IJPSR (2014), Vol. 5, Issue 11 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 19 April, 2014; received in revised form, 03 July, 2014; accepted, 31 July, 2014; published 01 November, 2014

ANTIMYCOBACTERIAL ACTIVITY OF FOLIOSE LICHENS ON PLANT AND ANIMAL PATHOGENS

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Keywords:

Sticta weigelii, *Heterodermia boryi*, *Dermatocarpon vellereum*, *Paeciliomyces variotii*

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ABSTRACT: Antimicrobial effects of different extracts of lichens were evaluated in this study. Acetone extract of *Heterodermia boryi* have shown maximum inhibitory activity against *Escherichia coli* (22mm) followed by *Staphylococcus aureus* (21mm) and *Pseudomonas fluorescenes* (20 mm). Among fungi, diethyl ether extract gave desirable sensitivity pattern against *Pestalotia foedans* was very sensitive (21mm) followed by *Paeciliomyces variotii* (20mm). Most of the extracts of *Sticta weigelii* were found to be inactive against *Fusarium oxysporum*. *Klebsiella pneumoniae* was found to most resistant bacteria against *Sticta weigelii*. Among the tested microbial strains, *Pseudomonas fluorescens* was mostly inhibited (26 mm) by chloroform extract of *Dermatocarpon vellereum*. *Serratia marcescens* was found to be the most resistant strain against the lichen, but was effectively inhibited only by ethanolic extract of *Dermatocarpon vellereum* (20 mm). Hexane extract *D. vellereum* have shown maximum inhibitory activity against *Staphylococcus aureus* (23 mm).

INTRODUCTION: Plant product drugs and herbal remedies have been employed since prehistoric times to treat human and animal diseases and several countries still rely on plants and herbs as the main sources of drugs[1]. Lichens are known to produce various secondary metabolites that are unique with respect to those of higher plants[2]. Lichens are complex organisms involved in symbiotic relationship between a phycobiont and a mycobiont, and have attracted considerable attention because of their perceived position on the ladder of evolution to land plants [3].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.5(11).4825-31

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.5(11).4825-31

The challenge for today's pharmaceutical industry lies in the discovery and development of new pharmacological active molecules[4]. Similar to higher plants, lichens were used since antiquity as natural drugs. Lichens, together with some marine organism and frog venom, are important sources of biologically active compounds[5]. These organisms produce secondary metabolites and many of them are known for presenting biological and/or pharmacological activities.

Lichen substances are extracellular products of relatively low molecular weight crystallized on the hyphal cell walls. Also, they are usually insoluble in water and can be extracted into organic solvents[6]. They make even more than 30% of the dry mass of thallus[7]. Many lichens species have been used for human nutrition, animal nutrition, for getting colors, perfumes, alcohol and in the medicine industry[8].

Lichens produce some characteristic anthraquinone and xanthone derivatives, which have yet to be found in higher plant [9, 10]. Anthraquinones and xanthones are also important constituents of plants, microorganisms and insects. Slow growth and often harsh living conditions make production of protective metabolites a necessity to lichens and many secondary constituents are believed to serve as antimicrobial or anti-herbivore agents [11, 12].

MATERIALS AND METHODS:

IDENTIFICATION AND PROCESSING OF LICHENS

The species were identified *Heterodermia boryi* (BSA/ACC/NO.8368), *Sticta weigelii* (BSA/ACC/NO.7623) and *Dermatocarpon vellereum* (BSA/ACC/NO.8369) were deposited at Botanical Survey of India, Allahabad.

Collected lichens were washed with clean water and dried under roof shadow for 15 days at 26˚C ± 1˚C. 20 grams portions of each lichen species were ground and added to 200 ml of each solvent including ethyl acetate, acetone, chloroform, diethyl ether, methanol, ethanol and hexane. The mixtures were extracted for 48 hours. The samples were left to stand for a while. Clear solvent content were collected and centrifuged at 2500 rpm. The supernatant were filtered in whatman No 1 filter paper and dried and suspended in sterile DMSO. The concentrations of extracts were adjusted to 50mg/ml.

CULTURES, MEDIA AND GROWTH CONDITIONS

Bacterial and fungal cultures were identified for these studies based on their pathogenicity, and were obtained from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh. Bacterial strains included were *Escherichia coli* (1650), *Staphylococcus aureus* (3160), *Klebsiella pneumoniae* (7028), *Pseudomonas fluorescens* (2268), *Serratia marcescens* (7103) and *Bacillus subtilis* (3053). The cultures were revived from lyophilized form and growth is initiated in nutrient broth for 24 hrs to get early mid log phase growth. Fungal strains included were *Pestalotia foedans* (934), *Phomopsis leptostromiformis var. occidentalis* (2382), *Fusarium oxysporum* (6338), *Paeciliomyces variotii* (1368). All cultures were stored at 4˚C and sub cultured once in every 15 days until analysis.

ANTIBACTERIAL ACTIVITY OF AGAR WELL DIFFUSION METHOD

The sensitivity of microorganisms to various solvents extracts of the lichen species and its components were tested by measuring the zone of inhibition at a given concentration of the extract. All the tested bacteria in this study were used and the bioactivity was based on agar well diffusion assay[13]. Mother inoculums were prepared from the bacterial strains incubated for 24 hour at 37°C on Muller-Hinton agar and brought up by dilution according to McFarland standards to adjust the final concentration at 108 CFU/ml.

Antimicrobial activity was done in Muller-Hinton agar by seeding with appropriate inoculums. Wells were made with 6mm diameter were made with sterile cork borer on inoculated substrate. Each well is aseptically filled with 20µl of extracts (1mg). Anti-bacterial activity was determined by measuring the diameter of the zone of inhibition around the well. Streptomycin (1mg/ml) was used as control. Plain 10% DMSO solution was used as negative control to check influence solvent influence on microbial growth. Each extract was evaluated in triplicates. Plates were incubated at 37˚C for 24 hours upright position and scored for zone of inhibition in millimeter.

ANTIFUNGAL ACTIVITY OF AGAR WELL DIFFUSION METHOD

The spore suspensions of fungal strains were prepared in 0.85% sterile normal saline containing 0.01% Tween 80. Fungal spore suspensions were prepared from fresh mature (seven days old) cultures grown at 25°C to 30°C on Potato Dextrose Agar (PDA), Oat meal agar & Czapek's agar substrates. Fungal spores were rinsed with sterile distilled water, to determine the population spectrophotometrically at 530 nm, and further diluted to adjust the final concentration at 106 CFU/ml[14].

The spore suspension of test fungi strains were swab inoculated aseptically on the sterile Sabouraud Dextrose Agar (SDA) medium followed by making wells of 6mm diameter using sterile cork borer. The wells are aseptically filled with 20

µl of extracts (1000µg). Antifungal activity was determined by measuring the diameter of the zone of inhibition around the well. Ketoconazole (1mg/ml) was used as control. Plain 10% DMSO was used as negative control to check the solvent influence[15]. Each extract was evaluated in triplicates. The plates were incubated at room temperature (25˚C ± 2˚C) for 5 days upright position and scored for zone of inhibition in millimeter).

RESULTS AND DISCUSSION:

This is the first attempt reported on the antibacterial and antifungal activity of solvent extracts of *Heterodermia boryi*, *Sticta weigelii* and *Dermatocarpon vellereum*.

Acetone extract have shown maximum inhibitory activity against most of the bacterial and fungal strains. Among bacteria, *E. coli* (22mm) was mostly inhibited followed by *S. aureus* (21mm) and *P. fluorescenes* (20 mm). Except *Serratia* most of the bacteria were found to be sensitive to acetone extract. All the fungal plant pathogens tested were inhibited by acetone fractions tested and the inhibition zone ranged from 12 - 16mm. Diethyl ether extract gave desirable sensitivity pattern against most of the micro organisms. Among fungi, *P. foedans* was very sensitive (21mm) against ethereal extract followed by the sensitivity of *P. variotii* (20mm). Comparatively, chloroform extract have less activity against *E. coli* (17mm), *S.aureus* (15mm), **(Table 1)** *P. foedans* (12mm). Chloroform extract shown significant inhibitory effect against most of the tested bacteria and fungi except *S. marcescens* and *P. variotii*. Chloroform fractions were found to be mostly effective against *E. coli* (17 mm). Ethanolic extract shown moderate activity against all the bacterial strains. The extracts of ethyl acetate, methanol and hexane possess no significant activity against the tested bacteria and fungi.

TABLE: 1. ANTIMYCOBACTERIAL ACTIVITY OF *HETERODERMIA BORYI*
(Extract volume - 20µl in each well containing 1000µg extract)

Bacteria	Zone of inhibition (against 108 CFU/ml cell counts)							
	1	2	3	4	5	6	7	S/K
Escherichia coli	14±1.74	22±1.22	17±0.90	14±0.26	18±0.80	13±0.62	15±1.45	17±1.24
Staphylococcus aureus	12±0.17	19±1.36	15±0.49	16±0.45	15±0.90	13±0.90	14±0.65	25±0.70
Klebsiella pneumoniae	11±0.80	17±0.32	13±0.85	16±1.17	15±0.61	14±0.40	14±0.47	20±0.85
Pseudomonas fluorescens	*	19±1.03	13±0.50	14±0.85	14±0.73	13±0.70	16±0.95	11±0.70
Serratia marcescens	*	*	*	12±0.70	*	13±0.60	*	18±0.90
Bacillus subtilis	12±0.40	16±0.75	13±0.58	16±0.75	14±0.61	16±0.35	16±0.52	11±0.76
Pestalotia foedans	8±0.20	12±0.20	12±0.30	21±0.35	*	*	*	12±0.95
Phomopsis leptostromiformis	*	15±0.77	13±0.65	18±0.35	*	*	*	12±0.40
Fusarium oxysporum	20±0.15	12±0.70	6±0.11	*	*	21±0.65	14±0.86	10±0.34
Paeciliomyces variotii	*	16±0.92	*	20±0.41	10±0.62	*	16±0.70	15±1.35

1 - Ethyl acetate, 2 - Acetone, 3 - Chloroform, 4 - Diethyl ether, 5 - Methanol, 6 - Ethanol, 7 - Hexane, S - Streptomycin (positive control for bacterial strains), K - Ketaconazole (positive control for fungal strains), *-No zone of inhibition.

The chloroform fractions of *S. weigelii* have shown significant activity against most of the tested bacteria and fungi. Among which *S. aureus* was mostly inhibited (21mm) followed by *P. leptostromiformis* (16mm). Acetone and diethyl ether fractions gave moderated effect against *E. coli* and *S. marcescens* (15mm). But *E. coli* was greater inhibited by methanolic extract (18mm). Among the tested bacteria, *P. fluorescens* was mostly inhibited by ethyl acetate extract. In case of fungi, *P. variotii* was inhibited (21mm) by chloroform extract of *S. weigelii*. Ethanolic and hexane extract of *S. weigelii* shown least inhibitory activity against *P. leptostromiformis* (8mm), *P. variotii* (9mm), *P. foedans* and *Bacillus cereus* (10mm). Most of the extracts of

Sticta weigelii were found to be inactive against *F. oxysporum* **(Table 2)**. *K .pneumoniae* was found to most resistant bacteria against *S. weigelii*.

TABLE: 2. ANTIMYCOBACTERIAL ACTIVITY OF *STICTA WEIGELII*
(Extract volume - 20µl in each well containing 1000µg extract)

Bacteria	Zone of inhibition (against 108 CFU/ml cell counts)							
	1	2	3	4	5	6	7	S/K
Escherichia coli	16±0.35	11±0.75	11±0.20	16±0.85	18±0.72	13±0.45	*	17±1.24
Staphylococcus aureus	*	17±1.60	21±0.55	13±0.25	*	16±0.61	12±0.68	25±0.70
Klebsiella pneumoniae	*	*	*	*	*	*	*	20±0.85
Pseudomonas fluorescens	20±0.57	10±0.40	12±0.43	15±0.43	14±0.11	12±0.65	*	11±0.70
Serratia marcescens	10±0.4	14±0.52	12±0.80	16±0.40	12±1.13	*	*	18±0.90
Bacillus subtilis	*	*	14±0.61	13±0.70	16±0.58	*	10±0.62	11±0.76
Pestalotia foedans	*	18±0.60	12±0.57	10±0.20	12±0.70	*	10±0.63	12±0.95
Phomopsis leptostromiformis	10±0.41	10±0.68	16±0.75	11±0.30	*	8±0.41	*	12±0.40
Fusarium oxysporum	8±0.36	15±0.65	*	*	*	*	*	10±0.34
Paeciliomyces variotii	*	*	21±0.95	*	*	9±0.25	13±0.30	15±1.35

1 - Ethyl acetate, 2 - Acetone, 3 - Chloroform, 4 - Diethyl ether, 5 - Methanol, 6 - Ethanol, 7 - Hexane, S - Streptomycin (positive control for bacterial strains), K - Ketaconazole (positive control for fungal strains), * - No zone of inhibition.

Among the tested microbial strains, *P. fluorescens* was mostly inhibited (26mm) by chloroform extract of *D. vellereum*. Methanolic extract have shown most inhibitory activity against *B. subtilis* (26mm). Secondly, acetone extracts shown effective inhibitory effect against *S. aureus* (25mm). Most of the bacterial strains were inhibited by diethyl ether extract **(Table 3)**. Among the tested bacterial strains, *S. marcescens* was found to be the most resistant strain against the lichen, but was effectively inhibited only by ethanolic extract (20mm). Hexane extract of *D. vellereum* was found to be least effective against most of the microbial strains. But shown a maximum inhibitory activity against *S. aureus* (23mm). *K .pneumoniae* was found to be the most least inhibited by ethanolic and hexane extracts (8mm) of *D. vellereum*.

TABLE: 3. ANTIMYCOBACTERIAL ACTIVITY OF *DERMATOCARPON VELLEREUM*
(Extract volume - 20µl in each well containing 1000µg extract)

Bacteria	Zone of inhibition (against 108 CFU/ml cell counts)							
	1	2	3	4	5	6	7	S/K
Escherichia coli	15±0.10	18±0.28	*	20±0.40	*	16±0.75	*	17±1.24
Staphylococcus aureus	16±0.20	24±0.83	15±0.55	14±0.35	12±0.51	18±0.26	24±1.95	25±0.70
Klebsiella pneumoniae	*	*	*	10±0.25	*	8±0.49	8±0.70	20±0.85
Pseudomonas fluorescens	10±0.56	18±0.50	26±0.66	15±0.45	*	*	*	11±0.70
Serratia marcescens	*	*	*	*	*	20±0.35	*	18±0.90
Bacillus subtilis	*	23±0.86	*	*	26±0.47	*	15±0.61	11±0.76
Pestalotia foedans	12±0.36	16±0.30	14±0.45	*	13±1.20	*	*	12±0.95
Phomopsis leptostromiformis	*	13±0.45	16±0.47	10±0.25	12±0.65	10±0.20	*	12±0.40
Fusarium oxysporum	*	10±0.37	18±0.30	*	*	*	8±0.10	10±0.34
Paeciliomyces variotii	14±0.40	*	18±0.23	12±0.61	11±0.40	*	9±0.15	15±1.35

1 - Ethyl acetate, 2 - Acetone, 3 - Chloroform, 4 - Diethyl ether, 5 - Methanol, 6 - Ethanol, 7 - Hexane, S - Streptomycin (positive control for bacterial strains), K - Ketaconazole (positive control for fungal strains), * - No zone of inhibition.

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 due to the emergence of multi drug resistance pathogens. Natural products, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of diversified chemical substances. The search for novel bioactive natural compounds to improve pharmaceutical, cosmetic and agricultural applications is an ancient one and currently it is regaining its potential importance and lichen compounds are not an exception.

Currently the interest on the lichen secondary compounds is again increasing because of previously reliable drug becoming in effective and since lichens are the reservoirs of pharmacologically relevant polyketide compounds. In the natural product research, drug formulating leads have been much better around 1% where as polyketides were screened compared to other compounds. Lichen substances as bioactive compounds now gaining a cutting edge over traditionally known chemicals due to their improved effectiveness over synthetic compounds. Extracts of lichen thalli proved to have strong antifungal activity against various plant pathogenic fungi.

In the present study, the crude extracts of *H. boryi* against Gram - positive, Gram - negative Bacteria and plant pathogenic fungi were tested and identified that acetone, chloroform and diethyl ether extracts were active against Gram negative bacteria, among which ether and acetone extracts were active even against the tested fungi. Chloroform extracts of *S. weigelii* have shown significant antimycobacterial effect in which it shows maximum activity against *S. aureus* and *P. leptostromiformis*.

Among three tested lichens extracts, the extracts of *D. vellereum* have least bioactivity against the tested microorganisms. The acetone extract of *D. vellereum* alone was found to have antimicrobial activity, which might due to relatively high content non polar 'Lichen substances' that might fail to diffuse in to bacterial and fungal cell wall. The selective antifungal effect of acetone, chloroform and ethereal extracts of the lichen species can be expected due to their differential solubility of constituent secondary metabolites in these extracts[16].

Acetone, methanol and light petroleum extracts of *Usnea ghattensis* showed activity against *B. subtilis* and *S.aureus*[17] which was found to be attributed that the lichen compound's chief function on inhibiting the bacterial/fungal growth was found to interfere within the ATP machinery of cell wall, and certain lichen derived ester compounds also reported for their ATP synthase inhibitory activity. It is commonly attributed that antimicrobial activities of lichen substances interferes with the ATP machinery of the cell wall. Lichen derived scabrosin esters have been reported for their ATP synthase inhibition activity and there by directs the influence on the production of ATP within mitochondrial apparatus. When the mitochondrial ATP synthase is inhibited , the mitochondrial membrane becomes hyperpolarized and finally apoptotic cell death occurs[18]. The process of H^+ dissociation leads to acidification of plasma membrane surface of microorganism, resulting in H^+ - ATPase rupture that is required for ATP synthesis[19]. Besides, it also causes intracellular coagulation of cytoplasmic constituents, leading to cell death, or by inhibiting its growth[20].

The antibacterial effect is relatively stronger than antifungal effect, which is due to the fact that numerous tests proved that bacteria are more sensitive to the antibiotics compared to fungi, because of the difference in sensitivity between fungi and bacteria can be found in transparency of the cell wall[21]. The greater resistance of Gram negative bacteria to most of the lichen extracts in this study indicates the presence of thick muerin layer in outer membrane which prevents the entry of active molecules inside the cell, and possessing lipopolysaccharide layer at the exterior, followed underneath by a thin layer of peptidoglycan[22]. The extracts used in this study had a stronger antibacterial than antifungal activity. This observation is in accordance with other studies[23].

Higher antibacterial activities might lies in the differences in composition and permeability of cell wall. The cell wall of Gram-negative bacteria is made of peptidoglycans, lipopolysaccharides and

lipoproteins, whereas in case of fungi, the cell wall is composed of thick polysaccharides including chitin and glucan that contributes poor permeability of the lichen substances diffuse into them. The data in this study have significant anti-mycobacterial influence relative to the tested bacteria and fungi. The wide variety of biological activities of lichens is generally correlated with their special ecological niche along with the production of 'Lichen substances' that are involved in their antimicrobial actions.

Further investigations on the antimicrobial activity as well as the economical and rapid, convenient isolation of the lichen substances is to be thoroughly investigated. Subsequently, the antimycobacterial effect of the lichens has to be tested on other bacteria and plant fungal pathogens in formulating the pharmacological tests for their efficiency in their growth inhibitory effect. Post clinical studies are mandatory for the search and isolation and characterization of principal substances from lichens, greater deal investigation in the action of lichen substances is required.

CONCLUSIONS: For certain plant fungal diseases, these above mentioned bioactive extracts of different lichen species can be formulated for improving the quality of bio fungicides which at both ways it kills plant insect pests as well as fungal pathogens. The anti-mycobacterial substances present in *Heterodermia boryi*, *Sticta weigelii* and *Dermatocarpon vellereum* were chiefly extracted with acetone and chloroform. The lead molecules of these foliose lichen extracts will provide the basis of future studies for the discovery of potential compounds for clinical treatments.

ACKNOWLEDGEMENTS: Authors thank the Department of Biotechnology and the Management of Nehru Arts and Science College for providing full support and encouragement to carry out this research work.

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How to cite this article:
Balasubramanian M and Nirmala P: Antimycobacterial Activity of Foliose Lichens on Plant and Animal Pathogens. Int J Pharm Sci Res 2014; 5(11): 4825-31.doi: 10.13040/IJPSR.0975-8232.5 (11).4825-31.