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ResearchArticle

EVALUATION OF THE *IN VITRO* ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF SECONDARY METABOLITES PRODUCED FROM LICHENS

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ABSTRACT

Lichens produce a great variety of secondary metabolites and most of them are unique. These chemically diverse lichen substances accumulate on the outer surfaces of the hyphae. Living cells may generate free radicals and other reactive oxygen species by-products as a results of physiological and biochemical processes. In the present study, the lichens *Cladomia fimbriata*, *Permilopsis ambigua*, *Punctelia subrudecta*, *Evernia mesomorpha* secondary metabolites were extracted in the two solvents methanol and water. The antimicrobial efficacy of extracted lichen compounds tested against bacteria.Total phenolic contents of the aqueous extracts of the plants were determined by the Folin-Ciocalteus reagent method.Proteins, carbohydrates, phenols, tannins, flavonoids, saponins, were detected in all of the lichens tested. The highest antibacterial activity was observed in sample C against *Bacillus* (1.9cm) in methanol extracts. The more total phenolic concentration was observed in methanol extract of *P. subrudecta* and followed by *P. ambigua*. The absorbance values of water extract extracted from *P. subrudecta,* sample-C (74.61±2.14) shows significantly higher values than the methanolic extract of *P. subrudecta* and followed by sample B, D and A respectively. The lichen extracts showed comparable and strong antioxidant activity, exhibited higher DPPH and hydroxyl radical scavenging activity. Among the tested lichen extracts, water extract of sample D (*Evernia mesomorpha*) gave highest reducing power, although the reducing activity was lower than the standard ascorbic acid. Our findings provided evidence that crude aqueous and organic solvent extracts of lichens contain medicinally important bioactive compounds and it justifies their use in the traditional medicine.

Keywords:: Secondary metabolites**,** antibacterial, antioxidant activity,phytochemicals, zone of inhibition.

INTRODUCTION

Lichens are self-supporting symbiotic associations formed by two living things. Most of the lichen is composed of fungal filaments, but living among the filaments are algal cells, usually from a green alga or a cyanobacterium. Lichens are valuable plant resources and are used as medicines, food, fodder, dyes perfume, spice, and for miscellaneous purposes. More than one thousand primary and secondary metabolites with identified structures are currently known as lichens. The use of lichens in medicine is based on the fact that they contain unique and varied biologically active substances, mainly with antimicrobial actions. These substances are used in lichen chemotaxonomy (i.e., their classification in terms of chemical features), and they are of interest as natural antibiotics. Lichen metabolites exert a wide variety of biological actions including antibiotic, antimycotic, antiviral, anti-inflammatory, analgesic and antipyretic, anti-proliferative and cytotoxic effects [1, 2 and 3]. Lichens have been found to contain a variety of secondary lichen substances with strong antioxidant activity. These are substances which have high ability to scavenge toxic free radicals due their phenolic groups. Even though these manifold activities of lichen metabolites have now been recognized, their therapeutic potential has not yet been fully explored and thus remains pharmaceutically unexploited. A large number of lichen species have been proven to be a source of these metabolites for food and pharmaceutical industries. Secondary lichen metabolites show a wide range of potentially useful biological activities [4, 5 and 6]. Most lichen substances with antibiotic activity are phenolic metabolites (e.g. usnic acid and the anthraquinone endocrocin) [7, 8].

Lichens produce a great number of various secondary metabolites, and most of them occur exclusively in these symbiotic organisms. They are produced by the mycobiont [9] and accumulate as extracellular tiny crystals on the outer surfaces of the hyphae. Approximately 1050 secondary compounds have been identified to date [10]. Substances extracted from lichens have previously been reported to possess antimicrobial activities against various groups of bacteria, fungi and viruses.

The aim of the present study was to investigate the presence of phytochemicals and to determine the secondary metabolites by thin layer chromatography, and their *in vitro* antioxidant, free radical scavenging activity by DPPH assay.

MATERIALS AND METHODS

Collection of lichens:

The lichen samples were collected form Goolapalli, Ramakuppam Mandal, Chittoor (District) during February, 2013.

Preparation of lichen extracts

Water extraction:

5gm of dried finely powdered lichen material was taken in a beaker and 200ml of distilled water was added. The mixture was heated on hot plate with continuous stirring at 30– 40^{o}C for 20 minutes. Then the water extract was filtered through filter paper and the filter was used for the phytochemical analysis. The water extract was kept in refrigerator when not in use [11].

Methanol extraction

Crude lichen extract was prepared by soxhlet extraction method. About 20gm powdered lichen material was uniformly packed into a thimble and extracted with 250ml of different solvents separately. In the present study solvent used was methanol. The process of extraction continues for 24 hours or till the solvent in siphon tube of an extract become colorless. After that the extract was taken in a beaker and kept on hot plate and heated at 30-40^{o}C till all the solvent got evaporate. Dried extract was kept in refrigerator at 4^{o}C for their future use in phytochemical analysis [11].

The yield of respective extract was calculated as:

Percentage yield (%) = (dry weight of extract/dry weight of samples) x100

Antimicrobial activity of lichen extracts

The antimicrobial efficacy of extracted lichen compounds tested against *Pseudomonas aeruginosa* (Gram-negative), *Escherichia coli* (Gram-negative), *Staphylococcus aureus* (Gram-positive), *Bacillus subtilis* (Gram-positive), by agar disc diffusion method [12]. 24 hours old LB broth cultures of tested bacteria were spreader on sterile LB agar plates using sterile spreader followed by placing the filter paper disks (5mm in diameter) on the surface of the inoculated plates using flame sterilized forceps. Simultaneously Streptomycin standard antibiotic disks can be placed as control. Using sterile micropipette 10µl (0.002mg) of the sample of lichen compounds was poured on to the each of the disk, and the plates were incubated at 37°C for 24 h. The zone of inhibition was measured. Experiments were carried in duplicate and average values were recorded.

Determination of the total phenolics

The total phenolics content determined using the Foline Ciocalteau method. Supernatant of lichen compounds was diluted to the concentration of 1 mg/ml, and aliquots of 0.5ml were mixed with 2.5ml of FC reagent (10 fold dilution with distilled water) and 2ml of NaH_2CO_3 (7.5%). After 15min of staying at the 45°C the absorbance was measured at 765nm on spectrophotometer versus blank sample. Total phenols were determined as gallic acid equivalents (mg GA/g extract), and the values were presented as means of triplicate analysis.

Thiobarbituric acid (TBA) Method

2ml of lichen compound was mixed with 1ml of 20% aqueous trichloroacetic acid and 2ml of 0.67% aqueous thiobarbituric acid. After boiling for 10min, the samples were cooled and then centrifuged at 3,000 rpm for 30min. Read the absorbance at 532nm in a spectrophotometer [13].The antioxidant activity was calculated by percentage of inhibition of this method is follows:

$$\% \text{ inhibition} = 100-[(A_1-A_0) \times 100]$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the lichen sample.

Determination of total antioxidant capacity

The total antioxidant capacity of the lichen samples was evaluated by the phosphomolybdenum method [14]. The assay is based on the reduction of Mo (VI)-Mo (V) by the anti-oxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at acid pH 0.3ml of lichen compound was combined with 3ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution wee incubated at 95°C for 90min. Then the absorbance of the solution was measured at 695nm using spectrophotometer against blank after cooling to room temperature. Methanol (0.3ml) in the place of extract was used as blank. Ascorbic acid (AA) was used as standard and the total antioxidant capacity is expressed as milligram of ascorbic acid per gram of the dry thalli.

Qualitative test for free Radical Scavenging

The supernatant solution of lichen compound was spotted on TLC plate as a spot (100µg/ml) for chromatography separation of the extract using the mobile phase methanol: chloroform (95:5, v/v). It was allow to the chromatogram for 30min after completion of the chromatogram the whole plates was sprayed with DPPH (0.15% w/v) solution using an atomizer. The color changes (Yellowish color development on pinkish back ground on the TLC plate) were noted as an indicator of the presence of antioxidant substances.

Quantitative test for free radical scavenging activity by DPPH

The free radical scavenging activity of lichen compounds was measured by the DPPH [15] with suitable modifications from kumaraswamy *et al* [16]. DPPH (8mg) was dissolved in methanol (100ml) to obtain a concentration of 80µg/ ml. serial dilutions were carried out with the stock solution (1 mg/ ml) of the lichen compounds. Solutions (2ml each) were than mixed with DPPH (2ml) and allowed to stand for 30 min for any reaction to occur, and the absorbance was measured at 517nm. Ascorbic acid (AA) was used as reference standards and dissolved in methanol to make the stock solution with the same concentration (1mg/ ml). Control sample was prepared containing same volume without test compounds or reference antioxidants. 95 % methanol used as blank. The DPPH free radical scavenging activity (%) was calculated using the following equation

$$\% \text{ inhibition} = \frac{Ac - As}{Ac} \times 100$$

Where Ac is the absorbance of the control and As is the absorbance of lichen sample.

Reducing power

The method of Oyaizu [17] was used to determine the reducing power of the lichen extracts. 1ml of tested extract was mixed with phosphate buffer (2.5ml, 0.2M, pH-6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixtures were incubated for 20 min at 50°C. Then, to the mixture TCA (10%, 2.5 ml) was added and centrifuged. At the end, the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%). The absorbance of the solution was measured at 700nm in spectrophotometer (ELICO-159). The reducing power is increased if absorbance of the reaction mixture increased.

Polysaccharide assay

The polysaccharide content in lichen extract was determined, using the phenol sulphuric acid method described by Dubois [18]. 1 ml of extract solution (20µg/ml) was added with 25µlof 80% phenol and 1ml sulphuric acid (H_2SO_4). Mixture was shaken and allowed to stand at 30°C for 30 minutes. The absorbance was measured by UV-Vis spectrophotometer at 490nm. Polysaccharide content was estimated by a standard curve using known amount of standard polysaccharide solution.

Statistical analysis

Experimental values are the mean ± standard deviation (SD). Statistical comparisons using one way analysis of variance (ANOVA) with $P < 0.05$ was regarded as significance and $p < 0.01$as very significant.

RESULTS

Identification of Lichen samples

The collected lichen samples were grown tight against the substrate (mango stem) with crust like appearance, some are leaf like, with flat sheets of tissue not tightly bound, slightly flattened pebble-like units and free-standing branching tubes with root like appearance. Based on the above morphological features the collected lichen samples were identified as *Cladomia fimbriata* (Sample A), *Permilopsis ambigua* (Sample B), *Punctelia subrudecta* (Sample C) *and Evernia mesomorpha* (Sample D) which are shown in figures 1 and 2.

Fig: 1 *Cladomia fimbriata* (Sample A), *Permilopsis ambigua* (Sample B), *Punctelia subrudecta* (Sample C) *and Evernia mesomorpha* (Sample D) growing on Stem of mango trees

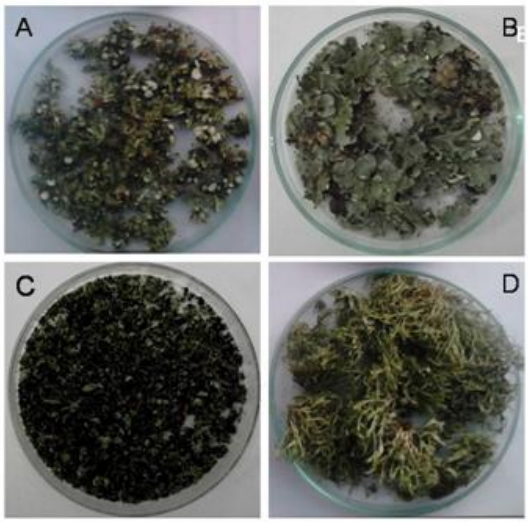


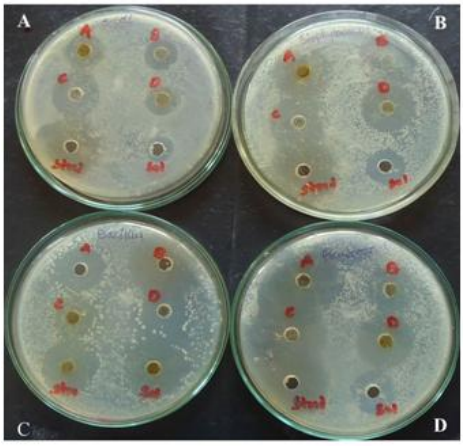
Fig: 2 Morphological appearance of *Cladomia fimbriata* (Sample A), *Permilopsis ambigua* (Sample B), *Punctelia subrudecta* (Sample C) *and Evernia mesomorpha* (Sample D) lichen samples.

Antibacterial activity of lichen compounds

The antibacterial activity of lichen extracts was investigated against various laboratory test organisms of Gram positive (*Staphylococcus, Bacillus*) and Gram negative strains (*E. coli, Pseudomonas,*) using disk diffusion technique (Figure-3). Control (Streptomycin) is also maintained. The diameter of inhibition zones around each well with lichen compound is represented in Table-1. The highest antibacterial activity was observed in sample C against *Bacillus* (1.9cm) in methanol extracts. Whereas water extracts showing minimum inhibition zone against bacteria samples.

Table: 1Methanol and water extract percentage of the four lichen samples

Lichen Sample	Extracts obtained in solvents (%)	
	Methanol Extract	Water Extract
Cladomia fimbriata	2.5	2.0
Permilopsis ambigua	3.75	3.5
Punctelia subrudecta	4.0	3.75
Evernia mesomorpha	3.0	2.5



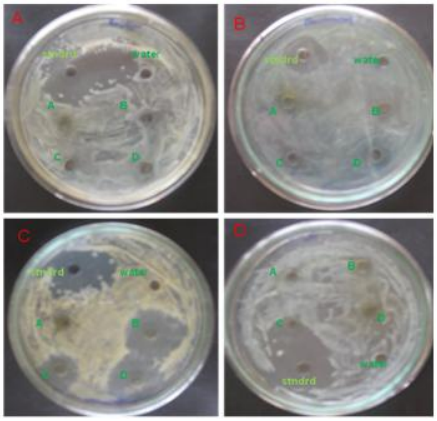


Fig: 3 *In vitro* antimicrobial activities of lichen extracts against bacterial cultures (A) *B. subtilis,* (B) *S. aureus*, (C) *P. aeruginosa*, (D) *P. putida* and (E) *E. coli*.

Determination of the Total Phenols

Phenolic compounds have been reported to be associated with antioxidative action in biological systems, mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching single and triplet oxygen, or decomposing peroxides [19]. The results for determination of total phenolic capacity were shown in figure 4. The more total phenolic concentration was observed in methanol extract of *P. subrudecta* and followed by *P. ambigua, C. fimbriata, E. mesomorpha* (19.5±1.62, 18.41±0.83, 15.6 ± 0.24, 11.51±2.45 mg GA/g) respectively, when compared with water extract of four lichen.

ME: *P. subrudecta* **>** *P. ambigua* **>***C. fimbriata* **>** *E. mesomorpha*

WE: *P. subrudecta* **>***C. fimbriata* **>***E. mesomorpha* **>***P. ambigua*

(**Note:** ME: Methanol Extract; WE: Water Extract)

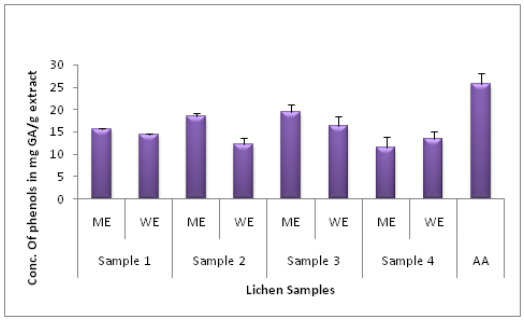


Fig: 4 Estimation of phenolic compounds in methanol extract and water extract of Lichen Samples.Results are mean ± S.D of three parallel measurements.

Determination of Total Antioxidant Capacity by Phosphomolybdenum method

Total antioxidant capacity by Phosphomolybdenum method assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) complex at acidic pH. The phosphor molybdenum method is quantitative since the total antioxidant activity is expressed as the number of equivalents of ascorbic acid. Extractions from different lichens samples showed very potent total antioxidant capacity.

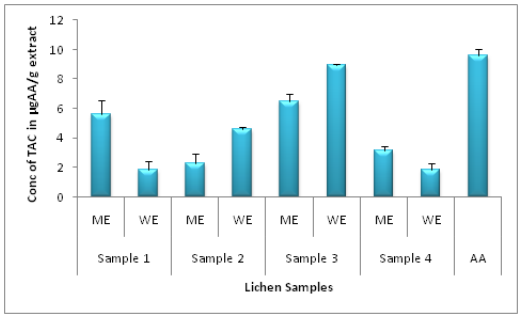


Fig: 5 Estimation of Total Antioxidant Capacity of methanolic extract and water extract of lichen samples. Results are mean ± S.D of three parallel measurements.

The optical density results and their ascorbic acid equivalents are presented in fig 5. The more total antioxidant capacity (6.49±1.47µg AA/g) was observed in water extract of sample C (*P. subrudecta*) and followed by sample B, A and D respectively.

ME: *P. subrudecta* **>** *C. fimbriata* **>** *E. mesomorpha* **>** *P. ambigua*

WE: *P. subrudecta* **>***P. ambigua* **>***C. fimbriata* **>***E. mesomorpha*

Lipid Peroxidation by Thiobarbituric acid (TBA) Method

Lipid Peroxidation results demonstrated that all tested extracts of lichen samples exhibited significant inhibitory activity towards lipid peroxidation. The absorbance values of water extract extracted from *P. subrudecta,* sample-C (74.61±2.14) shows significantly higher values than the methanol extract of *P. subrudecta* and followed by sample B, D and A respectively (Figure 6).

ME: *P. subrudecta* **<***E. mesomorpha* **<***C. fimbriata* **<***P. ambigua*

WE: *P. subrudecta* **<** *P. ambigua* **<** *E. mesomorpha* **<** *C. fimbriata*

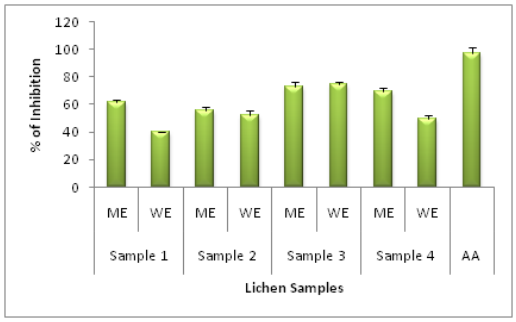


Fig: 6 Determination of lipid peroxidation in methanol extract and water extract of lichens samples. Results are mean ± S.D of three parallel measurements.

Qualitative test for Free Radical Scavenging Activity by TLC

After the completion of chromatogram the whole plate was sprayed with DPPH (0.15%W/V) solution. The development of yellowish colour spots on Figure 7 with Rf value 14, 13 and 10cm on the TLC plate was noted as an indicator of the presence of antioxidant substances.

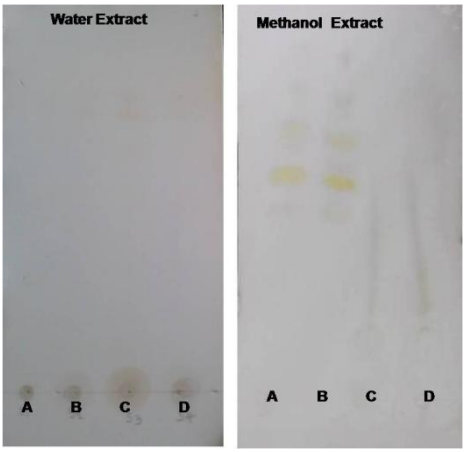


Fig:7Qualitative tests for Free Radical Scavenging Activity of lichens by TLC. Results are mean ± S.D of three parallel measurements.

Quantitative test for Free Radical Scavenging Activity by DPPH

The results of determination of hydroxyl radical scavenging activity of both methanol and water extracts in figure 8 showed that IC50 values of methanol extracts were 43.51±3.84, 29.62±2.37, 18.53±2.45 and 18.53±2.56µg/ml for all four methanol extracts of lichen samples respectively.

ME: *P. subrudecta* **>** *E. mesomorpha* **>***C. fimbriata* **>** *P. ambigua*

WE: *P. subrudecta* **>** *P. ambigua* **>***E. mesomorpha* **>***C. fimbriata*

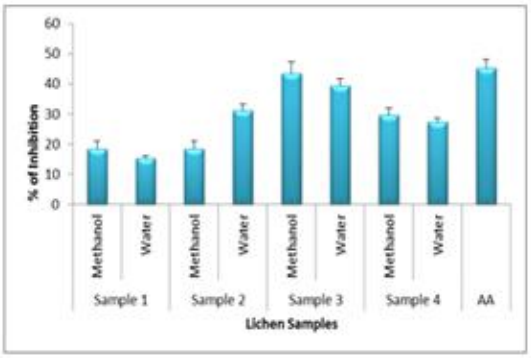


Fig: 8 Free radical scavenging activity of lichen compounds measured by the DPPH method. Results are mean ± S.D of three parallel measurements.

Reducing power

Reductive capabilities of both extracts along with ascorbic acid (AA) were shown in figure 9. High absorbance of extracts samples indicates a potent reducing power with increased concentration of extracts. Measured values of absorbance varied from 0.12 to 1.98. Among the tested lichen extracts, water extract of sample-D (*Evernia mesomorpha*) gave highest reducing power, although the reducing activity was lower than the standard ascorbic acid. The reducing capacity of the tested extracts decreased in the following order:

ME: *P. ambigua* **>** *C. fimbriata* **>***P. subrudecta* **>***E. mesomorpha*

WE: *E. mesomorpha* **>** *P. ambigua* **>***P. subrudecta* **>** *C. fimbriata*

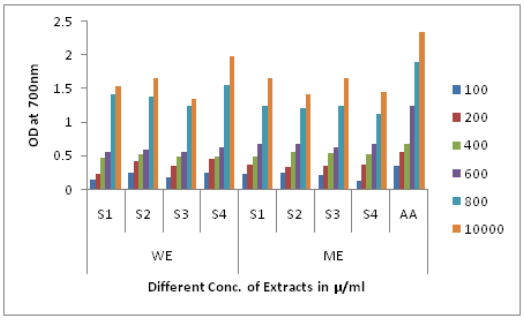


Fig: 9. Reducing power of methanol extract and water extract of Lichens.

Results are mean ± S.D of three parallel measurements.

Polysaccharide assay

Polysaccharide content in lichen extracts were shown in Figure 10, which indicates that the water extract of sample C (*P. subrudecta)* shows more concentrations of polysaccharides (24.39±1.85) when compared to remaining samples.

ME: *P. ambigua* **>***C. fimbriata* **>***P. subrudecta* **>***E. mesomorpha*

WE: *E. mesomorpha* **>** *P. ambigua* **>** *P. subrudecta* **>** *C. fimbriata*

The polysaccharide content obtained from the lichens was ranging from 9.89±0.26 to 18.23±0.68.

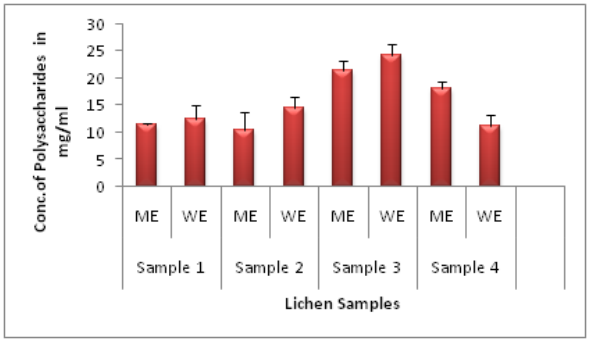


Fig:10Polysaccharides content in methanol extract and water extract of Lichens samples by Dubois method. Results are mean ± S.D of three parallel measurements.

DISCUSSION

Plants are known to produce certain secondary metabolites (bioactive molecules) which react with other organism in the environment including bacterial or fungal growth. Lichens which are the symbiotic organisms of both fungi and algae synthesize numerous metabolites, the lichen substances which comprise different compounds including amino acid derivatives, sugar alcohols, aliphatic acids, macrocyclic lactones, mono-cyclic aromatic compounds, quinones, chromones, xanthones, dibenzofuranes, depsides, depsidones, depsones, terpenoids, steroids, carotenoids and diphenyl ethers [20].

Secondary metabolites are not absolutely essential for the survival and growth of lichens nevertheless, their study has revealed many possible advantages. They may impact biotic and abiotic interactions of lichens with their environment. They may help to protect the thalli against herbivores, pathogens, competitors and external abiotic factors, such as high UV irradiation. Many of them exhibit multiple biological activities, such as the dibenzofuran usnic acid (e.g., antimicrobial and larvicidal effects, anticancer activities, known also for its UV absorption).

The antimicrobial activities were produced in different extents by the various fractions of the four different lichens studied. According to the result, it is shown that the antimicrobial inhibition vary with the different types of the lichens, the solvent used for extraction and the microbes tested. Phytoconstituents like flavonoids, in most lichens exhibit a wide range of biological activities like antimicrobial, anti-inflammatory, anti-angionic, analgesic, anti-allergic, cytostatic and antioxidant properties [21]. From the studies of Burkholder [22], indicated that the lichens inhibit mostly Gram positive bacteria. Even though most of the lichens have been reported to be active against Gram positive bacteria, but it is of great interest to note that the extracts of *E. nepalense* and *U. longifolia* equally inhibited the growth of both Gram negative and positive bacteria. A large body of evidences stated that the bacteria are more sensitive to antibiotics compared to fungi. The reason of difference in sensitivity between bacteria and fungi can be found in different transparency of the cell wall [23]. The study done by Marijiana *et al* [24] reported the fungi to be more resistant towards lichen extracts than bacteria. But the present study revealed that the lichen extracts showed the strong antifungal activity. The extracts of *Cetraria sp* and *P. milghenensis* showed the specific antifungal activity while least or ineffective toward bacterial pathogens. Kumar *et al* [25] also demonstrated antifungal activity of lichens. Isodivaricatic acid, 5-propylresorcinol, divaricatinic acid and usnic acid have been identified as antifungal agents [26].

Previous studies stated that the aqueous extracts of tested lichens did not show any antimicrobial activity [27, 28]. This is probably because the active components produced by lichens are either insoluble or poorly soluble in water.

Free radicals play an important role in many chemical processes in the cells, but they are also associated with unwanted side effects, causing cell damage. Since synthetic antioxidants are often carcinogenic, finding natural substitutes is of great interest. Lichens have been found to contain a variety of secondary lichen substances, which are strong antioxidant compounds. In the present study water extract of lichen sample-3 (*Punctelia subrudecta)* shows potent antioxidant activity and is more similar to that of standard antioxidant ascorbic acid. Total phenols and free radical activity were more in methanolic extracts of sample 3 (*P. subrudecta*)and total antioxidant capacity, TBA, polysaccharides were more in water extract of sample 3 (*P. subrudecta).*

It is well established that, the extreme conditions in lichens increase oxidative stress; consequently, lichens contain larger amounts of antioxidant substances and have higher antioxidant activity. Amo de Paz [29] reported that methanol extracts of *Xanthoparmelia camtschadalis* and *X. conspersa*, as well as their isolated lichen compounds (salazinic acid, stictic acid, and usnic acid) protected human astrocytes from hydrogen peroxide-induced damage. Astrocytes are the first line of defines in the brain against neurotoxicity of reactive oxygen species (ROS), thus salazinic acid, stictic acid, and usnic acid could act as antioxidant agents in those neurodegenerative disorders associated with oxidative damage (e.g., Alzheimer's disease and Parkinson's disease).

An easy, rapid and sensitive method for the antioxidant screening of plant extracts is to add free radical scavenging assay of 2, 2-diphenyl-1-picryl hydrazyl (DPPH) stable radical [30]. The DPPH model has been widely used as a quick, reliable and reproducible parameter to search for the *in-vitro* general antioxidant activity of pure compounds as well as plant extracts [31]. The decrease in absorbance in the DPPH assay with increase in concentration of the extract (Figure 8) which was accompanied with a rapid colour change of the purple DPPH, suggest that the methanol extract of *P. subrudecta* (sample 3) has antiradical activity. Antioxidant activity may be due to the presence of terpenes, tannins and flavonoids [32]. Based on the present results of phytochemical screening, DPPH antiradical activity, ferric antioxidant reducing ability and antibacterial activity, it is possible to affirm that *P. subrudecta* and other three lichen samples can be used as a source of natural antioxidants and alternative method for treatment of diseases caused by bacteria and prevention of diseases due to free radicals. According to Ebana *et al* [33] alkaloids inhibit pathogenic bacteria, and tannins are important in herbal medicine in treating wounds which includes severe burns and to arrests bleeding [34]. High absorbance of extracts samples indicates a potent reducing power with increased concentration of extracts. Similar results reported by

Rahman [35] that reducing power of *S. japonica* increased with increasing concentration of the sample.This confirms the use of lichens for wounds treatment in traditional medicine.

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