morphological features and deposited at the Department of Biotechnology, Maharaja Sriram Chandra Bhanja Deo University (MSCBU). Among all collected lichen species *Harpothellon rubrocincta*, *Arthonia polymorpha*, *Parmelia saxatilis* and *Parmotrema reticulatum* were used for experimental purposes.

Genomic DNA isolation

Lichen sample (10 mg) was placed in a micro-vial with three or four 2.5 mm sterile glass beads and submerged in liquid nitrogen. The sample was then disrupted using a Mini-Beadbeater-24 for 30 seconds. Following this, the sample was vigorously shaken about 20 times vertically with 300 µL of KCl extraction buffer. An equal volume of chloroform (300 µL) was added, and the mixture was kept inverted. The sample was centrifuged at 12,000 g for 1 minute at room temperature. The upper aqueous layer was transferred to a new microcentrifuge tube, and 180 µL of chilled isopropanol was added to reach a final volume of 60%. The mixture was gently combined and centrifuged again at 12,000 g for 1 minute at room temperature, after which the supernatant was discarded. The pellet was washed with 300 µL of 70% chilled ethanol, then dried in an oven at 55 °C for 5 minutes. Finally, 100 µL of TE buffer was added to resuspend the pellet. The purified DNA was stored at 4 °C [15].

PCR and sequencing

For the 18s, 28s and ITS sequencing, rDNA sequence generated by our research group, partial genomic DNA was isolated from the mycelium of the upper cortex region. Then the PCR amplification of ribosomal DNA (rDNA), including the internal transcribed spacer regions (ITS1 and ITS2), 5.8S rDNA (HRC1 and HRC2), and rDNA (PRA1 and PRA2), was performed using specific primers for different lichen strains followed by the method of White et al., [16]. For *Harpothellon rubrocincta* (strain CRSBR14), the forward primer HC1 (5′-ACGGCGCCCAGCTCCCCAA-3′; GC: 73.7%, Tm: 67.9ºC, ΔG: -47.02 kcal/mol) and the reverse primer HRC2 (5′-GAGTGGGGCTGCCCAACGG-3′; GC: 73.7%, Tm: 64.2ºC, ΔG: -43.57 kcal/mol) were used. For *Parmotrema reticulatum* (strain PRSBR04), the forward primer PRA1 (5′-TAGCGGAAATCCTCAGCATTTC-3′; GC: 45.5%, Tm: 55.2ºC, ΔG: -42.72 kcal/mol) and the reverse primer PRA2 (5′-TCGGATCAGGTAGGGACCC-3′; GC: 63.2%, Tm: 58.2ºC, ΔG: -38.6 kcal/mol) were used. For *Arthonia polymorpha* (strain APSBR04), the forward primer API1 (5′-GGCCCCGCCCGGGGTCCGA-3′; GC: 89.5%, Tm: 73.5ºC, ΔG: -52.3 kcal/mol) and the reverse primer API2 (5′-CACCAGAGTTTCCTCTGGC-3′; GC: 57.9%, Tm: 55.5ºC, ΔG: -35.9 kcal/mol) were used. For *Parmelia saxatilis* (strain PSSBR), the forward primer PSL1 (5′-CCTGAAGGCAATACGGGCA-3′; GC: 57.9%, Tm: 57.8ºC, ΔG: -40.47 kcal/mol) and the reverse primer PSL2 (5′-TGAAAGTTCGCCCTGTGGCCTG-3′; GC: 59.1%, Tm: 62.3ºC, ΔG: -46.45 kcal/mol) were used. The PCR products were visualized on a 1.5% agarose gel (120 V for 25 min) to confirm the presence and size of the amplicons. The products were then purified using Exonuclease I and recombinant Shrimp Alkaline Phosphatase, and sequenced bidirectionally on a Capillary Electrophoresis Genetic Analyzer (ABI 3730). The forward and reverse sequences were edited and assembled using MEGA and Sequencher v. 5, then deposited in GenBank.

To confirm the presence and size of the amplicons, the PCR bands were observed on a 1.5% agarose gel at 120 V for 25 minutes. The products were then purified by Exonuclease I and recombinant Shrimp Alkaline Phosphatase. The sequences read were edited and assembled with MEGA and Sequencher v. 5, System generated sequences are further BLAST (http://www. ncbi.nlm.nih.gov/ Basic Local Alignment Search Tool) analysis performed and sequence with 97% similarity are considered and submitted to NCBI (National Centre for Biotechnology Information) GenBank under accession numbers listed in Table 1, All alignments have been deposited in NCBI and obtained the accession numbers.

Preparation of the lichen extracts

Lichen samples were washed and grinded by using mortar and pistle into fine powder. This powder was Soxhlated (Barosil) at 45 °C for 24 hours with solvents such as acetone, methanol, and benzene. After 6 to 8 heat cycles, the solvent was evaporated in a hot air oven at 42-45°C, yielding around two grams of dry extract for each solvent. For the superoxide dismutase (SOD) and catalase (CAT) activities assay, 0.5 g of lichen cell extract was prepared under iced conditions with a pestle in the dark using 50 mM ethylenediaminetetraacetic acid (EDTA), 50 mM sodium phosphate buffer (pH 7.4), 10% (w/v) polyvinylpyrrolidone (PVP), and 2 mM phenylmethylsulfonyl fluoride (PMSF). The gelatinous mixture was centrifuged at 14,000 rpm for 20 minutes at 2º C. The supernatant was subjected to the SOD and CAT activity assay.

Antioxidant assay

DPPH and ABTS radicals scavenging assay

The radical scavenging activity of two test lichens was evaluated using the DPPH (1,1-diphenyl-2-picryl-hydrazyl) assay as described by Kosanic et al. [17]. One ml solution of DPPH (0.1 mM) was added to 3 ml of various concentrations (100-500 µg/ml) of lichen extract. The mixture was incubated at room temperature for 30 minutes, and the absorbance was measured at 517 nm using a UV-Visible Spectrophotometer (Systronics-119). The DPPH radical scavenging activity was calculated using the equation: {Activity (%) =} [\frac {control absorbance} (A_0) {sample absorbance} (A_1)} \ {control absorbance} (A_0)} \ 100 where, A0 is the absorbance of the negative control (2 ml of methanol solution of DPPH radical + 1 ml of 5% DMSO), and A1 is the absorbance of the reaction mixture. Butylated hydroxytoluene (BHT) was used as a standard, and ascorbic acid was used as a positive control. The ABTS [2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid] decolorization assay of lichen extracts was conducted in accordance with the method outlined by Re et al. [18]. The ABTS+ radical cation was generated by mixing 5 ml of ABTS solution (14 mM) with 5 ml of potassium persulfate ($K_2S_2O_8$) solution (4.9 mM), allowing the mixture to stand in the dark at room temperature for 16 hours. The resulting solution was diluted with ethanol to achieve an absorbance range of 0.7-0.02 at 734 nm prior to use, with ethanol serving as the blank. Absorbance readings were taken at 6-minute intervals. For the standard reaction mixture, 50 µl of BHT was mixed with 950 µl of ABTS+ solution. The inhibition percentage of ABTS+ radical was calculated using the formula [(A0 -

A1) / A0] × 100, where A0 represents the absorbance of the control and A1 represents the absorbance of the sample.

SOD activity

Superoxide dismutase (SOD) activity was assayed following the method of Das et al. [19], which involves measuring the inhibition of superoxide-driven nitrite formation from hydroxylamine hydrochloride. The reaction mixture, prepared in the dark, contained 1.11 ml of 50 mM phosphate buffer (pH 7.8), 0.075 ml of 10 mM hydroxylamine hydrochloride, 0.04 ml of 1% Triton X-100, 0.075 ml of 20 mM L-methionine, 0.1 ml of 50 mM EDTA, and 80 µl of 50 mM riboflavin. After preparing the mixture, it was exposed to light for 10 minutes to induce white fluorescence. Absorbance was then measured at 543 nm using the Griess reagent. One unit of SOD activity was defined as the amount that inhibits 50% of nitrite formation. Enzyme activity was calculated using the formula V0/V - 1, where V0 is the control absorbance and V is the sample absorbance, and expressed in units of nkat/mg.

CAT Assay

Catalase activity in the two lichen samples was measured using Aebi, [20] method. The reaction mixture included 2 ml of 0.1 M potassium phosphate buffer (pH 6.8), 500 µL of enzyme extract, and 500 µL of H_2O_2, making a total volume of 3 ml. Catalase activity was determined by monitoring the decrease in absorbance of H_2O_2 at 240 nm over a period of 3 minutes, compared to a blank.

Native-PAGE for SOD and CAT enzyme

Total SOD and catalase activities were analyzed using native-PAGE as described by Beauchamp and Fridovich [21]. A 10% resolving gel and 5% stacking gel were prepared, and samples were loaded at 4 °C with a constant current of 40 V for 12 hours. Gels contained 10% glycerol for support. SOD staining involved incubating the gel in the dark for 30 minutes with a solution of 50 mM sodium phosphate buffer (pH 7.8), 28 mM tetramethylethylenediamine, 0.003 mM riboflavin, 0.25 mM NBT, and 0.1 mM EDTA, followed by 30 minutes of light exposure to visualize protein bands. Catalase staining was performed by washing the gel with ddH2O, staining with 0.003% H_2O_2 for 10 minutes, and then added a mixture of 2% potassium ferricyanide, 2% ferric chloride, and 1% HCl to reveal dark green catalase isoforms. Enzyme activities were expressed in nkat/mg.

Phytochemicals

Phenol: Total phenolic content (%) was determined using the Folin-Ciocalteu reagent [22]. Lichen extract (100 µl) was mixed with 2ml of 2% sodium carbonate. After 10 minutes, 500µl of Folin's reagent was added. Absorbance was measured at 650 nm.

Flavonoid: Total flavonoid content was determined following Zhishen et al., [23]. Lichen extract was mixed with sodium nitrite (500 µl), aluminum chloride (10%, 300 µl), and sodium hydroxide (1ml). After incubation, absorbance was measured at 510 nm. Results were expressed as µg quercetin equivalent per gram dry extract.

Tannin: The total tannin content was determined using a modified method by Oyaizu, [24]. Tubes containing standard tannic acid solutions (100, 200, 300, 400, and 500 µg/mL) and lichen extract (200 µg/mL) were prepared. Distilled water, Folin Phenol reagent, and sodium carbonate solution were added, and the volume adjusted to 10 mL with distilled water. After incubation and measurement at 725 nm, tannin content was expressed as Tannic Acid Equivalent (TAE).

Terpenoid: Terpenoid content was quantified using Ghori et al. [25] method. Lichen extract (1 ml, 1:1 mg/ml) was mixed with chloroform (1 ml) and conc. H_2SO_4 (1 ml), and absorbance was measured at 538nm.

Steroid: Total steroid content was determined following Mital & Jha, [26] method. Lichen extract (1 ml) was mixed with 4N H_2SO_4 (2 ml), followed by Ferric Chloride (0.5%, 2 ml), and incubated at 70 °C for 30 min. Absorbance was read at 780 nm, expressed as Lichesterol equivalent (µg QE/g dry extract).

Alkaloid: Total alkaloid content was determined following the method of Mital & Jha, [26]. Lichen extract (100 µl) was mixed with ethanol (1 ml) and (20%) H_2SO_4 (1 ml). After centrifugation, (60%) H_2SO_4 (500 µl) was added, and absorbance was measured at 565 nm, expressed as Caffeine equivalent.

Antimicrobial Activity

Test Pathogens for MIC

The antimicrobial activity was evaluated using four human pathogenic bacteria collected from the Microbial Type Culture Collection (MTCC). These bacteria include *Escherichia coli* (MTCC-443), *Staphylococcus aureus* (MTCC-96), *Bacillus subtilis* (MTCC-441) and *Vibrio cholerae* (MTCC-3906). The cultures were maintained according to BSL-II lab facilities.

Minimum inhibitory concentration against pathogenic bacteria

The antimicrobial assay was performed using standard 96-well plates with Mueller Hinton broth (MHB) as the growth medium for bacteria. Lichen extracts were prepared at concentrations of 1000, 500, 250, 125, 62.5, 31.25, and 15.62 µg/mL by performing a two-fold serial dilution with MHB. The extracts and a control pathogen were used in the assay. Approximately 50 µL of bacterial inoculum, with a concentration of 10^6 CFU/mL, were added to each well. After 24 hours, the biofilms were examined using a microplate reader (Biorad, iMark-11457). The minimum inhibitory concentration (MIC) was defined as the lowest extract concentration in the broth that visibly inhibited microbial growth [27].

Gas chromatography mass spectrometry analysis (GC-MS)

Methanol was used as a solvent in Soxhlet extraction for GC-MS analysis since it exceeded acetone and benzene in gradient of biomolecule extraction. The liquid extract was filtered through Whatman's No. 1 filter paper, then dried at 45 °C for 48 hours to provide a dry extract for biomolecule characterization. GC–MS analyses were conducted on an Elite-wax Capillary-column (60.0 m × 250 µm × 0.25 µm) chromatography under specific temperature Injection in autosampler was used 1.5 µl of the sample split to 10:1. Oven: Initial temp 60 °C, ramp (temperature regulation) 7º 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.

Statistical analysis

The statistical analysis was conducted using Prism (8.0 software), Microsoft excel and design expert 13. The significance of antioxidant activity was assessed with Student's t-test. Results are presented as the mean ± standard deviation (SD) from three replicates.

RESULTS

Morphological identification of lichens

Morphological analysis of the lichen specimens confirmed their identification based on thallus type and reproductive features (Figure 1). *Harpothellon rubrocincta* and *Arthonia polymorpha* showed

crustose, corticolous, ecorticate thalli with cottony byssoid crusts and asci occurring in ascigerous areas or pseudisidia. *Parmelia saxatilis* and *Parmotrema reticulatum* exhibited foliose, heteromerous thalli with well-developed apothecia and characteristic rhizines patterns.

Molecular Identification

Sequencing, blast and phylogenetic analysis

Data validation: Sequencing was performed using the Sanger dideoxy sequencing method. The formation of the remaining nucleotides was analyzed by BLAST (Basic Local Alignment Search Tool) and submitted to GenBank, NCBI (National Center for Biotechnology Information), accessible from GenBank (OQ832495, OQ832494, OQ845612, and OQ825976). Forward and backward corrections were performed in MEGA and Sequencher v. 5. Edit and organize. For most of the lichen-forming fungi studied, nucleotide PCR products typically range in size from 400 to 1800 nucleotides. The evolutionary history was inferred using the Neighbor-Joining method (MEGA-11) (Figure 2).

Phytochemicals

A comparative phytochemical estimation from these four lichen species and fractions of different solvents indicates a huge variation in phytochemical extraction. All tested lichens have highest phytochemical content in Methalonic extract which are mentioned in (Table 2) *Harpothellon rubrocincta* had the highest phenol concentration in methanol (218.45 µg/g GAE) and significant flavonoid levels (43.81 µg/g QE). *Arthonia polymorpha* exhibited substantial phenol and flavonoid concentrations in methanol (245.51 µg/g GAE and 84.21 µg/g QE, respectively). *Parmotrema reticulatum* has the highest phenol content (316.95 µg/g GAE) and flavonoids (124.19 µg/g QE) when extracted with methanol, coupled with considerable tannin and terpene values. Upon exposure to methanol, *Parmelia saxatilis* produced high quantities of phenol (293.35 µg/g GAE) and flavonoid (96.55 µg/g QE). Methanol consistently increased phytochemical content among all species, particularly phenol, flavonoid, and alkaloid levels. Acetone and benzene demonstrated lower extraction efficiencies. These results thus indicated that the methanol has higher efficacy in extracting important compounds from lichen.

DPPH and ABTS Scavenging Assay

The free radical scavenging activity assayed from fraction of solvents of *P. reticulatum*, *A. polymorpha*, *P. saxatilis*, and *H. rubrocincta* to scavenge the DPPH and ABTS radical scavenging analysis, with BHT taken as the standard antioxidant. In DPPH assay analysis of *P. reticulatum*, the methanol extract was showing collectively significant radical scavenge to reaching 85.4% at final concentration but acetone and benzene extracts demonstrated lesser activity, with maximums of 75.06% and 65.18% in *A. polymorpha*. In *P. saxatilis* methanol extract had high activity, reaching 80.53%, whereas acetone and benzene extracts has lower activity i.e. 73.755% and 64.115%, respectively. In *H. rubrocincta* methanol extract demonstrated moderate activity i.e., 71.88% and acetone and benzene extracts indicated reduced activity of 59.16% and 54.28%, respectively (Figure 3). In the ABTS assay of *P. reticulatum*, methanol extract displays prominent activity, reaching 79.89% at 1000 µg/ml but acetone and benzene extracts demonstrated maximum activity of 80.84% and 77.89%, respectively. In *A. polymorpha* methanol extract achieved significant efficiency (82.94%) and alternatively acetone and benzene extracts demonstrated lower activity of 79.09% and 76.24%, respectively. The methanol extract of *P. saxatilis* demonstrated higher activity, reach 93.745% while, acetone and benzene extracts exhibited correspondingly 85.745% and 86.745% activity. Extract of *H. rubrocincta* in methanol revealed rising activity of 90.645% whereas, acetone and benzene extracts were comparatively less, i.e., 86.745% and 84.59% (Figure 4). In both DPPH and ABTS assays, methanol extracts outperformed acetone and benzene extracts for all examined lichens. Among the lichens, *P. saxatilis* and *H. rubrocincta* revealed the most promising antioxidant activity, notably in the ABTS test. The conventional antioxidant, BHT, consistently showed the highest activity in both trials.

Superoxide dismutase and catalase activity of lichen

The protein content and enzymatic activities in tested lichen species showing noticeable diversity of biomolecules. The superoxide dismutase (EC1.15.1.1) belongs to oxido-reductase family consists of metalo-enzymes that neutralize superoxide-anion free radicals, offering a physiological antioxidant defense machinery by converting superoxides into O_2 and H_2O_2. This process helps to remove the free radicals and suppressed ROS production and protect cells from oxidative damage. In eukaryotes, SODs are found in different organellar position, according to their metal ion co-factors, those are categorized into several types. Such types are copper-zinc superoxide dismutase (Cu/Zn SOD or SOD1) in the cytoplasm or extracellular fluid, manganese-containing superoxide dismutase (Mn-SOD or SOD2) in mitochondria, and extracellular superoxide dismutase (EC-SOD or SOD3). In present investigation *P. reticulatum* exhibits the highest protein content at 8.13 mg/g and the most substantial superoxide dismutase (SOD) activity at 8880.028 nkt/g (532.791 U/g), with isoenzymes including Mn, Zn, and Fe. *P. saxatilis* 7.02 mg/g protein and followed by SOD activity of 7905.691 nkt/g (474.332 U/g) containing Cu, Mn, Zn, and Fe isoenzymes (Figure 5). *H. rubrocincta* expressed a protein content of 6.25 mg/g and SOD activity of 6655.366 nkt/g (399.314 U/g), with Mn, Zn, and Fe iso-enzymes. *A. polymorpha* has the lowest protein content at 5.46 mg/g and SOD activity of 3593.505 nkt/g (215.606 U/g), with Mn, Fe, and Zn isoenzymes. While assayed the catalase activity, *P. reticulatum* also leads with 4591.675 nkt/g (275.495 U/g), followed by *P. saxatilis*, *H. rubrocincta*, and *A. polymorpha* (Table. 3). These findings concludes that the potential of these lichens for further pharmacological and enzymological treatment, particularly *P. reticulatum* and *P. saxatilis*, due to their high enzyme activities and diverse isoenzyme composition.

Antimicrobial Activity

Minimum Inhibitory concentration (MIC)

Antibacterial study evaluated in four lichen species by using acetone, methanol, and benzene solvents (crude extract) among four lichen species *Arthonia polymorpha* and *Cryptothecia rubrocincta* showing negligible antimicrobial potential. But *Parmelia saxatilis* and *Parmotrema reticulatum* with their respective extracts showing significant antibacterial activities. Among solvents methanol and acetone extract showed significant MIC against pathogens in a particular concentration range (Figure 6 & Table 4).

Gas chromatography-mass spectrometry

GC-MS characterization of *Parmelia saxatilis* and *Parmotrema reticulatum* (Figure 7 & 8) has screened a significant metabolite diversity from diverse metabolic classes, including polyphenols, hydroquinones, coumaric acids, alkaloids, flavonoids, arenes, meta divarinol, benzoquinones, terpenes, azaarenes, and aromatic ethers (Table 5 & 6). These compounds exhibit a wide range of pharmacological and nutritional properties such as strong antioxidant, antimicrobial, antigenotoxic, immunomodulatory, cytotoxic, anti-inflammatory, enzyme-suppressing, hepatoprotective, analgesic, antineuralgic, anticonvulsant, anti-tuberculosis, anticoagulant and herbicide effects. Detailed information about these compounds and their respective properties is presented in Table 5 & 6.

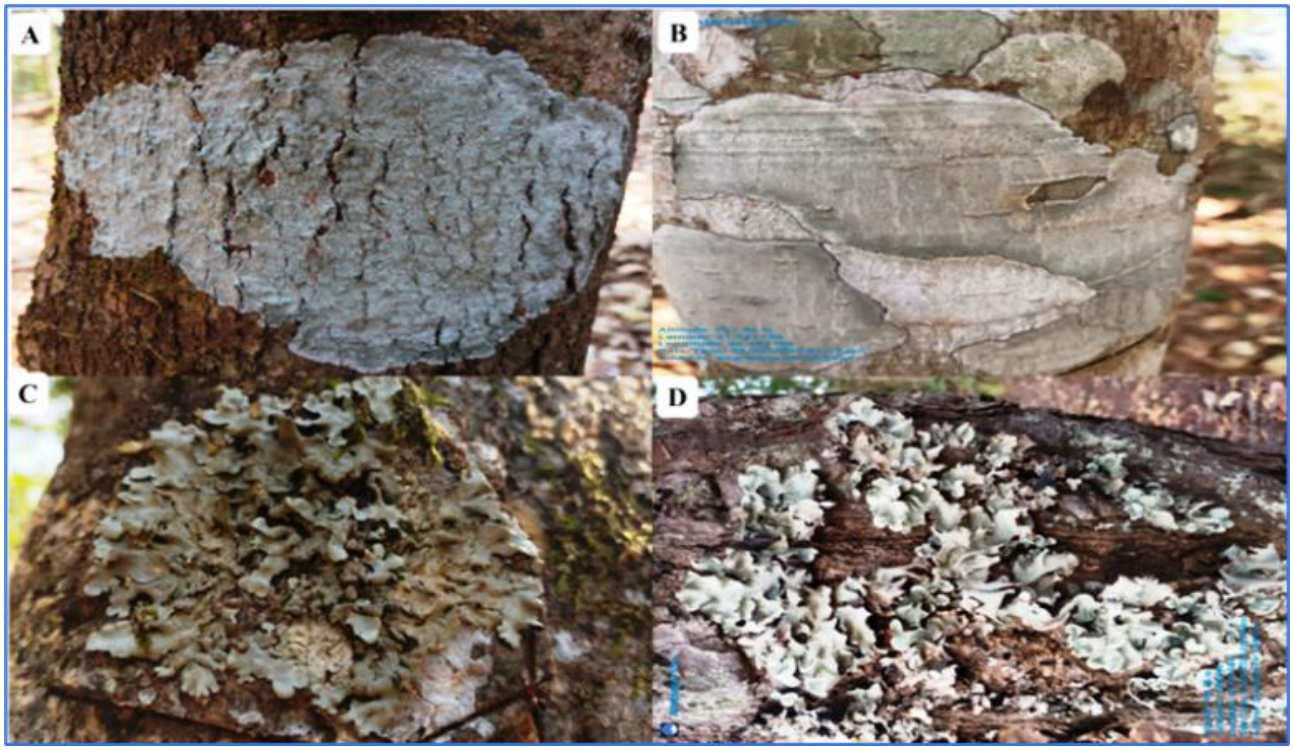

Figure 1: (A). ***Harpothellon rubrocincta***: The lichenized thallus is crustose, generally corticolous, and ecorticate with cottony-byssoid crusts. It is unusual because its asci do not develop in distinct ascomata but in ascigerous areas or solitary inside pseudisidia. The medulla is covered in ecorticate pseudisidia, and the ascigerous areas contain loosely aggregated asci, **(B).** ***Arthonia polymorpha*****:** The lichenized thallus is crustose, generally corticolous, and ecorticate, with cottony-byssoid crusts. Unusually, its asci do not develop in distinct ascomata but instead in ascigerous areas or solitary inside pseudisidia. The medulla is covered with ecorticate pseudisidia, and the ascigerous areas contain loosely aggregated asci, **(C).** ***Parmellia saxatilis*****:** Lichenised, foliose (leafy), grows 90 to 120 cm in gray-brown rosettes (5 to 10 centimetres across), rhizomatous, branched, heteromerous (Distinct cortex and medulla), disc, Apotheciate, maculate, grove attachment, The diameter flatcentral portion may die out (Fairy ring at central position), and **(D).** ***Parmotrema reticulatum***: Foliose lichen grows to 3–30 cm in diameter, with broad, dull to slightly shiny gray lobes that are 10-20 mm wide. It has apothecia (60-65 µm) and is pycnidiate with a thalline ridge. The underside is black with exposed brown areas and a central collection of simple rhizines, and it is heteromerous

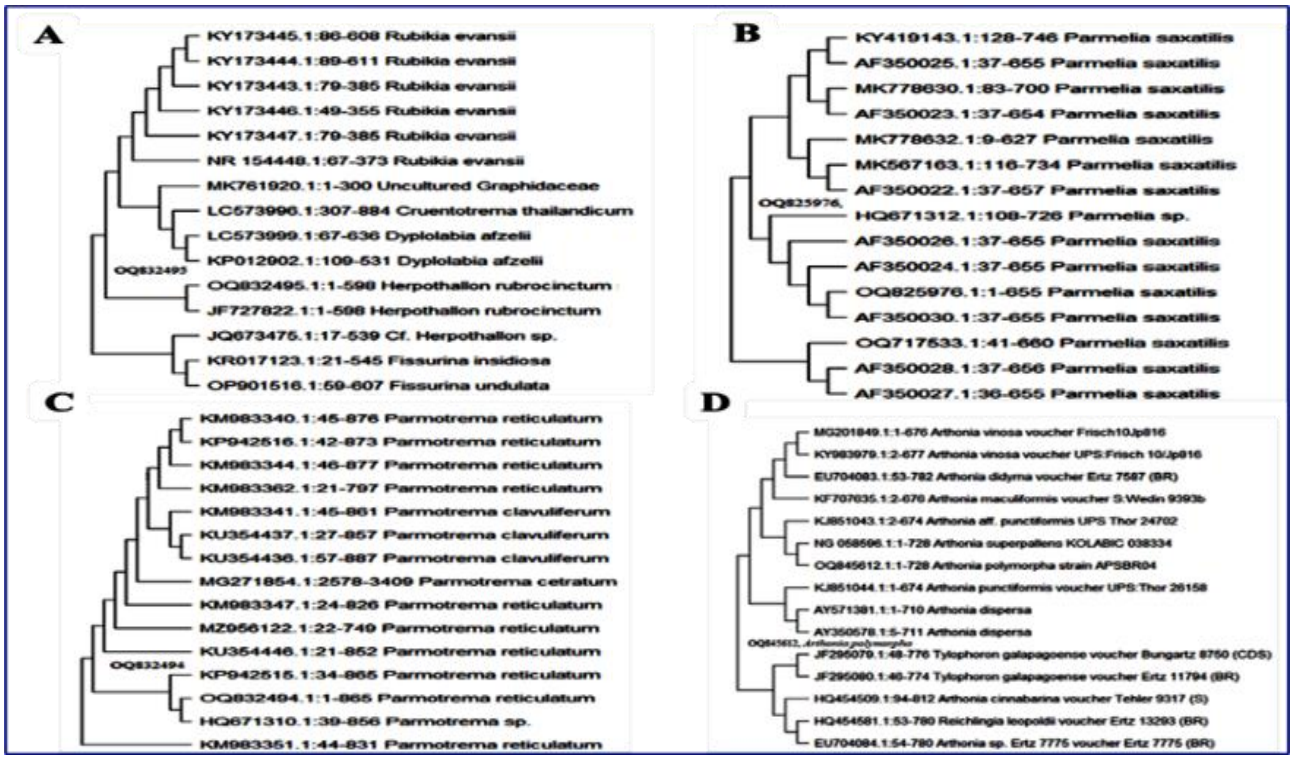

Figure 2: The evolutionary history was inferred using the Neighbor-Joining method. A bootstrap consensus tree from 600 replicates represents the taxa's evolutionary history. Branches with less than 50% bootstrap support are collapsed, and the percentage of replicate trees with associated taxa clustering together is shown below the branches. Evolutionary distances were computed using the Maximum Composite Likelihood method, measured in base substitutions per site. This analysis involved 15 nucleotide sequences, with ambiguous positions removed (pairwise deletion option). The final dataset included (A) 633 positions for *H. rubrocincta*, (B) 659 for *Parmelia saxatilis*, (C) 866 for *P. reticulatum* and (D) 734 for *Arthonia polymorpha*. Evolutionary analyses were conducted in MEGA11 [29].

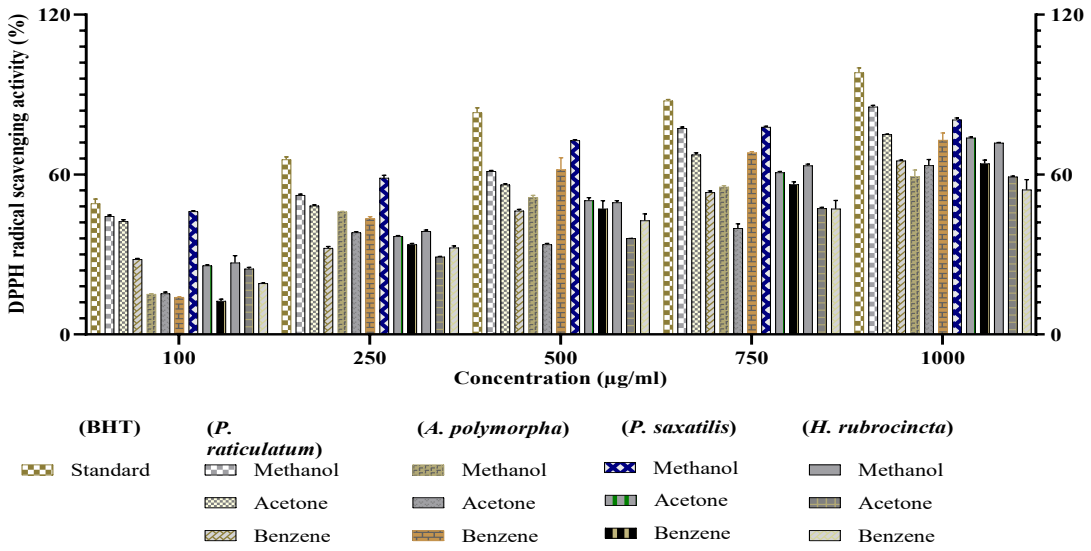

Figure 3: DPPH radical scavenging activities of lichens by using methanol, acetone and benzene solvent extraction, BHT- butylated hydroxy toluene taken as standard for DPPH assay

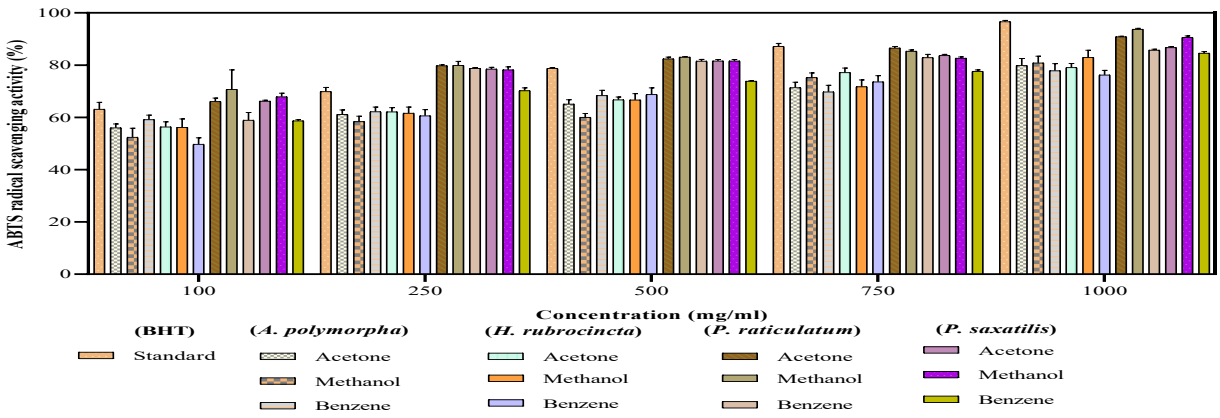

Figure 4: ABTS radical scavenging activities of lichens by using methanol, acetone and benzene solvent extraction, BHT- butylated hydroxy toluene taken as standard for ABTS assay

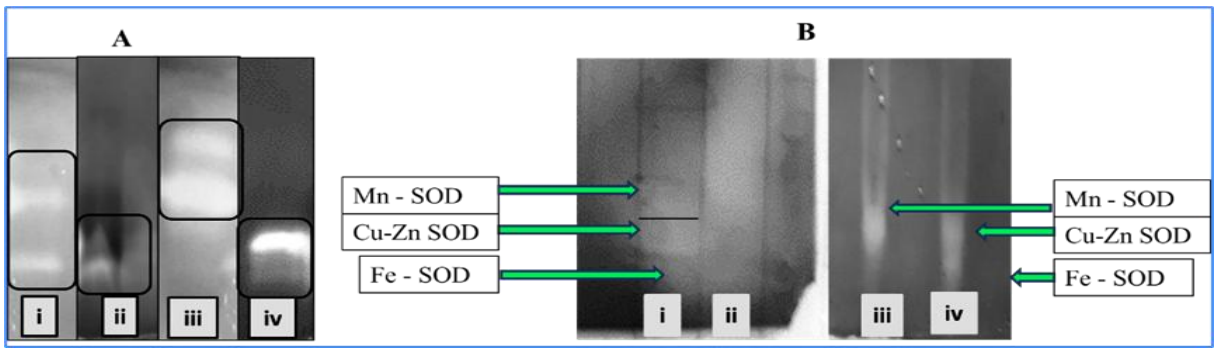

Figure 5a: Native-PAGE analysis of (A) isoforms of Catalase and (B) isoforms of superoxide dismutase enzyme in Lichens, (i) *H. rubrocincta*, (ii) *A. polymorpha*, (iii) *P. saxatilis* and (iv) *P. reticulatum*

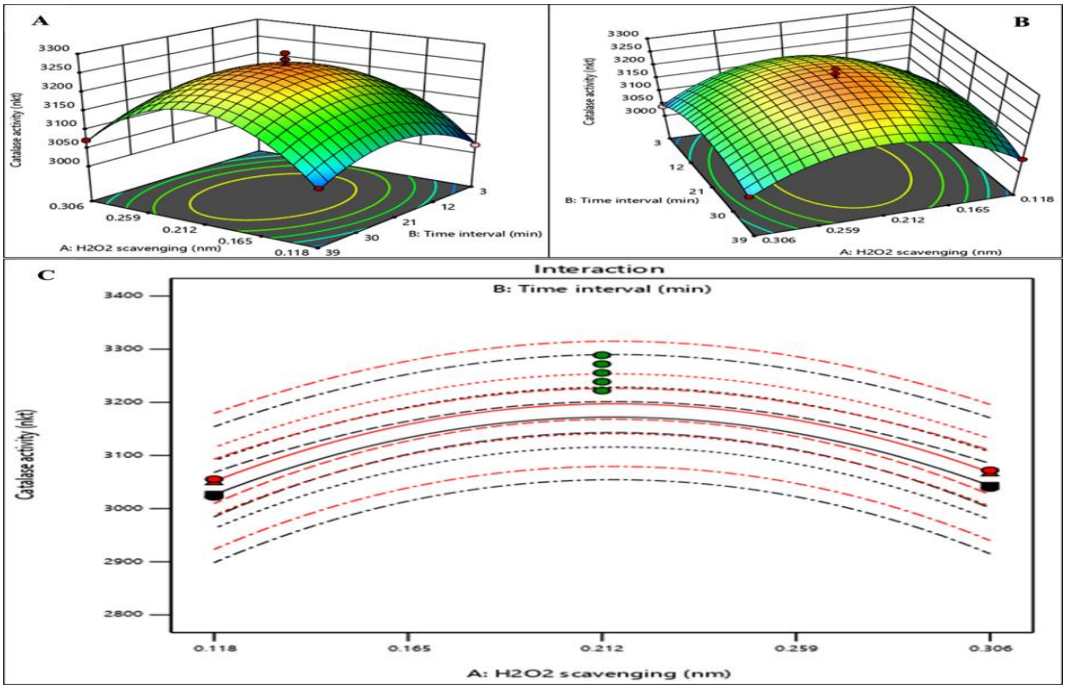

Figure 5b: (A&B) RSM plot of catalase activity showing enzymatic activity against substrate hydrogen peroxide (H_2O_2) across time interval (C) showing enzymatic order reaction, Km values for its catalase activity (high nkt to low nkt) in several time interval in *H. rubrocincta*

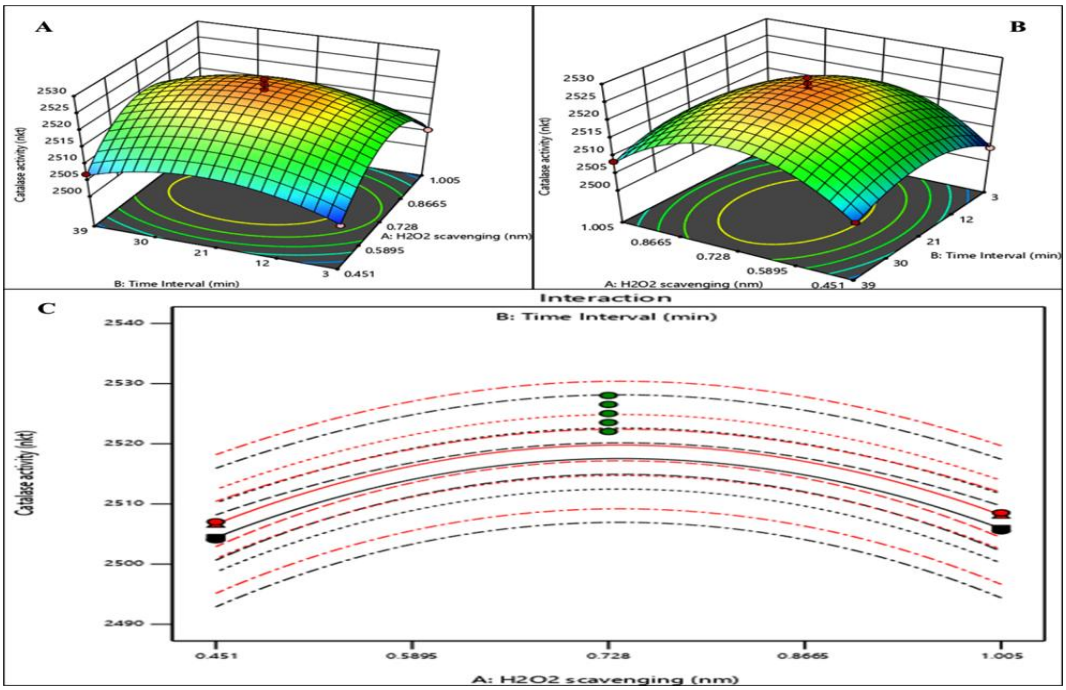

Figure 5c: (A&B) RSM plot of catalase activity showing enzymatic activity against substrate hydrogen peroxide (H_2O_2) across time interval (C) showing enzymatic order reaction, Km values for its catalase activity (high nkt to low nkt) in several time interval in *A. polymorpha*

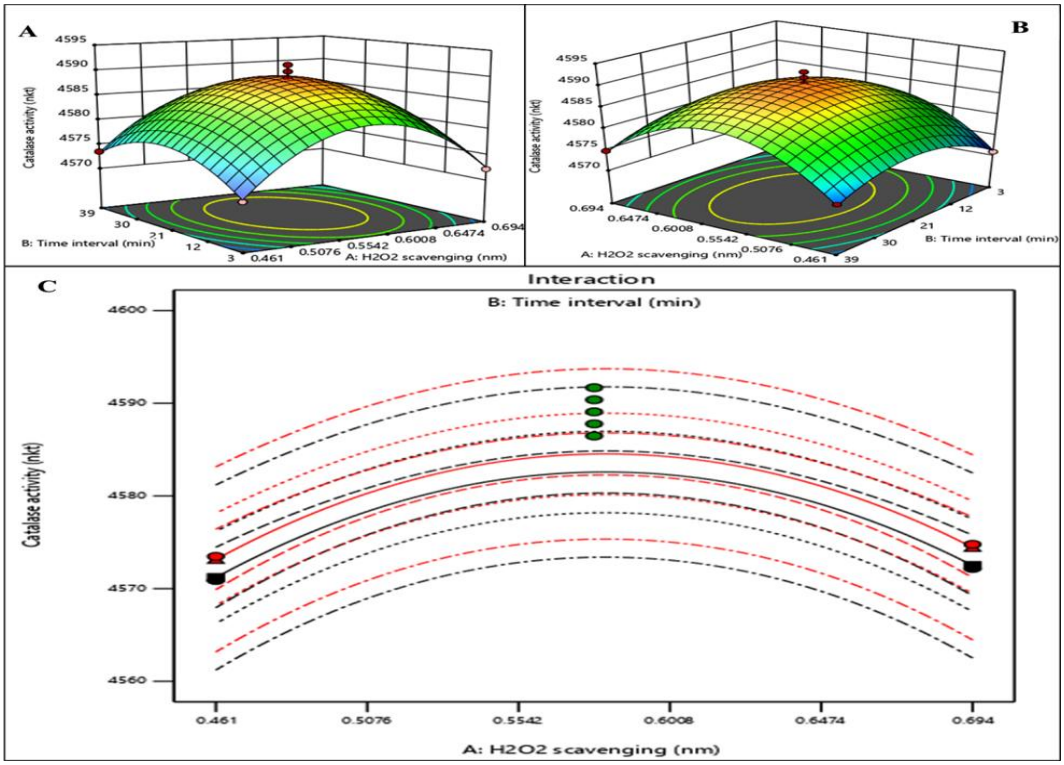

Figure 5d: (A&B) RSM plot of catalase activity showing enzymatic activity against substrate hydrogen peroxide (H_2O_2) across time interval (C) showing enzymatic order reaction in, Km values for its catalase activity (high nkt to low nkt) several time intervals in *P. reticulatum*

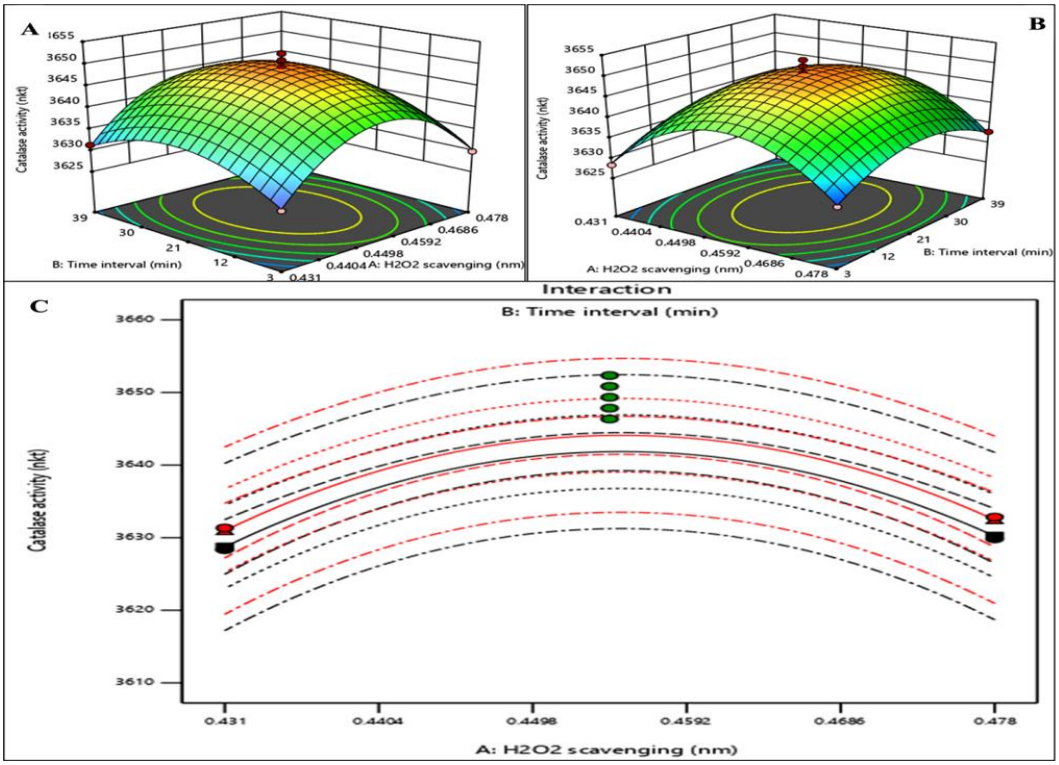

Figure 5e: (A&B) RSM plot of catalase activity showing enzymatic activity against substrate hydrogen peroxide (H_2O_2) across time interval (C) showing enzymatic order reaction, Km values for its catalase activity (high nkt to low nkt) in several time interval in *P. saxatilis*

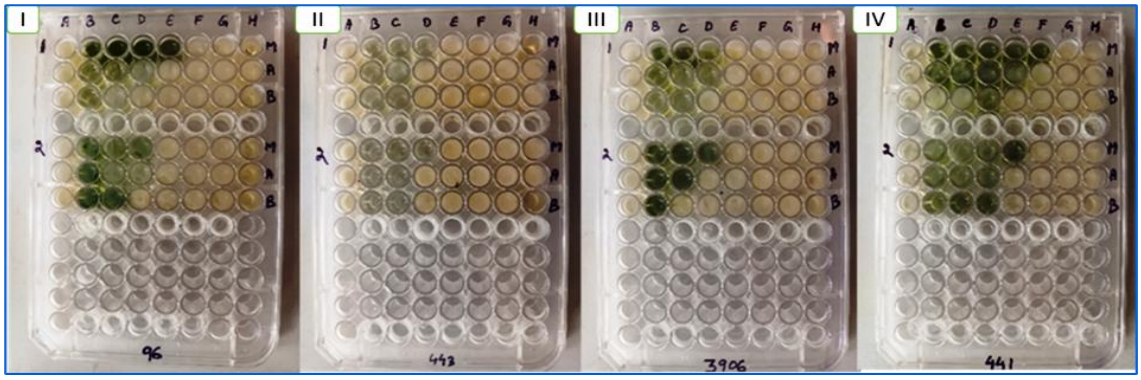

Figure 6: Here, (1) *Parmotrema reticulatum* and (2) *Parmelia saxatilis* [A- Control (medium without pathogen), B - 1000 µg/ml, C- 500 µg/ml, D – 250 µg/ml, E – 125 µg/ml, F - 62.5 µg/ml, G – 31.25 µg/ml, H – 15.62 µg/ml (concentrations of lichen extract)], (M- methanol, A- acetone and B- benzene (solvents used for extraction)), and (I) (MTCC-96, *Staphylococcus aureus*), (II) (MTCC-443, *Escherichia coli*), (III) (MTCC- 3906, *Vibrio cholerae*), and (IV) (MTCC-441, *Bacillus subtilis*)

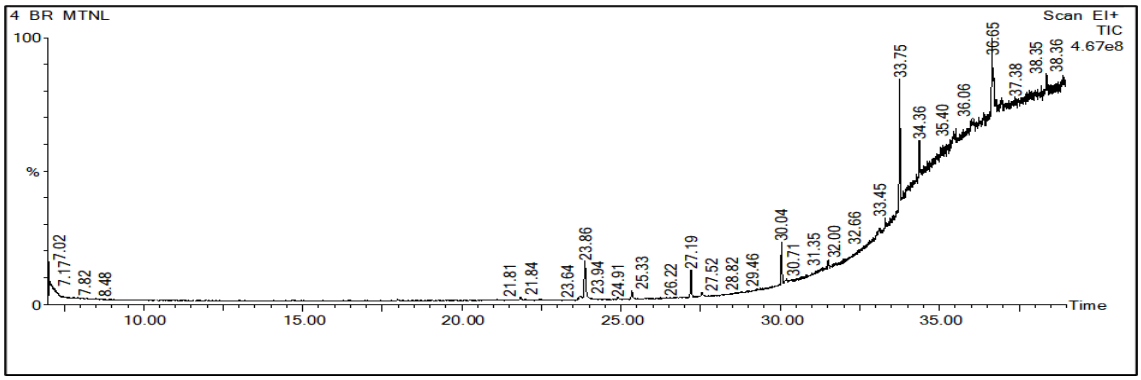

Figure 7: GCMS analysis of *P. reticulatum* showing major chromatograms with highlighted peaks

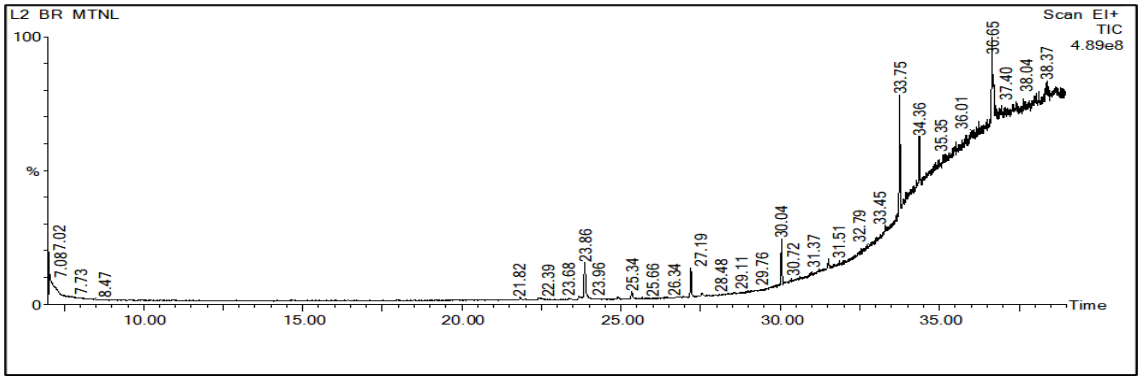

Figure 8: GCMS analysis of *P. saxatilis* showing major chromatograms with highlighted peaks

Table 1: Primer sequences used for the PCR analysis of lichens and GenBank accession no.

Lichen species	Primers	Sequence	Accession no.
H. rubrocincta (CRSBR14)	Forward HC1	5′-ACGGCGCCCAGCTCCCCAA-3′ (GC: 73.7%, Tm: 67.9ºC, ΔG: -47.02 kcal/mol)	OQ832495
	Reverse HRC2	5′- GAGTGGGGCTGCCCAACGG-3′ (GC: 73.7%, Tm: 64.2ºC, ΔG: -43.57 kcal/mol)	
P. reticulatum (PRSBR04)	Forward PRA1	5′-TAGCGGAAATCCTCAGCATTTC-3′ (GC: 45.5%, Tm: 55.2ºC, ΔG: -42.72 kcal/mol)	OQ832494
	Reverse PRA2	5′-TCGGATCAGGTAGGGACCC-3′ (GC: 63.2%, Tm: 58.2ºC, ΔG: -38.6 kcal/mol	
A. polymorpha (APSBR04)	Forward API1	5′-GGCCCCGCCCGGGGTCCGA-3′ (GC: 89.5%, Tm: 73.5ºC, ΔG: -52.3 kcal/mol)	OQ845612
	Reverse API2	5′-CACCAGAGTTTCCTCTGGC-3′ (GC: 57.9%, Tm: 55.5ºC, ΔG: -35.9 kcal/mol)	
P. saxatilis (PSSBR)	Forward PSL1	5′-CCTGAAGGCAATACGGGCA-3′ (GC: 57.9%, Tm: 57.8ºC, ΔG: -40.47 kcal/mol)	OQ825976
	Reverse PSL2	5′-TGAAAGTTCGCCCTGTGGCCTG-3′ (GC: 59.1%, Tm: 62.3ºC, ΔG: -46.45 kcal/mol)	

Table 2: Phytochemical estimation of lichens using acetone, methanol and benzene extracts.

Lichen species	Solvent	Phenol (µg/g GAE)	Flavonoid (µg/g QE)	Tannin (µg/g TAE)	Terpene (µg/g LE)	Alkaloid (µg/g CE)	Steroid (µg/g LSE)
H. rubrocincta	Acetone	178.65	33.51	31.513	121.2	128.7	8.4
	Methanol	218.45	43.81	48.47	153.8	218.7	11.4
	Benzene	113.35	11.84	20.93	104.7	98.7	7.4
A. polymorpha	Acetone	205.55	51.03	40.21	129.5	106.1	7.7
	Methanol	245.51	84.21	53.61	153.2	136.5	9.3
	Benzene	190.18	37.65	10.72	117.7	97.2	3.8
P. reticulatum	Acetone	288.88	87.39	73.32	152.2	135.6	10.2
	Methanol	316.95	124.19	91.04	188.3	241.7	13.5
	Benzene	248.65	68.38	51.15	130.3	115.4	8.7
P. saxatilis	Acetone	218.45	62.28	58.98	146.3	187.1	3.3
	Methanol	293.35	96.55	85.28	174.7	236.2	4.5
	Benzene	190.18	46.23	47.74	126.2	121.1	2.5

(Here, GAE- gallic acid equivalent, QE- quercetine equivalent, TAE- tannic acid equivalent, LE- linalool equivalent, CE- caffeic acid equivalent and LSE- lichesterol equivalent)

Table 3: SOD and CAT activities of lichens

Lichen species	Protein content (mg/g)	SOD activity (nkt/g)	SOD activity (U/g)	Isoenzymes of SODs	Catalase activity (nkt/g)	Catalase activity U/g Protein
H. rubrocincta	6.25	6655.366	399.314	Mn, Cu, Zn & Fe	3288.699	197.318
A. polymorpha	5.46	3593.505	215.606	Mn, Cu, Zn & Fe	2528.034	151.679
P. reticulatum	8.13	8880.028	532.791	Mn, Cu, Zn & Fe	4591.675	275.495
P. saxatilis	7.02	7905.691	474.332	Cu, Mn, Zn & Fe	3652.340	219.136

Here, mg/g- Milligram per gram, nkt/g- Nanokatal per gram, U/g- Unit per gram, Mn SODs- Manganese superoxide dismutase, Cu SODs- Cupper superoxide dismutase, Zn SODs- Zinc superoxide dismutase and Fe SODs- Iron superoxide dismutase

Table 4: Minimum inhibitory concentration of lichens

Pathogens	Minimum inhibitory concentration (µg/ml) (MIC)					
	Acetone		Methanol		Benzene	
	P. reticulatum	*P. saxatilis*	*P. reticulatum*	*P. saxatilis*	*P. reticulatum*	*P. saxatilis*
S. aureus	250	250	125	250	1000	500
E. coli	500	500	250	250	500	500
V. cholerae	250	500	250	250	500	1000
A. subtilis	125	250	62.5	125	250	250

Table 5: Bioactive compounds from *P. reticulatum* along metabolic classes, retention time, molecular weight, bioactivity with references

Compounds	IUPAC name	Classes	Retention time (minutes)	Molecular weight (g/mol)	Bioactivity	Reference
O-Methylorcinol ($C_8H_{10}O_2$)	3-methoxy-5-methylphenol	Phenols	23.860	138.16	Antioxidant	[45]
3-Methoxybenzyl alcohol ($C_8H_{10}O_2$)	(3-methoxyphenyl) methanol	Aromatic ether	23.860	138.16	Antioxidant	[46]
2,3-dimethylhydro Quinone ($C_8H_{10}O_2$)	2,3-dimethylbenzene-1,4-diol	Phenols (Hydroquinone)	23.860	138.16	Antioxidant	[47]
p-Hydroquinone ($C_6H_6O_2$)	Benzene-1,4-diol	Phenols (Hydroquinone)	23.860	138.16	Antioxidant	[48]
P-anisidine (C_7H_9NO)	4-methoxyaniline	Aromatic amine	23.860	123.15	Antioxidant	[49]
Benzenepropanoic acid ($C_9H_{10}O_3$)	3-(3-hydroxyphenyl) propanoic acid	Coumaric Acids (dihydrophenanthrenes)	27.191	166.17	Antibacterial and cytotoxic	[50, 51]
Benzeneethanamine ($C_8H_{11}N$)	2-phenylethanamine	Alkaloid (aralkylamine)	27.191	121.18	Anti-inflammatory and antimicrobial	[52, 53]
1-naphthalenol ($C_{10}H_8O$)	Naphthalen-1-ol	Naphthols (Aromatic phenol/Flavonoids)	27.191	144.17	Anti-Insecticidal and antimicrobial	[54]

Benzenepropanol ($C_9H_{12}O$)	3-phenylpropan-1-ol	Phenol (Arene)	27.191	136.19	Antimicrobial and antioxidant	[55]
Hydrocoumarin ($C_9H_8O_2$)	3,4-dihydrochromen-2-one	Benzopyran (Chromanes)	27.191	148.16	Antioxidant, antimicrobial, antiinflammatory and cytotoxic	[56, 57]
Hydrocinnamic acid ($C_9H_{10}O_2$)	3-phenylpropanoic acid	Phenol (Phenylpropionates)	27.191	150.17	Antioxidant, Anticancer, Antibacterial and antiinflammatory	[58-61]
Benzenemethanoic acid ($C_7H_6O_2$)	Benzoic acid	Hydrocarbons, Aromatic (Benzoates)	33.749	122.12	Antioxidant	[62]
Benzenepropionic acid ($C_9H_{10}O_2$)	3-phenylpropanoic acid	Phenol (Phenylpropionates)	33.749	150.17	Antioxidant, Anticancer, Antibacterial and antiinflammatory	[58-61]
Ethyl divaricatinate ($C_{13}H_{18}O_4$)	Ethyl 2-hydroxy-4-methoxy-6-propylbenzoate	Depsides (meta-Divarinol)	33.749	238.28	Antitumor, Anthelmintic and antimicrobial	[63,64]
n-methyl-4-morpholinoaniline ($C_{11}H_{16}N_2O$)	N-methyl-4-morpholin-4-ylaniline	Quinolones (Alkaloids)	33.749	192.26	EGFR inhibiter	[65]
5,7-dihydroxy-4-methylcoumarin ($C_{10}H_8O_4$)	5,7-dihydroxy-4-methylchromen-2-one	Phenol/ Coumarins (Benzopyrans)	33.749	192.517	Antiinflammatory and antioxidant	[66, 67]
4-tert-butylphenol, tms derivative ($C_{13}H_{22}OSi$)	(4-tert-butylphenoxy)-trimethylsilane	Monophenols	33.864	222.40	Antienzymatic activity	[68]
2-Propylpiperidine ($C_8H_{17}N$)	2-Propylpiperidine	Alkaloid	25.331	127.23	analgesics, antineuralgic, & teratogen properties	[69]
l-Pipecolic acid ($C_6H_{11}NO_2$)	(2S)-piperidine-2-carboxylic acid	Alkaloids	25.331	129.16	Antimicrobial	[70]
2-cyclohexylpiperidine ($C_{11}H_{21}N$)	2-cyclohexylpiperidine	Alkaloid	25.331	167.29	Antimicrobial	[71]
2-pyrrolidinecarboxylic acid-5-oxo-, ethyl ester ($C_7H_{11}NO_3$)	Ethyl 5-oxopyrrolidine-2-carboxylate	Alkaloid	25.331	157.17	Antimicrobial and antioxidant	[72]
Cyclopentanecarboxylic acid, 1-amino- ($C_6H_{11}NO_2$)	1-aminocyclopentane-1-carboxylic acid	Saturated hydrocarbons	25.331	129.16	Enzyme suppressor, antigenotoxic, cytostatic, immunosuppressive and antineoplastic	[73]
Benzoic acid, 2-hydroxy-4-methoxy-6-methyl-, methyl ester ($C_{10}H_{12}O_4$)	Methyl 2-hydroxy-4-methoxy-6-methylbenzoate	Benzoates	30.038	196.20	Antimicrobial and antioxidant	[75]
2-Isopropyl-5-methyl-1,4-benzoquinone ($C_{10}H_{12}O_2$)	2-methyl-5-propan-2-ylcyclohexa-2,5-diene-1,4-dione	Benzoquinones	30.038	164.20	Anticancer (colon, prostate, pancreatic and lung cancer), Antiinflammatory, antioxidant and anti-clastogenic, antibacterial, antimutagenic, and antigenotoxic activities	[76-78]
Ethyl everninate ($C_{11}H_{14}O_4$)	Ethyl 2-hydroxy-4-methoxy-6-methylbenzoate	Phenol (aromatic carboxylic acid)	30.038	210.23	Antimicrobial properties	[79]
Duroquinone ($C_{10}H_{12}O_2$)	2,3,5,6-tetramethylcyclohexa-2,5-diene-1,4-dione	Benzoquinones	30.038	164.20	Anticancer, Antioxidant etc.	[80,81]
Hinokitol ($C_{10}H_{12}O_2$)	2-hydroxy-6-propan-2-ylcyclohepta-2,4,6-trien-1-one	Terpenes	30.038	164.20	Antiimmflammatory, Antimicrobial etc.	[82, 83]

Table 6: Bioactive compounds from *P. saxatilis* along metabolic classes, retention time, molecular weight, bioactivity with references

Compounds	IUPAC name	Class	Retention time	Molecular weight	Bioactivity	Reference
O-Methylorcinol ($C_8H_{10}O_2$)	3-methoxy-5-methylphenol	Phenol (Orcinol derivative)	23.855	138.16	Anti-dermatophytic activity, antioxidant and anticancer	[45, 84]
3-methoxybenzyl alcohol ($C_8H_{10}O_2$)	(3-methoxyphenyl) methanol	Aromatic ether	23.855	138.16	Antioxidant	[85]
2,3-dimethylhydroquinone ($C_8H_{10}O_2$)	2,3-dimethylbenzene-1,4-diol	Phenols (Hydroquinone)	23.855	138.16	Antimicrobial and antioxidant	[47, 86]
7-hydroxyisotrichodermol ($C_{15}H_{22}O_4$)	(1R,2S,3R,7R,9R,10R,12S)-1,2,5-trimethylspiro [8-oxatricyclo [7.2.1.02,7] dodec-5-ene-12,2'-oxirane]-3,10-diol	Fungal toxin	23.855	266.33	Antifungal antibiotic	[87]
L-pipecolinic acid ($C_6H_{11}NO_2$)	(2S)-piperidine-2-carboxylic acid	Alkaloids (Alkaloids derived from lysine)	25.336	129.16	Antibacterial	[88]
Pyridine-d5- (C_5H_5N)	2,3,4,5,6-pentadeuteriopyridine	Azaarenes (Nitrogen-containing aromatic heterocycles)	25.336	84.13	Cytotoxic and Hepatoprotective	[89]
2-piperidinecarboxylic acid ($C_6H_{11}NO_2$)	Piperidine-2-carboxylic acid	Alkaloid	25.336	129.16	Anticonvulsant	[90]
Dl-proline, 5-oxo-, methyl ester ($C_6H_9NO_3$)	Methyl 5-oxopyrrolidine-2-carboxylate	Flavonoid Fraction	25.336	143.14	Antioxidant, Anti-inflammatory	[91]
4-methylproline ($C_6H_{11}NO_2$)	4-methylpyrrolidine-2-carboxylic acid	Amino acid derivative	25.336	129.16	Anti-tuberculosis	[92]
Piperidine ($C_7H_{13}NO_2$)	Piperidine	Alkaloid	25.336	143.18	Antimicrobial and antioxidant	[93]
2-chloro-2',6'-acetoxylidide ($C_{10}H_{12}ClNO$)	2-chloro-N-(2,6-dimethylphenyl) acetamide	Aniline Compounds (Acetanilides)	27.191	197.66	Herbicides	[94]
Hydrocoumarin ($C_9H_8O_2$)	3,4-dihydrochromen-2-one	Phenol (Coumarin)	27.191	148.16	Anticoagulant, Antitumor, immunomodulatory, anti-inflammatory	[95-97]
Sparassol ($C_{10}H_{12}O_4$)	Methyl 2-hydroxy-4-methoxy-6-methylbenzoate	Phenol (benzoic acids)	30.038	196.20	Antifungal, Insecticidal and Enzyme Inhibitory Activities	[98, 99]
2-Isopropyl-5-methyl-1,4-benzoquinone ($C_{10}H_{12}O_2$)	2-methyl-5-propan-2-ylcyclohexa-2,5-diene-1,4-dione	Benzoquinones	30.038	164.20	Antiinflammatory, anticancer,	[76, 100]
Triacontanoic acid, methyl ester ($C_{31}H_{62}O_2$)	Methyl triacontanoate	fatty acid methyl ester	30.038	466.8	Antifungal	[101]
Cyclopentaneundecanoic acid, methyl ester ($C_{17}H_{32}O_2$	Methyl 11-cyclopentylundecanoate	Methyl esters	30.038	268.4	Antioxidant	[102]

DISCUSSION

The differences in antioxidant potential among solvents can be explained by their varying capacities to extract bioactive compounds [89]. Aqueous extracts usually show lower antioxidant activity since many lichen metabolites are either only partially soluble or completely insoluble in water [90]. So, organic solvents are favoured due to their efficiency in isolation of a higher amount of bioactive metabolites. Several findings have demonstrated a correlation between the antioxidant activity in lichens and their phenolic contents [91]. Phenols are known for their strong antioxidant properties [92]. Sargsyan et al., [93] during phytochemical estimation showed, *P. sulcata* had the significant phenolic content, with 3811 mg/100 g GAE DE. In contrast, *P. subrudecta* showed relatively lower phenolic content at 608 mg/100 g GAE. Subsequently, *E. prunastri* was also observed to have a relatively high level of phenolic compounds of 3585 mg/100g GAE. Which evident to current findings that methanol extracts across all species: *H. rubrocincta* (218.45 µg/g GAE), *A. polymorpha* (245.51 µg/g GAE), *P. reticulatum* (316.95 µg/g GAE), and *P. saxatilis* (293.35 µg/g GAE). Flavonoids are known for their antioxidant and anti-inflammatory properties [94]. A similar trend was observed with methanol extracts yielding the highest flavonoid content. The methanolic extract of all lichens reported higher flavonoid content: *H. rubrocincta* (43.81 µg/g QE), *A. polymorpha* (84.21 µg/g QE), *P. reticulatum* (124.19 µg/g QE), and *P. saxatilis* (96.55 µg/g QE) and lower activity observed in benzene extract. Supported to the current findings Muthu et al., [95], found that the methanol extracts of *U. bismolluscula* had the highest flavonoid content (31.2 ± 1.10 mg RE/g extract), followed by *P. aurata* (26.9 ± 1.14 mg RE/g extract), *P. tinctorum* (25.8 ± 1.10 mg RE/g extract), and *R. taitensis* (17.04 ± 0.04 mg RE/g extract). In contrast, the aqueous extract of *P. aurata* exhibited significantly lower flavonoid content (3.6 ± 1.03 mg RE/g extract). Tannins, which have antioxidant and antimicrobial properties [96], were also highest in methanol extracts: *H. rubrocincta* (48.47 µg/g TAE), *A. polymorpha* (53.61 µg/g TAE), *P. reticulatum* (91.04 µg/g TAE), and *P. saxatilis* (85.28 µg/g TAE). Terpenes, has potential anti-inflammatory and anticancer properties and were quantify most abundant in methanol extracts, methanolic extract of *H. rubrocincta* (153.8 µg/g LE), *A. polymorpha* (153.2 µg/g LE), *P. reticulatum* (188.3 µg/g LE), and *P. saxatilis* (174.7 µg/g LE) [97] Alkaloids and steroids, widely recognized for their anti-inflammatory, anabolic, and other pharmacological effects [98]. The noticeable concentrations were estimated in methanol extracts: *H. rubrocincta* (218.7 µg/g CE), *A. polymorpha* (136.5 µg/g

CE), *P. reticulatum* (241.7 µg/g CE), and *P. saxatilis* (236.2 µg/g CE). Followed by steroids contents of *H. rubrocincta* (11.4 µg/g LSE), *A. polymorpha* (9.3 µg/g LSE), *P. reticulatum* (13.5 µg/g LSE), and *P. saxatilis* (4.5 µg/g LSE) (Table. 2). Overally, methanol and acetone solvent fraction proved to be the most effective solvents for extracting these phytochemicals from lichens, which, indicating their bio-potential for future pharmaceutical exploitation and applications.

The DPPH assay is performed to reduce, neutralize, or stabilize the free radicals by using different solvent fractions of lichens; the step involves a stable free radical (DPPH). In this process, the donation of a hydrogen atom causes the violet DPPH radical to become less powerful, which creates its reduced form, DPPH-H. The characteristic wide absorption band of DPPH is responsible for its deep violet color, and as antioxidants donate hydrogen atoms, the solution's color shifts from violet to pale yellow. This color change is due to the reduction of DPPH via hydrogen atom transfer, a reaction commonly recorded by UV-visible spectroscopy. This method is frequently applied to measure the antioxidant capacity of various substances, particularly plant extracts and phenolic compounds. It is a widely used technique for evaluating the effectiveness of antioxidants in neutralizing free radicals. Ganesan et al., [99], reported that *P. tinctorum* has very high antioxidant capacities, followed by *P. hababianum* and *P. austrosinense*. Acetone extracts of *P. tinctorum* showed the highest IC_{50} (18.41µg/mL), followed by methanol (60.3 µg/mL) and petroleum ether (74.5 µg/mL) extracts which was supported the investigation of Kumar et al., [100]. However, in recent investigation, for all tested lichens, the methanol extracts infer the highest RSA at 1000 µg/mL: *A. polymorpha* (59.35 ± 1.33%, IC_{50}- 101.309 µg/mL), *H. rubrocincta* (71.80 ± 0.17%, IC_{50}- 120.82 µg/mL), *P. reticulatum* (85.40 ± 0.566%, IC_{50}- 97.364 µg/mL), and *P. saxatilis* (80.53 ± 0.665%, IC_{50}- 81.494 µg/mL). In the recent findings evaluated the ABTS radical scavenging activity of acetone, benzene and methanolic extracts from various lichen species at a final concentration, *A. polymorpha* displayed 82.68% activity with an IC_{50} of 490.65 µg/ml followed by *H. rubrocincta* (84.88% of IC_{50} of 536.05 µg/ml), *P. reticulatum* (93.49%, IC_{50} of 535.04 µg/ml) and *P. saxatilis* (90.29%, IC_{50} of 471.73 µg/ml). Relatively, the acetone and benzene extracts of all tested lichen species demonstrated moderate to lower antiradical properties at final volume, suggesting the superior efficacy of methanolic extracts in antioxidant applications. In the present study, *P. reticulatum* demonstrated the most significant antioxidant activity which align to the findings of Manojlović et al., [101], Hawrył et al. [102], Sahoo et al., [103], Pradhan et al., [104], Pradhan et al., [105], Sahoo et al., [106].

Lichen polyphenols have antibacterial properties and are capable of eliminating a wide range of microorganisms. Polyphenols, flavanols, and phenolic acids exhibit the highest antibacterial activity due to their ability to inhibit bacterial virulence factors, interact with cytoplasmic membranes, suppress biofilm formation, and synergize with antibiotics [32,72]. Though flavonoids are synthesized via the CHS (chalcone synthase) pathway in lichens, CHS is one of several enzymes that use products from the upstream general phenylpropanoid pathway, which synthesizes *p*-coumaroyl-CoA from *p*-phenylalanine in three enzymatic steps. During flavonoid biosynthesis, several intermediates produced in the general phenylpropanoid pathway are diverted to synthesize benzoic acids such as benzoic acid, 2-hydroxy-4-methoxy-6-methyl-, methyl ester, salicylates, and coumarins, such as 3-(3-hydroxyphenyl) propionic acid, 5,7-dihydroxy-4-methylcoumarin, and Hydrocoumarin from lichens. Alternatively, flavonoids may have initially been used as signaling molecules or as antimicrobial lichenoalexins [42-46]. 2-Isopropyl-5-methyl-1,4-benzoquinone, detected in recent phytochemical screenings of lichens, is among other benzoquinones. 2-Isopropyl-5-methyl-1,4-benzoquinone generally has a prominent bactericidal property with significant antagonistic property over bacteria, i.e., bacterial cells treated with thymoquinone at MIC showed increased dichlorofluorescin fluorescence, suggesting that its probable mechanism of action against bacterial cells is due to the production of reactive oxygen species (ROS) [107]. Piperine, a piperidine-type alkaloid, exhibits high antimicrobial activity against both gram-positive and gram-negative bacteria, functioning as an EPI_S (Efflux pump inhibitors) in *S. aureus* when coupled with ciprofloxacin [79]. Terpenoids from lichens, such as 2,4,6-cycloheptatrien-1-one, 2-hydroxy-4-(1-methylethyl)- or hinokitiol, have considerable bactericidal properties by preventing plasma membrane degradation, anti-quorum sensing (QS) action, and inhibition of ATP and its enzyme [67,68]. Polyphenols, terpenoids, alkaloids, and flavonoids are the major metabolites in lichen phytochemicals with synergistic antibacterial properties. These metabolites are broadly biosynthesized in *P. saxatilis* and *P. reticulatum* and have several therapeutic interests (Tables 5 & 6).

The activation of Respiratory Burst Homolog (RBOH) proteins at the plasma membrane leads to the production of superoxide radicals in the apoplast. These superoxide radicals are then converted into hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD). The H_2O_2 can either pass into the cell through aquaporins or trigger cytosolic calcium signaling by interacting with a plasma membrane receptor known as HPCA1 (Hydrogen peroxide senor), which specifically detects H_2O_2 [108]. After H_2O_2 enters the cytoplasm, it can modify the cell's redox state, initiating acclimation and defense mechanisms, or it may be neutralized by the cell's antioxidant system. The swift cell-to-cell transmission of reactive oxygen species (ROS) signals is crucial for both local and systemic responses, facilitating stress adaptation and enhancing survival [109]. However, as discussed above, catalase (CAT) serves functions that go well beyond its antioxidant role in peroxisomes. CAT is the most abundant protein in plant peroxisomes, and in all ascomycetes lichenized fungi, the enzyme form is catalase A (catalase peroxidase) exhibits the chief catalytic activity [110]. Despite this, CAT exhibits a high capacity for H_2O_2 removal, with the Km values for its catalase activity varying from high nkt to low nkt across different systems (Figure 5 b-e). The enzymatic antioxidant activity of superoxide dismutase and catalase assay are preliminarily investigated in tested lichen species, while *P. reticulatum* and *P. saxatilis* showing promising SOD and catalase activity. GCMS analysis of two potent lichen *P. reticulatum* and *P. saxatilis* screened polyphenols, hydroquinones, coumaric acids, alkaloids, flavonoids, arenes, meta-divarinol, benzoquinones, terpenes, azaarenes, and aromatic ethers. These compounds have a variety of pharmacological and therapeutic properties, including strong antioxidant, antimicrobial, antigenotoxic, immunomodulatory, cytotoxic, anti-inflammatory, enzyme-suppressing, hepatoprotective, analgesic, antineuralgic, anticonvulsant, antituberculosis, anticoagulant, and herbicide effects (Table 5 and 6), This investigation highlights the pharmaceutical properties and secondary bioactive compounds present in lichens, suggesting their potential as alternative sources for future therapeutic applications.

CONCLUSION

This study assessed the pharmaceutical potential of four lichen species from the Similipal Biosphere Reserve. All tested lichens showed significant antioxidant activity as evident from DPPH and ABTS non enzymatic assays, alongwith SOD and CAT enzymatic assays confirmed robust antioxidant properties. Antimicrobial tests revealed potent bactericidal effects from *P. reticulatum* and *P. saxatilis.* GC-MS profiling of these lichens detected major compounds i.e. polyphenols, flavonoids, and terpenes, which are known for their pharmacological properties. The findings thus suggest that these lichens can act as promising biosource for therapeutic applications due to their strong antioxidant and antimicrobial activities.

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Data Availability Statement

The nucleotide sequences generated and analyzed during the current study have been submitted to the NCBI GenBank database. The corresponding accession numbers are as follows: *Heterodermia rubrocincta* (CRSBR14) - OQ832495, *Parmotrema reticulatum* (PRSBR04) - OQ832494, *Arthonia polymorpha* (APSBR04) OQ845612, and *Parmelia saxatilis* (PSSBR) - OQ825976. All primer sequences used for amplification are detailed in the manuscript.

Conflict of interest

The authors declared no conflict of interest.

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