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Antimicrobial activities of 2-Propanol crude extract from lichen *Parmotrema tinctorum* (Despr.ex. Nyl.) Hale, collected from Eastern Ghats, India

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Abstract

The present study was conducted to evaluate the *in vitro* antimicrobial activity of 2Propanol extract of *Parmotrema tinctorum* (Despr. ex Nyl.) Hale, against each ten bacterial and fungal pathogens. Secondary compound of the species was extracted using Soxhlet apparatus and antimicrobial activity was carried out by using Bauer-Kirby disc diffusion method. The extract was found more effective against ten bacterial and eight fungal pathogens. The highest zones of inhibition in bacterial pathogens were noted against *Escherichia coli* (14.66 ± 0.57), *Bacillus subtilis* (13.0 ± 2.99), *Salmonella abony* (12.33 ± 2.51) and *Corynebacterium rubrum* (11.33 ± 0.57) followed by lowest inhibition zones were recorded in *Streptococcus pyogenes* (9.66 ± 0.57), *Bacillus cereus* (8.66 ± 1.15) and Streptomycin was taken as standard control found more effective against all the bacterial pathogens. In case of fungal pathogens the highest zones of inhibition were noted against *Aspergillus flavus* (10.0 ± 1.0) followed by *Colletotrichum falcatum*, *Fusarium oxysporum* and *Penicillium chrysogenum* (7.33 ± 0.57 each), *Trichoderma lignorum* (7.0 ± 1.53) and *Fusarium moniliforme* (6.0 ± 5.2) while commercially available synthetic antifungal drug Ketoconazole was taken as standard control found more effective against eight fungal pathogens. The study revealed that extracts obtained from *P. tinctorum* are having potential compounds which in turn useful to control human pathogenic microorganisms.

Keywords – Macrolichen **–** antibacterial **–** antifungal activity **–** disc diffusion **–** bio-prospection

Introduction

Lichen is a stable self-supporting association of a mycobiont and a photobiont in which the mycobiont is the exhabitant (Hawksworth 1988). Lichens are known for their medicinal abilities and used in treating cutaneous diseases (Perez-Llano 1944) and lichen acids used by the tribals contained antibacterial and antiviral properties (Asahina and Shibata 1954). It has been documented that more than 800 secondary metabolites were found (Huneck and Yoshimura, 1996) so far and among them 550 are unique in lichens with respect to those of higher plants. Secondary metabolites of lichens are called "lichen substances," that produces depsides, depsidones, dibenzofurans, anthroquinones, xanthones, pulvinic acid derivatives and napthoquinones (Rankovic et al. 2011).

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The use of lichens in folk medicine is as old as Chinese and Egyptians civilizations. Their utilization in folklore as medicine has been cited in different pharmacopoeias of the world. During the middle-ages lichens figured prominently among the herbs used by medicinal practitioners (Hale 1983). In India lichens are being used as major ingredients in Ayurvedic and Unani system of medicines (Upreti et al. 2005). The use of lichens and their products in medicine still hold a considerable interest as alternative medicine to cure various diseases in different parts of the world. Numerous lichens in European countries have been used for the treatment of diabetes, whooping cough, pulmonary diseases, tuberculosis, cancer treatment, stomach disorders, fever, diarrhoea, infections, skin diseases, epilepsy, convulsions, hydrophobia, asthma, haemorrages, and pulmonary diseases (Perez-Llano 1944, Vartia 1973). Several reviews are available dealing with biological activities of lichens and their compounds (Miao et al. 2001, Mitrovic et al. 2011, Zambare and Christopher 2012, Shrestha and Clair 2013) which clarifies beyond doubts the potential of lichens and their compounds.

Parmotrema tinctorum (Despr. ex Nyl.) Hale, is common Parmelioid lichen found growing luxuriantly over rock and tree trunks in Eastern Ghats parts of Andhra Pradesh. It is characterized by shining, whitish to mineral grey upper surface, up to 10 cm across, isidiate, lobes rotund, up to 15 mm wide, black lower surface with wide bare brown marginal zone, contains secondary compounds such as atranorin, lecanoric acid and traces of orsellinic acid. The species also has a cosmopolitan distribution in the world (Divakar 2005, Awasthi 2007) and found to be very useful to the mankind. The lichen species *P. tinctorum* is used as a spice and flavouring agent to increase the taste of food by the tribal people (Brij lal et al. 1985). Abo-Khatwa et al. (1997) reported that *P. tinctorum* is called as Al-Sheba in Arabic and used as food spice by the people. The lichen compound lecanoric acid extracted from *P. tinctorum* by using acetone and reported antibacterial activity against Gram positive and Gram negative bacterial pathogens by Alcier et al. (2003). The lichenic secondary metabolite atranorin isolated from this lichen was used in folk medicine as antinociceptive and anti-inflammatory substances by Melo et al. (2011). The studies of *P. tinctorum* extracts of dichloromethane, hexane, ethyl acetate and acetone showed their results against the *Mycobacterium tuberculosis* H37RV and their toxicity against *Artemia salina* as anti-tuberculosis by Kusumaningrum et al. (2011). The studies of Verma et al. (2008a, 2008b) with *P. tinctorum* cultured cell aggregates of natural thallus in methanol extracts and lecanoric acid isolates exhibited antioxidant activity. The extract of *P. tinctorum* showed their potential to inhibit the tyrosinase activity which is helps in whitening of skin colour (Jennifer et al. 2012, Santos et al. 2004). Keeping in view of bioactive potential of *P. tinctorum* in the present study antimicrobial activities of the lichen extract is tested.

Materials and methods

Collection and identification of sample

The lichen *P. tinctorum* thalli (Fig. 1A) were collected from the bark of trees at Horsley hills of Chittoor district, Andhra Pradesh at an altitude of 1265 m, latitude13°39.123′ N and longitude 078° 34.117′ E. Lichen identification was carried out by following the standard procedures given by Nayaka (2014). The literature of Awasthi (2007) was referred for taxonomic characters and Orange et al. (2001) was followed for chemical analysis. The voucher specimens were deposited at the Department of Botany, Yogi Vemana University, Herbarium, Kadapa, Andhra Pradesh, India.

Extraction of bioactive compounds

Lichen thalli were washed using distilled water and dried at room temperature for 24 hrs to remove the moisture contents completely, subsequently it was powdered using a pestle and mortar. Around 10 g of powdered lichen samples was wrapped in Whatman No.1 filter paper which was kept inside the extractor tube of Soxhlet apparatus. 150 ml of 2-Propanol was used in Soxhlet apparatus for the extraction of bioactive secondary metabolites from lichen thallus. Few drops of the extract were used for Thin Layer Chromatography (TLC) to identify the secondary metabolites

present in it. The extract obtained was concentrated in vacuo at 40°C using a Heidolph rota vapour. The residues so obtained were kept in a Deep Freezer at -80°C until they are used for future study.

Microorganisms and media

The microbes used in the present study were procured form NCIM (Pune). Ten bacterial pathogens viz. *Bacillus subtilis, B. cereus, Corynebacterium rubrum Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Salmonella abony, S. typhimurium, Staphylococcus aureus, Streptococcus pyogenes* were used and maintained on Muller- Hinton agar (MHA) media at 37°C. As well as same number of fungal pathogens *viz. Aspergillus niger, A. flavus, Botrytis allii, Colletotrichum falcatum, Fusarium moniliforme, F. oxysporum, Mucor* sp., *Penicillium chrysogenum, P. notatum,* and *Trichoderma lignorum* were used and maintained on Potato dextrose agar (PDA) at 27°C.

Screening of antimicrobial activity

The antimicrobial activity of crude lichen extracts was studied by Kirby-Bauer disc diffusion method using pathogenic bacterial and fungal microorganisms (Bauer et al. 1966). Bacterial cultures (1.5×10^8 CFU/ml) were seeded onto the Muller-Hinton Agar plates with the help of L – spreader. The fungal mat of 2-3 days old cultures grown on Potato Dextrose Broth were collected. These cultures were inoculated onto PDA plates for fungal pathogens and MHA for bacterial pathogens. On the seeded plates the sterile filter paper discs of 5 mm soaked with 15 µL of lichen extract (100mg/1ml) concentration were placed. The inoculated plates with bacteria were incubated at 37°C for 24 hrs while fungal plates were incubated at 27°C for 2-3 days.

Streptomycin (10µg/ml) for bacterial and Ketoconazole (15µg/ml) for fungal pathogens were taken as a standard positive and solvent alone as negative control. All the experiments were carried out in triplicates. Growth was evaluated after 18-24 hrs of incubation in the case of bacterial cultures and similarly, in the case of fungal cultures the plates were observed after 2-3 days. The diameter of the inhibition zone was measured in millimeter (including the disc size 5mm) and the mean, standard deviations are calculated.

Results

Identification of lichen secondary compounds

The results of thin layer chromatography of *P. tinctorum* showed presence of lecanoric acid with grey colour at R*f* class 3 including orsellanic acid traces and atranorin with yellow orange colour at R*f* class 7. In spot test, Potassium hydroxide showed yellow colour on thallus indicates the presence of atranorin in cortex while medulla gives rose red colour by adding C (liquid solution of bleaching powder) indicates the presence of lecanoric acid.

Antibacterial activity of extract

Kirby-Bauer disc diffusion assay with 2-Propanol extract of *P. tinctorum* showed their effective results against all the bacterial pathogens. Bacterial pathogens showed their maximum zone of inhibition against *Escherichia coli* (14.66±0.57), *Bacillus subtilis* (13.0±2.99), *Salmonella abony (*12.33±2.51), *Klebsiella pneumonia* (12.0±1.0), *Corynebacterium rubrum* (11.33±0.57), *Staphylococcus aureus* (11.0±2.64), *Salmonella typhimurium* (10.66±0.57), *Pseudomonas aeruginosa* (10.66 ± 2.30) and minimum zone of inhibition against *Streptococcus pyogenes* (9.66±0.57) and *Bacillus cereus* (8.66±1.15). Against the standard drug Streptomycin bacterial pathogen showed their highest zone of inhibition against *E. coli* (29.33±0.57), *S. aureus* (21.0±1.0), *Corynebacterium rubrum* (20.33±0.57), and their minimum zone of inhibition against *B. cereus* (18.0±1.0), *S. abony* (17.66±1.15) and *B. subtilis* (17.33±1.52) (Fig. 1B–L and Table 1). Inhibition zone of all the bacterial pathogens are provided with their mean values and standard deviations in Fig. 3.

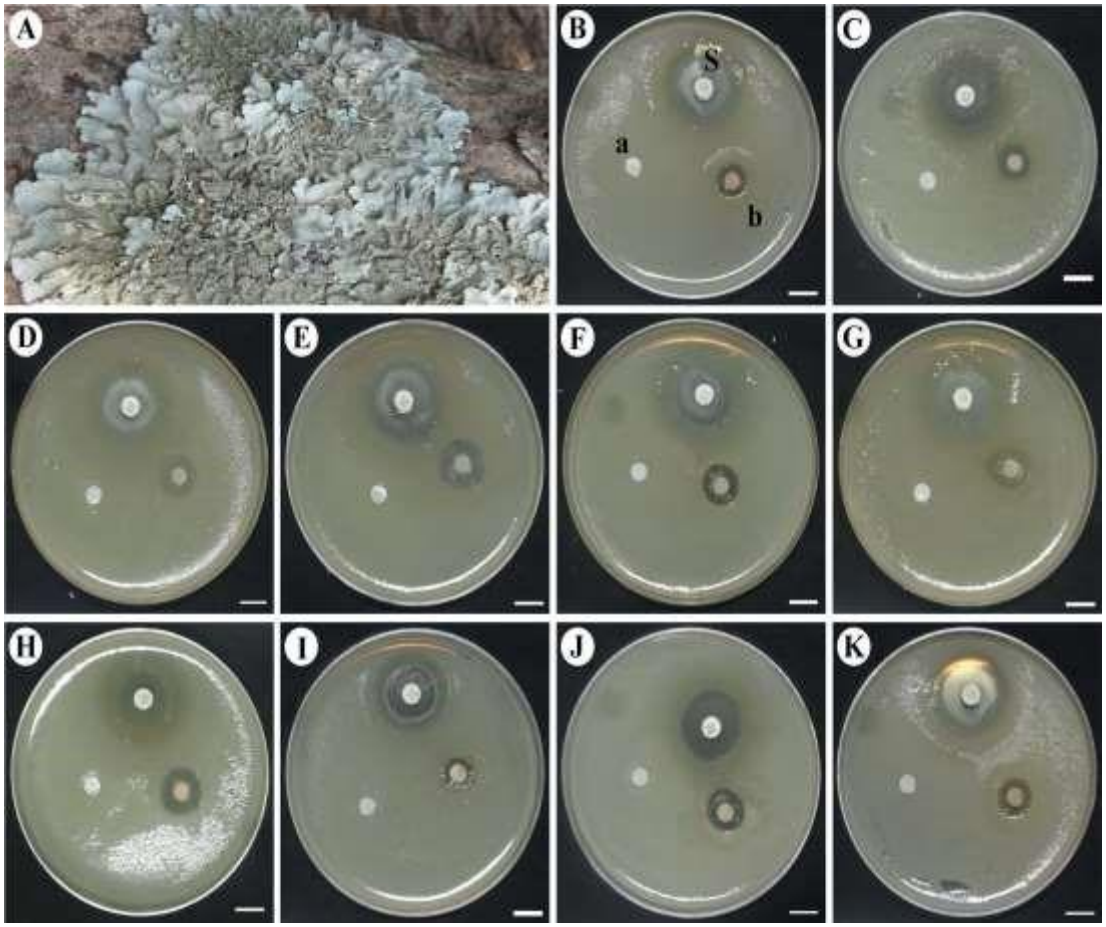

Fig. 1 – The inhibition zones of *P. tinctorum* extract against tested bacterial pathogens and Streptomycin (**S**), **A.** Habit of *P. tinctorum*, **B.** *Bacillus cereus*, **C.** *B. subtilis*, **D.** *Corynebacterium rubrum* **E.** *Escherichia coli*, **F.** *Klebsiella pneumonia*, **G.** *Pseudomonas auerginosa*, **H.** *Salmonella abony*, **I.** *S. typhimurium*, **J.** *Staphylococcus aureus*, **K.** *Streptococcus pyogene;*, **a** = water extract, **b** = 2-Propanol extract, **Scales: A** = not to scale, B–K =10 mm.

Table 1 Inhibition of lichen species *P. tinctorum* against tested bacterial pathogens

S. No.	Bacterial pathogens	Streptomycin	2-Propanol
1	*Bacillus cereus*	18.0±1.0	8.66±1.15
2	*Bacillus subtilis*	17.33±1.52	13.0±2.99
3	*Corynebacterium rubrum*	20.33±0.57	11.33±0.57
4	*Escherichia coli*	29.33±0.57	14.66±0.57
5	*Klebsiella pneumonia*	19.66±0.57	12.0±1.0
6	*Pseudomonas aeruginosa*	19.33±1.15	10.66±2.30
7	*Salmonella abony*	17.66±1.15	12.33±2.51
8	*Salmonella typhimurium*	20.0±1.0	10.66±0.57
9	*Staphylococcus aureus*	21.0±1.0	11.0±2.64
10	*Streptococcus pyogenes*	18.33±3.21	9.66±0.57

(*values are in mean ± standard deviation)

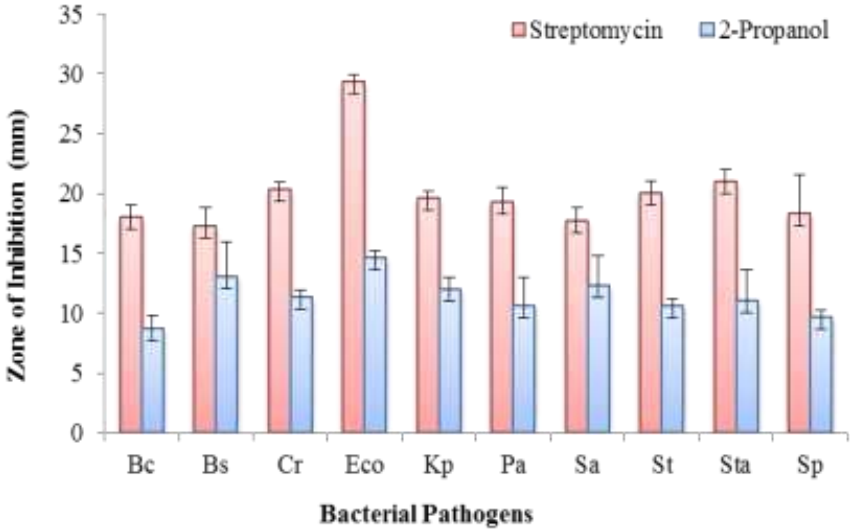

Fig. 2 – Figure showing inhibition zone of *P. tinctorum* extract against bacterial pathogens. Streptomycin = control, 2-propanol = extract, *Bc = B. cereus, Bs = B. subtilis, Cr = Corynebacterium rubrum, Eco = Escherichia coli, Kp = Klebsiella pneumonia, Pa = Pseudomonas aeruginosa, Sa = Salmonella abony,* St= *S. typhimurium*, Sta = *Staphylococcus aureus, Sp= Streptococcus pyogenes.*

Antifungal activity of extract

In case of fungal pathogen *Aspergillus flavus* (10.0±1.0) and *A. niger* (8.66±0.57) showed their maximum zone of inhibition followed by *Fusarium oxysporum* (7.33±0.57), *Colletotrichum falcatum* (7.33±0.57), *Penicillium chrysogenum* (7.33±0.57), *Fusarium moniliforme* (6.0±5.2) while *Mucor* sp. (8.0±1.0) and *Trichoderma lignorum* (7.0±0.57) showed minimum zone of inhibition activity. The positive control Ketoconazole showed highest zone of inhibition against *Trichoderma lignorum* (20.66±4.61) *Fusarium oxysporum* (15.33±1.52) and *Colletotrichum falcatum* (15.0±1.0) while lowest inhibition zone was reported against *Aspergillus niger* (9.0±2.64), *Mucor* sp. (10.33±1.24) and *Fusarium moniliforme* (11.0±4.0). (Fig. 3A–H and Table 2.). Inhibition zone of eight fungal pathogens are provided with their mean values and standard deviations in Fig. 4.

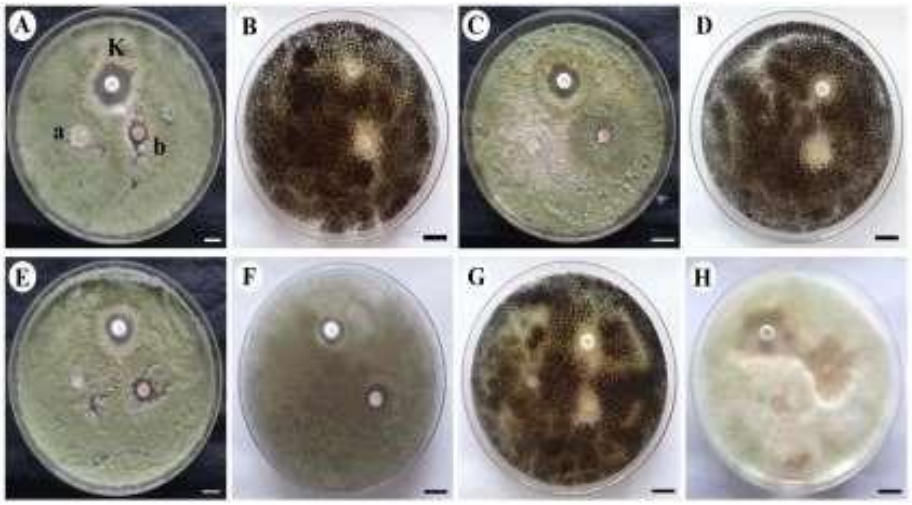

Fig. 3 – The inhibition zones of *P. tinctorum* extract against tested fungal pathogens and Ketoconazole (**K**), **A.** *Aspergillus flavus*, **B.** *A. niger*, **C.** *Colletotrichum falcatum*, **D.** *Fusarium moniliforme*, **E.** *F. oxysporum*, **F.** *Mucor* sp. **G.** *Penicillium chrysogenum*, H. *Trichoderma lignorum;* **a** = water extract, **b** = 2-Propanol extract, **Scale:** A-F = 10 mm.

Table 2 Inhibition zone of lichen species *P. tinctorum* against tested fungal pathogens

S. No.	Fungal pathogens	Ketoconazole	2-Propanol
1	*Aspergillus flavus*	14.0±2.64	10.0±1.0
2	*Aspergillus niger*	9.0±2.64	8.66±0.57
3	*Colletotrichum falcatum*	15.0±1.0	7.33±0.57
4	*Fusarium moniliforme*	11.0±4.0	6.0±5.2
5	*Fusarium oxysporum*	15.33±1.52	7.33±0.57
6	*Mucor* sp.	10.33±1.24	8.0±1.0
7	*Penicillium chrysogenum*	12.66±4.04	7.33±0.57
8	*Trichoderma lignorum*	20.66±4.61	7.0±1.53

(*values are in mean ± standard deviation)

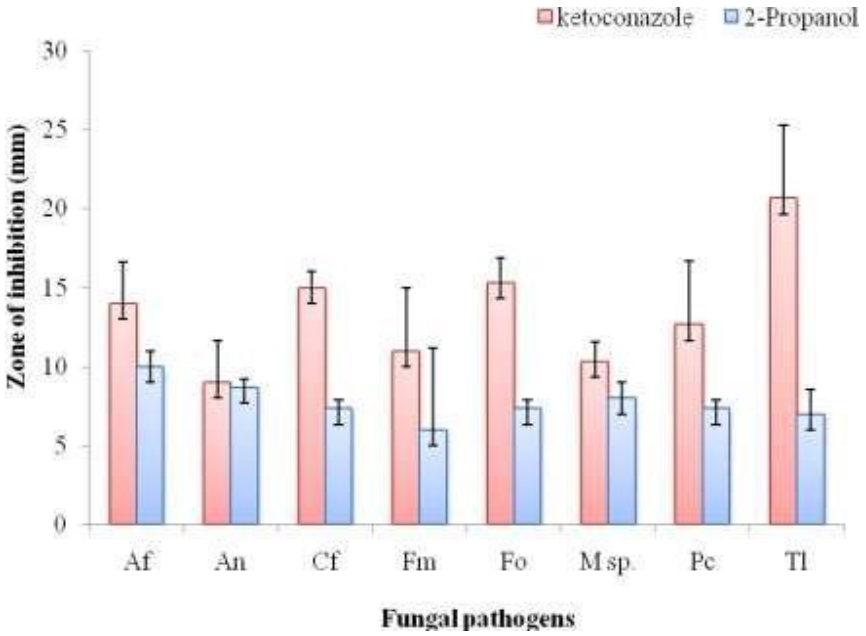

Fig. 4 – Figure showing inhibition zone of *P. tinctorum* extract against tested fungal pathogens. Ketoconazole = control, 2-propanol = extract, Af = *Aspergillus flavus*, An = *A. niger*, Cf = *Colletotrichum falcatum*, Fm = *Fusarium monilliforme*, Fo = *F. oxysporum*, M sp. = *Mucor* sp., Pc = *Penicillium chrysogenum*, Tl = *Trichoderma lignorum*.

Discussion

Earlier, it has been proved that 2-propanolic extract of *Roccella montagnei* Bél collected from Horsley hill, Chittoor district can be used for the antimicrobial activities against human pathogenic microorganisms reported by Anjali et al. (2014). The present study is undertaken on similar line, but with extract of *P. tinctorum.* Recently, Vinayaka et al. (2013) reported that methanolic extracts of *P. tinctorum* exhibited inhibition of amylase activity against fungus while in another study the extract was active against *Staphylococcus aureus* and *Streptococcus mutans* (Vivek et al. 2014). Similarly, the phenolic compounds such as lecanoric acid and methyl orsellinate of the same species were found less active against any of the strains (HEp-2 MCF7, 7860 or B16-F10) that cause different types of carcinomal diseases reported by Bogo et al. (2010). A study on fungal efficacy against the *Fusarium oxysporum* F. Sp. Capsici with methanolic, ethyl acetate and acetone extracts of different species of lichen *P. tinctorum* showed activity against methanolic (11.3±1.1) and ethyl acetate (14.3±0.5) extracts only and not with the acetone extract reported by Rashmi et al. (2014) but in the present study with 2-Propanol the fungal species *Fusarium oxysporum* (7.33±0.57) showed less zone of inhibition. In a study done by Kumar and Muller (1999a,b)

resulted that some of the metabolites isolated from this lichen species found to be potent antiproliferative agents against human keratinocyte line HaCaT and also inhibited the leukotriene B4 biosynthesis by a non-redox mechanism. Christine et al. (2011) reported antioxidant and total phenolic content on four lichen genera belonging to *Ramalina, Parmotrema, Bulbothrix* and *Cladia* from Malaysia resulted that the total phenolic content and the percentage of yield in *P. tinctorum* is high with acetone and methanol when compare to the other three lichen species.

Tiwari et al. (2011) reported antifungal activity of *P. tinctorum* with acetone, methanol and chloroform extracts against ten fungal pathogens in which methanol extract was effective only against all the fungal pathogens that were taken for the study. Their zones of inhibition against *Aspergillus flavus, Aspergillus niger* and *Fusarium oxysporum* were more with methanol, acetone and chloroform than with 2-Propanol in the present study. Transmittable mediators viz. bacteria, fungi, viruses and parasites have threatened mankind throughout history and caused millions of deaths. Discovery of antibiotics during 20th century is one of the important milestones in the history of precautionary chemotherapy. The use of these wonder drugs saved countless lives during the past years. However, overuse and misuse of these wonder drugs resulted in the development of resistance in pathogens. *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Penicillium chrysogenum and Trichoderma lignorum* are among the important drug resistant pathogens. The development of antimicrobial resistance presents a major hazard to public health as it reduces the effectiveness of treatment, resulting in increased illness, mortality, and health care expenditure. Moreover, these pathogens have the ability to acquire and transmit resistance Smith and Coast, (2002); Davies and Davies, (2010), Giedraitiene et al. (2011); Kekuda et al. (2012), Kekuda et al. (2013). High cost, possible side effects and development of resistance in pathogens against antibiotics encouraged researchers to investigate antimicrobials from natural sources. Lichens are among the gifted sources of chemotherapeutic agents that are active against pathogenic organisms including clinical and drug resistant strains Kekuda et al. (2012), Chauhan and Abraham (2013), Javeria et al. (2013), Kekuda et al. (2013). In the present study it can be noted that the effect of both the standards (streptomycin and ketoconazole) are invariably stronger than that of the 2-Propanol extract. The extract performed comparatively well only against *A. flavus.* Nevertheless, the 2-Propanol extract has substantial antimicrobial activity which can be exploited for bioprospection programmes.

Conclusion

Some of the scientists worked on *P. tinctorum* but the use of 2-propanol has not been reported. Results obtained in the present study can be concluded that *P. tinctorum* has a very broad antimicrobial activity with the 2-Propanol extracts. However, these activities of the extracts are comparatively lesser than that of the control used. The study encourages exploring for novel antimicrobial bio-molecules within lichen biodiversity. The lichen *P. tinctorum* may yield potential antimicrobial compound in some other organic solvent systems. It is very useful for the pharmaceutical and food industries. Hence, the need for further investigations of pure compound extraction and isolation of it is necessary.

Acknowledgments

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