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# Studies on Antibacterial, Anthelmintic and Antioxidant activities of a Macrolichen *Parmotrema pseudotinctorum* (des. Abb.) Hale (Parmeliaceae) from Bhadra wildlife sanctuary, Karnataka

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**ABSTRACT:** The present study was carried to determine the antibacterial, anthelmintic and antioxidant activity of a macrolichen *Parmotrema pseudotinctorum* (des. Abb.) Hale (Parmeliaceae) collected from forest area of Bhadra wildlife sanctuary. The extract exhibited marked antibacterial activity. The minimum inhibitory concentration of the extract was found to be lesser in case of Gram negative bacteria than Gram positive bacteria. The lichen extract exhibited a dose-dependent inhibition of spontaneous motility. At doses of 15 and 20mg/ml, the effects were comparable with that of standard anthelmintic. The lichen extract exhibited marked antioxidant activity in a dose dependent manner in DPPH free radical scavenging assay and $Fe^{+3}$ reducing assay. The methanol extract exhibited marked antioxidant activity by scavenging DPPH* (free radical) and converting into DPPHH. The absorbance was found to increase with the dose of methanolic extract and standard which is suggestive of reducing power. Preliminary phytochemical analysis showed the presence of secondary metabolites namely tannins and steroids in the extract. Thin layer chromatography revealed the presence of Atranorin and Lecanoric acid in the lichen material. The efficacy of the solvent extract may be due to the presence of various constituents. Further studies on isolation of secondary metabolites and their biological activities *in vitro* and *in vivo* are to be carried out.

**Keywords:** *Parmotrema pseudotinctorum* (des. Abb.) Hale, Macrolichen, Agar well diffusion, *Pheretima pasthuma*, DPPH radical scavenging assay, $Fe^{+3}$ reducing assay.

## INTRODUCTION

Infectious diseases caused by bacteria, fungi, viruses, and parasites remain a major threat to public health, despite tremendous progress in human medicine. Their impact is particularly great in developing countries because of the relative unavailability of medicines and the emergence of widespread drug resistance [1]. Interest in natural products with antimicrobial properties has revived as a result of current problems associated with the use of antibiotics [2]. Helminth infections are among the most common infections in man, affecting a large proportion of the world's population. During the past few decades, despite numerous advances made in understanding the mode of transmission and the treatment of these parasites, there are still no efficient products to control certain helminthes and the indiscriminate use of some drugs has generated several cases of resistance. Furthermore, it has been recognized recently that anthelmintic substances having considerable toxicity to human beings are present in foods derived from livestock, posing a serious threat to human health [3]. Indigenous system of medicine reports a number of natural sources for their anthelmintic efficacy.

Free radicals are found to be a product of normal metabolism. Although oxygen is essential for aerobic forms of life, oxygen metabolites are highly toxic. As a consequence, reactive oxygen species (ROS) are known to be implicated in many cell disorders and in the development of many diseases including cardiovascular diseases, atherosclerosis, chronic inflammation etc [4,5]. Although organisms have endogenous antioxidant defences produced during normal cell aerobic respiration against ROS, other antioxidants are taken both from natural and synthetic origin [6]. Antioxidants that can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, therefore, appear to be very important [7]. Synthetic antioxidants are widely used but their use is being restricted nowadays because of their toxic and carcinogenic effects. Thus, interest in finding natural antioxidants, withough any undesirable effect, has increased greatly [6].

India is a rich center of lichen diversity, contributing nearly 15% of the 13,500 species of lichens so far recorded in the world [8]. Lichens and lichen products have been used in traditional medicines for centuries and still hold considerable interest as alternative treatments in various parts of the world. In various systems of traditional medicine worldwide, including the Indian system of medicine, these lichen species are said to effectively cure dyspepsia, bleeding piles, bronchitis, scabies, stomach disorders, and many disorders of blood and heart [9-11]. They produce characteristic secondary metabolites that are unique with respect to those of higher plants [12]. Lichen metabolites exert a wide variety of biological actions including antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic effects [13]. The utility of lichens is due of range of secondary compounds produced by them. A wide range of secondary metabolites of lichens were characterized. According to their chemical structure, most lichen substances are phenolic compounds, dibenzofuranes, Usnic acids, depsidones, depsones, lactones, quinines and pulvunic acid derivatives [14].

Bhadra reserve area $75^0$15'-$75^0$50' E and $13^0$25'-$13^0$50' N latitude. The area comprises the forests of Western Ghats and its fringes. Sanctuary being situated in the south interior Karnataka, with cool climate throughout the year and affords pleasant days during the hot months. *Parmotrema pseudotinctorum* (des. Abb.) Hale (Parmeliaceae) is a foliose lichen with thallus loosely adnate to the substratum, corticolous, up to 6.0 cm across [15]. The present study describes the antibacterial, anthelmintic and antioxidant activity of a macrolichen *P. pseudotinctorum* collected from forest area of Bhadra wildlife sanctuary.

## MATERIALS AND METHODS

### Collection and identification of Lichen material

The lichen *P. pseudotinctorum*, growing on barks of trees, was collected from the forest area of Bhadra wildlife sanctuary, Karnataka. The voucher specimen of lichen (Voucher no. KSV/KU01130) was deposited in the Department of Applied Botany, Shankaraghatta for future reference. The dried lichen material was identified based on morphological, anatomical and color tests [15]. Thin layer chromatography in solvent A (180 ml toluene: 60 ml 1,4, dioxine: 8 ml acetic acid) was performed to detect secondary metabolites [16,17].

### Extraction of powdered material using methanol

For extraction, 20g of powdered lichen material was added to 100 ml methanol, sonicated for 30 minutes and left at room temperature overnight. The extract was filtered over Whatman No 1 filter paper and the filtrate was concentrated under reduced pressure to pasty mass [18]. The solvent extract was subjected to phytochemical screening [19].

### Screening lichen extract for Antibacterial activity

The antibacterial activity was tested against *Staphylococcus aureus* MTCC-902, *Clostridium perfringens* MTCC-450, *Escherichia coli* MTCC-405 and *Pseudomonas aeruginosa* MTCC-1934 by Agar well diffusion method [20]. The test bacteria were obtained from IMTECH, Chandigarh, INDIA Twenty

four hours old Muller-Hinton broth cultures of test bacteria were aseptically swabbed on sterile Muller-Hinton agar plates. Wells of 6 mm diameter were made aseptically in the inoculated plates and the methanol extract (20mg/ml of 10% DMSO), Standard (Chloramphenicol, 1mg/ml) and Control (10% DMSO) were added into the respectively labeled wells. The plates were incubated at 37$^{o}$C for 24 hours in upright position. The experiment was carried in triplicates and the zone of inhibition was recorded.

**Determination of Minimum inhibitory concentration (MIC)**

Sterile nutrient broth tubes containing different dilutions of extract (0.05mg to 1.0mg/ml) were specifically inoculated with 0.1 ml of standardized inoculum ($10^{7}$cfu/ml). The tubes were incubated aerobically at 37$^{o}$C for 18-24 h. Two control (tube containing the growth medium, saline and the inoculum) tubes for each organism were maintained. The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (no turbidity) when compared with the control tubes were regarded as MIC [21,22].

**Screening lichen extract for Anthelmintic activity**

The anthelmintic assay was performed on adult Indian earthworm *Pheretima pasthuma* due to its anatomical and physiological resemblance with the intestinal roundworm parasite of human beings. Standard drug (Piperazine citrate, 1%) and different concentrations of methanol extract of lichen (5, 10, 15 and 20mg/ml) were prepared in normal saline (0.85%) and poured into respective labeled petriplates (50 ml). Six worms of nearly equal size were introduced into each of the plates. Observations were made for the time taken to paralysis and death of individual worm. Paralysis was said to occur when the worms were not able to move even in normal saline. Death was concluded when the worms lost their motility followed with fading away of their body colors [23]. Death was also confirmed by dipping the worms in slightly warm water. The mortality of parasite was assumed to have occurred when all signs of movement had ceased [24].

**Antioxidant activity by DPPH radical scavenging assay**

The antioxidant activity of different concentrations, namely 0.125, 0.250, 0.5 and 1.0 mg/ml, of methanol extract and the Ascorbic acid was tested on the basis of the radical scavenging effect of the stable DPPH free radical activity. 0.002% of DPPH in methanol was used as the free radical. In clean and labeled test tubes, 2ml of DPPH solution was mixed with 2ml of different concentrations of methanol extract and standard separately. The tubes were incubated at room temperature in dark for 30 minutes and the optical density was measured at 517nm using UV-Vis Spectrophotometer. The absorbance of the DPPH control (containing no sample) was also noted [25,26]. The scavenging activity of the extract against the stable DPPH* was calculated using the equation.

Scavenging activity (%) = A –B / A x 100

Where A was the absorbance of DPPH solution and B was the absorbance DPPH* solution with extract.

**Antioxidant activity by $Fe^{+3}$ reducing assay**

Different concentrations of Methanolic extracts and tannic acid (0.125, 0.250, 0.5 and 1.0 mg/ml of methanol) were mixed in separate tubes with 2.5ml of phosphate buffer (200mM, pH 6.6) and 2.5ml of 1% potassium ferricyanide. The mixture was placed in a water bath for 20 min at 50$^{o}$C, cooled rapidly and mixed with 2.5ml of 10% trichloroacetic acid and 0.5ml of 0.1% Ferric chloride. The amount of iron (II)-ferricyanide complex formed was determined by measuring the formation of Perl's Prussian blue at 700nm after 10min. The higher absorbance of the reaction mixture indicates increased reducing power [27].

**RESULTS**

Preliminary phytochemical analysis and thin layer chromatography were employed to detect various secondary metabolites in the methanol extract of *P. pseudotinctorum*. Preliminary phytochemical analysis detected tannins and steroids in the extract. Further, thin layer chromatography revealed the presence of Atranorin and Lecanoric acid in the lichen material.

The result of antibacterial activity of lichen extract is shown in Table-1. Results were recorded as presence or absence of zones of inhibition around the well. The inhibitory zone around the well indicated the absence of bacterial growth and it as reported as positive and absence of zone as negative [28]. It was found that extract and standard (Chloramphenicol) have shown inhibition of all tested bacteria. Among bacteria, *P. aeruginosa* and *E. coli* were found to be more sensitive to lichen extract as revealed by larger inhibition zones. The minimum inhibitory concentration of the extract was found to be lesser in case of Gram negative bacteria namely *P. aeruginosa* (0.35mg/ml) and *E. coli* (0.40) as compared to Gram positive bacteria *S. aureus* (0.50mg/ml) and C. *perfringens* (0.45mg/ml).

The different concentrations of methanol extracts of lichen species were evaluated for anthelmintic activity using adult Indian earthworm model. The lichen extract exhibited a dose-dependent inhibition of spontaneous motility (paralysis). With higher doses (15 and 20mg/ml) the effects were comparable with

that of 1% piperazine (Table-2). The results show that extract possesses wormicidal activity and thus, may be useful as an anthelmintic.

DPPH free radical scavenging activity of different concentrations of methanol extract and standard (Ascorbic acid) is presented in Table-3 and Fig-1. The methanol extract exhibited marked antioxidant activity by scavenging DPPH* (free radical) and converting into DPPHH. A dose dependent radical scavenging activity was observed. The scavenging activity of standard (ascorbic acid) was greater than that of solvent extracts. The result of reducing power of different concentrations of methanol extract of lichen species and tannic acid is represented in Table-4 and Fig-2. In this study, the absorbance was found to increase with the dose of methanolic extract and standard which is suggestive of reducing power.

## DISCUSSION

Lichens are self-supporting symbiotic associations of a fungus and one or several algal or cyanobacterial components. Since the fungal constituent is unique in that symbiosis and usually dominates the association, lichens traditionally have been considered a type of fungus. The lichenous lifestyle is maintained by one-fifth of all fungi, including more than 40% of ascomycetes [29]. Lichens have diversified extensively during the past 600 years [30], and occur over >10% of the terrestrial surface. Humans have exploited lichens for several purposes among which most important use has been for dyeing textiles. Besides dyeing, lichens were used extensively in traditional medicines and for cosmetic purposes [31-38]. Lichen substances exhibit a great diversity of biological effects, including antimicrobial, anti-inflammatory, analgesic, antipyretic, and antiproliferative and cytotoxic activities, and there has been a growing interest in the pharmaceutical properties of compound derived from lichens [14]. However, relatively few lichen substances have been screened in detail for their biological activity and therapeutic potential, due principally to difficulties in obtaining them in quantities and purities sufficient for structural elucidation and pharmacological testing. Most known lichen substances are phenolic compounds, anthraquinones, dibenzofurans, depsides, depsidones, depsones, gamma lactones and pulvinic acid derivatives. Lichens had to evolve diverse biosynthetic pathways to produce such complex arrays of secondary metabolites. The polyketide biosynthetic pathway appears to be responsible for most of the classes of lichen compounds, whereas pulvinic acids are shikimate derivatives, and the abundance of di- and triterpenoids found in lichens are formed via the mevalonate pathway [39,40]. In this study, secondary metabolites namely Tannins, steroids, Atranorin and Lecanoric acid were detected in the lichen material which might be responsible for the biological efficacy of the lichen.

Infectious diseases are the leading cause of death across the world. As a global concern the antibiotic resistance by pathogens has emerged. Many of the antibiotics have been out of use as multidrug resistant pathogens have emerged. Natural products, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and reemerging infectious diseases. Therefore, much attention has been given to folk medicine in order to look for new leads to develop better drugs to treat resistant bacteria [41-45]. In this study, the methanol extract of *P. pseudotinctorum* has shown promising antibacterial activity against bacteria which are known to cause hospital infections, food poisoning, gastroenteritis, burn infections etc. The extract could be useful against these pathogenic bacteria.

Helminthes are recognized as a major problem to livestock production throughout the tropics. Parasitic helminthes affect human being and animals by causing considerable hardship and stunted growth. Most diseases caused by helminthes are of a chronic and debilitating in nature [46]. The origin of many effective drugs is found in the traditional medicine practices and in view of this several workers have undertaken studies pertaining to testing of natural compounds for their proclaimed anthelmintic activity. The traditional medicines hold a great promise as a source of easily available effective anthelmintic agents to the people, particularly in developing countries, including India [24]. Indigenous system of medicine reports a number of natural sources for their anthelmintic efficacy. However, their scientific evaluation as compared to commercial anthelmintics is limited. Many plants have proven to possess anthelmintic activity *in vitro* and *in vivo*. In this study, tannins were detected in the methanol extract. Tannins were found to possess anthelmintic activities. Reported anthelmintic effect of tannins is that they can bind to free proteins in the gastrointestinal tract of host animal or glycoprotein on the cuticle of the parasite and may cause death [47,48]. In light of this, the results of the present study suggest that the extract of *P. pseudotinctorum* could be used in the control of helminthic infections namely Ascariasis, hookworm infections etc as the worms used in the study are in resemblance with the intestinal parasitic worms.

Free radicals are chemical species containing one or more unpaired electrons that makes them highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability [49]. Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS [50,51]. In recent years much attention has been devoted to natural antioxidant and their association with health benefits [49]. There are several methods available to assess antioxidant activity of compounds. An easy, rapid and sensitive method for the antioxidant screening of plant extracts is free radical scavenging assay using 1,1, diphenyl-2-picryl hydrazyl (DPPH) stable radical spectrophotometrically. In presence of an antioxidant, DPPH radical obtains one more electron and the absorbance decreases [52]. In this study, the scavenging activity of methanol extract was found to be dose dependent i.e., higher the concentration, more was the scavenging activity. Though the DPPH radical scavenging abilities of the extracts were less than that of ascorbic acid, the study showed that the extract has the proton-donating ability and could serve as free radical inhibitors or scavenger, acting possibly as primary antioxidant. In the $Fe^{+3}$ reducing assay, the reducing power of crude solvent extract was found to increase with the dose. The reducing capacity of compound may serve as significant indicator of its potential antioxidant activity [53]. The antioxidant activities have been reported to be the concomitant development of reducing power [54].

## CONCLUSION

In this study, a marked antibacterial, anthelmintic and antioxidant potential of methanol extract of *P. pseudotinctorum* was observed. The lichen studied could find ethno- medicinal use in the prevention and control of bacterial, helminthic infections and damage caused by free radicals. The efficacy of the solvent extract may be due to the presence of constituents such as tannins, Atranorin, Lecanoric acid etc. Further studies on isolation of secondary metabolites and their biological activities *in vitro* and *in vivo* are to be carried out.

**Table-1: Antibacterial activity and MIC of methanol extract of lichen**

| Test bacteria | Zone of inhibition in cm | | MIC (mg/ml) |
|---|---|---|---|
| | Methanol extract | Standard | |
| *S. aureus* | 1.9 | 2.3 | 0.50 |
| *C. perfringens* | 1.6 | 2.8 | 0.45 |
| *P. aeruginosa* | 2.4 | 2.4 | 0.35 |
| *E. coli* | 2.1 | 2.3 | 0.40 |

**Table-2: Anthelmintic activity of methanol extract and standard drug**

| Extract | Concentration | Paralysis time | Death time |
|---|---|---|---|
| Methanol extract | 05 mg/ml | 113 | 138 |
| | 10 mg/ml | 89 | 109 |
| | 15 mg/ml | 79 | 96 |
| | 20 mg/ml | 66 | 84 |
| Piperazine citrate | 1% | 78 | 96 |

**Table-3: DPPH free radical scavenging activity of different concentrations of methanol extract and Ascorbic acid**

| Concentration (mg/ml) | Radical scavenging activity (in %) | |
|---|---|---|
| | Methanol extract | Ascorbic acid |
| 0.125 | 62.14 | 81.69 |
| 0.250 | 68.83 | 88.01 |
| 0.500 | 79.61 | 91.66 |
| 1.000 | 89.41 | 97.08 |

**Fig-1: Radical scavenging activity of DPPH and methanol extract**

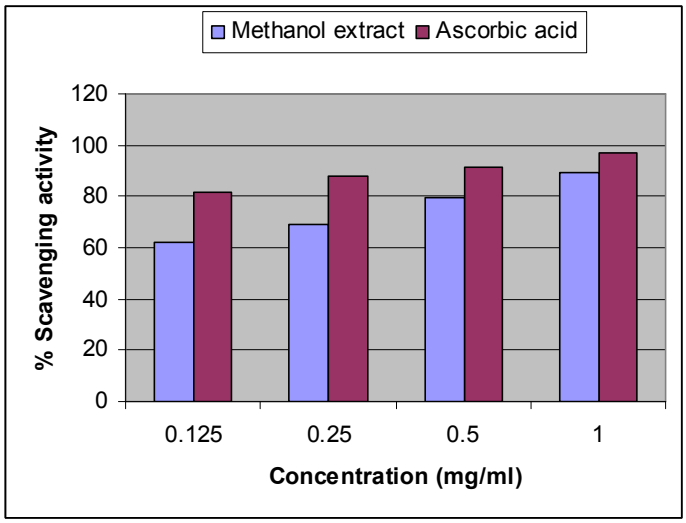


**Table-4: Antioxidant activity of different concentrations of methanolic extract and Tannic acid by $Fe^{+3}$ reducing power assay**

| Concentration (mg/ml) | Absorbance at 700nm | |
|---|---|---|
| | Methanol extract | Tannic acid |
| 0.125 | 0.265 | 0.363 |
| 0.250 | 0.321 | 0.440 |
| 0.500 | 0.551 | 0.789 |
| 1.000 | 0.776 | 1.220 |

**Fig-2: $Fe^{+3}$ reducing activity of tannic acid and methanol extract *A. pulchellus***

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