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Evaluation of Antimicrobial Prospective of *Parmotrema perlatum* Hexane Extract

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Abstract

The aim of the study was to test the antimicrobial potential of *Parmotrema perlatum* (*P. perlatum*) against various bacterial and fungal pathogens. Hexane was used for the extraction of bioactive components from *P. perlatum*. The efficacy of the bacterial and fungal pathogens against *P. perlatum* was tested by measuring the zone of inhibition (ZOI) using disc diffusion method and by determining the minimum inhibitory concentration (MIC) by micro-broth dilution method. Hexane extract of *P. perlatum* was found as a potent inhibitor against the tested microorganisms. The extract showed significantly high antibacterial property as compared to antifungal activity.The results of the present study indicated that *P. perlatum* has potential antimicrobial compounds which can be further characterized for identifying new lead molecules against various pathogenic microorganisms. Emergence of drug resistance and reduced efficiency of most of the antibacterial and antifungal drugs necessitates identification of potential lead molecules with new molecular targets and mechanism of action.

Key words: *Parmotrema perlatum, Antimicrobial, Zone of Inhibition, Minimum Inhibitory Conc.*

Introduction

Infectious diseases caused by pathogenic and opportunistic microorganisms remain a major threat to public health, in-spite of tremendous progress in antimicrobial drug discovery. Indiscriminate use of antibiotics, have lead to the emergence of multidrug resistant pathogens that are progressing towards final line of antibiotic defence. This has lead to the search of new lead molecules and targets. Plant derived antimicrobial compounds and leads have been the source of novel therapeutics since many years[1]. This may be due to the fact that these compounds show structural intricacy and chemical diversity required to interact with antibacterial protein targets and provide vast opportunities for new drug development[2, 3].

Lichens and their products have been traditionally used as medicine for centuries in various parts of the world. They are dynamic sources of natural antimicrobial drugs due to the presence of various secondary metabolites as phenolic compounds, dibenzofuranes, depsidones, usnic acids, lactones, depsones, pulvunic acid derivatives and quinines[4]. A lichen is a composite organism that emerges from algae or cyanobacteria (or both) living among filaments of fungus in a mutually beneficial (symbiotic) relationship. The present study was done on the lichen *Parmotrema perlatum* which belongs to the family Parmeliaceae categorized under ascomycota[5.] The genus *Parmotrema* is usually characterized by large foliose thalli with broad lobes, commonly with a broad erhizinate marginal zone on the lower surface, pored epicortex, thick-walled hyaline ellipsoid ascospores, sublageniform or filiform conidia and with or without marginal cilia[6]. Hale and Ahti in 1986 gave earlier name *Lichen chinensis* which was universally accepted. Later *P. perlatum* was given a valid name by Hawksworth in 2004. *P. perlatum* is distinguished by the presence of stictic acid[7].

The objective of the study was to investigate the *in-vitro* antimicrobial property of *P. perlatum* against common bacterial and fungal pathogens. Three bacterial pathogens used in the study were *Escherichia coli, Pseudomonas sp.* and *Bacillus subtilis.* Among fungal pathogens *Cryptococcus neoformans, Candida albicans, Aspergillus niger* and *Aspergillus fumigatus* were used. *E. coli* is a gram negative bacterium that can be categorized as commensal, extra intestinal pathogenic and intestinal pathogenic strain. Extra intestinal infections are common which include urinary tract infection, abdominal infection, meningitis and pneumonia. Intestinal pathogens are obligate pathogens causing gasteoenteritis or

colitis[8]. *Pseudomonas* is also a genus of gram negative bacteria that commonly infects patients with burn wounds, cystic fibrosis, acute leukemia, organ transplants and intravenous-drug addiction. Meningitis, septicemia, endocarditis, pneumonia, malignant external otitis and endophthalmitis are the most serious infections[9]. *B. subtilis* is a ubiquitous gram positive bacterium that adapts to various environmental changes by initiating survival mechanism as biofilm formation and sporulation[10.] It is not a potent pathogen but is associated with outbreaks of food poisoning and infection of the eye as reviewed by Frangois in 1934[11]. *C. neoformans* is a pathogenic encapsulated fungus that severely affects immuno-compromised patients, especially those with AIDS. The most serious clinical symptom of cryptococcal infection is Meningoencephalitis[12, 13]. *C. albicans* is a dimorphic yeast that causes mucocutaneous or genitourinarty candidiasis[14], superficial dermatitis mostly in neonates[15]. *A. niger* is the 3rd most common disease causing *Aspergillus* that has been associated with otomycosis[16] and cutaneous infections[17]. It is an unusual causative agent of invasive pulmonary aspergillosis and pneumonia[18]. *A. fumigatus* is an opportunistic pathogen which majorly causes Invasive Aspergillosis (IA) in immunocompromised patients[19]. The sensitivity of pathogens against *P. perlatum* was examined by calculating zone of inhibition (ZOI) by disc diffusion method and by measuring minimum inhibitory concentration (MIC) using micro-broth dilution method.

Materials and methods

Microorganisms:

The bacteria used as the test organisms in the study were *Escherichia coli, Pseudomonas sp.* and *Bacillus subtilis.* The fungi used were *Cryptococcus neoformans* (ATCC 66031), *Candida albicans* (ATCC 10231), *Aspergillus niger* (ATCC 16404) and *Aspergillus fumigatus* (NAIMCC-F-02473). The bacterial cultures were taken from Plant Secondary Metabolite Laboratory, at Amity Institute of Biotechnology, Amity University, Noida, India. *A. fumigatus* was procured from NBAIM, Mau, India. *C. albicans*, *C. neoformans* and *A. niger* were procured from Himedia Laboratories, Mumbai, India. All bacterial cultures were maintained on Nutrient Agar/Broth (Himedia). *C. albicans* culture was maintained on Yeast Peptone Dextrose media (Himedia), *C. neoformans* and *A. niger* was cultured on Potato Dextrose Agar/Broth (Himedia). The culture of *A. fumigatus* was maintained on Czapek Yeast Agar/Broth (Himedia).

Sample collection:

Lichen *Parmotrma perlatum* was collected from local market, Noida, U.P., India. Sample was cleaned under running tap water and dried at room temperature.

Preparation of *P. perlatum* extract:

100g of lichen was ground using mortar and pestle to make fine powder and sieved. Powdered sample was extracted in 400 ml hexane (Himedia) for 72 hours under constant shaking at 100 rpm. The extract was filtered out using a three layered muslin cloth followed by Whatmann no.1 (Himedia) and dried under vacuum in a rotary evaporator. The dried extract was re-suspended in 5% Di-Methyl Sulfoxide (DMSO) (Himedia) at a final concentration of 100µg/µl and stored at 4ºC. Hexane extract was used to perform further experiment.

Sub-culturing the microorganisms in liquid media:

For bacterial culture 100 ml of Nutrient Broth (NB) was prepared and autoclaved. 10 ml of NB was transferred into 25 ml conical flasks. 50 µl of each bacterial culture was inoculated in to the media and sealed with a cotton plug. The flasks were then placed in an incubator at 37°C for 24 hours. All procedures were carried out under aseptic conditions.

For fungal cultures 100 ml of Yeast Peptone Dextrose Broth (YPDB) was prepared and sterilized. 20ml of YPDB was poured into 50 ml conical flasks and 50 µl of each fungal culture was inoculated. The conical flasks inoculated with *A. fumigatus* and *A. niger* was kept at 28ºC for 7 days, *C. albicans* and *C. neoformans* were kept at 30º C for 48 hours.

Sub culturing the microorganisms in solid media:

100 ml of Nutrient Agar (NA), Yeast Peptone Dextrose Agar (YPDA), Czapek Yeast Agar (CzA), Potato Dextrose Agar (PDA) prepared for bacterial and fungal culture respectively. Each media was sterilized by autoclaving at 121ºC for 15 minutes. 20 ml media was poured into autoclaved glass petri plates and cooled. Cultures were then streaked using a nichrome wire loop (Himedia) and then kept in incubator at respective temperatures.

Screening the *P. perlatum* Extract for Antimicrobial Assay:

The antimicrobial activity of the extract against the microbial pathogens was determined by Kirby-Bauer disc diffusion method[20]. NA, YPDA, CzA and PDA plates were spread with 100 µl of bacterial and fungal suspension of $1*10^6$cells/ml

respectively using sterile L-shaped spreader. Sterile paper discs (Himedia) were placed on each petri plates and 5 µl of extract was impregnated on the discs. Penicillin, 1mg/ml (Himedia) was kept as positive control for bacterial culture where as fluconazole, 1mg/ml (Himedia) was used as positive control for fungal strain. 5% DMSO was kept as negative control. These plates were incubated for respective time and temperatures in the incubator. The antimicrobial activity was evaluated by measuring the zone of inhibition (ZOI) in mm around the discs formed at the end of the incubation period.

Minimum Inhibitory Concentration (MIC):

The extract was tested for the lowest concentration at which it is able to inhibit any visible microbial growth. This was done using dose dependant micro-broth dilution method in a 96 well plate[21]. 180µl of freshly prepared and autoclaved fresh culture broth was added to 1st well of each row. Further 20 µl of the extract was added making the final volume 200 µl. The well contents were mixed thoroughly. 100 µl of culture broth was added to rest of the wells. Serial dilution was performed along the row using 100 µl contents from the 1st well of each row. 100 µl of freshly prepared microbial culture was then added to all wells. The final concentration of the culture was kept at 10^6 cells / ml. In the 12th column, 100 µl of microbial suspension and 100 µl culture broth was added, which acted as (+) Control. The final concentration of the extract was 1mg-1.8µg per well. The plate was incubated at respective temperature. After incubation period, the plate was observed visually. The MIC was determined by measuring the optical density at 530 nm using Bio-Rad micro plate reader Model 680. The non inoculated broth was kept as reference.

Results

Antimicrobial assay:

The antimicrobial activity of hexane extract of the lichen *P. perlatum* against the test microorganisms was estimated on the basis of presence or absence of inhibitory zones and their diameters. Antibacterial activity of the extract is shown in Table 1 and antifungal activity in Table 2. The standards (Penicillin and Fluconazole) have shown inhibition against all tested pathogens. The hexane extract of *P. perlatum* proved to be most effective against *B. subtilis* among bacteria. Among fungi the extract showed most inhibition against *A. fumigatus* and least against *C. neoformans*.

Table 1: Antibacterial activity of the hexane extract of *P. perlatum* against Bacterial Pathogens tested using disc-diffusion assay. Results are shown in mm

Test Bacteria	Zone of Inhibition (mm)	
	Hexane extract (100µg/µl)	Standard(Penicillin,1 mg/ml)
E. coli	20	22.5
Pseudomonas sp.	18.4	19.6
B. subtilis	21.2	21

Table 2: Antifungal activity of the hexane extract of *P. perlatum* against the fungal pathogens tested using disc-diffusion assay. Results are indicated in mm

Test Fungi	Zone of Inhibition (mm)	
	Hexane extract (100µg/µl)	Standard (Fluconazole, 1 mg/ml)
C. neoformans	9	21.6
C. albicans	10.4	22
A.niger	10.1	16.4
A. fumigatus	14	18.2

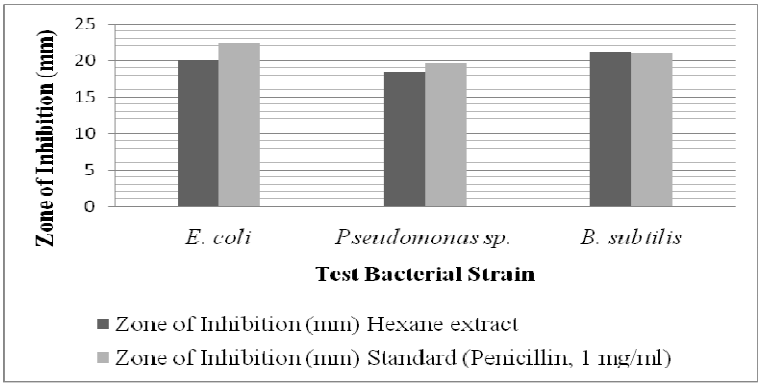

Figure 1: Graph representing antibacterial activity of the hexane extract of *P. perlatum* against Bacterial pathogens

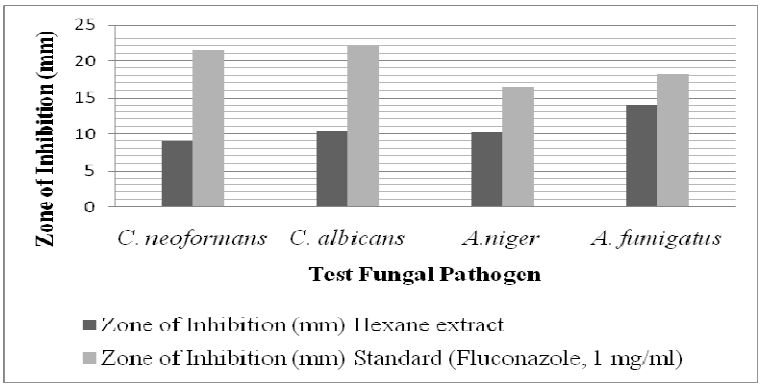

Figure 2: Graph representing antifungal activity of the hexane extract of *P. perlatum* against fungal pathogens tested in the present study

Minimum Inhibitory Concentration (MIC):

MIC of the hexane extracts of *P. perlatum* was measured by visualizing the 96 well plates. Minimum Inhibition is the minimum concentration of the compound or drug that can inhibit 100% microbial growth. The results were calculated both visually and through ELISA reader.

Table 3: MIC calculated in (mg/ml) against all the bacterial Pathogens used in the present study.

Test Bacteria	MIC (mg/ml)
E. coli	0.625
Pseudomonas sp.	1.25
B. subtilis	0. 312

Table 4: MIC calculated in (mg/ml) against all the fungal pathogens used in the study

Test Fungi	MIC (mg/ml)
C. neoformans	2.5
C. albicans	2.5
A.niger	2.5
A. fumigatus	1.25

Discussion

Infectious diseases are the second leading cause of death worldwide and are a major cause of concern both in developing and developed countries. Since, 1940s antimicrobial drugs have been used to treat various bacterial and fungal infections. However, indiscriminate use of these life saving antibiotics have led to emergence of Multi Drug Resistance (MDR) in these microorganisms. In 2012, there were about 4,50,000 new cases of MDR tuberculosis. Resistance to earlier generation anti malarial drug is reported in most malarial endemic countries. MDR *Klebsiella* and *E. coli* have been isolated in hospitals throughout United States. Antibiotic resistance is also been reported among some fungi that leads to Invasive fungal diseases in immunocompromised patients[22]. Natural products provide unlimited opportunities for new lead molecules with vast chemical diversity. Most clinically used antibiotics are either natural products or semi synthetic derivatives of these molecules. High through put technologies in the area compound purification and structural elucidation have led to considerable advancement in the drug discovery process[2].

Lichens have been reportedly used in dyes in the earlier 17th and 18th century. The antimicrobial property of lichen are well documented in folklore, but till date none of the lichen derived drugs are approved in the market [23] Various studies have confirmed that Lichens are the store house of pharmacologically relevant unique polyketide compounds[23, 24]. In this study *in-vitro* antimicrobial potential of hexane extract from the lichen *P. perlatum* has been examined against Gram positive and Gram negative bacterial as well as fungal pathogens. The extract revealed varying degree of antimicrobial activity. The Hexane extract of *P. perlatum* was tested against three bacterial pathogens *E. coli*, *Pseudomonas sps*, *B. subtilis* and four fungal pathogens *C. neoformans*, *C. albicans*, *A. niger* and *A. fumigatus.* The efficacy of the extract against these micro organisms was tested by ZOI and MIC studies. The results indicated high efficacy of the extract against the bacterial pathogens. *B. subtilis* showed maximum zone of inhibition 21.2 mm and *Pseudomonas sps* gave the minimum zone of 18.4mm. The fungal pathogens were also tested for ZOI using disc diffusion assay. *A. fumigatus* showed high inhibition with 14mm Zone. The MIC was calculated using micro broth dilution assay. MIC of the extract was estimated at 0.312µg/ml for *B. subtilis* and 0.625µg/ml for *E.coli*. The MIC for fungal pathogens was high with *A. fumigatus* showing minimum inhibitory concentration of 1.25 mg/ml and other fungal pathogens showing an inhibition at 2.5 mg/ml. It has been reported in a study that *Parmotrema praesorediosum* methanol, acetone, hexane and dichloromethane extract showed inhibitory activity against some bacteria. Crude extracts of *P. praesorediosum* tested using disc diffusion technique showed antimicrobial activity[25]. Aqueous extracts of the lichen *Nephroma articum* showed no antifungal activity[26].

It has been concluded from our experiments that the tested lichen extract has significant antibacterial property in comparison to antifungal activity. This result is in accordance with other studies[27, 28]. Gulluce *et al* also found that the methanol extract of the lichen *Parmelia saxatilis* had stronger antibacterial than antifungal activity[29]. Similar finding was observed in a study where extract of *Parmelia perlata* was prepared by hot and cold extraction method using various solvents[30]. Bacteria are more sensitive than fungi because of the differences between the composition and permeability of the cell wall. Gram-positive bacteria have cell wall made of peptidoglucanes and teichoic acids, where as that of Gram-negative bacteria is made of peptidoglucanes, lipopolysacharides and lipoproteins[31]. Fungal cell wall is poorly permeable and consists of polysaccharides as chitin and glucan[32]. The present study implies that the bioactive compound can be obtained from hexane extract of *P. perlatum* which can be used as natural antimicrobial agents. These can be utilized for the formulation of new drug to fight against pathogens.

Conclusion

The present work concludes that the hexane extract of *Parmotrema perlatum* is more potent against the bacterial cultures used in the study. The extract did not show high efficacy against the fungal strains used except *Aspergillus fumigatus.* Hence, it can be summarised that the extract can be used as an antibacterial agent. Further purification and identification of the antimicrobial compound is needed.

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