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Original Research Article

Biological and Chemical Analysis of Five Selected Lichen Species from Sagarmatha National Park of Nepal

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ABSTRACT

Lichens are unique organisms consisting of fungi and algae in a symbiotic association. Lichens have been used as medicines for a long time. The purpose of this study was to profile the chemical constituents and biological activities of methanol extract of five lichens; *Lobaria japonica, Lobaria retigera, Heterodermia leucomela, Heterodermia speciosa* and *Ramalina spp.* collected from Sagarmatha National Park, Solukhumbu District, Nepal. Total phenolic content was evaluated by Folin Ciocalteu method while total flavonoid content was evaluated by aluminum chloride colorimetric method. Antioxidant activity was evaluated by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay and *α*-amylase inhibitory activity was evaluated by starch-iodine method. Antibacterial activity was evaluated by agar-well diffusion method. The highest phenolic content was observed in *L. retigera* and the lowest was in *H. speciosa*. Similarly, the highest flavonoid content was observed in *L. retigera* and lowest in *Ramalina spp*. The highest DPPH radical scavenging activity was shown by *L. retigera* and lowest by *H. speciosa*. The methanol extract of *L. japonica* exhibited the highest *α*-amylase inhibitory activity. The lichens possessed high antimicrobial properties against *E. coli, S. aureus* and *B. subtilis.* Metabolites profiling of selected three lichens species by Gas Chromatography-Mass Spectrometry revealed the presence of atraric acid, methyl haematomate, orcinol and methyl everniate as the major components. Among the five lichens, *L. japonica and L. retigera* were found to possess potent biological activities as well as higher phenolic and flavonoid contents. These lichens can be further studied and processed for the commercial production of polyphenols.

Keywords: Antioxidant, Antibacterial, *α*-amylase, GC-MS, Secondary metabolites.

Introduction

Lichens are unique organisms consisting fungi and algae in symbiotic association.[1] The bioavailability of lichen is diverse; they are found in high mountains and desert as well and some can make stones and trees as their substratum. Lichens have been used by human from the ancient time to fulfill their basic needs such as food, medicines, decoration, making dyes, cosmetic products etc.[2] Lichens produce several secondary metabolites which exhibit important biological activities such as antioxidant, antimicrobial, cytotoxic, antidiabetic and antiproliferative.[3-6] Due to these diverse activities, many scientists around the world are being attracted towards the identification, isolation and purification of lichen metabolites. The major secondary metabolites found in lichens are dibenzofurans (e.g., usnic acid), depsides (e.g., gyophoric acid), depsidones (norstictic acid), xanthones and terpene derivatives.[7]

Phenolic compounds like phenolic acids, flavonoids, and vitamins are also the major secondary metabolites in lichens which exhibit potential antioxidant activities. These secondary metabolites can scavenge harmful free radicals such as reactive oxygen species which are one of the major causes of ageing, cell proliferation and cancer. Nowadays people are being more conscious about their health and prefer foods containing extra nutrition such as vitamins, flavonoids, minerals and so on.[8] Lichens can be the source of these additive nutrition and may serve as the potential source for their commercial production.

α-Amylase is one of the main enzymes responsible for breakdown of carbohydrates in the form of starch found in food materials into small molecules like glucose and helps in increasing blood glucose level. Different researches have been performed revealing the potential of different lichens to inhibit *α*-amylase.[9,10] Furthermore, inhibitions of other enzymes such as *α*, *β*-glucosidase and lipase by different lichen species have also been studied which directly or indirectly play roles in developing diabetes.[11,12] Although Nepal is rich in biodiversity, diverse group of organisms along with different species of lichens with medicinal properties are abundant in large scale. Very few researches have been carried out in lichens and no significant efforts have been made till now to make the lichens commercialization while advanced researches have been done and still going on in other parts of the world. This study might be a step forward to attract researchers for the further studies in lichens and to let people know about the importance of lichens.

Furthermore, resistance of pathogenic microbes against synthetic drugs are the great problems which might be due to continuous and uncontrolled uses of antibiotics.[13] Synthesis of natural products having potential antimicrobial activities with minimal side effect is one of the major concern of the scientist these days.[7] So this study mainly focused on secondary metabolite profiling; antioxidant and antibacterial potential of methanol extracts.

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Materials and Methods

Collection and Identification of Lichens

Five different lichens, *Lobaria japonica*, *Lobaria retigera*, *Heterodermia leucomela*, *Heterodermia speciosa* and *Ramalina spp* were collected from Sagarmatha National Park in the month of May-June 2016. All samples were scientifically identified by Prof. Dr. Chitra Bahadur Baniya at Tribhuvan University Central Herbarium (TUCH), Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal. The plant voucher specimens (L01 for *L.retigera* and L02 for *L. japonica*, H01 for *H. speciosa*, H02 for *H. leucomela* and R01 for *Ramalina spp*) were deposited at National Herbarium and Research Laboratory, Department of Plant Resources, Ministry of Forests and Soil Conservation, Godawari, Kathmandu, Nepal.

Extraction

Samples were dried in the shade and ground to powder form for the extraction. All the dry powered of respective lichens were extracted using methanol at room temperature. The extract was filtered then concentrated under reduced pressure rotary evaporator. This process was repeated 2-3 times to yield methanol extract.

Determination of total phenolic contents

The amount of total phenolic contents (TPC) on lichens extract was determined by Folin Ciocalteu (FC) assay described previously[6] with slight modification using gallic acid as standard. Methanol extracts of all five lichens were prepared at concentration of 1 mg/mL. Briefly, 0.1 mL of FC reagent and 0.8 mL deionized water were added to 0.5 mL of the prepared extracts. After 3 min of incubation, 0.3 mL of Na_2CO_3 (75 mg/mL) was added and allowed to stand for 30 min at room temperature. Absorbance was measured at 765 nm against the methanol as blank in 96 wells plate ELISA reader (BIOTEK, US). All the tests were done in triplicates and TPC was expressed as the milligram gallic acid equivalent per gram of dry weight (mg GAE/g DW).

Determination of total flavonoid content

The total flavonoids content (TFC) was determined by Aluminum chloride colorimetric method as previously described[14] with slight modification using quercetin as standard. All the five lichens extracts of 1 mg/mL was prepared and 1.5 mL of methanol extracts was mixed with 75 µl of 5% $NaNO_2$ and incubated for 6 min. Then 150 µl of 10% $AlCl_3$ was added to the mixture and incubated for 6 min and 0.5 mL of 1M NaOH was added after that distilled water was added to make up the final volume to 3 mL and the reaction mixture was mixed gently. All the experiments were performed in triplicates and absorbance measured at 510 nm in 96 wells plate ELISA reader (BIOTEK, US). TFC was expressed as the milligram of quercetin equivalent per gram of dry weight (mg QE/ g DW).

Antioxidant assay

DPPH free radical scavenging assay

DPPH assay was performed by using a previously described procedure[15] with some modification. Briefly, 2.5 mL of different concentrations (10-200 µg/mL) of methanol extracts of all five lichens were mixed with 1 mL of 0.3 mM freshly prepared DPPH in methanol, mixed well and incubated in the dark for 30 min. After incubation, the absorbance was measured at 518 nm against methanol as blank in 96 wells plate ELISA reader (BIOTEK, US). Ascorbic acid was used as a standard. The experiments were performed in triplicate and percentage Radical Scavenging Activity (RSA) was calculated by using the following formula. Inhibitory Concentration 50 (IC_{50}) was calculated on the basis of average RSA values.

$$\%RSA = \frac{absorbance\ of control - absorbance\ of sample}{absorbance\ of control} \times 100$$

ABTS free radical scavenging assay

For ABTS assay, the procedure described by Saeed *et. al*.[16] was used with slight modifications using ascorbic acid as a standard. Briefly, 7 mM ABTS and 2.45 mM dipotassium sulfonatooxy sulfate ($K_2S_2O_8$) solution were prepared separately in distilled water mixed properly and kept for 16 h in the dark for free radical generation. The next day, the dark green colored mixture formed was diluted with Phosphate Buffer Saline (pH 7.4) and absorbance was measured at 734 nm. Then 1 mL of the diluted ABTS free radical solution was added to the 0.5 mL of the different concentrations (2.5-20 µg/mL) of methanol extract of lichens. The mixture was incubated for 15 min and absorbance was measured at 734 nm. The IC_{50} was calculated from average RSA values and percentage RSA was calculated by using following equation.

$$\%RSA = \frac{absorbance\ of\ control - absorbance\ of\ sample}{absorbance\ of control} \times 100$$

α-Amylase inhibitory assay

α-Amylase inhibitory assay of all five extracts were performed according to the method adopted by Hossain *et. al*.[17] with slight modifications. Briefly, 390 µL of different concentrations (100, 200, 300, 400, 500 µg/mL) of lichen extracts were prepared from stock solution (1 mg/mL) and in 99% (v/v) DMSO. Then 50 µL of fungal *α*-amylase (diastase) (1 IU/mL) prepared in 0.02 M sodium phosphate buffer (pH 6.9) was added and incubated for 10 min at 37˚C. After incubation, 100 µL of 1% (w/v) starch solution prepared in 0.02 M Sodium phosphate buffer (pH 6.9) was added to the mixture and incubated further for 30 min at 37˚C. Then, 100 µL of iodine reagent (5mM I_2 and 50 mM KI) was added, mixed thoroughly, diluted with 3 mL water and absorbance was measured at 565 nm. Substrate blank and *α*-amylase blank were also prepared in the same condition. Percentage inhibition of *α*-amylase was calculated according to the formula.

$$\text{Percentage inhibition} = \frac{A-C}{B-C} \times 100$$

where A is the absorbance of sample along with substrate and *α*-amylase, B is the absorbance of blank (no *α*-amylase), and C is the absorbance of control (no starch). All the experiments were performed in triplicates.

Gas Chromatography- Mass Spectrometry

On the basis of thin layer chromatography (TLC) pattern at different solvent system only three extracts namely *L. retigera*, *H. speciosa*, and *Ramalina spp* were selected for metabolites profiling by gas chromatography (Agilent technologies 7890A) connected with mass spectrometer (Agilent technologies) as described previously.[18] The carrier gas was Helium with constant flow rate of 1 mL/min. The column used in GC-MS was Agilent 19091S-433: 1887.08794 HP-5MS with stationary phase 5% Phenyl Methyl Silox and the size of column used for analysis was of 30 m×250 µm×0.25 µm. The injection volume was 2 µL with syringe size of 10 µl and split ratio was 25:1. The initial temperature of oven was 40˚C held for 1 min for injecting sample and then raised up to 250˚C at the rate of 15˚C/min which was held for 5 min and the total run time for each sample was 20 minutes.

Antibacterial assay

Bacteria and culture medium

Seven different bacterial strains *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), *Enterobacter faecalis* (ATCC 29212), *Bacillus subtilis* (ATCC 6051) and *Salmonella typhi* (clinical isolate) were used for antibacterial assay. Antibacterial activities were performed by agar well diffusion technique[19] for the preliminary screening. The bacteria were grown in Nutrient agar and bacterial suspension for antibacterial test was prepared in Muller Hinton Broth.

Minimum inhibitory concentration (MIC)

The Minimum inhibitory concentration was determined by broth micro dilution method according to the CLSI guidelines.[20] The extracts

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of five lichens were prepared by two-fold dilutions (25, 12.5, 6.25, 3.125, 1.56, 0.78 µg/mL) in 96 well micro-titration plate. Then, each well was inoculated with a microbial inoculum prepared in the same medium after dilution of standardized microbial suspension. After well-mixing, the inoculated 96-well micro-titration plate were incubated at 37˚C for 24 h and analyzed for determining MIC. Streptomycin was used as a positive control while DMSO as a negative control.

Statistical analysis

All the analyses were performed with Excel, origin Pro 6.1 and SPSS version 20. All the experiments were carried out in triplicate and the results are expressed in mean ± standard deviation. Significance of all the values obtained were tested by one way ANOVA at $P<0.05$.

Results and Discussion

Yield of Extracts

All five lichen samples were extracted with methanol and the yield of extracts and percentage of extract are tabulated in Table 1. The yield of the extract of Ramalina was highest (7.25%) while that of *H. leucomela* was the least (2.92%).

Total Phenolic and Flavonoid Contents

The overall results on total phenolic contents and flavonoid contents were summarized in Table 2. The highest content of phenolic compound was found in *L. retigera* (89.97± 1.66 mgGAE/g DW) followed by *L. japonica* (72.30± 0.93 mgGAE/g DW). Similarly, *L. retigera*was found to containe the highest amount of flavonoids (677.50± 2.39 mgQE/g DW) followed by *L. japonica* (473.50± 3.17 mgQE/g DW). This study showed that the two lichens *L. retigera* and *L. japonica* are good source of phenolics and flavonoids. Phenolic compounds such as depsides, depsidones and dibenzofuran are present in lichens and these compounds might be responsible for the antioxidant, antimicrobial, antitumor, antiviral and ant-allergicactivity since these compounds have capacity to donate hydrogen to free radicals.[21, 22]

Antioxidant activity

The antioxidant activity of the methanol extracts of all five lichens are tabulated in Table 3. The antioxidant capacity of the lichen extracts were compared with the standard ascorbic acid. The IC_{50} value of the standard ascorbic acid for DPPH and ABTS were found to be 6.23±0.03 µg/mL and 1.8±0.3 µg/mL, respectively. *L. retigera* showed the highest DPPH free radical scavenging activity among the five lichens with IC_{50} value of 23.36±1.61 µg/mL followed by *L. japonica* with $IC_{50}$of 40.26±2.84 µg/mL. Three lichens; *H. leucomela*, *H. speciosa* and *Ramalina* spp. showed relatively low activity with IC_{50} values of 255.89±1.65, 410.65±15.23 and 153.80±3.97 µg/mL, respectively. Furthermore, similar results were obtained with ABTS free radical scavenging activity of *L. japonica* and *L. retigera* with IC_{50} values of 7.31±0.14µg/mL and 9.86±0.26 µg/mL, respectively.

The highest DPPH and ABTS free radical scavenging activity of both *Lobaria* spp. is related to their high content of phenolic compounds and flavonoids which is also justified by the Pearson's correlation coefficient. Since *Lobaria* spp. has shown potential antioxidant activity, these species can serve as the promising source of natural antioxidants. The highest amount of phenolic compounds and flavonoids in these two species might be the reason for the highest antioxidant activities. There are strong relationships between the TPC, TFC and antioxidant activity (AA) values of methanol extracts of these species. Different studies also proved that phenolic constituents and flavonoid contents in the sample is mainly responsible for their potential antioxidant activity.[23,24] The Pearson's correlation coefficient (Table 4) showed that both DPPH and ABTS are correlated with TPC and TFC. Although our results shows positive correlation with TPC and TFC, antioxidant activity is not necessarily always correlated with the amount of phenolic and flavonoid compounds present in the crude extracts.[16] It might be due to the compounds other than phenolics and flavonoids present in the extract.[25,26]

α-Amylase inhibitory assay

The *α*-Amylase inhibitory activity of the selected lichen extracts were carried out using the standard protocol. Among the analyzed lichen extract, *L. japonica* showed the highest *α*-amylase inhibitory activity as indicated by its lowest IC_{50} value (989.56±9.45 µg/mL) and followed by *H. leucomela* (1063±34.96 µg/mL) and *H. speciosa* (1222±13.06 µg/mL). Inhibitory activity of all the lichens is shown in Table 3. The *Ramalina* spp showed the lowest inhibitory activity.

Further purification and identification of the bioactive constituents might significantly increase the inhibitory activity against *α*-amylase. Numerous scientific reports revealed that lichen metabolites possess significant *α*-amylase inhibitory activity.[27-29]

Minimum inhibitory concentration (MIC)

The anti-bacterial activity of all the five selected lichens was determined by measuring the minimum inhibitory concentration (MIC) of extracts against seven different pathogenic organisms. The results were summarized in Table 5. All lichens showed antibacterial activity with the MIC range of 0.78- 25 mg/mL. *L. japonica* showed potential antibacterial activity against *E. coli* and *P. aeruginosa* with MIC of 1.56 mg/mL and 3.12 mg/mL against *K. pneumonia* and *S. aureus*. The lowest MIC of 3.12 mg/mL was observed for *L. retigera* extract and the MIC against other organisms was relatively higher (6.25 mg/mL for *B. subtilis* and *S. aureus* and 12.5 mg/mL for *E. faecalis*, *S. typhii*, *K. pheumoniae*, *P.aeruginosa*). Similarly, *H. leucomela* was active against *B. subtilis* with MIC of 3.125 mg/mL and 1.56 mg/mL against *E.coli* and *S.aureus*. Furthermore, MIC of 3.12 mg/mL was observed against *E.coli* and *S. aureus* for *H. speciosa*. In this study the lowest MIC was found with the extract of *Ramalina* spp. against *E. coli* (0.78 mg/mL).The MIC value of standard antibiotic Streptomycin were 1.9 µg/mL for *S. aureus*, and *K. pneumoniae*, 3.9 µg/mL for *E. faecalis*, 7.8 µg/mL for *E. coli*, *B. subtilis* and *P. aeruginosa* and 15.6 µg/mL for *S. typhi*.

Although the results revealed that lichen extracts are not as potent as the standard antibiotics, but it is an evident that these crude extract are potent antimicrobial agents. Further, we can assume that purification of the compounds from extract might significantly increase the antibacterial activity. Out of the seven bacterial strains, *E. coli*, *S. aureus* and *B. subtilis* were found to be more susceptible than the other four bacteria with comparatively low MIC values. The antibacterial activity of the extracts depend upon various parameters such as types of lichens used and their chemical constituents, solvent used for extraction and the amount of extracts used for the activity.[29]

Table 1: Yield of lichen extracts obtained by cold extraction with methanol.

Name of Lichens	Weight of extracts (g)	Yield of extract (%)
L. japonica	5.58	6.06
L. retigera	3.69	3.96
H. leucomela	1.20	2.92
H. speciosa	4.92	4.27
Ramalina spp	3.41	7.25

Table 2: Total phenolic content (TPC) and total flavonoid content (TFC) of methanol extract of lichens.

Lichens	TPC (mg GAE/g DW)	TFC (mg QE/g DW)
L. japonica	72.30±0.93	473.50±3.17
L. retigera	89.97±1.66	677.50±2.39
H. leucomela	50.88±0.73	95.10±3.17
H. speciosa	33.86±2.88	73.83±8.22
Ramalina spp	44.21±0.64	58.65±2.91

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The antimicrobial activity of the pure isolated compounds of lichens such as Usnic acid, physodic acid, atranorin and gyrophoric acid, norstictic acid and protocetraric acid have also been reported to have antibacterial activity.[31,32]

GC-MS analysis of methanol extracts of lichens

The major metabolites present in three lichens extracts were identified on the basis of their GC chromatogram and mass fragmentation pattern and comparing the peaks and retention time with NIST database. They were further confirmed by comparing with previously reported metabolites in lichens analyzed by GC-MS.[32] The major metabolites found in these lichens are methyl orsellinate, methyl-haematommate, atraric acid, methyl orsellinate, orcinol, and chloroatronorin. All the metabolites of these lichens as detected by GC-MS are listed in Table 6. Some of the previously identified compounds are methyl orsenillate, orsenillic acid, atranorin, and lecanoric acid. These compounds were screened for antioxidant activity and showed moderate antioxidant activity. Other compounds such as atranorin, hopane-6 α, 22-diol, usnic acid and vulpinic acid are reported and tested for biological activities such as antimicrobial, antitubercular and anticancer activities.[34,35]

Table 3: IC_{50} values for DPPH, ABTS and α-amylase inhibitory assay of selected lichen species

Lichens	Antioxidant Activity		α-amylase inhibitory activity (µg/mL)
	DPPH (µg/mL)	ABTS (µg/mL)	
L. japonica	40.26±2.84	7.31±0.14	989.56±9.45
L. retigera	23.36±1.61	9.86±0.26	1470.02±88.12
H. leucomela	255.89±1.65	23.36±1.41*	1063±34.96
H. speciosa	410.65±15.23	42.96±2.77	1222±13.06
Ramalina spp	153.80±3.97	20.72±2.23*	1576.8±56.02
Ascorbic acid	6.23±0.03	1.83±0.10	-

All the values are significantly different at $p < 0.05$ except the values marked with asterisk*

Table 4: Correlation coefficient showing the correlation between TPC, TFC and antioxidant activities

	TPC	TFC	DPPH	ABTS
TPC	1	0.968**	0.875	0.855
TFC	0.968**	1	0.796	0.748
DPPH	0.875	0.796	1	0.979**
ABTS	0.855	0.748	0.979**	1

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

Table 5: Minimum Inhibitory Concentration of lichens against different pathogenic bacteria

Organisms	Minimum Inhibitory Concentration					
	L. japonica	*L. retigera*	*H. leucomela*	*H. speciosa*	*Ramalina spp*	*Streptomycin*
B. subtilis	6.25	6.25	3.12	6.25	3.12	7.80
E. coli	1.56	3.12	1.56	3.12	0.78	7.80
E. faecalis	12.50	12.50	6.25	12.50	12.50	3.90
K. pneumonia	3.12	12.50	6.25	12.50	25.00	1.90
P. aeruginosa	1.56	12.50	6.25	6.25	25.00	7.80
S. aureus	3.12	6.25	1.56	3.12	6.25	1.90
S. typhi	12.50	12.50	6.25	6.25	25.00	15.60

MIC values are in mg/mL for lichen extracts and µg/mL for Streptomycin.

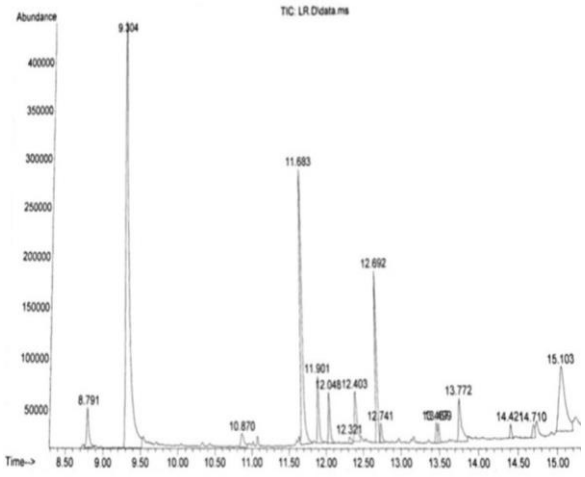

Figure 1: GC Chromatogram of *L. retigera*

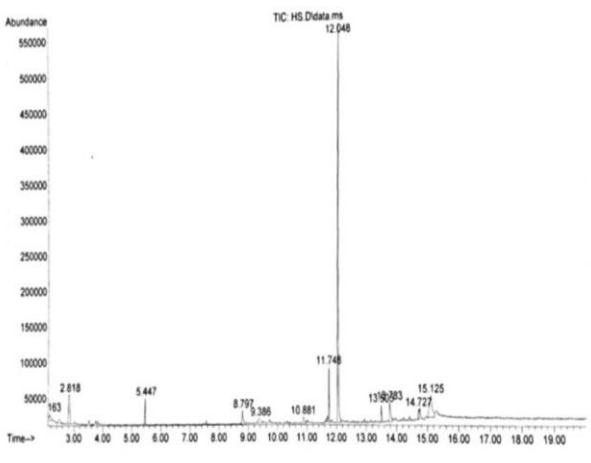

Figure 2: GC Chromatogram of *H.speciosa*

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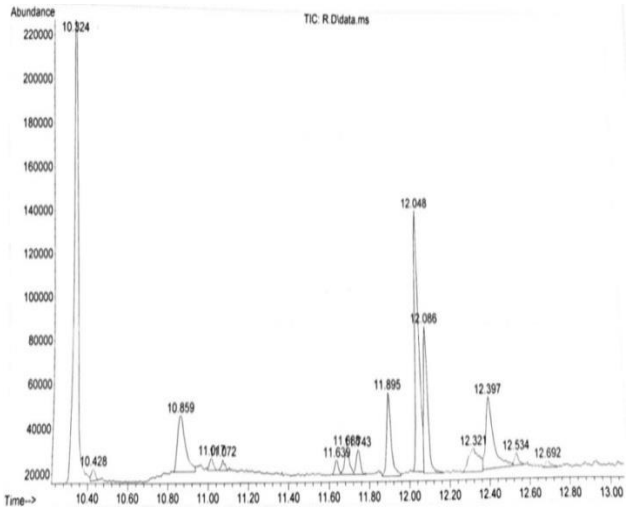

Figure 3: GC Chromatogram of *Ramalina* spp

Conclusion

Results obtained from this study revealed that of the five lichen species, *L. japonica* and *L. retigera* have potent antioxidant activity along with high content of phenolic and flavonoid contents. All five lichens extract were found to possess moderate *α*-amylase inhibitory activity. Potential antibacterial activity was also observed in all the lichens against *E. coli*, *S. aureus* and *B. subtilis*. Further, GC-MS analysis revealed the presence of methyl orsellinate, atraric acid, olivetol in *L. retigera*, methyl linoleate in *H. speciosa* and O-methyl orcinol, methyl orsellinate, olivetol in *Ramalina spp* as major metabolites. Furthermore, our results open up possibility in future to purify the extracts with a view to isolating the bioactive compounds.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Table 6: Metabolites from GC-MS Analysis

Lichens	Compounds	RT (min)	Molecular formula	Molecular weight (g/mol)
L. retigera	*O*-methyl orcinol	8.791	$C_8H_{10}O_2$	138.166
	Methyl orsellinate	11.683	$C_9H_{10}O_4$	182.175
	Dihydrocarvone	11.901	$C_{10}H_{16}O_4$	153.237
	Atraric acid	12.048	$C_{10}H_{12}O_4$	196.20
	Olivetol	12.403	$C_{11}H_{16}O_2$	180.247
	Methyl haematommate	12.692	$C_{10}H_{10}O_5$	210.185
	Palmitic acid	13.772	$C_{16}H_{32}O_2$	256.43
	Oleic acid	15.103	$C_{18}H_{34}O_2$	282.47
H. speciosa	Glycolic acid, ethyl ester	2.163	$C_4H_8O_3$	104.105
	Thiazolidine	2.818	C_3H_7NS	89.159
	O- methyl orcinol	8.797	$C_8H_{10}O_2$	138.166
	Methyl haematommate	11.748	$C_{10}H_{10}O_5$	210.185
	Atraric acid	12.048	$C_{10}H_{12}O_4$	196.20
Ramalina spp	*O*-methyl orcinol	8.791	$C_8H_{10}O_2$	138.166
	Everninic acid	8.791	$C_9H_{10}O_4$	182.175
	Dimethyl resorcinol	10.324	$C_8H_{10}O_2$	138.16
	Methyl orsellinate	11.688	$C_9H_{10}O_4$	182.175
	Methyl haematommate	11.743	$C_{10}H_{10}O_5$	210.185
	Atraric acid	12.048	$C_{10}H_{12}O_4$	196.20
	Chloroatranorin	12.048	$C_{19}H_{17}ClO_8$	408.787
	Palmitic acid	13.767	$C_{16}H_{32}O_2$	256.43

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References

1. Nugraha AS, Pratoko DK, Damayanti YD, Lestari ND, Laksono TA, Addy HS, Untari LF, Kusumawardani B, Wangchuk P. antibacterial and anticancer activities of nine lichens of Indonesian Java Island. J Biol Act Prod Nat. 2019; 9(1):39-46.
2. Manojlovic NT, Vasiljevic PJ, Maskovic PZ, Juskovic M, Bogdanovic-Dusanovic G. Chemical Composition, Antioxidant, and Antimicrobial Activities of Lichen *Umbilicaria cylindrica* (L.) delise (Umbilicariaceae). Evid Based Compl Altern Med. 2012; 2012: 1-8.
3. Gülçin İ, Oktay M, Küfrevioğlu Öİ, Aslan A. Determination of antioxidant activity of lichen *Cetraria islandica* (L) Ach. J Ethnopharmacol. 2002; 79(3):325-329.
4. Bézivin C, Tomasi S, Lohézic-Le Dévéhat F, Boustie J. Cytotoxic activity of some lichen extracts on murine and human cancer cell lines. Phytomedicine. 2003; 10(6):499-503.
5. Zhang Y, Shi J, Zhao Y, Cui H, Cao C, Liu S. An investigation of the anti-diabetic effects of an extract from *Cladonia humilis*. Pak J Pharm Sci. 2012; 25(3):509-512.
6. Mitrović T, Stamenković S, Cvetković V, Tošić S, Stanković M, Radojević I, Stefanović O, Comić L, Dačić D, Curčić M, Marković S. Antioxidant, antimicrobial and antiproliferative activities of five lichen species. Int J Mol Sci. 2011; 12(8):5428-5448.
7. Ranković BR, Kosanić MM, Stanojković TP. Antioxidant, antimicrobial and anticancer activity of the lichens Cladonia furcata, Lecanora atra and Lecanora muralis. BMC Complement Alternat Med. 2011; 11(1):97.
8. Yokohira M, Yamakawa K, Saoo K, Matsuda Y, Hosokawa K, Hashimoto N, Kuno T, Imaida K. Antioxidant effects of flavonoids used as food additives (purple corn color, enzymatically modified isoquercitrin, and isoquercitrin) on liver carcinogenesis in a rat medium-term bioassay. J Food Sci. 2008; 73(7):C561-C568.
9. Valadbeigi T, Shaddel M. Amylase inhibitory activity of some macrolichens in Mazandaran province, Iran. Physiol Pharmacol. 2016; 20(4):215-219.
10. Salin Raj P, Prathapan A, Sebastian J, Antony AK, Riya MP, Rani MP, Biju H, Priya S, Raghu KG. *Parmotrema tinctorum* exhibits antioxidant, antiglycation and inhibitory activities against aldose reductase and carbohydrate digestive enzymes: an *in vitro* study. Nat Prod Res 2014; 28(18):1480-1484.
11. Lee K-A, Kim M-S. Glucosidase inhibitor from *Umbilicaria esculenta*. Can J Microbiol. 2000; 46 (11) : 1077-1081.
12. Anil KH, Prashith KT, Vinayaka K, Swathi D, Venugopal T. Anti-obesity (Pancreatic lipase inhibitory) activity of *Everniastrum cirrhatum* (Fr.) Hale (Parmeliaceae). Phcog J. 2011; 3(19):65-68.
13. Davies J and Davies D. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev. 2010; 74(3):417-433.
14. Wong JY, Matanjun P, Ooi YBH, Chia KF. Evaluation of antioxidant activities in relation to total phenolics and flavonoids content of selected Malaysian wild edible plants by multivariate analysis. Int J Food Prop. 2014; 17(8):1763-1778.
15. Mensor LL, Menezes FS, Leitão GG, Reis AS, Santos TCd, Coube CS, Leitão SG. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother. Res. 2001; 15(2):127-130.
16. Saeed N, Khan MR, Shabbir M. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla* L. BMC Compl Altern Med. 2012;12(1):221.
17. Hossain SJ, Tsujiyama I, Takasugi M, Islam MA, Biswas RS, Aoshima H. Total phenolic content, antioxidative, anti-amylase, anti-glucosidase, and antihistamine release activities of Bangladeshi fruits. Food Sci Technol Res. 2008; 14(3):261-268.
18. Pratibha P and Mahesh CS. GC-MS analysis and biological activities of medicinally important lichen: *Parmelia perlata*. IJPPR. 2016; 8(12):1975-1985.
19. Selvamohan T, Ramadas V, Kishore SSS. Antimicrobial activity of selected medicinal plants against some selected human pathogenic bacteria. AdvApplSci Res. 2012; 3(5):3374-3381.
20. Balouiri M, Sadiki M, Ibnsouda SK. Methods for *in vitro* evaluating antimicrobial activity: A review. J Pharm Anal. 2016; 6(2):71-79.
21. Odabasoglu F, Aslan A, Cakir A, Suleyman H, Karagoz Y, Halici M, Bayir Y. Comparison of antioxidant activity and phenolic content of three lichen species. Phytother Res 2004; 18(11):938-941.
22. Kosanić M, Ranković B, Vukojević J. Antioxidant properties of some lichen species. J Food Sci Technol. 2011; 48(5):584-590.
23. Ristić S, Ranković B, Kosanić M, Stanojković T, Stamenković S, Vasiljević P, et al. Phytochemical study and antioxidant, antimicrobial and anticancer activities of *Melanelia subaurifera* and *Melanelia fuliginosa* lichens. J Food Sci Technol. 2016; 53(6):2804-2816.
24. Behera B, Verma N, Sonone A, Makhija U. Evaluation of antioxidant potential of the cultured mycobiont of a lichen *Usnea ghattensis*. Phytother Res. 2005; 19(1):58-64.
25. Rice-evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radic Res. 1995; 22(4):375-383.
26. Ranković B, Kosanić M, Stanojković T, Vasiljević P, Manojlović N. Biological activities of *Toninia candida* and *Usnea barbata* together with their norstictic acid and usnic acid constituents. Int J Mol Sci. 2012; 13(11):14707-14722.
27. Hengameh P, Rashmi S, Rajkumar H. *In vitro* inhibitory activity of some lichen extracts against α-amylase enzyme. EJBPS. 2016; 3(5):315-318.
28. Shivanna R, Parizadeh H, Garampalli RH. Screening of lichen extracts for *in vitro* antidiabetic activity using alpha amylase inhibitory assay. Int J Biol Pharm Res. 2015; 6(5):364-367.
29. Thadhani VM and Karunaratne V. Potential of lichen compounds as antidiabetic agents with antioxidative properties: A review. Oxid Med Cell Longev. 2017; 2017: 1-10.
30. Srivastava P, Upreti D, Dhole T, Srivastava AK, Nayak MT. Antimicrobial property of extracts of Indian lichen against human pathogenic bacteria. Interdiscip Perspect Infect Dis. 2013; 2013:704348.
31. Ranković B, Mišić M, Sukdolak S. The antimicrobial activity of substances derived from the lichens *Physcia aipolia*, *Umbilicaria polyphylla*, *Parmelia caperata* and *Hypogymnia physode*s. World J Microbiol Biotechnol. 2008; 24(7):1239-1242.
32. Tay T, Türk AÖ, Yılmaz M, Türk H, Kıvanç M. Evaluation of the antimicrobial activity of the acetone extract of the lichen *Ramalina farinacea* and its (+)-usnic acid, norstictic

acid, and protocetraric acid constituents. Z Naturforsch C J Biosci. 2004; 59(5-6):384-388.
33. Stojanović IŽ, Radulović NS, Mitrović T, Stamenković S, Stojanović G. Volatile constituents of selected Parmeliaceae lichens. J Serb Chem Soc. 2011;76(7):987-994.
34. Jayaprakasha GK and Rao LJ. Phenolic constituents from the lichen *Parmotremas tuppeum*(Nyl.) Hale and their antioxidant activity. Z Naturforsch C J Biosci. 2000;55(11-12):1018-1022.
35. König GM and Wright AD. ^{1}H and ^{13}C-NMR and biological activity investigations of four lichen-derived compounds. Phytochem Anal. 1999;10(5):279-284.