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Antimicrobial and antioxidant activities of a macrolichen *Usnea pictoides* G. Awasthi (Parmeliaceae)

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ABSTRACT

Lichens are self-sufficient symbioses between an alga and a fungus. In the present study, we have determined total phenolic content, antimicrobial and antioxidant efficacy of a macrolichen *Usnea pictoides* G.Awasthi (Parmeliaceae) collected at Mullayanagiri, Western Ghats of Chikmagalur, Karnataka, India. The lichen was powdered and extracted sequentially using solvents of increasing polarity *viz*., petroleum ether, chloroform, ethyl acetate and methanol. Total phenolic content of solvent extracts was estimated by Folin-Ciocalteau reagent method. Antimicrobial activity of solvent extracts was tested against two bacteria *viz*., *Staphylococcus aureus* and *Pseudomonas aerugionsa* and two fungi *viz*., *Candida albicans* and *Cryptococcus neoformans* by Agar well diffusion assay. Antioxidant activity of solvent extracts was determined by DPPH free radical scavenging assay and Ferric reducing assay. Thin layer chromatogram showed the presence of usnic acid. The total phenolic content was highest in methanol extract followed by ethyl acetate, chloroform and petroleum ether extracts. *S. aureus* and *C. neoformans* showed high susceptibility to solvent extracts among bacteria and fungi. A dose dependent scavenging of DPPH radicals by solvent extracts was observed. The scavenging potential of methanol extract was higher than other extracts. In ferric reducing assay, methanol extract showed stronger reducing power than other extracts. Overall, extracts containing high phenolic contents exhibited stronger antioxidant activity. The inhibitory potential of the lichen extracts might be attributed to the presence of usnic acid. The radical scavenging and ferric reducing potential of solvent extracts could be attributed to the phenolic compounds. A positive correlation was observed between total phenolic content and the antioxidant activity of lichen extracts. The lichen *U. pictoides* can be a potential candidate for the development of bioactive agents.

INTRODUCTION

The lichens are symbioses between an alga and a fungus. The alga (photobiont) provides the lichen with nutrients by photosynthesis, and the fungus (mycobiont) helps in absorption of water and nutrients from surroundings. As a result, lichens are self-sufficient and can grow on a variety of habitats such as rocks, roofs, tree trunks etc. Since lichens have no roots, they absorb nutrients from the air, instead of doing so from the soil as the plants do. Lichens have different growth forms *viz*., crustose, foliose and fruticose. Together with bryophytes and liverworts, lichens form a unique community on trees and/or rocks. Lichens synthesize a variety of secondary metabolites termed the 'lichen substances' unique with respect to those of higher plants. These metabolites differ in chemical structure and have strong biological activities such as antibiotic, antimycobacterial, antiviral, antiinflammatory, antioxidative, analgesic, antipyretic, antiproliferative and others. Lichen secondary compounds are important for the taxonomy of lichens. Lichens have been used as food and folk medicine since ancient times (Palo, 1993; Bernasconi *et al*., 2000; Turk *et al*., 2006; Vinayaka *et al*., 2009; Behera *et al*., 2009; Temina *et al*., 2010; Kowalski *et al*., 2011; Verma *et al*., 2012). Diagnostic features of the genus *Usnea* include a fruticose shrubby to pendant thallus with a cortex, medulla, and a cartilaginous central axis, and the presence of usnic acid in the cortex (Clerc, 1998; Canarasan *et al*., 2006; Ohmura, 2012). *Usnea pictoides* G.Awasthi (Parmeliaceae) is an endemic fruticose lichen of Western Ghats and is found distributed in Kerala, Karnataka and Tamil Nadu. The lichen grows at high altitudes 1900-2200 m and has an erect, brown corticolous thallus with sympodial branching.

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Lateral branchlets are sparse, central axis is solid, surface has crackes, hypothecium is colorless, isidea, soredia and apothecia are absent. Iodine test for thallus shows blue color which later turns black. Usnic acid is present (Awasthi, 2000). On searching the literatures, it was found that the biological activities of the lichen U. pictoides have not been investigated, particularly from Karnataka, India. Hence, in the present study, we have determined bioactivities namely antimicrobial and antioxidant activities of solvent extracts of U. pictoides.

MATERIALS AND METHODS

Collection and identification of U. pictoides

The lichen, growing on the barks of trees, was collected at Mullayanagiri, Western Ghats of Chikmagalur district, Karnataka during the month of June 2012. Mullayanagiri is the highest peak in Karnataka, India and is one of the best trekking places in Karnataka and South India. The lichen was identified by morphological, anatomical, chemical tests (Awasthi, 2000). The shade dried and powdered lichen material was extracted with methanol, spotted on the silica plate and developed with solvent system that consisted of 180 ml of toluene, 60 ml of 1-4, dioxine and 8 ml of acetic acid to detect secondary metabolites (Kumar et al., 2011).

Sequential extraction of powdered lichen

For extraction, about 25g of dried and powdered lichen sample was taken and sequentially extracted with petroleum ether, chloroform, ethyl acetate and methanol in soxhlet apparatus. The extracts were filtered over Whatman No. 1 filter paper. The solvent extracts were concentrated in vacuum under reduced pressure and dried in the desiccator (Kekuda et al., 2012).

Total phenolic content of solvent extracts of U. pictoides

The Folin-Ciocalteau reagent (FCR) method was employed to estimate total phenolic content of solvent extracts of U. pictoides. In brief, a dilute concentration of extract (0.5ml) was mixed with 0.5ml of FC reagent (1:1 diluted) and 4ml of sodium carbonate (1M). The reaction mixtures were allowed to stand for 30 minutes and the optical density was read colorimetrically at 765nm. A standard curve was plotted using different concentrations of Gallic acid (standard, 0-1000µg/ml) and the phenolic content of extracts was expressed as µg Gallic acid equivalents (GAE) from the graph (Junaid et al., 2013).

Antimicrobial activity of solvent extracts of U. pictoides

Agar well diffusion assay was carried out in order to investigate antibacterial (against Staphylococcus aureus NCIM-2079 and Pseudomonas aeruginosa NCIM-2242) and antifungal (against Candida albicans NCIM-3466 and Cryptococcus neoformans NCIM-3378) activity of solvent extracts of U. pictoides. The test bacteria and fungi were inoculated into sterile Nutrient broth (HiMedia, Mumbai) and Sabouraud dextrose broth (HiMedia, Mumbai) respectively. On inoculation, the nutrient broth tubes were incubated for 24 hours at 37oC and Sabouraud dextrose broth tubes were incubated at 37oC for 48 hours. The broth cultures of bacteria and fungi were aseptically swabbed on sterile Nutrient agar (HiMedia, Mumbai) and Sabouraud dextrose agar (HiMedia, Mumbai) respectively using sterile cotton swabs followed by punching wells of 6mm diameter using sterile cork borer. 100µl of solvent extracts (20mg/ml of 25% DMSO), standard antibiotic (1mg/ml of sterile distilled water) and DMSO (25%, in sterile water) were transferred into respectively labeled wells. Streptomycin and Fluconazole were used as standard antibacterial antifungal antibiotics. The plates were incubated at 37oC aerobically for 24 hours (for bacteria) and 48 hours (for fungi) and the zone of inhibition (cm) formed around the wells was measured (Kekuda et al., 2012).

Antioxidant activity of solvent extracts of U. pictoides

DPPH free radical scavenging assay

The free radical scavenging effect of various concentrations of solvent extracts was studied by employing DPPH free radical scavenging assay. In brief, 1 ml of different concentrations (2.5-100µg/ml of methanol) of solvent extracts and reference standard (ascorbic acid) was mixed with 3 ml of DPPH solution (0.004% in methanol) in clean and labeled tubes. The tubes were incubated at room temperature in dark for 30 minutes and the optical density was read at 517nm using UV-Vis spectrophotometer. The absorbance of the DPPH control (1ml methanol + 3ml DPPH solution) was also noted. The scavenging activity of the extracts was calculated using the formula:

Scavenging activity (%) = [(Ao – Ae) / Ao] x 100, where Ao is absorbance of DPPH control and Ae is absorbance of DPPH and extract/standard combination (Elmastas et al., 2006).

Ferric reducing assay

The reducing potential of solvent extracts of the lichen was determined by employing ferric reducing assay. Here, different concentrations (2.5-100µg/ml) of solvent extracts and reference standard (ascorbic acid) in 1 ml of methanol were mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (1%) in separate tubes. The tubes were then placed in water bath for 20 minutes at 50oC, cooled and mixed with 2.5 ml of trichloroacetic acid (10%) and 0.5 ml of Ferric chloride (0.1%). The amount of iron (II)-ferricyanide complex formed was determined by measuring the formation of Perl's Prussian blue at 700 nm after 10 minutes. The increase in absorbance of the reaction mixtures indicates increased reducing power (Junaid et al., 2013).

RESULTS

Thin layer chromatogram showed the presence of usnic acid in the lichen material. The content of total phenolics, as estimated by FCR method is shown in Figure 1. The quantity of phenolics was high in methanol extract (ME) followed by ethyl acetate extract (EAE), chloroform extract (CE) and petroleum

ether extract (PEE). The efficacy of solvent extract to inhibit *S. aureus* and *P. aeruginosa* was tested by Agar well diffusion method and the presence of zone of inhibition of test bacteria around the well was considered positive for antibacterial activity. It was observed that both the test bacteria were susceptible to solvent extracts. Inhibition produced by standard antibiotic was higher than that of solvent extracts. Among the bacteria, *S. aureus* exhibited highest sensitivity to solvent extracts as well as standard antibiotic. Chloroform and petroleum ether extracts have caused higher inhibition of *S. aureus* and *P. aeruginosa* respectively. There was no inhibition in case of DMSO which was used to prepare extracts **(Table 1)**.

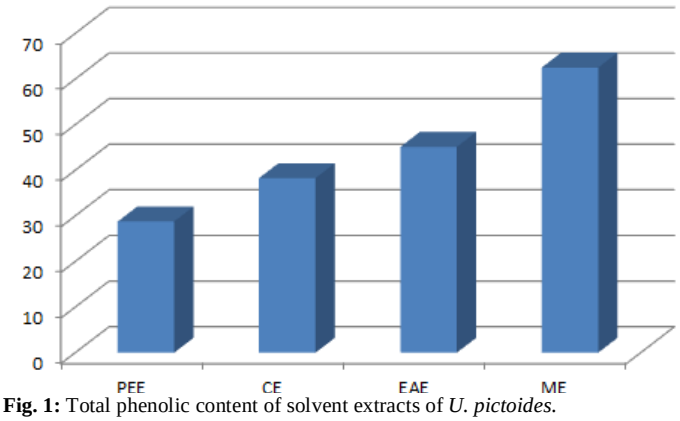

Fig. 1: Total phenolic content of solvent extracts of *U. pictoides.*

Table. 1: Antibacterial activity of solvent extracts of *U. pictoides*

Extract	Zone of inhibition in cm	
	S. aureus	***P. aeruginosa***
PEE	2.6±0.2	2.4±0.3
CE	2.8±0.6	2.0±0.5
EAE	2.7±0.2	2.0±0.1
ME	2.4±0.1	2.2±0.1
Streptomycin	3.9±0.2	3.1±0.3
DMSO	0.0±0.0	0.0±0.0

Table 2: Antifungal activity of solvent extracts of *U. pictoides*

Extract	Zone of inhibition in cm	
	C. albicans	***C. neoformans***
PEE	0.8±0.2	1.3±0.1
CE	1.4±0.1	2.1±0.2
EAE	1.2±0.1	1.9±0.2
ME	0.8±0.1	1.3±0.1
Fluconazole	3.5±0.2	4.1±0.2
DMSO	0.0±0.0	0.0±0.0

Two human pathogenic fungi *C. albicans* and *C. neoformans* were used to screen their susceptibility to solvent extracts of the lichen. Both the fungi were inhibited to varied extent by the solvent extracts. Petroleum ether and methanol extracts were least inhibitory to both the fungi. Chloroform extract caused marked inhibition of test fungi followed by ethyl acetate extract. Fluconazole was more inhibitory to test fungi than solvent extracts. Overall, *C. neoformans* exhibited high susceptibility than *C. albicans* to solvent extracts and as well as antibiotic. There was no inhibition in case of DMSO (Table 2).

The efficacy of solvent extracts of *U. pictoides* to scavenge free radicals was assessed by DPPH radical scavenging model and the results are shown in Figure 2. Extent of bleaching of color of DPPH solution in the presence of different concentrations of solvent extracts was determined at 517nm and the scavenging activity of extracts was compared with reference standard ascorbic acid. The scavenging effect was highest in case of methanol extract followed by ethyl acetate, chloroform and petroleum ether extracts. At high concentration (100µg/ml), only methanol extract produced >50% scavenging of radicals. The scavenging effect of extracts was lesser than that of ascorbic acid.

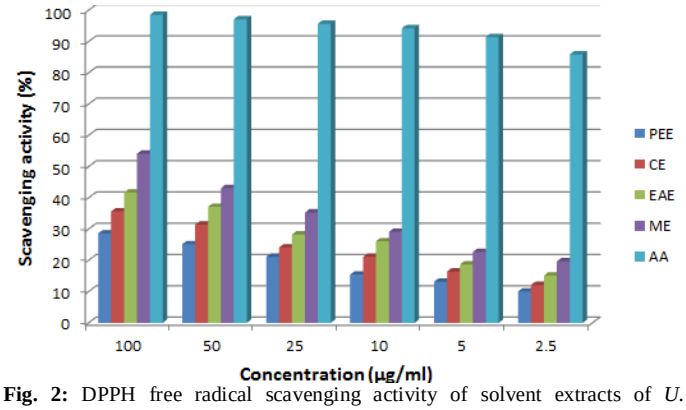

Fig. 2: DPPH free radical scavenging activity of solvent extracts of *U. pictoides.*

Ferric reducing assay was conducted to determine the reducing potential of solvent extracts of *U. pictoides*. It was observed that the absorbance of reaction mixture at 700nm increased on increasing the concentrations of extracts. The reducing power was high in methanol extract followed by ethyl acetate, chloroform and petroleum ether extracts. The reducing powers of solvent extracts were lesser than that of ascorbic acid (Figure 3).

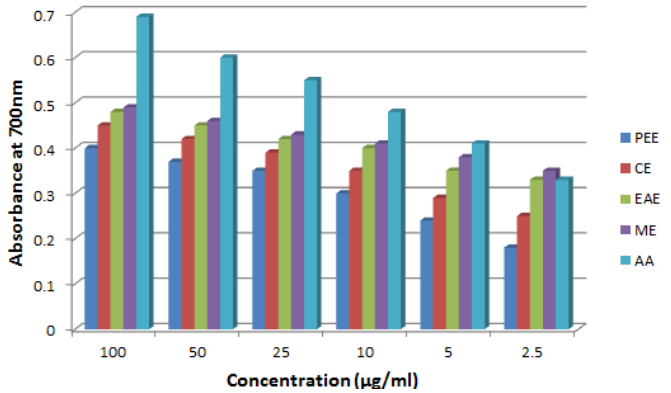

Fig. 3: Ferric reducing activity of solvent extracts of *U. pictoides.*

DISCUSSION

The infectious diseases are caused by a number of bacteria, fungi, viruses and parasites. The discovery of Antibiotics has revolutionized the field of medicine in many respects and saved countless lives and their discovery is considered as a turning point in human history. However, the use of these wonder drugs has come out with the rapid appearance of microbial strains being resistant to most of the currently used antibiotics. The multidrug-

resistant pathogens have emerged and are continuously emerging due to an extensive use of antibiotics such as penicillin, methicillin, vancomycin, Streptomycin etc., both in prophylaxis and long-term therapy. Microorganisms such as *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* are few among the antibiotic resistant bacteria that have become resistant to a number of antibiotics, of which *Staphylococcus aureus* is the most important (Hancock and Speert, 2000; Springer *et al*., 2001; Dominguez *et al*., 2002; Francolini *et al*., 2004; Hemaiswarya *et al*., 2008; Davies and Davies, 2010). Like bacteria, many of the fungi have also developed resistance against commonly used antimycotic drugs. The two most common fungi are *Candida albicans* and *Cryptococcus neoformans* that have become major fungal pathogens in HIV-infected patients and those receiving immunosuppressive treatment for cancer, organ transplantation, and other serious medical conditions. *Candida albicans* is the most common etiological agent of fungal bloodstream infections accounting majority of nosocomial fungal infections. *Cryptococcus neoformans* is an encapsulated yeast (Basidiomycetes) causing fatal infection in both immunocompetent and immunocompromised patients, including a prevalence of infection of up to 15% of patients with AIDS. These fungi have shown to be resistant to commonly used antifungal agents such as Fluconazole, Itraconazole, Amphotericin B etc. (Xu *et al*., 2001; Perea and Patterson, 2002). This alarming situation triggered an immense interest in scientific community to search alternatives for the prevention and control of infectious organisms.

Usnic acid, a low molecular weight dibenzofuran derivative produced by the fungal partner, is one of the important lichen substances found in abundance especially in genera such as *Alectoria*, *Cladonia*, *Usnea*, *Lecanora*, *Ramalina* and *Evernia*. It presents as a yellowish cortical pigment and occurs in two enantiomeric forms differing in the orientation of the methyl group located in the stereogenic centre at the 9b position. Lichens and their extracts containing usnic acid have been utilized for several purposes such as medicinal, perfumery, cosmetic etc. As a pure substance, Usnic acid has been formulated in creams, toothpaste, mouthwash, deodorants and sunscreen products. It exhibits several important biological and pharmacological properties (Yilmaz *et al*., 2004; Ribeiro-Costa *et al*., 2004; Behera *et al*., 2009). Lichens have been recognized as potent antimicrobial agents since ancient times and many studies conducted all over the world showed the potential of lichen extracts and purified metabolites to inhibit a wide range of bacteria and fungi (Yilmaz *et al*., 2004; Turk *et al*., 2006; Canarasan *et al*., 2006; Candan *et al*., 2006; Vinayaka *et al*., 2009; Kekuda *et al*., 2011).

In the present study, the solvent extracts of *U. pictoides* displayed marked inhibition of test bacteria. It was observed that Gram positive bacterium *S. aureus* exhibited high susceptibility than Gram negative bacterium *P. aeruginosa*. The lesser inhibitory activity of solvent extracts against the gram negative bacterium could be ascribed to the presence of an outer membrane that possess hydrophilic polysaccharides chains and forms an additional barrier for extracts as well as antibiotics (Lodhia *et al*., 2009; Nalubega *et al*., 2011). Antifungal activity of solvent extracts of *U. pictoides* was tested against *C. albicans* and *C. neoformans*. It has been shown that usnic acid possess marked antimicrobial activity. Usnic acid isolated from *Ramalina terebrata* exhibited marked inhibition of *S. aureus* and *B. subtilis* (Paudel *et al*., 2010). Usnic acid from *Cladonia foliacea* has shown inhibition of a panel of bacteria and fungi that included *S. aureus* and *C. albicans* (Yilmaz *et al*., 2004). It has been found that higher amount of usnic acid, from *Usnea* species of Anatolia, resulted in increased antimicrobial activity against *S. aureus* and other bacteria (Cansaran *et al*., 2006). In the present study, thin layer chromatograph revealed the presence of usnic acid. The observed antimicrobial activity could be attributed to the presence of usnic acid.

Reactive Oxygen Species (ROS) such as superoxide radical, hydroxyl radical, hydrogen peroxide, peroxyl radical, singlet oxygen are formed and degraded by all aerobic organisms. However, excessive production of ROS leads to a condition called oxidative stress (Nodberg and Arner, 2001; Blokhina *et al*., 2003). The formation of ROS is implicated in the onset of several diseases or disorders such as cancer, rheumatic arthritis, cirrhosis, arteriosclerosis, neurodegenarative diseases and others (Barros *et al*., 2008). The free radicals may come from endogenous sources through normal physiological and metabolic processes such as mitochondrial respiration or could result from exogenous sources such as exposure to toxic pollutants and radiations (Barros *et al*., 2008). To overcome the lethal effects of ROS, cells have been equipped with various defence system *viz*., antioxidant enzymes (Superoxide dismutase, Catalase) and chemical compounds such as ascorbic acid, α-tocopherol, carotenoids, glutathiones, polyphenol compounds including flavonoids etc (Barros *et al*., 2008). Many synthetic antioxidant namely BHA, BHT, PG have been used to retard oxidation process, however, their safety is doubtful (Stoilova *et al*., 2007). Therefore attention has been focused on antioxidants from natural sources.

Phenolic compounds are a large and diverse group of metabolites and are known to possess a broad range of biological activities including antioxidant activity. Phenolic compounds have strong antioxidant properties both *In vitro* and *in vivo* and the antioxidant efficacy is associated with their ability to scavenge free radicals, break radical chain reactions and chelate metal ions. Increased consumption of phenolic compounds is found to be associated with a reduced risk of many diseases and disorders such as cardiovascular diseases and certain types of cancer (Kaisoon *et al*., 2011). In this study, the total Phenolic contents of lichen extracts were estimated by FCR method. This method was initially intended for the analysis of proteins and later, the method was adopted to estimate in wine and plants. Despite the undefined chemical nature of FCR, the total phenolic assay by FCR method is convenient, simple and reproducible assay for studying phenolic antioxidants in plant extracts. Phenolic compounds in plant extracts react with FCR under basic conditions (pH~10, adjusted by Na_2CO_3). Dissociation of a phenolic proton leads to a phenolate

anion, which is capable of reducing FCR and blue colour is formed (Huang *et al*., 2005). The content of total phenolics was higher in methanol extract followed by ethyl acetate and other extracts. There are many reports which correlate the total phenolic content of plants and their antioxidant activity (Coruh *et al*., 2007; Poornima *et al*., 2012; Dileep *et al*., 2012).

DPPH is one of the few, stable, N-centred, commercially available organic free radical and has an UV-Visible absorption maxima at 515-517 nm in methanol. On accepting hydrogen from a corresponding donor, the solution of DPPH loses the characteristic deep purple colour and becomes yellow coloured diphenylpicryl hydrazine. DPPH radical scavenging activity is one of the widely used assays to determine antioxidant activity of many compounds including plant extracts (Tirzitis and Bartosz, 2010; Huang *et al*., 2005). In the present study, the model of scavenging of stable DPPH free radicals showed a dose dependent radical scavenging activity of Lichen extracts. In this assay, a decrease in absorbance was observed as a result of a colour change from purple to yellow. The radical nature of DPPH was lost due to the donation of hydrogen by Lichen extract leading to the formation of the stable DPPH-H molecule (Conforti *et al*., 2008). The radical scavenging activity of methanol extract was stronger when compared to other solvent extracts. The extracts possessing high phenolic content displayed stronger scavenging of DPPH radicals. The results obtained are similar to previous studies where extracts containing high phenolic contents exhibited stronger scavenging activities (Poornima *et al*., 2012; Dileep *et al*., 2012; Rekha *et al*., 2012). Although the scavenging abilities of lichen extracts were lesser than that of ascorbic acid, it was evident that the extracts showed hydrogen donating ability and could serve as free radical scavengers, acting possibly as primary antioxidants (Chung *et al*., 2006). Previous studies on antioxidant potential of *Parmotrema pseudotinctorum* (Kumar *et al*., 2010) and *Everniastrum cirrhatum* (Kekuda *et al*., 2011) showed similar result where the extract was found to exhibit lower scavenging potential when compared to reference standard.

The presence of reductants (antioxidants) in extracts causes the reduction of ferric complex to ferrous form. In the present study, Fe^{3+}-Fe^{2+} transformation was investigated to determine the reductive ability of solvent extracts of *U. pictoides*. The amount of Fe^{+2} can be monitored by measuring the formation of Perl's Prussian blue coloration at 700 nm followed the addition of excess ferric ions. In this assay, an increase in the absorbance of reaction mixture on increasing the concentration of the extracts/purified compounds indicates the reducing ability. Though the reducing potential of lichen extracts were lesser than that of ascorbic acid, it is evident from the study that the extracts have electron donating potential and thereby neutralize the free radicals and terminate radical chain reactions (Chung *et al*., 2006; El-Haci *et al*., 2009; Gulcin *et al*., 2010). Methanol extract showed higher reducing potential than that of other solvent extracts. The total phenolic content of methanol extract was also higher when compared to other extracts. Similar results were observed in other studies where a positive correlation has been made between the total phenolic content of extracts and the ferric reducing potential (Poornima *et al*., 2012; Dileep *et al*., 2012).

Lichens and their purified metabolites are proven to be good source of antioxidants and a plenty of literatures have supported the antioxidant action of lichens and their metabolites (Behera *et al*., 2006; Behera *et al*., 2009; Kekuda *et al*., 2011; Behera *et al*., 2012; Verma *et al*., 2012). Usnic acid is also found to exhibit antioxidant activity. It has been reported that usnic acid, isolated from the lichen *U. longissima* is found to exhibit gastroprotective effect which can be attributed to its reducing effect on the oxidative damage and neutrophil infiltration in tissues (Odabasoglu *et al*., 2006). Usnic acid, isolated from the lichen *Usnea complanata* showed antioxidative action in terms of radical scavenging activity and lipid peroxidation inhibition (Behera *et al*., 2012). In the present study, the observed antioxidative effect of *U. pictoides* might be attributed to the presence of usnic acid and phenolic compounds.

CONCLUSION

A marked antimicrobial activity of solvent extracts of the lichen *U. pictoides* was observed in this study. The inhibitory potential of the extracts could be attributed to the presence of usnic acid in the lichen. The radical scavenging and ferric reducing potential of solvent extracts may be attributed to the presence of phenolic compounds**.** The results show that the lichen *U. pictoides* can be a potential candidate for the development of antimicrobial agents and antioxidants. Further, toxicity studies and isolation of active principles from the lichen extracts are to be carried out.

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