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Research Article

Antimicrobial activity of lichen *Everniastrum* sp, its synergism with *Sapindus mukrossi* and *Panax pseudoginseng*: LC-MS identification of active compounds

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Abstract

A fruticose lichen, *Everniastrum*, was screened for its antimicrobial activity against eight microorganisms in this study, and its synergistic antimicrobial activity was tested with two commonly known medicinal plants of the Darjeeling Hills. Identification of the active principle in the lichen extract and the probable active compounds was performed by LC-MS analysis. The list of names, classes, mass spectrum, and occurrence of lichen substances obtained from LC-MS Chromatogram of the methanolic extract of *Everniastrum* sp. revealed the presence of mainly orcinol, depsidones, and depside compounds with some aliphatic acids, xanthones, and usnic acid derivatives. The compounds present were namely hypostitic acid, salazinic acid, norstictic acid, alectoronic acid, thiomelin, conloxodin, usnic acid, and 2'-O-methylnobarbatic acid. The findings of this research indicate that the presence of distinct groups of phytochemicals in *Everniastrum* sp. may be explored for the development of widely acceptable agents to combat disorders without side effects.

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Introduction

Lichens are organisms that may hold the potential for medical exploration. The word "lichen" is derived from the Greek word "Leprous" and refers to the use of lichens in treating skin diseases due to the peeling-skin appearance. Lichens comprise a unique group that consists of two unrelated organisms, a fungus and an alga, growing together in a symbiosis. Lichens with blue-green algae symbionts contribute significantly to forest nitrogen fixation. They are distributed universally and occur in varied climatic conditions ranging from the poles to the tropics. They may look like crust, spreading rapidly over the surface (crustose), or leafy and loosely attached to the surface (foliose), and branched and shrubby, hanging from tree twigs or branches, with a single attachment (fruticose). Besides many other uses, lichens are also used as pollution monitors (Nash and Wirth, 1988; Richardson, 1992; Stolte *et. al*., 1993; Slack, 1998; Garty *et. al.*, 2000; Nash and Gries, 2002; Nimis *et al.*, 2002 and Kumar, 2009). In the folklore of many European countries, lichens were used as a remedy for pulmonary tuberculosis and in the treatment of wounds and disorders. These medicinal uses to some extent, been confirmed by studies which showed that many lichen metabolites such as depsides, depsidones and usnic acid are active against mycobacteria and gram-positive bacteria (Vartia,1973). Lichens like *Lobaria pulmonaria* (Stictaceae) and *Parmelia sulcata* (Parmeliaceae) have been used in the treatment of pulmonary and cranial diseases, respectively. Similarly, *Xanthoria parietina* (Lobariaceae), being yellow, was used to cure jaundice (Bown, 2001). Various biological activities of some lichens are known, such as: antimicrobial, antiviral, anti-tumor, anti-inflammatory, analgesic, antipyretic, antiproliferative and antiprotozoal (Lawrey, 1986; Huneck, 1999; Halama and Van, 2004). The lichen compounds are not an exception in this field. Currently, the interest in lichen secondary compounds is increasing because of the

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ineffectiveness of some previously reliable drugs (Huneck, 1999). India is a rich center of lichen diversity contributing about 15% of the 13,500 species of lichens so far recorded in the world (Negi, 2000). In India parmeloid lichens are extensively used in traditional medicine to treat several diseases and disorders e.g., headache, skin diseases, urinary trouble, boils, vomiting, diarrhoea, dysentery, heart trouble, cough, fever, leprosy and as blood purifier (Chandra and Singh, 1971). Lichens of Eastern Himalayan regions, particularly of Darjeeling District have been studied extensively by Chopra (1934) and Awasthi and Agarwal (1970) from taxonomic point of view, but the antimicrobial and antioxidant properties of these lichens have not yet been explored to that extent. Gupta and Paul (1995) reported the antimicrobial properties of *Usnea floria*, *Physcia* sp, *Usnea pendulata*, *Cladonia cristatella*, *Parmelia perforata*, and *Ramalina calicaris* collected from Darjeeling Hills against *Bacillus megaterium and Staphylococcus aureus.* In another study from Darjeeling Hills, Ray *et. al*. (2003) screened the extracts of *Usnea articulate, Ramalina jamesii,* and *Parmelia tinctorum* against both Gram-positive and Gram-negative bacteria, and antimicrobial activity was reported to most of the tested microorganisms. The extracts were also found to be inhibitors of protein synthesis, energy metabolism, and growth of selected bacteria.

Everniastrum sp belongs to the family Parmeliaceae. Very little research has been undertaken to determine its antimicrobial properties and the identification of the active principal compound from this lichen. Hence, the present work is to estimate the antimicrobial activity, synergistic antimicrobial activity and the identification of active compounds from lichen extracts.

Material and methods

Collection of lichen and medicinal plant samples

The lichens and medicinal plants under study were collected from profusely grown places of Darjeeling and the surrounding area (Figure 1). Each specimen was preliminarily identified with the help of available literature and the Key to Macrolichens (Awasthi, 1988). The taxonomic identity of lichen samples was confirmed by the Lichenology Laboratory, National Botanical Research Institute, Lucknow, Uttar Pradesh, India, and the voucher specimens were deposited in the Herbarium of the Postgraduate Department of Botany, Darjeeling Government College, Darjeeling, India.

Extraction of samples

Each lichen and medicinal plant sample was washed to remove debris, dried and ground to powder, and stored in a sterile glass bottle in the refrigerator. The 10g portions of sieved powder were added to 100 ml of solvents (ethanol and methanol), sonicated for 30 min, and left overnight at room temperature. The crude extract was prepared by decanting, followed by filtration through muslin cloth, and further filtered with Whatman No. 1 filter paper to obtain a clear filtrate. Fifty ml of the filtrate was evaporated to obtain 10 ml of concentrated extract and sterilized by membrane filtration using 450 nm bacteriological filters. Such sterilized filtrate was stored in screw-capped airtight containers in the refrigerator and used for antimicrobial screening, belongs to the family Parmeliaceae. Very little research has been undertaken to determine its antimicrobial properties and the identification of the active principal compound from this lichen. Hence, the present work is to estimate the antimicrobial activity, synergistic antimicrobial activity, and identify active compounds from lichen extracts.

Test microorganisms: Test microorganisms (seven bacteria and one fungus) were obtained from the Institute of Microbial Technology, Chandigarh, India (Table 2). The bacterial culture was preserved in N.A medium and fungal culture in King's medium B.

Screening of antimicrobial activity

This procedure is based on the disc diffusion method of Bauer *et al.* (1966). Overnight-grown bacterial cultures (approximately 0.1 mL) were spread-plated on nutrient agar plates to achieve semi-confluent growth. Sterile filter paper discs were soaked in concentrated extracts, allowed to dry between applications, and placed on plates, which were then incubated at 37°C for 24 hrs. *Streptomycin* (10 µg/mL) and sterile distilled water were taken as positive and negative controls, respectively. Growth was evaluated, and inhibition zones were measured. All the experiments were repeated thrice, and the data presented are the average of three independent readings.

Table 1. List of lichens and medicinal plant samples

Sl No.	Name of lichen and medicinal plants	Extraction solvent	Extract code
1	*Everniastrum* sp	ethanol	EVRE
		methanol	EVRM
2	*Panax pseudoginseng*	ethanol	PNXE
		methanol	PNXM
3	*Sapindus mukrossi*	ethanol	SAPE
		ethanol	SAPM

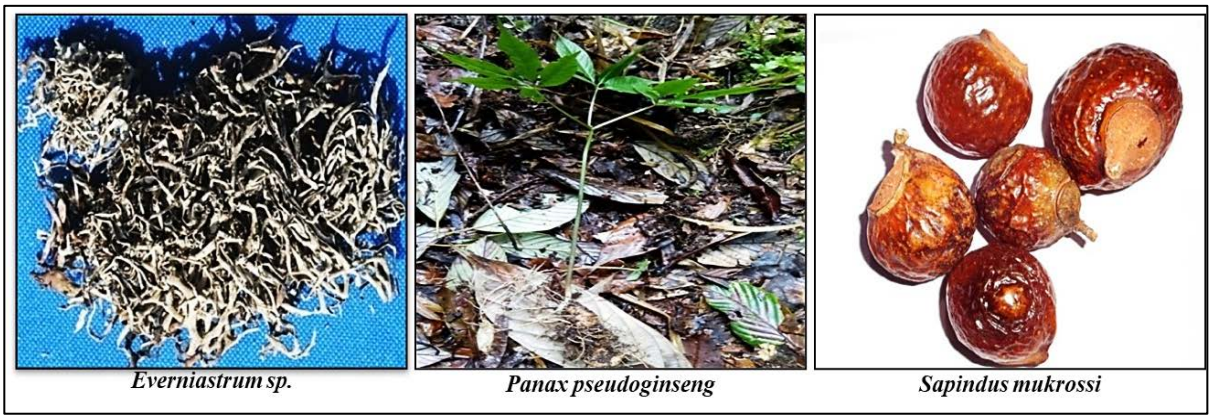

Figure 1. a) *Everniastrum* sp; b) *Panax pseudoginseng*, and c) *Sapindus mukrossi*.

Table 2. Test microorganisms under study

Sl. No.	Test Microorganisms	Gram nature	MTCC Code
1	*Alcaligenes faecalis*	Gram negative	MTCC9780
2	*Bacillus megaterium*	Gram positive	MTCC 7192
3	*Bacillus subtilis*	Gram positive	MTCC 3972
4	*Candida albicans*	-	MTCC 4748
5	*Escherichia coli*	Gram negative	MTCC 6365
6	*Enterobacter aerogenes*	Gram negative	MTCC 111
7	*Staphylococcus aureus*	Gram positive	MTCC 7443
8	*Pseudomonas aeruginosa*	Gram negative	MTCC 424

Identification of the active principle in lichen extract

Four lichen samples were air-dried at room temperature (26°C) until complete drying, and then they were ground into powder. Powdered lichen material (10g) was added to 100 mL of methanol, sonicated, and shaken for 7 days in a shaking incubator at room temperature. The extract was filtered through Whatman filter paper no 42 and was concentrated using a rotary evaporator. The obtained extracts were sent to SAIF, CDRI, Lucknow, for LC-MS analysis.

The mass spectrum as an LC-MS chromatogram of EVRM obtained from SAIF was studied following the literature - A catalogue of standardized chromatographic data of synthetic relationship for lichen substances (Elix, 2014), and lichen substances were determined.

Statistical analysis

Experimental values are the mean ± standard deviation (SD), which was calculated using EXCEL 2007. The graphs were designed using Excel software.

Results and Discussion

Ethanolic extract of *Everniastrum* sp. was very active against all the microorganisms tested, whereas the methanolic extract was active against *A. faecalis* (10 mm), *B. megaterium* (13 mm), *C. albicans* (14 mm), *E. aerogenes*, and *S. aureus* (10 mm). *C. albicans* and *B. megaterium* were highly sensitive to the ethanolic extract of *Everniastrum* sp. The methanolic extract of *Everniastrum* sp was unable to inhibit the growth of *B. subtilis*, *E. coli*, and *P. aeruginosa.*

Generally, the ethanolic extracts of *Everniastrum* sp were active against all the test microorganisms. Turkey lichens *Evernia prunastri*, *Pseudoevernia furfuraceae* and *Alectoria capillaries* (Rowe *et al*., 1989) were active against Gram-positive bacteria and *C. albicans*. The present study also showed that the ethanolic and methanolic extracts of *Everniastrum* sp were mostly active against Gram-positive bacteria and *C. albicans* (Table 3).

Table 3. Antimicrobial activity of extracts of *Everniastrum* sp by the disc diffusion method

Sl No.	Test organisms	Inhibition zone (mm)			
		SDW	EVRE	EVRM	Streptomycin
1	*A. faecalis*	0	8	10	16
2	*B. subtilis*	0	9	0	15
3	*B. megaterium*	0	15	13	14
4	*C. albicans*	0	19	14	9
5	*E. aerogenes*	0	8	13	16
6	*E. coli*	0	14	0	10
7	*P. aeruginosa*	0	12	0	12
8	*S. aureus*	0	11	10	16

[SDW = sterile distilled water]

All these studies indicate that the lichens inhibit mostly Gram-positive bacteria. It is of great interest to note that *Everniastrum* sp inhibited the growth of both Gram-positive and Gram-negative bacteria. Studies on 100 species of American lichens (Burkholder *et al*., 1944) showed that 52% of the lichens in America were active against Gram-positive bacteria.

Lichen *Rocella belangeriana* had antimicrobial activity against both Gram-positive and Gram-negative bacteria (Dahake *et al.,* 2010)**.** The ethanolic extract of *Everniastrum* sp also manifested antibacterial activity against these two Gram-negative and Gram-positive bacteria. The extracts of *Everniastrum nepalense* and *Usnea longifolia* equally inhibited the growth of both Gram-negative and Gram-positive bacteria (Baral *et al*., 2011).

Table 4. Antimicrobial activity of extracts of *Panax pseudoginseng* by the disc diffusion method

Sl No.	Test organisms	Inhibition zone (mm)			
		SDW	PNXE	PNXM	Streptomycin
1	*A. faecalis*	0	0	8	18
2	*B. subtilis*	0	17	19	19
3	*B. megaterium*	0	17	19	19
4	*C. albicans*	0	11	0	9
5	*E. aerogenes*	0	11	13	19
6	*E. coli*	0	0	9	18
7	*P. aeruginosa*	0	14	0	19
8	*S. aureus*	0	13	10	19

SDW-sterile distilled water

It could be observed that the methanolic extract of *P. pseudoginseng* was better than the ethanolic extract (Table 4). Both ethanolic and methanolic extracts were highly active against Gram-positive bacteria. However, the lichen extracts were completely unable or weakly able to restrict the growth of Gram-negative bacteria.

Antimicrobial activity may be due to numerous free hydroxyl ions that have the capability to combine with the carbohydrates and proteins in the cell wall of bacteria and may attach to the enzyme site, making the microorganisms inactive**.** Similarly, the antimicrobial activity as observed may be due to the presence of free hydroxyl ions in *P. pseudoginseng*. *P. pseudoginseng* showed no antibacterial activity against *Chromobacterium violaceum* CV026 and *P. aeruginosa* PAO1 (Siew *et al*., 2012).

The results proved that bacteria are more sensitive to the antimicrobial compound than fungi (Hugo *et al.,* 1983). The reason for the different sensitivity between fungi and bacteria may be due to the different transparency of the cell wall (Yang *et al.,* 1999). The cell wall of the Gram-positive bacteria consists of peptidoglycans (mureins) and teichoic acids; the cell wall of the Gram-negative cells consists of lipopolysaccharides and lipopoliproteins (Hugenholtz, 2002), whereas the cell wall of fungi consists of polysaccharides such as chitin and glucan (Griffin, 1994).

Table 5. Antimicrobial activity of extracts of *Sapindus mukrossi* by disc diffusion method

Sl. No.	Test organisms	Inhibition zone (mm)			
		SDW	SARE	SARM	Streptomycin
1	*A. faecalis*	0	0	0	18
2	*B. subtilis*	0	0	0	19
3	*B. megaterium*	0	0	0	19
4	*C. albicans*	0	18	0	9
5	*E. aerogenes*	0	0	0	19
6	*E. coli*	0	11	10	18
7	*P. aeruginosa*	0	0	12	19
8	*S. aureus*	0	0	0	19

SDW-sterile distilled water

It was observed (Table 5) that the ethanolic and methanolic extracts of *S. mukrossi* have weak antimicrobial activity against most of the bacteria and test fungi. Ethanolic extract of *S. mukrossi* was active against *C. albicans* and *E. coli* with an inhibition zone measuring 18 mm and 11 mm, respectively. Growth of *A. faecalis, B. subtilis, B. megaterium, C. albicans, E. aerogenes,* and *S. aureus* was not inhibited on exposure to the methanolic extract of *S. mukrossi*.

Thota *et al.* (2012) found that methanol, acetone, and 1,4-dioxan extract of *S. saponaria* showed good inhibitory activity against *S. aureus, P. vulgaris, P. aerogenes,* and *Micrococcus albus*. The methanolic leaf extract of *S. saponaria* produced an inhibition zone measuring 11 mm approx against *E. coli* (Table 5). An inhibition zone measuring 12 mm was produced by the methanolic extract of *S. mukrossi* against *P. aeruginosa. S. emarginatus* (soapnut tree) belongs to the family Sapindaceae found to contain saponins and sugar in the pericarp, which may be one of the factors for growth inhibition (Gupta and Ahmed, 1990).

S. emarginatus showed antimicrobial activity against *C. albicans, Trichophyton rubrum*, and *Epidophyton flocossum* (Manjulata *et al*., 2012). Our report is very much consistent with this result, in which the ethanolic extract of *S. mukrossi* is highly active against *C. albicans* with an inhibition zone measuring 18 mm.

It was evident that the combined methanolic extract of *S. mukrossi* and *Everniastrum* sp was strongly active towards 90% of the microorganisms under test except *B. megaterium* (Table 6). The combined methanolic extract was moderately active against the bacteria and fungi under test. Out of four Gram-negative bacteria, three Gram-positive bacteria, and a fungus under test, the growth of *A. faecalis* (17.2 mm), *C. albicans* (16.2 mm), *E. coli* (14.72 mm), *P. aeruginosa* (16.74 mm), and *S. aureus* (15.84 mm) was greatly inhibited. Ethanolic extract of *S. mukrossi* was weak, as it could inhibit the growth of

C. albicans and *E. coli* (Table 5), but when combined with *Everniastrum* sp it inhibited the growth of both Gram-positive (*B. subtilis* and *S. aureus*) and Gram-negative bacteria. (*A. faecalis*, *E. aerogenes*, *E. coli*, and *P. aeruginosa*) The weak antibacterial activity of *S. mukrossi* was increased by the presence of *Everniastrum* sp. Similarly, the methanolic extract of *S. mukrossi* was also weak, as it was active against Gram-negative bacteria, but its combination with *Everniastrum* inhibited the growth of 90% of the microorganisms under test, except *B. megaterium*.

Table 6. Antimicrobial activity of combined extracts of *Sapindus mukrossi* with *Everniastrum sp* by disc diffusion method

Sl. No.	Test organisms	Inhibition zone (mm)	
		SAPE+EVRE	SAPM+EVRM
1	*A. faecalis*	17	11
2	*B. subtilis*	12	12
3	*B. megaterium*	-	-
4	*C. albicans*	16	11
5	*E. aerogenes*	12	7
6	*E. coli*	14	9
7	*P. aeruginosa*	16	6
8	*S. aureus*	15	13

Table 7. Antimicrobial activity of combined extracts of *Panax pseudoginseng* and *Everniastrum* sp by disc diffusion method

Sl. No.	Test organisms	Inhibition zone (mm)	
		PNXE+EVRE	PNXM+EVRM
1	*A. faecalis*	0	10
2	*B. subtilis*	12	20
3	*B. megaterium*	14	11
4	*C. albicans*	20.7	17.1
5	*E. aerogenes*	9	14
6	*E. coli*	18	18.9
7	*P. aeruginosa*	17.1	18.9
8	*S. aureus*	22.05	10.8

Combined ethanolic extract of *P. pseudoginseng* and *Everniastrum* was active against all the microorganisms except *A. faecalis*. where the highest inhibition zone was observed against *S. aureus* (22.05 mm) and the lowest against *E. aerogenes* (9 mm). Methanolic extract inhibited all the test organisms. A mixture of ethanolic extract of *P. reticulatum* and *Everniastrum* sp was observed to produce an even larger inhibition zone against *S. aureus* (22.05 mm) than that of *Streptomycin* (19 mm) (Table 7).

A good synergism was observed as both ethanolic and methanolic extracts of *P. pseudoginseng* and *Everniastrum* sp greatly inhibited the growth of test microorganisms. This combination could be exploited as a more potent antimicrobial agent than S*treptomycin*.

Table 8. List of names, classes, mass spectrum of lichen substances identified from LC-MS chromatogram, methanolic extract of *Everniastrum* sp (SAIF 7904)

Sl. No.	Compound	Class	Mass spectrum (nm)	Also occurs in
1.	Hypostictic acid	â-Orcinol Depsidones	372,354,328 327	*Xanthoparmelia quintaria*
2.	Salazinic acid	â-Orcinol Depsidones	388,370,354,179	*Parmelia sulcata*
3.	Norstictic acid	â-Orcinol Depsidones	372,354,179,177	*Xanthoparmelia substrigosa*
4.	Thiomelin	Xanthones	342,340,327,325	*Rinodina thiomela*
5.	Conloxodin	Orcinol Depsidones	428,396,384,370	*Xanthoparmelia xanthofarinosa*
6.	Usnic acid	Usnic acid derivatives	344,260,233,217	*Usnea* sp
7.	Alectoronic acid áAlectoronic acid	Orcinol Depsidones	494,468,450,370	*Parmotrema rigidum*
8.	2'Omethylnobarbatic acid	â-Orcinol Depsides	360, 197, 196, 180	*Pseudocyphellaria norvegica*

Lichen substances are secondary metabolites of lichens, like Dibenzofurans, Depsidones, Xanthones, and Terpene derivatives (Kosanic *et al*., 2013). The LCMS chromatogram of the studied lichen *Everniastrum* sp revealed the presence of mainly Orcinol Depsidones and Depside compounds with some Aliphatic acids, Xanthones, and Usnic acid derivatives. The compounds present are, namely, Hypostitic acid, Salazinic acid, Norstictic acid, Alectoronic acid, Thiomelin, Conloxodin, Usnic acid, and 2'-O-Methylnobarbatic acid (Figures 2 & 3; Table 8).

It has been studied earlier that the salazinic acid constituent and *Parmelia sulcata* showed antimicrobial properties against food-borne bacteria and fungi (Candan *et al*., 2007), hence, salazinic acid identified from the methanolic extract of *Everniastrum* sp may also possess such antimicrobial ability. Similarly, usnic acid was obtained from the methanolic extract of *Everniastrum* sp and the methanolic extract of *Usnea baileyi*, which is known to possess antiprotozoal, antiviral, antiproliferative, anti-inflammatory, analgesic, antipyretic, and antitumour activities (Cocchietto *et al.,* 2002; Ingolfsdpttir, 2002).

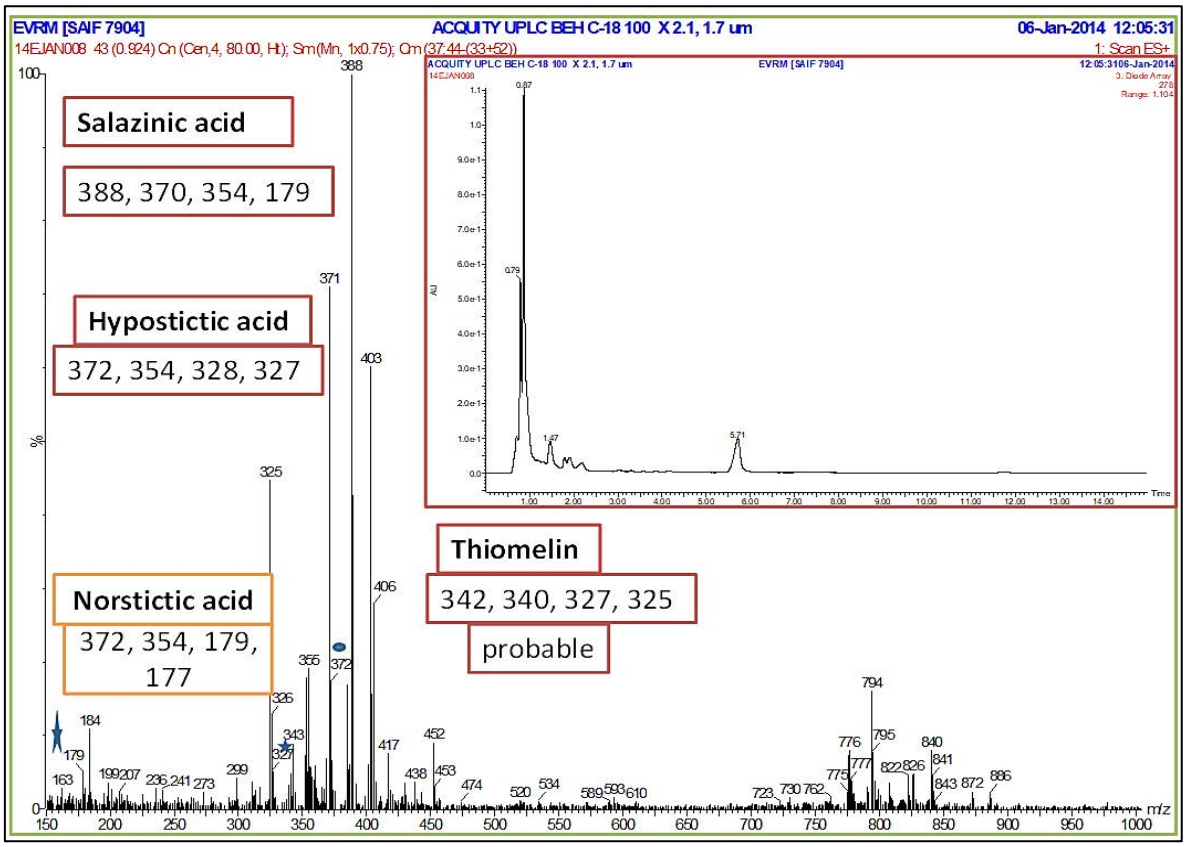

Figure 2: LCMS spectral peaks and respective compounds of the methanolic extract of *Everniastrum* sp

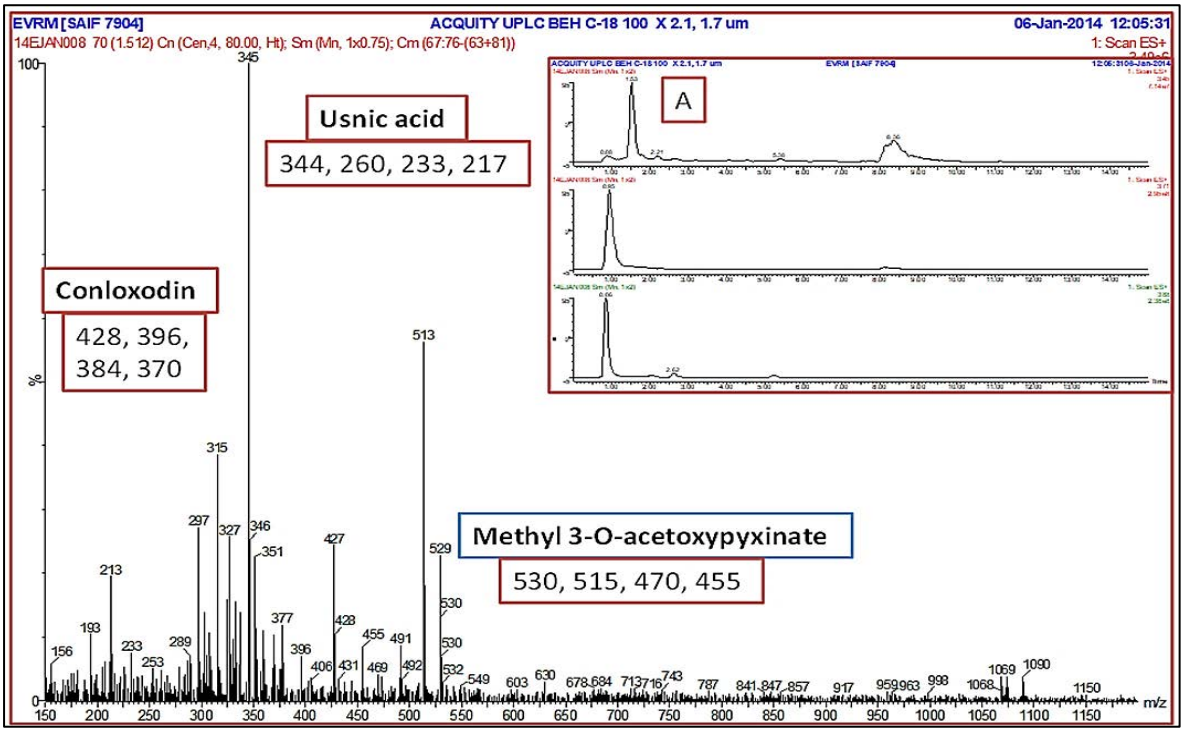

Figure 3. LCMS spectral peaks and respective compounds of the methanolic extract of *Everniastrum* sp

Conclusion

The LCMS chromatograms of *Everniastrum* sp (methanolic extract) have been found to contain a variety of secondary lichen substances, which are responsible for antimicrobial activity. The property of synergism observed between lichen and medicinal plants opens the door for further research in this field. The results obtained in the present study could be used as a database for further use of lichens for medicinal purposes.

The antimicrobial effect of lichens and plants tested can be further authenticated with new studies by taking other clinical pathogens and conducting pharmacological tests. The findings of this study are a database for further research for the search and isolation of lichen metabolites; a more detailed investigation into the action of lichen substances for their application is essential.

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