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Antioxidant and Phytochemical analysis of selected lichen species from Mizoram, India

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ABSTRACT

Lichens are an important source of biologically active substances, primarily phenols, which are well known for their antioxidant properties. The aim of the present study was to evaluate the phytochemical constituents (flavonoids and phenols), and the antioxidant activity of the methanol extract of the selected lichens species viz. *Usnea baileyi*, *Hypotrachyna cirrhata* and *Lobaria pulmonaria* were collected from Tawi Wildlife Sanctuary, Mizoram, India. The total phenolic content and total flavonoid of the various extracts varied from 31.11 to 67.84 mg of Gallic acid equivalent per gram dry weight (GAE/g DW) and from 27.43 to 37.06 mg of Quercetin equivalent per gram dry weight (QE/g DW) respectively. DPPH free radical scavenging activity of the methanol extract of tested samples showed a concentration-dependent manner with an IC₅₀ value ranging from 133.6 µg/ml in *L. pulmonaria* to 243.9 µg/ml in *U. baileyi*. A comparison between lichens extract and positive control (BHT) showed a strong antioxidant capacity (IC₅₀ = 10.81 µg/ml) of positive control than the lichens methanolic extract. A high negative and significant negative correlation (P < 0.05) was observed between total phenol content and antioxidant activity IC₅₀ of DPPH assay. Moreover, a positively high significant correlation was also obtained between total flavonoid and phenol content (P < 0.01). The present study showed that bioactive compounds present in the lichen thallus may be used as good sources of natural antioxidants and a potential candidate for curing several ailments.

KEYWORDS: Antioxidant, Flavonoids, Phenols, Phytochemical, Mizoram

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INTRODUCTION

Lichens are organisms showing symbiosis between a fungal partner with an alga or a cyanobacterium (Bates *et al.*, 2011). There is a common belief that lichens contain medicinal properties against various diseases and are often used in folk medicines (Rankovic *et al.*, 2007). There are about 800 secondary metabolites identified from lichens and these are unique concerning those produced by higher plants (Hunneck & Yoshimura, 1996). Many useful secondary metabolites are synthesized by lichens which are antiviral, anti-microbial, antitumor, anti-allergic, and have an inhibitory effect on the growth of plants (Halama & van Haluwin, 2004; Rankovic *et al.*, 2007). This makes them an interesting subject for research as lichens have been shown to contain active antioxidants (Behera *et al.*, 2006; Gulcin *et al.*, 2002). The several bioactive compounds extracted from lichens are showing promising avenues for biopharmaceutical applications in the development of new formulations or technologies including antimicrobial, antioxidant, and cytotoxic agents (Zambare *et al.*, 2012).

Usnea baileyi, *Hypotrachyna cirrhata* and *Lobaria pulmonaria* were selected for the study of antioxidant property and phytochemical content. They have been widely used in folk remedies for the treatment of various diseases such as eczema, respiratory and pulmonary diseases, arthritis, bone fractures, strains, ulcers, and other stomach problems (Chopra *et al.*, 1958; Turner, 1983; Brij & Upreti, 1995; Huneck, 1999; Müller, 2001; Süleyman *et al.*, 2003; Nayaka *et al.*, 2010; Rajeswari, 2019). It has been established that natural antioxidants containing natural products show antifungal, antibacterial, antiviral, anti-inflammatory, and anti-allergic properties (Muanda *et al.*, 2010). Since different ethnic groups in the North-eastern part of India and the tribes in the Indo-Burman region, in particular, have hugely benefitted from various lichens as a source of medicine and as ingredients in flavouring agents for their local cuisines, therefore, buoyed by the long-standing traditional uses, three lichen species were selected to quantify their phytochemical content and antioxidant properties.

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MATERIALS AND METHOD

Reagents and Chemicals

All the chemical used in this study were of analytical grade and were purchased from Hi – media, India: Methanol HPLC grade, Butylated hydroxytoluene (BHT) LR, 2,2- Diphenyl-1-picrylhydrazyl (DPPH), Galic acid monohydrate, Folin-ciocalteau's reagent LR, Quercetin, Sodium carbonate, Sodium chloride AR, Aluminium chloride AR, Potassium acetate ACS.

Lichen Samples Collection and Identification

Fresh specimens of selected lichens (Figure. 1) were collected from Tawi Wildlife Sanctuary (23°30' North Latitude and 93°00' East longitude), Aizawl District, Mizoram, Northeast India during January- November 2018. The specimens were identified based on morphology, anatomy and chemical test following the literature on lichen (Awasthi, 1991; Awasthi, 2007) at the Botanical Survey of India, Allahabad. Voucher specimens were deposited at the Department of Botany, Mizoram University under the reference numbers (Table 1).

Preparation of Lichen Extracts

The collected lichens specimens (Figure 1) were air-dried at room temperature in a well-ventilated area and ground to a fine powder in a mixer grinder. The powder form thalli of lichens species were extracted in methanol for 72 hours using a Soxhlet apparatus. The extract was evaporated to dryness in a water bath to form a crude extract. The lichen extracts were refrigerated at 4°C until further use.

PHYTOCHEMICAL ANALYSIS

Determination of Total Phenolic Content (TPC)

The total phenolic content of methanol extracts was determined by using Folin-Ciocalteau's assay (Mc Donald *et al.*, 2001) with slight modification. 1 ml of extract (1 mg/ml) was mixed with 5 ml of folin-ciocalteau reagent and kept for 3 minutes at room temperature. Thereafter, 4 ml of 0.7 M Na₂CO₃ was added and the solution was kept at room temperature for 1 hour. Absorbance was measured at 750 nm using UV Vis-spectrophotometer. Gallic acid (50 µg/ml- 500 µg/ml) was used to prepare the standard curve, showing linear regression of $r^2 = 0.999$. The amount of total phenolic content in the given sample was expressed as Gallic Acid equivalent per gram dry weight (GAE/g DW).

Determination of Total Flavonoids Content

The total flavonoids content was determined by the Aluminum chloride method (Chang *et al.*, 2002). Briefly, 0.5 ml of plant extract (1 mg/ml) was mixed with 1.5 ml of methanol. After 5 minutes, 0.1 ml each of 10% Aluminium chloride (AlCl₃) and Potassium acetate was added and the final volumes were made up to 5 ml with methanol and incubated at room temperature for 30 minutes. Absorbance was measured at 415 nm using a UV Vis-spectrophotometer. The total flavonoid content in the given samples was determined from the standard curve of Quercetin (10µg/ml-100µg/ml) which showed linear regression of $r^2 = 0.996$. The amount of total flavonoids content in the given sample was expressed as Quercetin equivalent per gram dry weight (QE/g DW).

Table 1: Lists of lichen species collected, sampling area, habitat, and altitude

Voucher Number	Lichen species	Sampling area	Habitat	Altitude
17-00154 (MZUBOT)	<i>Usnea baileyi</i> (Stirton) Zahlbr	Southern part of Tawi Wildlife Sanctuary (Hmuntha)	Twig of <i>Litsea</i> <i>monopetala</i>	1435 m asl.
16-00039 (MZUBOT)	<i>Hypotrachyna cirrhata</i> (Fr.) Divakar <i>et al.</i>	Eastern part of Tawi Wildlife Sanctuary (Maite)	Bark of <i>Schima</i> <i>wallichii</i>	1377 m asl.
16-00112 (MZUBOT)	<i>Lobaria pulmonaria</i> (L.) Hoff.	Central part of Tawi Wildlife Sanctuary	Bark of <i>Cordia</i> <i>dichotoma</i>	1107 m asl.



Figure 1: Lichens species selected for phytochemical and antioxidant activity

DETERMINATION OF ANTIOXIDANT ACTIVITY

DPPH Free Radical Scavenging Assay

The methanol extract of the lichens, ability to scavenge the free radical was determined by using a stable 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) according to the method described by Blois (1958) with minor changes. 3 ml of the extract (1 mg/ml) was mixed with 0.5 ml of 0.1 mM DPPH solution dissolved in methanol. It was allowed to incubate at room temperature for 30 minutes. The decline of DPPH concentration was measured by a decrease in absorbance at 517 nm in a UV-Vis spectrophotometer. Butylated hydroxytoluene (BHT) was used as a positive control, methanol as a negative control.

The scavenging effect of DPPH free radical was calculated using the following equation: Percentage of scavenging activity = $\frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$. The IC_{50} value which represents the concentration of the extract that inhibits the formation of DPPH radicals by 50% was also calculated.

Statistical Analysis

All the results analysed were expressed in means of triplicate (Mean \pm SD). For comparison of statistical significant ($P < 0.05$), one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests was used. IC_{50} was calculated using Graph Pad Prism software (version 6.04; Graph Pad Software, Inc., La Jolla, CA, USA). Pearson correlation coefficients were calculated to know the relationship between variables using PASW statistical software version 18.

RESULTS

Determination of Total Phenolic Content (TPC) and Flavonoid Content (TFC)

The total phenolic content (TPC) of selected lichens extracts exhibited a significant variation between the plant extract ($p < 0.05$). The result ranges from 31.11 to 67.84 mg of GAE/g DW. The maximum amount of TPC was determined in methanol extract of *L. pulmonaria* (67.84 mg of GAE/g DW) followed by *U. baileyi* and *H. cirrhata* with 39.17 mg of GAE/g DW and 31.11 mg of GAE/g DW respectively (Table 2). The total flavonoid content (TFC) of selected methanol extract of lichens ranges from 27.43 to 37.06 mg of QE equivalent/g DW. The highest TFC was determined in *L. Pulmonaria*

Table 2: Total phenolic (TPC) and flavonoid content (TFC) of selected lichen species

Lichen species	TPC (mg GAE/g)	TFC (mg QE/g)
<i>Hypotrachyna cirrhata</i>	31.11 \pm 1.1 ^c	27.43 \pm 1.43 ^c
<i>Usnea baileyi</i>	39.17 \pm 2.02 ^b	31.10 \pm 0.43 ^b
<i>Lobaria pulmonaria</i>	67.84 \pm 2.28 ^a	37.06 \pm 0.91 ^a

Means followed by different letters within same column are significantly different according to Duncan's multiple range comparisons (DMRTs) ($P < 0.05$)

(37.06 mg of QE/g DW) which was followed by *U. baileyi* (31.11 mg of QE/g DW) and the lowest content was observed in *H. cirrhata* with a TFC content of 27.43 mg of QE/g DW. TFC also revealed a significant difference between the plant extract ($p < 0.05$) (Table 2).

Determination of DPPH Radical Scavenging Activity

The percentage of free radical scavenging activity of the selected lichens extract was in a dose-dependent manner (Figure 2). The antioxidant activity detected by the DPPH assay also exhibited extremely large significant variation of antioxidant capacities among the species ($p < 0.05$) (Table 3). The IC_{50} value ranges from 133.6 μ g/ml in *L. pulmonaria* to 243.9 μ g/ml in *U. baileyi* (Table 3). The lower the IC_{50} values of the lichen extract, the stronger was their free radical scavenging activity. Among the three lichens extract, *L. pulmonaria* showed a lowest DPPH IC_{50} value (133.6 μ g/ml) which indicates a potent antioxidant property in the species of *L. pulmonaria*. However, a comparison between IC_{50} values of lichens extract with the positive control (BHT IC_{50} = 10.81 μ g/ml), showed that positive control antioxidant had stronger activity than the lichen extracts (Figure 2).

Correlation coefficient between antioxidant activity, total phenol and flavonoid contents

To evaluate the reliability of DPPH assay, Pearson's correlation coefficient (R) between antioxidant activity, total phenol and flavonoid content was performed (Table 4). The results

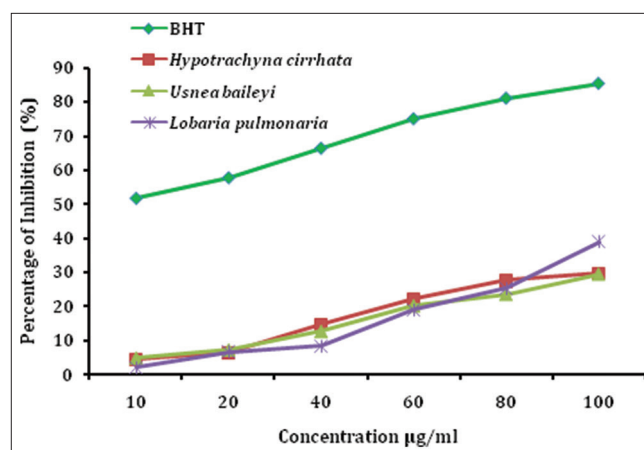


Figure 2: Comparison between percentages of DPPH free radical scavenging activity of lichens extract with positive control

Table 3: IC_{50} value of antioxidant activity assay of the selected lichens species

Name of the lichens species	$IC_{50} \pm SD$ (μ g/ml)
<i>H. cirrhata</i>	188.4 \pm 2.7 ^b
<i>U. baileyi</i>	243.9 \pm 4.3 ^a
<i>L. pulmonaria</i>	133.6 \pm 7.00 ^c
BHT	10.81 \pm 0.7 ^d

Means followed by different letters within same row are significantly different according to Duncan's multiple range comparisons (DMRTs) ($P < 0.05$)

Table 4: Pearson's correlation coefficients between antioxidant activity, total phenol and flavonoid content

Antioxidant assay	Total phenol		Total flavonoid	
	R value	P- Value	R value	P value
DPPH radical scavenging activity	-0.725*	0.027	-0.589	0.095
Flavonoid	0.964**	0.00	-	-

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

further indicate total phenolic content of the lichens extract had a negative and significant correlation with IC₅₀ of DPPH (R= - 0.725*, P<0.05). This suggests that phenol present in the methanol extract of lichens sample might be the major contributor of antioxidant activity by DPPH method. A positive and significant correlation was obtained between total flavonoid and total phenol content (R= 0.964**, P<0.01). This reveals that flavonoids present in the methanol extracts of the lichens might be the main components of total phenols obtained in this study.

DISCUSSION

Lichen possesses valuable compounds that are not found in higher plants. Phenolic compounds possess amazing antioxidant capability through free radical scavenging activity (Kaushik et al., 2010; Leopoldini et al., 2011). Since, methanol is well known as one of the best solvents for extracting compounds such as phenolic, flavonoid, and other polar materials in plants (Velioglu et al., 1998), methanol was selected as a solvent for the extraction of lichens in the present study. The present study of lichen species exhibited varying degrees of antioxidant activity. TPC in this study was determined by Folin-Ciocalteu method, ranging between 31.11 to 67.84 mg GAE/g, which was much higher than the value (18.20 to 40.57 mg PE/g) reported by Kosanic et al. (2012). The results further revealed that *L. pulmonaria* contained highest TPC (67.84 ± 2.28 mg GAE/g DW), which was lower than the previously reported value of 87.9 mg GAE/g of methanol extract (Odabasoglu et al., 2004). The methanol extract of *H. cirrhata* contained 31.11 mg GAE/g DW of TPC in this study, while Fernández-Moriano et al. (2016) reported the TPC of 60.6 mg GAE/g in the methanol extract of the same species. In the case of *U. baileyi*, the TPC (39.17 mg GAE/g DW) was in accordance with the study reported by Santiago et al. (2021). However, it was much higher than the previously reported value of 10.5 mg GAE/g (Saidi et al., 2018). While, Nguyen et al. (2019) reported TPC of 101.2 µg GAE/g from methanol extract of *U. baileyi*.

Flavonoids are biologically active phytochemicals having a wide range of biological and pharmacological activities including anti-allergic, antioxidant, anti-inflammatory, and anti-microbial activities (Trease & Evans, 2002; Bender & David, 2007). The present study highlights TFC of methanol extract of different samples varied significantly (P<0.05), from 27.43 to 37.06 mg QE/g DW, which was higher than the previous work of Aoussar et al. (2017) and Santiago et al. (2021) (1.288–3.957 mg QE/g). TFC content of *U. baileyi* in the

present study (31.10 mg QE/g DW) was lower than the study of Nguyen et al. (2019) who reported the TFC as 66.87 mg QE/g. However, it was higher than the recent work of Santiago et al. (2021). The flavonoid contents of the selected lichens which were being utilized by some tribes as an ethno-medicinal plant may prove to be useful for treating complicated diseases. Moreover, to the best of our knowledge, this study proved to be the first report of *L. pulmonaria* and *H. cirrhata* in determining their TPC.

The reduction capability of DPPH was induced by antioxidants present in lichens. In the present study, the in vitro antioxidant properties were evaluated by DPPH assay. The selected lichens species of the present study revealed variable antioxidant capacity. Antioxidant activity of selected lichens methanol extract showed a dose-dependent manner, which indicated that with the increase in concentrations, the percentages of free radical scavenging activity was increased. This finding was also in accordance with similar studies of various lichens (Mitrović et al., 2011; Kosanic et al., 2014). *L. pulmonaria* exhibited the highest free radical-scavenging activity (DPPH IC₅₀ of 133.6 µg/ml). This activity may be due to the high content of phenolic and flavonoid compounds in the lichen thallus of *L. pulmonaria* (Odabasoglu et al., 2004). The IC₅₀ value of *H. cirrhata* in this study was 188.4 µg/ml, while Fernández-Moriano et al. (2016) reported DPPH EC₅₀ of 946 µg/ml from methanol extract of the same species. In the case of *U. baileyi*, DPPH IC₅₀ in the present study was found lower than the previously reported value of 281.1 µg/ml (Nguyen et al., 2019). On the contrary, Santiago et al. (2021) reported *U. baileyi* IC₅₀ value of 13.24mg/ml. These suggested that all the extracted samples were able to scavenge the free radical even at low concentrations. This result further showed that the strong free radical scavenging activity of *L. pulmonaria* may be due to the presence of more antioxidant compounds than other phytochemicals which helps them to scavenge the free radical.

Pearson's correlation (R) was used to study the relationship between antioxidant activity and secondary metabolites of lichens species. The present study reveals a strong negative and significant correlation between antioxidant activity and TPC (P<0.05), which was in accordance with the previously reported value of R= -0.744 (Fidrianny et al., 2020). On the contrary, a positive and significant correlation was observed between antioxidant activity and TPC of various lichen extracts with an R-value of 0.706 (Fernández-Moriano et al., 2016) and 0.921* (Ranković et al., 2011). A positive significant correlation (P<0.01) was observed between TPC and TFC of the selected lichens species, the same result was also observed by Kosanic et al. (2012) (R= 0.84). This suggests that flavonoids present in the methanol extract of the lichen understudy might be the major constituents in the phenol obtained. However, no correlation was observed between antioxidant activity and TFC in the present study, which was also in agreement with the previous finding from various lichens extract (Nguyen et al., 2019). This result further indicated that many other secondary metabolites plant products, other than flavonoids, might be involved in the antioxidant capacities of the present study lichens species.

CONCLUSION

Exploration research is crucial in the discovery of new drug compounds having different mechanisms of action. The present investigations explored the different varieties of lichens for their potential as antioxidants and concluded with the observation of secondary metabolites of potent antioxidant activity from the thallus of lichens that may have potential sources of new drug synthesis. Future research on the identification, isolation, and assessment of the biological activity of the active compounds can give us new insight and impact in the discovery of new drugs.

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