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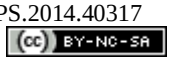
ISSN 2231-3354 

Radical scavenging and antibacterial activity of three *Parmotrema* species from Western Ghats of Karnataka, India

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ABSTRACT

Western Ghats are one of the biodiversity hotspots in the world. The present study was conducted to determine antibacterial and radical scavenging potential of three *Parmotrema* species *viz*., *P. tinctorum*, *P. grayanum* and *P. praesorediosum* from Maragalale and Guliguli Shankara, Western Ghats of Karnataka, India. The powdered lichen materials were extracted using methanol. Antibacterial activity of lichen extracts was tested against three Gram positive and five Gram negative bacteria by Agar well diffusion assay. Radical scavenging activity of lichen extracts was determined by DPPH free radical scavenging assay. Total phenolic content of lichen extracts was estimated by Folin-Ciocalteau reagent method. The lichen extracts showed dose dependent antibacterial activity. Overall, the lichen extracts were more inhibitory to Gram positive bacteria than Gram negative bacteria. *P. grayanum* displayed high inhibitory activity against test bacteria. Scavenging of DPPH radicals by lichen extracts was concentration dependent. Among the lichen species, *P. grayanum* showed higher scavenging potential as indicated by lower IC_{50} value. Total phenolic content was also high in *P. grayanum*. Thin layer chromatogram revealed the presence of Lecanoric acid, Orsellinic acid, Protolichesterinic acid, Chloroatranorin, Protopraesorediosic acid and Praesorediosic acid in lichen samples. The observed bioactivities of lichens could be ascribed to the presence of secondary metabolites. These lichens can be considered as suitable candidates for development of bioactive agents active against pathogenic microbes and oxidative damage.

INTRODUCTION

Lichens are stable, ecologically obligate, self supporting composite organisms comprised of a fungal partner (mycobiont) and an algal partner (photobiont). Lichens are known to be the earliest colonizers of terrestrial habitats with a worldwide distribution from artic to tropical regions and from the plains to the highest mountains. Lichens have been used in food and in folk medicine in many countries over a considerable period of time. All over the world, lichens are used as medicine to treat dyspepsia, bleeding piles, diabetes, bronchitis, pulmonary tuberculosis, spermatorrhoea and other diseases. These lichens have been considered as bioindicators of air pollution. The lichens represent taxonomically and physiologically a diverse group of organisms. Lichens produce a number of characteristic secondary metabolites called lichen substances, which seldom occur in other organisms. Depsides and depsidones are among the most common secondary metabolites produced by the fungal symbiont. The lichens and their metabolites are shown to possess various biological activities such as antimicrobial, antiviral, antiprotozoal, enzyme inhibitory, insecticidal, antitermite, cytotoxic, antioxidant, antiherbivore, wound healing, analgesic and anti-inflammatory (Perry *et al*., 1999; Bombuwala *et al*., 1999; Conti and Cecchetti, 2001; Oh *et al*., 2006; Kekuda *et al*., 2011; Kumar *et al*., 2011; Mitrovic *et al*., 2011; Kekuda *et al*., 2012; Barreto *et al*., 2013).

Western Ghats of India represents one of the biodiversity hotspots in the world and covers an area (1,80,000 km^2) which is just under 6% of the land area of India. Western Ghats harbors >30% of all plant, fish, herpeto-fauna, birds, and mammal species found in India. The mountain ranges of Western Ghats runs through states namely Gujarat, Maharashtra, Goa, Karnataka and Kerala. The area represents the 'gene pool' and harbors numerous species of plants, animals and microbes including a number of globally threatened and endemic species of plants and animals (Nampoothiri *et al*., 2013). Studies have been carried out on distribution and biological activities of macrolichens of Western Ghats of Karnataka

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(Vinayaka *et al*., 2010; Vinayaka *et al*., 2011; Vinayaka and Krishnamurthy, 2012; Vinayaka *et al*., 2012; Kumar *et al*., 2011; Kekuda *et al*., 2011; Kekuda *et al*., 2012; Pavithra *et al*., 2013). In the present study, we report antibacterial and radical scavenging activity of three *Parmotrema* species *viz*., *P. tinctorum*, *P. grayanum* and *P. praesorediosum* collected at Maragalale and Guliguli Shankara, Western Ghats of Karnataka, India.

MATERIALS AND METHODS

Collection and identification of lichens

P. tinctorum and *P. praesorediosum* were collected at a place called Maragalale, Thirthahalli taluk of Shivamogga district; Karnataka and *P. grayanum* was collected at Guliguli Shankara, Hosanagara taluk, Shivamogga district, Karnataka. The lichens were collected in the month of September 2013. The collected lichens were identified by morphological, anatomical and chemical tests. Color reactions were performed on the cortex and medulla by using 10% potassium hydroxide (K), Steiner's stable paraphenylenediamine solution (P) and calcium hypochlorite solution (C). Thin layer chromatography (TLC) for lichen extracts was performed using solvent system A (Benzene:1,4-Dioxane:Acetic acid in the ratio 90:25:4). The spots were marked out, Rf values were calculated and the compounds were identified (Awasthi, 2000; Culberson and Kristinsson, 1970; Culberson, 1972).

Extraction

The dried lichens were powdered using a blender. For extraction, about 25g of each of the lichen material was subjected to Soxhlet extraction and extracted using methanol (HiMedia, Mumbai). The methanol extract was filtered through sterile Whatman No. 1 filter paper and concentrated in vacuum under reduced pressure (Kekuda *et al*., 2012). The lichen extracts were stored in amber colored containers until use.

Test bacteria

Inhibitory potential of lichen extracts was tested against three Gram positive bacteria *viz*., *Staphylococcus aureus* NCIM-2079, *S. epidermidis* MTCC-3382, *Bacillus cereus* NCIM-2016 and five Gram negative bacteria *viz*., *Klebsiella pneumoniae* NCIM-2957, *Enterobacter aerogenes* NCIM-2695, *Shigella flexneri* NCIM-4924, *Salmonella typhi* MTCC-734 and *Escherichia coli* NCIM-2685.

Antibacterial activity of lichen extracts

We employed Agar well diffusion assay in order to screen antibacterial efficacy of methanol extract of lichens. The test bacteria were inoculated into test tubes containing sterile Nutrient broth (HiMedia, Mumbai) and incubated for 24 hours at 37 ^{o}C. The broth cultures were then swabbed aseptically on sterile Nutrient agar (HiMedia, Mumbai) plates. Using a sterile cork borer, wells of 6mm diameter were punched in the inoculated plates and 100µl of lichen extract (2.5, 5.0, 10 and 20mg/ml of dimethyl sulfoxide [DMSO; HiMedia, Mumbai]), reference antibiotic (Chloramphenicol, 1mg/ml of sterile water) and DMSO (25%, in sterile water) were transferred into labeled wells. The plates were incubated at 37^{o}C for 24 hours in upright position and zones of inhibition formed around wells were measured using a ruler (Kekuda *et al*., 2012).

DPPH free radical scavenging assay

DPPH assay was conducted to evaluate the radical scavenging effect of lichen extracts (Rekha *et al*., 2012). Here, 2ml of different concentrations of lichen extracts (5-200µg/ml of methanol) was mixed with 2ml of DPPH solution (0.002% in methanol). The tubes were incubated in dark for 30 minutes at room temperature and the optical density was measured at 517 nm using UV-Visible spectrophotometer (ELCO, SL159). The absorbance of the DPPH control (2ml of DPPH+2ml of methanol) was also noted. Ascorbic acid was used as reference standard. The scavenging activity of each concentration of the extracts was calculated using the formula:

Scavenging activity (%) = [(A–B) /A] x100, where A is absorbance of DPPH and B is absorbance of DPPH and extract/standard combination. The IC_{50} value for the extract was calculated. IC_{50} represents the concentration of extract required to scavenge 50% of DPPH free radicals.

Total phenolic content of lichen extracts

Total phenol content was estimated by Folin-Ciocalteu reagent (FCR) method. A dilute concentration of each of the lichen extract (0.5ml) was mixed with 0.5ml diluted Folin-Ciocalteu reagent (1:1) and 2 ml of sodium carbonate (7%). The mixtures were left at room temperature for 30 minutes and the absorbance was measured at 765nm using UV-Visible spectrophotometer (ELCO, SL159).

A standard curve was plotted using different concentrations of Gallic acid (standard, 0-1000µg/ml). The concentration of total phenolics was determined as µg Gallic acid equivalents (GAE) from the graph (Kekuda *et al*., 2013).

Statistical analysis

The experiments were conducted in triplicates. The values are represented as Mean±Standard Deviation (SD). The IC_{50} value for the extract was calculated by Origin 6.0 software.

RESULTS

The details of habitat, the thallus characteristics, color tests and secondary metabolites (TLC) of the lichen selected in this study are shown in Table 1.

The result of antibacterial activity of lichen extracts is shown in Table 2. The lichen extracts displayed dose dependent inhibitory activity against test bacteria. Overall, the lichen extracts caused higher inhibition of Gram positive bacteria than Gram negative bacteria.

Table. 1: Description of *Parmotrema* species selected in this study.

Lichen	Habitat	Thallus	Color test	TLC
P. tinctorum	Corticolous	Large loosely adnate, membranous, broad, lobes irregular, rotund; margins crenate, eciliate; upper surface grey, smooth, isidiate; lower surface minutely wrinkled, rough, black, erhizinate; rhizines sparse, coarse at the centre	Cortex K+ yellow; Medulla K-, C +red, KC +red, Pd -	Lecanoric acid, Atranorin, Orsellinic acid
P. grayanum	Saxicolous	Adnate; lobes rotund; margins ascending, crenate, ciliate; cilia dense and thick; upper surface ashy grey; lower surface wrinkled, black, erhizinate, rhizinate at the centre; rhizines sparce, black and simple	Cortex K+ yellow; Medulla K-, C -, KC -, Pd -	Atranorin, Protolichesterinic acid
P. praesorediosum	Saxicolous	Thallus coriaceous, adnate to substratum; lobes rotund; margins crenate; upper surface grey, smooth; lower suface minutely wrinkled, black; rhizines sparse, simple	Cortex K+ yellow; Medulla K-, C -, KC -, Pd -	Atranorin, Chloroatranorin, Protopraesorediosic acid, Praesorediosic acid

Table. 2: Antibacterial activity of extracts of *Parmotrema* species.

Lichen/ Antibiotic	Conc. (mg/ml)	Inhibition of test bacteria (Zone of inhibition in cm)							
		Sa	***Se***	***Bc***	***Kp***	***Ea***	***Sf***	***St***	***Ec***
Pt	20.0	1.9±0.1	2.1±0.2	1.5±0.1	1.5±0.1	1.7±0.1	1.2±0.0	2.1±0.2	1.2±0.0
	10.0	1.5±0.0	1.8±0.1	1.2±0.2	1.2±0.0	1.5±0.0	0.8±0.0	1.5±0.1	0.8±0.0
	5.0	1.0±0.2	1.3±0.0	0.9±0.0	0.8±0.1	1.0±0.1	0.0±0.0	1.2±0.0	0.0±0.0
	2.5	0.8±0.0	1.1±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	1.0±0.0	0.0±0.0
Pg	20.0	2.1±0.1	2.3±0.1	2.4±0.2	1.9±0.2	1.8±0.0	1.6±0.1	2.1±0.2	1.4±0.1
	10.0	1.8±0.0	1.9±0.1	2.2±0.1	1.4±0.1	1.5±0.1	1.3±0.1	1.6±0.0	1.1±0.1
	5.0	1.3±0.1	1.5±0.0	1.7±0.0	1.1±0.0	1.1±0.0	0.8±0.0	1.3±0.1	0.8±0.0
	2.5	1.0±0.0	1.2±0.1	1.5±0.2	0.8±0.0	0.8±0.0	0.0±0.0	1.0±0.1	0.0±0.0
Pp	20.0	1.9±0.1	1.8±0.1	2.5±0.1	1.4±0.0	1.8±0.2	0.8±0.0	1.7±0.1	1.1±0.1
	10.0	1.7±0.1	1.6±0.1	2.3±0.1	1.2±0.1	1.6±0.0	0.0±0.0	1.6±0.0	0.8±0.0
	5.0	1.4±0.1	1.2±0.0	1.8±0.2	0.0±0.0	1.1±0.1	0.0±0.0	1.2±0.0	0.0±0.0
	2.5	0.8±0.0	0.8±0.0	1.3±0.0	0.0±0.0	0.8±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Chl	1.0	3.8±0.2	3.9±0.3	3.6±0.1	2.9±0.0	2.3±0.1	2.4±0.2	3.1±0.1	2.2±0.0

Pt- P.tinctorum; Pg- P.grayanum; Pp- P.praesorediosum; Sa- S.aureus; Se- S.epidermidis; Bc- B.cereus; Kp- K.pneumoniae; Ea- E.aerogenes; Sf- S.flexneri; St- S.typhi; Ec- E.coli ; Chl- Chloramphenicol

Table. 3: Total phenolic content (mg GAE/g) in extracts of *Parmotrema* species.

Lichen	Total phenolic content
P. tinctorum	25.68±5.6
P. grayanum	56.48±8.8
P. praesorediosum	35.33±6.1

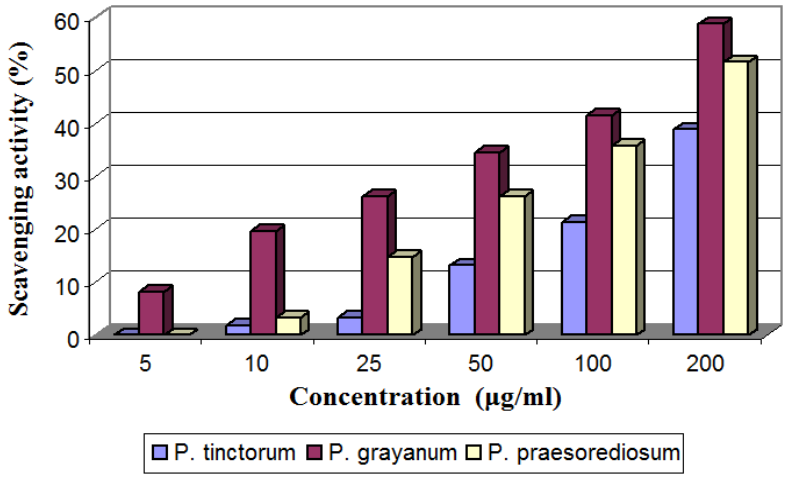

Fig. 1: DPPH radical scavenging activity of extracts of Parmotrema species.

Among lichens *P. grayanum* caused high inhibition of test bacteria followed by other two lichen extracts. Reference antibiotic showed high inhibitory efficacy when compared to lichen extracts. DMSO did not cause inhibition of any bacteria (not shown in table). Scavenging of DPPH free radicals by extracts of three *Parmotrema* species was concentration dependent i.e., scavenging potential was found to increase on increasing the concentration of extracts (Figure 1). Among three species, *P. grayanum* (IC_{50}148.39µg/ml) showed higher scavenging potential followed by *P. praesorediosum* (IC_{50} 179.81µg/ml) and *P. tinctorum* (IC_{50} 439.06µg/ml). However, the radical scavenging efficacy of extracts was lesser than that of ascorbic acid (IC_{50} 2.3µg/ml). The phenolic content of extracts of lichens was estimated by FCR method and the results were taken as mg GAE/g of extract. Extract of *P. grayanum* contained high phenolic content followed by *P. praesorediosum* and *P. tinctorum* (Table 3).

DISCUSSION

The lichens of the genus *Parmotrema* (Parmeliaceae) are foliose and characterized by large thalli with broad rotund lobe apices, the absence of pseudocyphellae, broad erhizinate marginal zone on the lower surface, marginal cilia, simple rhizines and thick walled ellipsoid ascospores. The genus is best developed in tropical regions of the world. Over 220 species are known, out of which 46 species are distributed in India (Divakar and Upreti, 2005; Benatti *et al*., 2013; Jayalal *et al*., 2013). In the present study, we evaluated antibacterial efficacy of three *Parmotrema* species collected at Western Ghats of Karnataka, India. We observed a marked dose dependent inhibition of test bacteria by lichen extracts. It has been found that lichens of the genus *Parmotrema* are promising antimicrobial agents. Balaji and Hariharan (2007) reported marked antimicrobial efficacy of dichloromethane extract of *P. praesorediosum* collected from silicious rocks of Western Ghats of Tamil Nadu. Kumar *et al*. (2010) showed the antibacterial activity of methanol extract of *P. pseudotinctorum* from the Western Ghats of Karnataka. Sinha and Biswas (2011) reported the antibacterial efficacy of solvent extracts of *P. reticulatum* from Sikkim, India. Verma *et al*. (2011) found antibacterial efficacy of solvent extracts of *P. nilgherrensis* and *P. sancti-angelii* collected from Karnataka, India. Chauhan and Abraham (2013) showed the inhibitory effect of methanol extract of *Parmotrema* sp. collected from Kodaikanal forest, India against clinical isolates of bacteria. Javeria *et al*. (2013) showed the inhibitory efficacy of solvent extracts of *P. nilgherrense* collected from Nainital, India against drug resistant bacteria.

The secondary metabolites in the lichens of the present study were detected by TLC. Thin layer chromatogram developed with solvent A showed the presence of atranorin in all the lichen specimens. Lecanoric acid and orsellinic acid were detercted in *P. tinctorum*. Protolichesterinic acid was present in *P. grayanum*. Compounds *viz*., Chloroatranorin, Protopraesorediosic acid and Praesorediosic acid were detected in *P. praesorediosum*. Most of these secondary metabolites from lichens have been shown to possess inhibitory activity against bacteria and fungi. Atranorin was shown to exhibit inhibitory activity against Gram positive and Gram negative bacteria (Thadhani *et al*., 2012). Atranorin isolated from the lichen *Cladonia foliacea* was shown to possess marked antibacterial activity (Yilmaz *et al*., 2004). Chloroatranorin from *Pseudevernia furfuracea* is found to exhibit inhibitory activity against bacteria and yeasts (Turk *et al*., 2006). Lecanoric acid showed inhibitory activity against bacteria and fungi (Misic *et al*., 2008). Protolichesterinic acid from *Cetraria aculeate* showed antibacterial activity (Turk *et al*., 2003). Protolichesterinic acid from the lichen *Cetraria islandica* showed anti-*Helicobacter pylori* activity (Ingolfsdottir *et al*., 1997). Atranorin and lecanoric acid from macrolichens of Karnataka were shown to possess inhibitory activity against bacteria (Verma *et al*., 2011). Oxidative stress results when the production of potentially harmful free radicals exceeds antioxidant defense of the body. These free radicals in particular reactive oxygen species such as superoxide radical, hydroxyl radical, singlet oxygen, and hydrogen peroxide play a pivotal role in the pathogenesis of diseases such as atherosclerosis, neurodegenerative diseases, rheumatoid arthritis, age-related degeneration and cancer initiation. These effects of free radicals are through their action on proteins, lipids and DNA. Cells have the innate antioxidant defense system which protects against the dreadful action of free radicals. However, during oxidative stress, there is extra need for antioxidants from external sources. Synthetic antioxidants like BHT, BHA and PG have been extensively used but they are suspected to have some adverse effects. Hence, antioxidants from natural sources are of immense interest (Kulisic *et al*., 2004; Katalinic *et al*., 2006; Letelier *et al*., 2008; Junaid *et al*., 2013). Lichens have been shown promising as they possess various bioactivities including antioxidant activity (Jayaprakasha and Rao, 2000; Kekuda *et al*., 2009; Stanly *et al*., 2011; Kekuda *et al*., 2011; Sharma and Kalikoty, 2012). DPPH is a stable, organic, nitrogen centered free radical with absorption maxima at 515-520 nm in alcoholic solution. On accepting an electron or hydrogen atom, it becomes a stable diamagnetic molecule. In the presence of a compound (antioxidant) capable of donating a hydrogen atom, the free radical nature of DPPH is lost and the purple color changes to yellow (diphenylpicrylhydrazine). The DPPH free radical scavenging assay is one of the most widely used assays to evaluate the antioxidant activity of several kinds of samples including lichen extracts. The method is simple, rapid, sensitive and requires small amount of samples (Kulisic *et al*., 2004; Kaviarasan *et al*., 2007; Letelier *et al*., 2008; Kekuda *et al*., 2011; Junaid *et al*., 2013; Vivek *et al*., 2013; Kekuda *et al*., 2013). In our study, we monitored the decrease in DPPH absorption in the presence of varying concentrations of lichen extracts at 517nm. Extract of *P. grayanum* showed high scavenging activity followed by *P. praesorediosum* and *P. tinctorum* as indicated by lower IC_{50} value. Though the scavenging of free radicals by lichen extracts was lesser than ascorbic acid, it is evident that the extracts showed hydrogen donating ability and therefore the extracts could serve as free radical scavengers, acting possibly as primary antioxidants (Chung *et al*., 2006). It has been found that *Parmotrema* species possess radical scavenging activity. Kekuda *et al*. (2009) observed dose dependent DPPH radical scavenging activity in the lichen *P. pseudotinctorum*. Methanol and ethanol extract of *P. reticulatum* have shown DPPH radical scavenging activity (Sharma and Kalikoty, 2012). Phenolic constituents are among the most effective antioxidants. Hence, it is important to estimate the phenolic contents in order to justify their contribution to antioxidant activity (Choi *et al*., 2007). FCR method is one of the oldest and most commonly employed methods being used to estimate total phenolic contents in a variety of samples. The phenolic compounds react with FCR under basic conditions and produce blue complex with absorption maxima near 750nm. The assay of total phenolic content by FCR is convenient, simple, and reproducible (Chung *et al*., 2006; Harish and Shivanandappa, 2006; Coruh *et al*., 2007; Kekuda *et al*., 2011; Rekha *et al*., 2012; Vivek *et al*., 2013). In the present study, the phenolic content of lichen extract is in the order *P. grayanum* > *P. praesorediosum* >

P. tinctorum. Studies have shown a direct correlation between the phenolic content and the antioxidant activity (Tilak *et al*., 2004; Coruh *et al*., 2007; Rekha *et al*., 2012; Poornima *et al*., 2012). In our study also, *P. grayanum* with high phenolic content displayed marked scavenging of DPPH radicals when compared to other lichens. Stanly *et al*. (2011) reported no such correlation between total phenolic content and antioxidant activity of selected lichens species from Malaysia.

CONCLUSION

In the present study, the species of *Parmotrema* from Western Ghats of Karnataka have shown radical scavenging and antibacterial activity. The observed bioactivities can be ascribed to the presence of secondary metabolites in the lichen materials. These lichens can be the potential candidates for the development of agents active against infectious agents and free radical induced oxidative damage.

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