SHORT COMMUNICATION

Estimation of trace elements and *in vitro* biological activities of lichens extracts

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Abstract: Infectious diseases caused by etiological agents are still a major threat to public health. Their impact is particularly large in developing countries due to relative unavailability of medicine and the emergence of widespread drug resistance. In the current research, trace metals were detected in lichens species through inductively coupled plasma atomic emission spectroscopy. The antimicrobial potency of *Pseudevernia furfruracea*, *Physcia species*, *Dermatocarpon vellerum* and *Parmellia species* (lichens) extracts have been investigated against three local clinical bacterial isolates i.e. *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* through various agar disc and well diffusion methods. The antioxidant potential effect was also evaluated by DPPH and $ABTS^{+}$ free radical scavenging methods. Phytochemical constituents were screened through thin layer chromatography (TLC) and qualitative methods. Methanolic extract of *P. furfruracea, Physcia spp,* and *D. vellerum* showed a significant inhibition of *S. epidermidis* (14.3±1.7mm, 12.3±2.0mm, and 11.3±0.9mm) by pouring method of disc diffusion. Moderate zone of inhibition (8.0±1.4 mm) against *S. epidermidis* was observed by methanolic extract of *Parmellia spp,* through spreading method. All the results were evaluated by ANOVA and LSD tests at $p<0.05$. The diethyl ether extracts showed considerable antioxidant potential activity with 80%, 81%, 79% and 66%. Thin layer chromatography profiling gave us the idea about the presence of phytochemical constituents such as tannins, phenols, saponins, and terpenoids. Various R_f values on silica gel plates provided the valuable clues about polarity and the selection of solvents for separation of phytochemicals. Significant inhibition of *E. coli* was also observed through TLC-Bioautography. The findings revealed the considerable inhibitory and antioxidant effect of lichens may be due to the presence of bioactive compounds. Therefore, lichens could be a potential source of new antimicrobial and antioxidant agents.

Keywords: Antibacterial activity, antioxidant assay, phytochemical screening, tlc-bioautography, lichens, antibiogram, estimation of trace metals.

INTRODUCTION

Lichens are symbiotic organisms consisting of fungi and a photosynthetic partner that can be an alga or a cyanobacterium (Ahmadjian, 1993). Lichens and Lichen products have been used in traditional medicines for centuries. Almost 20,000 plant species have been reported by World Health Organization (WHO) to be used currently for medicinal purposes (Karthikaidevi *et al*., 2009). Various biological activities such as antiviral, anti-tumor, anti-inflammatory, anti-allergic, antipyretic, anti-proliferative, antiprotozoal of some lichens and their components are known (Halama, 2004). Many species of lichens are used for human nutrition, animal nutrition, for getting colors, perfumes, alcohol and in the medical industries (Kirmizigül *et al.,* 2003). Numerous researchers also have justified the use of some lichens in traditional medicine which proved their antimicrobial activity (Choudhary *et al*., 2005; Cansarana *et al*., 2006; Rankovic *et al*., 2007b). Rankovic *et al.* (2011) and Branislav *et al*. (2011) claimed that lichens can be used in curing bacterial and cancer diseases. Lichens produce a wide range of organic compounds that can be grouped as primary metabolites and secondary metabolites. Due to the presence of wide range of substances these medicinal herbs can be used to treat chronic as well as infectious diseases (Brantner, 1994; Gnanamani *et al.,* 2003; Yasunaka *et al.,* 2005). Rankovic *et al.* (2007a) screened the antimicrobial properties of acetone, methanol and aqueous extracts of lichens. The antibacterial activity may be possessed by many lichen species against both Gram-negative and Gram-positive bacteria (Retallack, 2007).

On the other hand, lichens are used as bioindicators of air pollutants (Balabanova *et al*., 2012). So, it has been proved that the lichen has a distinguished antimicrobial activity and used as the best air pollutant indicator (Gulluce *et al*., 2006; Rankovic *et al*., 2007a; Balabanova *et al*., 2012). Lichens could be used as a natural treatment for human bacterial diseases. However, there is a lack of

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scientific research regarding lichens from Azad Jammu and Kashmir, Pakistan. Therefore, the aim of the current study is to evaluate the antibacterial and antioxidant activities of various lichens extract, could be a potential source of medicine.

MATERIALS AND METHODS

Lichens collection and identification

Four Lichens were collected from Nagdar village, District Neelum of Azad Jammu and Kashmir (AJ&K), Pakistan. They were collected using paper bags and collected material was transported to the Microbial Biotechnology Laboratory at Department of Zoology, Azad Jammu and Kashmir, Muzaffarabad, Pakistan. All the characters including morphological features and habitat were also recorded. Lichens were rinsed with running tap water in order to remove sand particles. After washing photographs were taken by a digital camera. All samples were shade dried and packed into sealed bags. The identification was done by the experts from the Department of Botany, Punjab University, Lahore, Pakistan on the basis of external morphological features.

Extract preparation

Lichen powder having 5 gm weight was soaked into 100 ml of polar and non-polar solvents (methanol, acetone, chloroform, and diethyl ether) few days and used as 100% stock extract (50mg/ml). The conventional solvent extraction method was used for the extract preparation. Extracts were kept at room temperature for few days to ensure solubility. After few days the crude extract was filtered and the filtrate was subjected to biological studies.

Estimation of trace metals

Atomic absorption cross checked by inductively coupled plasma atomic emission spectroscopy (ICP-AES) was used for the detection of trace metals. Standard procedure was adopted for the acid digestion. Dried lichens were crushed into fine powder and solid samples were digested in Aqua Regia (HCl: HNO_3, 3:1) for at least 3 days. After digestion, evaporation was done on a hot plate till complete removal of acid. Sodium (Na), calcium (Ca), iron (Fe), manganese (Mn), copper (Cu), Cobalt (Co), chromium (Cr), Molybdenum (Mo), zinc (Zn), potassium (K) and magnesium (Mg) were detected in µg g^{-1} units.

$ABTS^{\cdot+}$ free radical scavenging activity

$ABTS^{\cdot+}$ (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) free radical scavenging activity was carried out to evaluate the antioxidant potential of extracts (Re *et al*., 1999). The ABTS stock solution was prepared by reacting potassium persulphate (2.5mM) and ABTS (7mM), then the mixture was kept for at least 16 h to generate $ABTS^{+}$ free radicals and their absorbance was recorded at 734 nm ($Ao_{Control}$). For tests, 1ml of ABTS solution was mixed with different extracts (100µl). The absorbance of test samples (Ai_{Sample}) was also observed at 734 nm. The percentage radical scavenging activity (% RSC) was calculated using the formula: % RSC = [($Ao_{Control}$ − Ai_{Sample}) / $Ao_{Control}$] ×100%.

DPPH free radical scavenging activity

DPPH [(di (phenyl)-(2, 4, 6-trinitrophenyl) iminoazanium] free radical scavenging activity of lichen extracts was determined with slight modifications (Braca *et al*., 2001). All extracts (0.25 ml and 0.5 ml) were mixed with 3 ml of a 0.004 % methanol solution of DPPH. Water (0.1 ml) was used as a control in place of extracts. Absorbance was recorded after 30 min at 517 nm. The percentage scavenging activity was calculated by using the formula: %=[(Ao-Ai)/Ao]*100; where Ao is the absorbance of control and Ai is the absorbance of extract.

Test microorganisms

Both Gram-positive and Gram-negative bacteria *Pseudomonas aeruginosa, Staphylococcus epidermidis* and *Escherichia coli* were isolated from human infected samples (Blood, urine, and pus; Awan *et al*., 2013). Different antiseptic techniques like sterile cotton swab, needle aspiration, were used for the isolation of these bacterial pathogens. All the necessary precautions were adopted to avoid contamination.

Antibacterial assay

In order to acquire the antibacterial activity of lichens extracts different agar disc and agar well diffusion methods were used (Rios *et al*., 1988; Prescott *et al*., 1999; Jorgensen and Turnidge, 2007). To activate microorganisms, a loop of bacterial strains was inoculated in nutrient broth medium (NBM; CM1) and then incubated at 37°C on a rotary shaker for 24 h.

Pouring method with disc diffusion method

In the case of this method, the overnight bacterial culture was mixed with nutrient agar medium (NAM; CM003), when the temperature reached up to 45°C and was poured the sterilized plates. All plates were placed at room temperature in a laminar flow to solidify. The discs of 5 mm were prepared as follows: 200µl of a particular extract and the control solvents was applied onto a disc and then allowed to dry for the assay. Presoaked discs were placed in the Petri dishes at their labeled position. These prepared plates were left for incubation at 37°C for 24-48 h. Discs of diethyl ether, chloroform, acetone, and methanol were also used as a control. All solvents were also used as a negative control. Microbial growth was determined by measuring the diameter of the zone of inhibition after 24h in millimeter (Seeley *et al*., 2001). The diameter of the clear zones (if greater than 5 mm) around each well was measured with the help of scale (Hammer *et al*., 1999). The results of the sensitivity tests were expressed as (0) for no sensitivity, *(below 1-5) for low sensitivity, **(<6-10) for moderate sensitivity and ***(<11-20) for high sensitivity.

Streaking method with disc diffusion method
In this case, the overnight bacterial culture was streaked on the solidified nutrient agar plates, already prepared at room temperature in a laminar flow. The other process is same as indicated in pouring method with disc diffusion method.

Agar well diffusion method
The overnight culture was mixed with freshly prepared nutrient agar medium (NAM) at 45°C and was poured into the sterilized Petri dishes. All Petri dishes were kept at room temperature in laminar flow for solidification. In each plate, three wells of 5 mm diameter were made using a 1 ml of sterilized micropipette tip and sterilized needle was used for the removal of agar plug. Approximately 30 µl of each crude extract and control solvent samples were placed in each prepared wells and placed at 37°C for 24-48 h. All solvents were also used as a negative control. Microbial growth was determined by measuring the diameter of the zone of inhibition after 24 h in millimeter (Seeley *et al*., 2001). The diameter of the clear zones (if greater than 5 mm) around each well was measured with the help of scale (Hammer *et al.,* 1999). The results of the sensitivity tests were expressed as (0) for no sensitivity, *(below 1-5) for low sensitivity, **(<6-10) for moderate sensitivity and ***(<11-20) for high sensitivity.

Antibiogram analysis
The sensitivity of antibiotics against test microbial strains was assessed by agar disc diffusion method (Prescott *et al.,* 1999; Preeze *et al*., 1990). Sensitivity was predicted with a degree of the clear zone surrounding the disc. The results of the sensitivity tests were expressed as (0) for no sensitivity, *(below 1-5) for low sensitivity, **(<6-10) for moderate sensitivity and ***(<11-20) for high sensitivity.

Phytochemical screening
Phytochemical screening of lichen extracts is done by determining the phytoconstituents like amino acid, flavonoids, glycosides, alkaloids, tannins, steroids, and carbohydrates (Harborn, 1993; Sofowora, 1993; Iyengar, 1995; Siddiqui and Ali, 1997; Trease and Evans, 2002). Different solvents were used to assess the presence of phytoconstituents.

Chemical screening through thin layer chromatography
The presence or absence of phytochemical constituents can be verified by TLC precoated Silica gel 60F264 plates (Wagner and Bladt, 2004). In order to get better resolution of the components different screening systems diethyl acetate, benzyl ethyl acetate and acetone ethyl acetate were used. The developed plates were observed under visible as well as UV light (734 nm). The R_f value of each spot was calculated as:

$$RF = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by the solvent}}$$

TLC bio-autography for antioxidants
The antioxidant constituents were evaluated using TLC-developed plates followed by DPPH (2, 2- Diphenyl-1-picrylhydrazyl) spray technique. DPPH solution (0.05%) in methanol was sprayed on the surface of developed TLC plates and incubated at room temperature for 10 min. The active antioxidant constituents of lichen were detected as radish brown spots on the TLC plates by resolving bands.

Direct bioautography
In direct bio-autography, agar overlay technique was used with slight modifications as demonstrated by Slusarenko *et al*. (1998). 10 ml of lichens extract were used to spot on the silica gel plates. For the separation chloroform and methanol 1:1 was used as a solvent system. The developed chromatogram was placed in sterilized Petri plates. After that, the fresh overnight grown culture of *S. epidermidis* was mixed with nutrient agar and then poured over the chromatogram as a thin layer. The plates were left at room temperature for 5 minutes then left for incubation at 37PC for overnight. The zone of growth inhibition was recorded around the active chromatogram spot.

STATISTICAL ANALYSIS

To compare the antibacterial activity of lichen species with standard antibiotics, the activity index (AI) has been determined (Shekhawat and Vijayvergia, 2010). For measuring activity index the following formula was used:

$$\text{Activity Index} = \frac{\text{Zone of inhibition of extract}}{\text{Zone of inhibition of antibiotic}}$$

RESULTS

Identification of lichens
The identified lichens were *Pseudevernia furfuracea, Physcia species, Dermatocarpon vellerum,* and *Parmellia species* belong to the division of Ascomycota. The physical appearance of identified lichens is shown in fig. 1. These lichen species were associated with different families such as *Furfuraceae, Physciaceae, Verrucariaceae,* and *Parmeliaceae.* Most of these usually grow upon stones, bark trees including birch, pine, and spruce, marshy stones and trunk of trees etc. All these lichens grow during the period of January to March. None of these lichens have been reported edible. The scientific names, habitat, division, family and growing period are described in table 1.

Estimation of essential and non-essential trace metals
The elemental analysis was carried out to evaluate the essential and non-essential metals (Mg, Ca, Na, Fe, Mn, Cu, Ca, Cr, Mo, Co, Zn, and K) in lichen species *Pseudevernia furfuracea, Physica spp.*, *Dermatocarpon vellerum* and *Parmellia spp,* (table 2). The contents of

magnesium, calcium, sodium, iron, manganese, zinc and potassium in lichen species were found to be 10.0-101.0, 2.9-113.0, 13.4-33.0, 61.0-103.0, 0.016-2.5, 1.0-2.9 and 19-<1000 µg/g respectively. Moreover, the elements like cobalt, chromium, and molybdenum were not detected in any lichen species. It was also observed that the concentration of trace metals in lichens was found to be varying from species to species. The highest level of potassium was found in *Pseudevernia furfuracea, Physica spp*, and *Dermatocarpon vellerum* whereas lowest level was found in *Parmellia spp*. Similarly, magnesium, calcium, and iron were found to be in highest concentration in *Pseudevernia furfuracea.* Lichen *Parmellia* showed higher concentrations of sodium and zinc among all other tested lichen species.

Antibacterial activity of lichen extracts

The antibacterial activity of lichen species *P. furfruracea (L1), Physcia species (L2), Dermatocarpon vellerum (L3)* and *Parmellia species* (L4) were determined through three different diffusion methods, namely pouring method of agar disc diffusion, spreading method of agar disc diffusion and agar well diffusion method against three human pathogenic bacteria *E. coli, S. epidermidis* and *P. aeruginosa* (table 3).

It has been observed that methanolic extract of *P. furfruracea* (L1) showed a significant zone of inhibition (14.3±1.7 mm) against *S. epidermidis* by pouring method and moderate activity of (9.0±0.8 mm) by spreading method against *S. epidermidis*. The good method of disc diffusion showed resistance against all pathogens. Similarly, the acetonic extract of *P. furfruracea* (L1) showed no zone of inhibition against all three human pathogenic bacteria by using three methods. The extract of *P. furfruracea* (L1) species showed resistance against all pathogens. It has also been observed that diethyl ether extract of *P. furfruracea* (L1) showed a moderate zone of inhibition (7.0±0.0mm) against *S.epidermidies* by pouring method of disc diffusion and a moderate zone of inhibition (6.6±0.9mm) against *P. aeruginosa* by agar well method. The species also showed resistance against *E. coli* by spreading method. Moreover, it has been observed that chloroform extract of *P. furfruracea* (L1) showed no zone of inhibition against human pathogenic bacteria by all three methods of disc diffusion.

The results obtained for the lichen *Physcia species* (L2) reveal that methanolic extract of (L2) *Physcia species* showed a significant zone of inhibition (12.3±2.0mm) against human pathogenic bacteria *S. epidermidis* by pouring method of disc diffusion. But another two methods showed no zone of inhibition. The acetonic extract of (L2) showed no zone of inhibition against any human pathogenic bacteria by three disc diffusion methods. Whereas the diethyl ether extract of (L2) *Physcia species* showed a moderate zone of inhibition (6.0±0.8mm) against *S. epidermidis* pathogen and low zone of inhibition (5.0±1.6 mm) against pathogen *P. aeruginosa* by the well disc diffusion. Similarly, the chloroform extract of (L2) *Physcia species* showed a moderate zone of inhibition (7.3±1.2mm) against *E. coli* human pathogenic bacteria whereas no zone of inhibition was shown against *P. aeruginosa* and *S. epidermidis* by all three methods of disc diffusion.

The results tabulated in table 3 showed that the methanolic extract of *Dermatocarpon vellerum* (L3) showed a significant zone of inhibition (11.3±0.9mm) against *S. epidermidis* pathogen by pouring method along with a moderate zone of inhibition (5.6±0.4mm) against *S. epidermidis* by spreading method of disc diffusion. It can be seen that the acetonic extract of *Dermatocarpon vellerum* (L3) showed a moderate zone of inhibition (8.6±0.4 mm) against *P. aeruginosa* pathogen by the well method and no zone of inhibition was shown against *S. epidermidis* and *E. coli* by three methods of disc diffusion. The diethyl ether extract of *Dermatocarpon vellerum* (L3) showed a moderate zone of inhibition (6.0±2.1mm) against *S. epidermidis* human pathogenic bacteria by the the well method and no zone of inhibition against *P. aeruginosa* and *E. coli* by three methods of disc diffusion methods. Similarly, the chloroform extract of *Dermatocarpon vellerum* (L3) showed resistance or no zone of inhibition against *S. epidermidis*, *P. aeruginosa* and *E. coli* by three methods of disc diffusion

It has been observed that methanolic extract of *Parmellia species* (L4) showed a moderate zone of inhibition (8.0±1.4 mm) against *S. epidermidis* by spreading method. Whereas against the pathogens *P. aeruginosa* and *E. coli*, all three methods of disc diffusion show only resistance. It has also been observed that acetonic extract of *Parmellia species* (L4) showed resistance against all pathogens by using three methods of disc diffusion. The diethyl ether extract of *Parmellia species* (L4) showed a moderate zone of inhibition (6.0±0.8mm) against *E. coli* by pouring method and (7.3±1.2) against spreading method. Similarly, the chloroform extract of *Parmellia species* (L4) also showed a moderate zone of inhibition (5.6±1.8mm) against *E. coli* by pouring method and (6.3±0.4) against *P. aeruginosa* by the well disc diffusion.

Antibiogram analysis

The results revealed that Tetracycline, Penicillin G, Oxytetracycline, Ampicillin, and Amoxicillin had no effect upon *E. coli* strain (table 2). These antibiotics showed moderate inhibition of *P. aeruginosa* (6.0±0.0 mm, 6.0±0.0mm, 6.0±0.0mm) and *S. epidermidis* (10.0±0.0mm, 10.0±0.0mm, 10.0±0.0mm). From activity index analysis of lichen extracts may have potential compounds that showed the strong inhibition of tested pathogens and may be used at the place of antibiotic (table 4).

Table 1: Identification of collected lichens from Neelum Valley, Azad Jammu and Kashmir, Pakistan

Lichens identified	Division	Family	Habitat	Growing period
Pseudevernia furfuracea	Ascomycota	*Furfuraceae*	Bark trees, including birch, pine and spruce	January to March
Physcia spp.		*Physciaceae*	Marshy stones, trunk of trees etc.	January to March
Dermatocarpon vellerum		*Verrucariaceae*	Stones	January to March
Parmellia spp.		*Parmeliaceae*	Marshy stones, trunk of trees etc.	January to March

Table 2: Estimation of essential and non-essential metals in mushrooms

Lichens	Trace metals through ICP-AES (µg/g)										
	Mg	Ca	Na	Fe	Mn	Cu	Co	Cr	Mo	Zn	K
Pseudevernia furfuracea	101.0	113.0	26.0	103.0	2.0	ND	ND	ND	ND	1.1	<1000
Physica spp.	11.3	2.9	13.4	101.0	0.016	0.25	ND	ND	ND	1.0	<1000
Dermatocarpon vellerum	14.2	5.6	16.7	84.0	2.2	0.25	ND	ND	ND	1.3	<1000
Parmellia spp.	10.6	19.5	33.0	61.0	2.5	ND	ND	ND	ND	2.9	19

ND indicates not detected; Magnesium (Mg), Calcium (Ca), Sodium (Na), Iron (Fe), Manganese (Mn), Copper (Cu), Cobalt (Co), Chromium (Cr), Molybdenum (Mo), Zinc (Zn), Potassium (K).

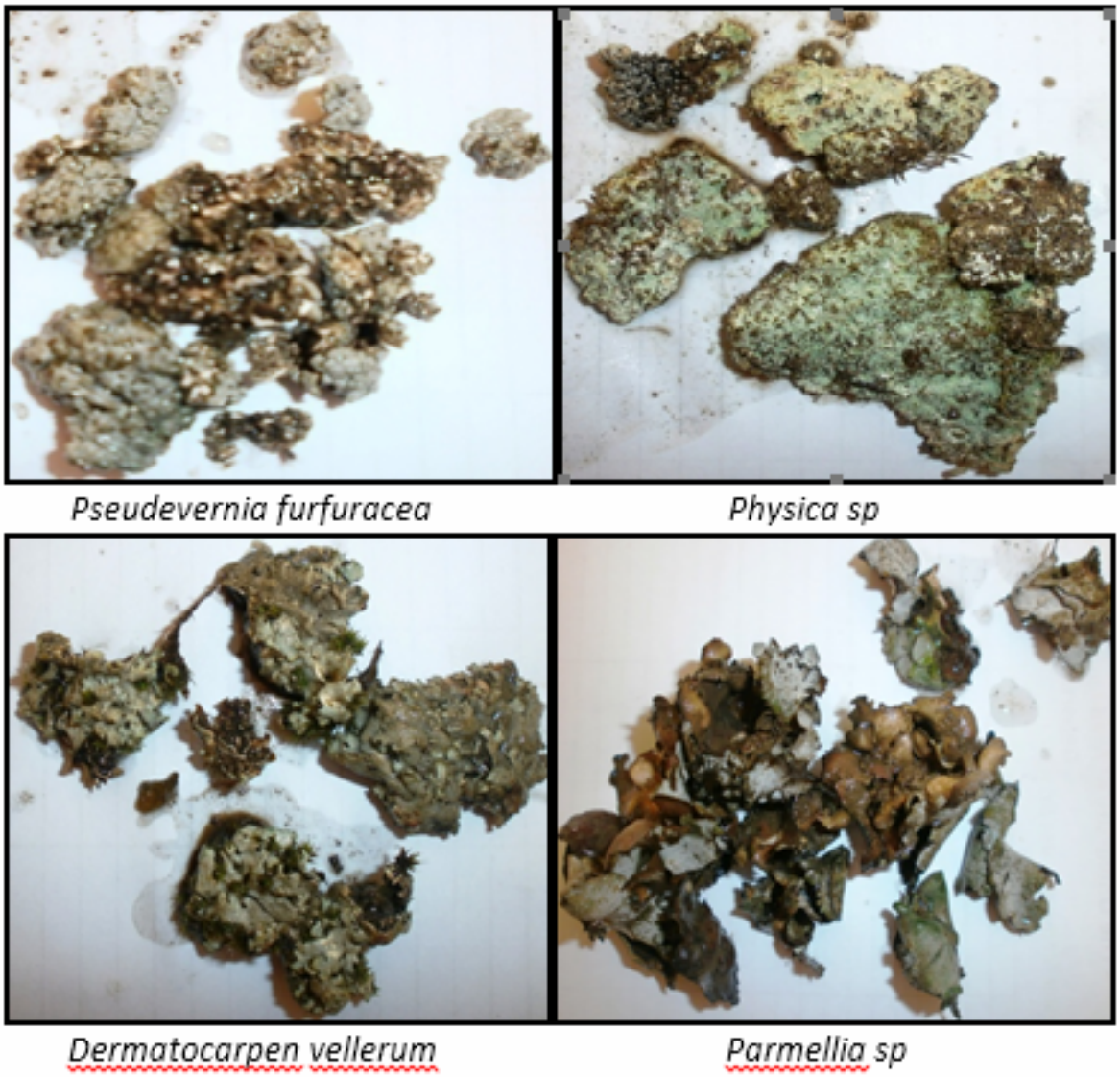

Fig. 1: Identification of lichens species collected from Neelum valley, Azad Jammu and Kashmir, Pakistan.

Table 3: Antibacterial activity of lichen extracts by different methods of diffusion

Antibacterial activity methods	Lichens spp.	Extracts	*Escherichia coli*	*Staphylococcus Epidermidis*	*Pseudomonas aeruginosa*
			Zone of inhibition (M±SD) mm		
Pouring method	L1	Methanol (Met)	3.00±0.82*	14.33±1.70***	0.00±0.00
		Acetone (Ace)	0.00±0.00	0.00±0.00	0.00±0.00
		Diethyl ether (Dee)	7.00±0.00**	5.33±1.70**	2.00±2.16*
		Chloroform (Chl)	2.00±0.82*	0.00±0.00	0.00±0.00
	L2	Methanol (Met)	1.33±0.47*	12.33±2.05***	0.00±0.00
		Acetone (Ace)	0.00±0.00	0.00±0.00	0.00±0.00
		Diethyl ether (Dee)	6.00±0.82**	1.00±0.00*	2.00±0.82*
		Chloroform (Chl)	1.67±0.47*	0.00±0.00	0.00±0.00
	L3	Methanol (Met)	2.67±1.70*	11.33±0.94***	0.00±0.00
		Acetone (Ace)	0.00±0.00	0.00±0.00	0.00±0.00
		Diethyl ether (Dee)	4.00±2.16*	1.00±0.82*	3.00±1.63*
		Chloroform (Chl)	3.00±0.82*	0.00±0.00	0.00±0.00
	L4	Methanol (Met)	3.00±0.82*	13.33±1.25***	0.00±0.00
		Acetone (Ace)	0.00±0.00	0.00±0.00	0.00±0.00
		Diethyl ether (Dee)	6.00±0.82**	2.00±0.82*	1.67±0.47*
		Chloroform (Chl)	2.67±1.25*	0.00±0.00	0.00±0.00
Spreading method	L1	Methanol (Met)	1.67±0.47*	9.00±0.82**	4.67±3.09*
		Acetone (Ace)	0.33±0.47*	0.00±0.00	0.00±0.00
		Diethyl ether (Dee)	2.33±0.47*	2.00±0.82*	2.67±0.94*
		Chloroform (Chl)	0.33±0.47*	2.00±0.00*	1.00±0.00*
	L2	Methanol (Met)	1.33±0.47*	7.00±1.41**	5.33±1.70**
		Acetone (Ace)	0.67±0.47*	0.67±0.94*	0.00±0.00
		Diethyl ether (Dee)	2.00±0.82*	1.33±0.47*	3.00±0.82*
		Chloroform (Chl)	0.67±0.47*	3.00±0.82*	1.00±0.82*
	L3	Methanol (Met)	1.00±0.00*	5.67±4.03**	3.67±2.49*
		Acetone (Ace)	1.00±0.82*	0.33±0.47*	0.00±0.00
		Diethyl ether (Dee)	0.33±0.47*	0.00±0.00	0.67±0.47*
		Chloroform (Chl)	1.00±0.82*	1.67±0.47*	0.67±0.47*
	L4	Methanol (Met)	0.00±0.00	8.00±1.41**	4.76±1.70*
		Acetone (Ace)	0.67±0.47*	1.00±0.00*	0.00±0.00
		Diethyl ether (Dee)	1.33±0.47*	1.00±0.82*	2.00±0.82*
		Chloroform (Chl)	3.67±1.25*	2.33±0.47*	0.33±0.47*
Well diffusion method	L1	Methanol (Met)	0.00±0.00	0.00±0.00	0.00±0.00
		Acetone (Ace)	0.00±0.00	0.00±0.00	6.67±0.94**
		Diethyl ether (Dee)	1.33±0.94*	3.33±1.25*	7.33±1.25**
		Chloroform (Chl)	1.33±1.25*	0.00±0.00	0.33±0.47*
	L2	Methanol (Met)	0.00±0.00	0.00±0.00	0.00±0.00
		Acetone (Ace)	0.00±0.00	0.00±0.00	7.00±0.82**
		Diethyl ether (Dee)	4.00±2.16*	4.00±2.45*	5.00±1.63*
		Chloroform (Chl)	7.33±1.25**	0.67±0.47*	1.67±0.94*
	L3	Methanol (Met)	0.00±0.00	0.00±0.00	0.00±0.00
		Acetone (Ace)	2.33±1.70*	0.00±0.00	8.67±0.47**
		Diethyl ether (Dee)	6.00±2.16**	0.00±0.00	3.33±0.47*
		Chloroform (Chl)	4.33±1.70*	1.00±0.82*	2.67±1.70*
	L4	Methanol (Met)	0.00±0.00	0.00±0.00	0.00±0.00
		Acetone (Ace)	1.67±0.94*	0.00±0.00	0.33±0.47*
		Diethyl ether (Dee)	6.00±0.82**	1.00±0.82*	5.00±1.63*
		Chloroform (Chl)	5.67±1.89**	0.33±0.47*	6.33±0.47**

Growth inhibition was expressed as (0) for no sensitivity, *(below 1-5) for low sensitivity, **(<6-10) for moderate sensitivity and ***(<11-20) for high sensitivity. *Pseudevernia furfuracea* (L1), *Physcia species* (L2), *Dermatocarpon vellerum* (L3), and *Parmellia species* (L4).

Table 4: Activity index analysis of lichens extracts and standard antibiotics

Pathogens	*Staphlococcus epidermidis*				Antibiotics	*Pseudomonas aeruginosa*				Antibiotics	*Escherichia coli*				Antibiotics
Lichens	Met	Ace	Dee	Chl	(mm)	Met	Ace	Dee	Chl	(mm)	Met	Ace	Dee	Chl	(mm)
	AI of pouring method														
L1	2.85	A>E	1.60	A>E	Oxy (5)	A>E	A>E	0.4	A>E	Oxy (0)	E℘A	1.50	E℘A	E℘A	Oxy (0)
	1.43	A>E	0.53	A>E	Tet (10)	A.>E	A>E	0.2	A.>E	Tet (2)	%	E ℜA	%	%	Tet (2)
	%	%	E>A	%	Pen (0)	%	A.>E	%	%	Pen(0)	E℘A	3.50	E℘A	E℘A	Pen(0)
	%	%	E>A	%	Amp (0)	%	%	%	%	Amp (0)	E℘A	1.00	E℘A	E℘A	Amp (0)
	7.15	A>E	A>E	A.E	Amx (2)	A>E	A>E	1	A>E	Amx (0)	A℘E	2.00	%	A℘E	Amx(0)
L2	2.40	A>E	O.50	A>E	Oxy (5)	A>E	A>E	0.4	A>E	Oxy (0)	E℘A	66.5	E℘A	E℘A	Oxy(0)
	1.20	A>E	0.10	A>E	Tet (10)	A>E	A>E	0.2	A>E	Tet (2)	%	A℘E	%	%	Tet(2)
	E>A	A>E	0.20	A>E	Pen (0)	%	%	E>A	E>A	Pen(0)	E℘A	3.00	E℘A	E℘A	Pen(0)
	E>A	%	%	%	Amp (0)	%	%	E>A	%	Amp (0)	E℘A	%	E℘A	E℘A	Amp(0)
	A>E	A>E	A>E	A>E	Amx (2)	A>E	A>E	1.00	E>A	Amx (0)	%	1.00	%	%	Amx (0)
L3	2.21	A>E	0.20	A>E	Oxy (5)	A>E	A>E	3.31	A>E	Oxy (0)	E℘A	E℘A	%	%	Oxy (0)
	1.23	A>E	0.10	A>E	Tet (10)	A>E	A>E	O.16	A>E	Tet (2)	A℘E	A℘E	E℘A	E℘A	Tet (2)
	E>A	%	E>A	A>E	Pen (0)	%	%	%	%	Pen(0)	1.30	%	2.00	1.50	Pen(0)
	E.A	%	E>A	%	Amp (0)	%	%	%	%	Amp (0)	E℘A	%	E℘A	E℘A	Amp (0)
	A>E	A>E	A>E	A>E	Amx (2)	A>E	A>E	0.83	A>E	Amx (0)	E℘A	%	E℘A	E℘A	Amx (0)
L4	2.60	A>E	0.40	A>E	Oxy (5)	0.81	E<A	0.51	0.20	Oxy (0)	E℘A	%	E℘A	E℘A	Oxy (0)
	1.30	A.>E	0.20	A>E	Tet (10)	0.46	E<A	0.26	0.10	Tet (2)	1.50	A℘E	3.00	1.30	Tet (2)
	E>A	%	E>A	%	Pen (0)	E>A	%	E>A	E>A	Pen(0)	E℘A	%	E℘A	E℘A	Pen(0)
	E>A	%	E>A	%	Amp (0)	E>A	%	E>A	E>A	Amp (0)	E℘A	%	E℘A	E℘A	Amp (0)
	A>E	A>E	A>E	A>E	Amx (2)	2.30	A>E	1.33	0.50	Amx (0)	E℘A	%	E℘A	E℘A	Amx (0)
	AI of spreading method														
L1	A>E	0.40	0.41	1.40	Oxy (5)	1.60	E<A	0.60	0.20	Oxy (0)	E℘A	E℘A	E℘A	E℘A	Oxy (0)
	A>E	0.20	0.23	0.70	Tet (10)	0.53	E<A	0.30	0.10	Tet (2)	8.00	0.15	1.50	0.15	Tet (2)
	%	E>A	E>A	E>A	Pen (0)	E>A	%	E>A	E>A	Pen(0)	E℘A	E℘A	E℘A	E℘A	Pen(0)
	%	A>E	E>A	E>A	Amp (0)	E>A	%	E>A	E>A	Amp (0)	E℘A	E℘A	E℘A	E℘A	Amp (0)
	A>E	1.00	1.00	3.50	Amx (2)	2.65	E<A	1.50	0.50	Amx (0)	E℘A	E℘A	E℘A	E℘A	Amx (0)
L2	1.52	0.12	0.21	0.60	Oxy (5)	0.61	A>E	A>E	0.10	Oxy (0)	E℘A	E℘A	E℘A	E℘A	Oxy (0)
	0.76	0.60	0.13	0.30	Tet (10)	0.36	A>E	A>E	0.11	Tet (2)	6.50	0.30	1.00	3.35	Tet (2)
	E>A	E>A	E>A	E>A	Pen (0)	E>A	%	%	0.67	Pen(0)	E℘A	E℘A	E℘A	E℘A	Pen(0)
	E>A	E>A	E>A	E>A	Amp (0)	E>A	%	%	E>A	Amp (0)	E℘A	E℘A	E℘A	E℘A	Amp (0)
	3.80	0.30	0.52	1.50	Amx (2)	1.80	A>E	A>E	E>A	Amx (0)	E℘A	E℘A	E℘A	E℘A	Amx (0)
L3	1,20	0.60	A>E	3.20	Oxy (5)	0.33	0.33	0.84	A.>E	Oxy (0)	E℘A	E℘A	E℘A	E℘A	Oxy (0)
	0.56	0.30	A>E	1.60	Tet (10)	0.12	0.12	0.47	A>E	Tet (2)	0.50	0.15	0.50	5.00	Tet (2)
	E>A	E>A	%	E>A	Pen (0)	0.67	0.67	E>A	%	Pen(0)	E℘A	E℘A	E℘A	E℘A	Pen(0)
	E>A	E>A	%	E>A	Amp (0)	E>A	E>A	E>A	%	Amp (0)	E℘A	E℘A	E℘A	E℘A	Amp (0)
	2.80	0.15	A>E	0.80	Amx (2)	E>A	E>A	2.38	A>E	Amx (0)	E℘A	E℘A	E℘A	E℘A	Amx (0)
L4	1.60	0.20	0.20	4.60	Oxy (5)	0.40	0.66	A>E	1.10	Oxy (0)	%	E℘A	E℘A	E℘A	Oxy (0)
	0.80	0.10	0.20	2.30	Tet (10)	0.20	0.33	A>E	0.60	Tet (2)	A℘E	0.33	6..50	1.80	Tet (2)
	E>A	E>A	E>A	E>A	Pen (0)	E>A	E>A	%	E>A	Pen(0)	%	E℘A	E℘A	E℘A	Pen(0)
	E>A	E>A	E>A	E>A	Amp (0)	E>A	E>A	%	E>A	Amp (0)	%	E℘A	E℘A	E℘A	Amp (0)
	4.00	0.50	0.50	1.15	Amx (2)	1.00	0.16	A>E	3.30	Amx (0)	%	E℘A	E℘A	E℘A	Amx (0)
	AI of Well diffusion method														
L1	E>A	E<A	0.60	E<A	Oxy (5)	1.20	6.60	A>E	1.40	Oxy(0)	%	%	E℘A	E℘A	Oxy (0)
	A>E	E>A	0.30	E>A	Tet (10)	0.73	0.33	A>E	0.70	Tet (2)	EℜA	EℜA	2.00	3.65	Tet (2)
	%	%	E>A	%	Pen (0)	E>A	E>A	%	E>A	Pen(0)	%	%	E℘A	E℘A	Pen(0)
	%	%	E>A	%	Amp (0)	E>A	E>A	%	E>A	Amp (0)	%	%	E℘A	E℘A	Amp (0)
	E<A	E<A	1.50	A>E	Amx (2)	3.60	1.60	A>E	3.50	Amx (0)	%	%	E℘A	E℘A	Amx (0)
L2	E<A	E<A	0.80	E<A	Oxy (5)	1.00	0.32	A>E	1.70	Oxy (0)	%	%	E℘A	E℘A	Oxy (0)
	E<A	E<A	0.40	E<A	Tet (10)	0.50	0.17	A>E	0.86	Tet (2)	A℘E	A℘E	2 .40	3.33	Tet (2)
	%	%	E>A	%	Pen (0)	E>A	E>A	%	E>A	Pen(0)	%	%	E℘A	E℘A	Pen(0)
	%	%	E>A	%	Amp (0)	E>A	E>A	%	E>A	Amp (0)	%	%	E℘A	E℘A	Amp (0)
	E<A	E<A	2.00	E<A	Amx (2)	0.25	0.83	A>E	4.33	Amx (0)	%	%	E℘A	E℘A	Amx (0)
L3	E<A	E<A	E<A	0.20	Oxy (5)	6.60	5.10	A>E	0.60	Oxy (0)	%	E℘A	E℘A	E℘A	Oxy (0)
	E<A	E<A	E<A	0.10	Tet (10)	0.66	0.21	A>E	0.30	Tet (2)	A℘E	1 .15	3.10	2.65	Tet (2)
	%	%	%	E>A	Pen (0)	E>A	E>A	%	A>E	Pen(0)	%	E℘A	E℘A	E℘A	Pen(0)
	%	%	%	E>A	Amp (0)	E>A	E>A	%	A>E	Amp (0)	%	E℘A	E℘A	E℘A	Amp (0)
	E<A	E<A	E<A	A>E	Amx (2)	1.66	1.33	A>E	0.16	Amx (0)	%	E℘A	E℘A	E℘A	Amx (0)
L4	E<A	E<A	E>A	E<A	Oxy (5)	O.60	1.30	6.50	1.20	Oxy (0)	%	E℘A	E℘A	E℘A	Oxy (0)
	E<A	E<A	A>E	E<A	Tet (10)	0.30	0.30	0.30	A>E	Tet (2)	EℜA	5.1	3.00	2.80	Tet (2)
	%	%	%	%	Pen (0)	%	E>A	E>A	%	Pen(0)	%	E℘A	E℘A	E℘A	Pen(0)
	%	%	%	%	Amp (0)	%	E>A	E>A	%	Amp (0)	%	E℘A	E℘A	E℘A	Amp (0)
	A>E	A>E	0.50	A>E	Amx (2)	A>E	1.60	1.00	2.10	Amx (0)	%	E℘A	E℘A	E>A	Amx (0)

E > A & > 1 indicate extracts has higher effect against bacterial pathogens compared to antibiotics; A>E & <1 indicate antibiotics has higher effect against bacterial pathogens compared to extracts; 1 indicates both have equal effect; % indicates both have no effect.

Antioxidant activity

Antioxidant activity of diethyl ether extracts of lichens species showed considerable results with values of 80%, 81%, 79% and 66%. In methanol solution it show 11%, 29%, 76%, and 58%, in acetone solution it show 2%, 12%, 13% and 44% and in chloroform it show 71%, 76%, 82% and 62% . It can be found that lichen extracts are excellent sources of antioxidant. Hence, apart from their medicinal properties, these lichen extracts can also be used as antioxidant supplements.

Phytochemical and TLC analysis

To perform the phytochemical analysis, the phytochemicals were obtained from fresh and naturally dried lichen species through various phytochemical screening methods. All tested phytochemicals were found to be the most abundant in lichen species such as flavonoids, Cardiac glycoside Terpenoid, Steroids, and Phenol, whereas saponins and tannins showed a negative result. The chemical screening performed by Thin Layer Chromatography (TLC) on the crude extracts of lichen showed an impressive diversity of the chemical constituents. In TLC analysis, different absorbing bands were observed under short and long wavelength of UV i.e., 254nm and 356nm, respectively (Park *et al*., 2002). Prominent colored bands of the different crude extracts were observed by staining with anisaldehyde/ H_2SO_4 and also observed under UV. The colored bands red, orange, brownish to yellow, red to brown indicated the presence of different types of amines etc. Similarly, yellow, purple, blue and brown colored bands indicated the presence of the functional groups like phenols, steroids, terpenoids, and flavonoids in the crude extracts. In all lichen extracts, the most promising diversity of the colored bands was seen in the crude diethyl ether and methanolic extracts, whereas the chloroform and acetone solvent system extracts show low bands.

DISCUSSION

Lichens are good accumulators of many elements (Bingöl et al. 2009), particularly heavy metals and radionuclides (Nayaka *et al*., 2003; Aslan *et al*., 2010). Current research indicated that magnesium, calcium, sodium, iron, manganese, zinc and potassium are accumulated by various species of lichens such as *Dermatocarpon vellerum, Pseudovernia furfuracea, Physica sp* and *Parmelia sp*. Natural products have been investigated for centuries for the cure and prevention of a wide range of ailment. Lichens are more important therapeutic agents. The current study confirmed the antibacterial effect, antioxidant potential, essential and non-essential elements, and phytochemical constituents of lichens extracts. Four lichen species namely *Dermatocarpon vellerum, Pseudovernia furfuracea, Physica sp* and *Parmelia sp* were used against three human pathogenic bacteria such as *Staphylococcus epidermidis, Escherichia coli,* and *Pseudomonas aeruginosa,* by using different solvents (methanol, acetone, diethyl ether and chloroform).

The tested lichen extracts showed a relatively strong antibacterial activity. The intensity of result depends upon the sort of concentration of extracts and tested microorganisms. The difference between the results of antibacterial activity of turkey lichens *Pseudovernia furfuracea* that were active against Gram-positive bacteria depends upon the solvents used for extraction. Rowe *et al.* (1989) demonstrated that the *Pseudovernia furfuracea* were active against Gram-positive bacteria. Therefore current study indicates that lichens inhibited mostly Gram-positive bacteria. The actual factors that affect the selective antibiotic activity have not been identified. However biochemical and physiological variation between Gram-positive and Gram-negative bacteria have been demonstrated. The cell wall of Gram-positive bacteria is made of peptidoglycan and teichoic acids while the cell wall of Gram-negative bacteria is made of polysaccharides and lipoprotien (Hugenholtz, 2001).

Pharmacological effects of most natural products used for medicinal purposes have been correlated to their possession of antioxidant activity (Sofidiya *et al*., 2006). Antioxidant capacity is the ability to reduce oxidative reactions within the human body. It is mainly due to their redox properties which play a significant role in neutralizing and absorbing free radicals (Louli *et al*., 2004). Phytochemicals are specialized metabolic compounds that act as antioxidants (Oktay *et al*., 2003; Wangensteen *et al.,* 2004). They participate in redox systems which allow them to act as hydrogen donors, electron donors, and singlet oxygen quenchers (Kahkonen *et al*., 1999). The DPPH model has been widely used as a reliable, quick, and reproducible parameter to search for the *in vitro* general antioxidant activity of lichen extracts (Kähkonen *et al*., 1999; Maestri *et al*., 2006). The decrease in absorbance in the DPPH assay with an increase in the concentration of the extracts which was accompanied by a rapid color change of the purple DPPH suggest that the ethanol extract has antiradical activity. Antioxidant activity may be due to the presence of terpenoids, tannins, and flavonoids (El-Massy *et al*., 2009; Maestri *et al*., 2006).

On the basis of the current results of the phytochemical screening, DPPH antiradical activity, ferric antioxidant reducing ability and antibacterial activity, it is possible to confirm that *Physica sp* 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*. (1991), alkaloids inhibit pathogenic bacteria, and tannins are important in herbal medicine in treating wounds which includes severe burns and to arrests bleeding (Nguyi,

1988). This confirms the *Physica* sp was used for wounds treatment in traditional medicine in Zimbabwe. The reason for the different sensitivity of the extracts towards the selected bacteria can be rationalized as due to morphological differences between the organisms, for example, differences in the porosity of the cell walls (Rankovic *et al*., 2008). The present study shows that lichens extracts of different species consist of appreciable quantities of phytochemicals, significant antioxidant, and antibacterial activity thereby supporting its use in traditional medicines by herbalists in Zimbabwe. It can be used as a source of antioxidant to prevent food rancidity and prevention of diseases. It is suggested that future studies should focus on the use of other models of *in vitro* antioxidant assessment, separation of active components from the extracts, structural elucidation, synthesis and antifungal screening as well as their possible use in the reduction of food rancidity in actual food samples.

The results obtained in the current study are consistent with those reported by Sun *et al*. (2005), where hexane-ethyl ether solvent system was comparatively more effective in extracting phenolic components from lichen extracts. In the present study, the content of phenolic components extracted by hexane-ethyl ether system was about higher than that extracted by acetone ethyl ether solvent system having different polarity had a significant effect on polyphenol content (Siddhuraju *et al*., 2003; Sultana *et al*., 2007). Current results also reveal that methanolic solvent system is much better for separation of components than other less polar or non-polar systems.

CONCLUSION

On the basis of obtained findings, it was concluded that the lichens could be used to monitor the air pollution as well as could be used as a potential source of antibacterial agent. Further studies required to determine the antimicrobial agents for the treatment of both human and plants infectious diseases.

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