Journal of Applied Pharmaceutical Science Vol. 5 (10), pp. 067-072, October, 2015
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2015.501012
ISSN 2231-3354

Evaluation of fungistatic potential of lichen extracts against *Fusarium solani* (Mart.) Sacc. causing Rhizome rot disease in Ginger

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ARTICLE INFO

Article history:
Received on: 05/07/2015
Revised on: 22/07/2015
Accepted on: 07/08/2015
Available online: 28/10/2015

Key words:
Lichens, *Parmotrema tinctorum*, *Flavoparmelia caperata*, *Fusarium solani*.

ABSTRACT

Eight lichens were collected, identified and solvent extract were obtained using methanol and ethyl acetate in soxhlet apparatus. Antifungal properties of lichens were determined by Agar well diffusion method, Microdilution assay and TLC- Bioautographic technique. The largest zone of inhibition in well diffusion method was recorded (in mm) with the ethyl acetate extract of *Parmotrema tinctorum* with 18.6±1.15 followed by *Teloschistes flavicans* with 18.6±0.5. The maximum antifungal activity was found in the extracts of lichen *Parmotrema tinctorum* and *Flavoparmelia caperata* with low MIC value of 1.562mg/ml. *Flavoparmelia caperata* extract was active with a clear zone on TLC bioautogram of the tested organism indicating two zones of growth inhibition at an Rf value of 0.46, 0.6. Comparatively ethyl acetate extract showed a strong antifungal activity than methanolic extract. The results were promising with potential drug candidate having a fungitoxic effect to reinforce bioprospecting of lichens as a bio-fungicide.

INTRODUCTION

Lichen is a stable, ecologically obligate, self-supporting mutualism between an exhabitant fungus (the mycobiont) and one or more inhabitant, extracellulary located unicellular or filamentous photoautotrophic partners (the photobiont: alga or cyanobacterium) (Molnár and Farkas, 2010).Lichens are valuable plantresources and are used as medicine, food, fodder, perfume, spice, dyes and for miscellaneous purposes throughout the world. Lichens are inherently resistant to microbial infection due to the production of large numbers of unique secondary metabolites (Huneck, 1999). These lichens produce a diverse range of primary and secondary metabolites (Hale, 1983). Slow growth and often harsh living conditions, make the production of protective metabolites a necessity to lichens, and many secondary constituents are believed to serve as antigrowth, antimicrobial or antiherbivore agents (Rankovic *et al.*, 2008). The lichen secondary metabolites show a wide range of potentially useful biological activities like antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic properties (Boustie and Grube, 2005).Lichens and their secondary metabolites have shown great potential as antifungal source (Muller, 2002). But as far as plant diseases are concerned the lichens are very less explored and, especially, against fungal plant pathogens there is very little information reported till yet (Goel *et al.*, 2004; Halama and Haluwin, 2004;Babiah *et al.*, 2014a;Vivek *et al.*, 2014; Kekuda *et al.*, 2014). Plant diseases may result in less food or of food in poor quality or may sometimes be poisonous and unfit for consumption. Some plant diseases may wipe out entire plant species. Ginger (*Zingiber officinale* Rosc.) is an important commercial crop cultivated throughout India for its rhizome as a spice. India is the largest producer of ginger accounting for about 1/3rd of total world output. It is an important crop that earns a sizeable amount of foreign exchange for the country (Tarafdar and Saha 2007). It has high medicinal value, hence it is used in traditional Ayurvedic medicine. It was classified as a stimulant and carminative and used frequently for dyspepsia, gastroparesis, slow motility symptoms, constipation and colic. Ginger is affected by several fungal pathogens (Dohroo, 1993). Among which, rhizome rot caused by *Fusarium solani* is most common (Kumar, 1977). Crop loss due to rot-causing fungal pathogens is a significant problem. Controlling plant disease may result in better more food of better quality (Agrios, 2005). Since a decade people are using pesticides of toxic chemicals and even synthetic fungicides to control pathogens. The control of these fungi by synthetic fungicides is clouded by lots of drawbacks such as severe human and environmental toxicity effects and high costs.

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Their high costs make them less accessible particularly by rural farmers. These setbacks warrant an alternative source of fungicide. The aim of the present investigation is to evaluate the fungitoxic effect of lichens in the management of rhizome rot of ginger caused by *Fusarium solani* (Mart.) Sacc.

MATERIALS AND METHODS

Collection and identification of lichen material

The lichens were collected from in and around Madikeri and Chamarajanagar districts in Karnataka state, India. Identification of lichens were done based on morphology and biochemical tests, e.g., (1) growth form: Foliose or fruticose length of thallus; (2) color and texture of thallus; (3) presence or absence of reproductible structures (isidia, soredia, apothecium or perithecium); (4) thalline spot test (K, C, KC, P- test); (5) Thin layer chromatography (TLC). For the thalline spot test, the upper cortex of each specimen was initially scraped off with a razor blade to expose the medulla. Then, different chemical reagents, e.g., potassium hydroxide (K) and Calcium hypochlorite (C), p-phenylenediamine (P)were spotted directly onto the exposed medulla. Any immediate change in color of the thallus indicates a positive reaction (Awathi 1988, 2000; Goward *et al.* 1994; Malcolm and Galloway 1997).

Solvent Extraction

Collected samples were washed thoroughly with distilled water and shade dried at room temperature. The dried lichens were milled to a fine power with the help of a blender. Powdered lichen samples of about 10 gms from each species were extracted by standard Soxhlet extraction procedure using Ethyl acetate and Methanol solvents. The lichen extracts were filtered through sterile Whatman No. 1 filter paper and concentrated in vacuum under reduced pressure and the weight/yield of the crude extracts were determined and preserved at 5 ℃ in airtight bottles until further use.

Fungal Test Organism

The fungus *Fusarium solani*, a causal organism of Rhizome rot in ginger was isolated from the diseased rhizome part of ginger by standard blotter method and the fungi were identified on the basis of their typical structure and basic characters (Domsch and Gams, 1972). The isolated fungus from the diseased ginger samples was maintained on potato dextrose agar (Himedia) at 26-28℃. The standard culture inoculum was prepared on Potato Dextrose Broth by adjusting the spore range of 1×10^6 - 5×10^6 spores /ml (Aberkene *et al*., 2002).

Antifungal Activity Assay

Agar well diffusion assay

100 µl of fungal suspension was spread on the solidified Potato dextrose agar medium and wells were punched using 5mm cork borer, a concentration of 30 mg/ml extracts of 100 µl was loaded into the wells and the solvents of the same were used as a negative control. The plates were kept for incubation for 4-7 days; the diameter of the zone of inhibition of the tested microorganism by the given extract was measured in millimeters. All experiments were performed in triplicate. To every sample tested, a set of positive control (Bavistin) and negative control (respective solvents) was run parallel.

Microdilution assay

Determination of Minimum inhibitory concentration [MIC] was carried out by microdilution method. The MIC is to determine the lowest concentration of an antifungal agent that appears to inhibit growth of the fungus (Andrews, 2011). MICs were calculated for the extract that had antifungal activity. Residues of different extracts were dissolved in respected solvents to a concentration of 50mg/ml. The plant extracts (100µl) were serially diluted 50% with solvents in 96 well flat bottomed microtitre plates. Fungal cultures were transferred into fresh Potato dextrose broth, and 100µl of this was added to each well, 40µl of 2, 3, 5- triphenyltetrazolium chloride [TTC] dissolved in water was added to each of the micro plate wells, as growth indicator. Appropriate solvent blanks as control were included. The micro plates were covered with a cling film and incubated for 2-3 days at 26℃ and at 100% relative humidity (Eloff, 1998). The MIC was recorded by visual analysis in microtitre plate wells, where the lowest concentration of the lichen extract that inhibited fungal growth after 48 to 72 hours of incubation will not change its colour to formazen dye.

TLC- Bioautography method

Bioautographic method was developed to determine active compounds. Aluminium-backed TLC plates (Aluchrosep Silica Gel $60/UV_{254}$ for TLC) were loaded with 20µl of 100µg extracts. The TLC plate was developed in solvent system A (180 ml toluene: 60 ml 1-4, dioxin: 8 ml acetic acid) (Culberson, 1972). The chromatogram was dried for complete removal of solvents. Fungal inoculum solution was prepared containing approximately 3×10^4 spores/ml of actively growing fungi. Developed chromatogram was placed in petridish molten potato dextrose agar seeded with fungal inoculum was poured on the chromatogram. After agar got solidified the petri plates were kept at 4^oC for diffusion for 3 hours and plates were incubated at room temperature for 4 days. Fungal growth inhibition appeared as clear zones against a dark background. The R_f value of the zone of inhibition is recorded.

RESULTS

A total of eight lichens were identified in which, six lichens were of foliose growth form and two lichens were of fruticose growth form. Five species *Flavoparmelia caperata* (L.) Ach., *Parmotrema austrosinensis* (Zahlbr.) Hale, *Parmotrema grayanum* (Hue) Hale, *Parmotrema reticulatum* (Taylor) Choisy and *Parmotrema tinctorum* Nyl. belongs to family Parmeliaceae, *Physcia aipolia* (Ehrh. ex Humb.) Furnr. belongs to family

Physciaceae, *Roccella montagnei* Bèl. Emend. Awas. belongs to family Roccellaceae and *Teloschistes flavicans* (Swartz) Norm. Belongs to family Teloschistaceae (Table 1).

Extraction of lichens with Ethyl acetate and methanol extracts yielded a mass from 332 to 1330 mg/ml. Well diffusion assay was carried out to test the inhibition of lichens against the fungi. The zones of inhibition for both extracts ranged from 12.3±0.5 to 18.6±1.15 mm to inhibit the phytopathogenic fungi *Fusarium solani* (Mart.) Sacc., a causal organism of rhizome rot of Ginger. Comparatively ethyl acetate extract showed a strong antifungal activity than methanolic extract. The largest zone of inhibition was recorded with the ethyl acetate extract of *Parmotrema tinctorum* with 18.6±1.15 mm followed by *Teloschistes flavicans* with 18.6±0.5 mm. Methanol extract showed activity in only two lichens *Flavoparmelia caperata* and *Roccella montagnei* with 12.6±0.5 and 11.6±0.5 mm respectively (Table.2). The MIC was carried out in 96 well flat bottom microtitre plates to record the minimum inhibition concentration of extract at which the activity of the pathogen will be inhibited. The lower the MIC value, the more active is the extract at a particular concentration. The MIC of both the extracts related to the tested fungi were 1.562 -12.25 mg/ml. The maximum antifungal activity was found in the extracts of lichen *Parmotrema tinctorum* and *Flavoparmelia caperata* with low MIC value of 1.562mg/ml. There was no inhibition of growth in wells of methanol and ethyl acetate used as our solvent blank, which means these solvents did not have effect on the tested organism, proving it good solvent system for bioassays. Bioautography was carried out to test the zone of inhibition on TLC plates. *Flavoparmelia caperata* extract was active with a clear zone on TLC bioautogram of the tested organism indicating lack of spore germination of the pathogenat an $R_f$value of 0.46, 0.6 (Fig 1). *Parmotrema tinctorum* at 0.67, *Teloschistes flavicans* at 0.61, *Parmotrema grayanum* at 0.64 and *Physcia aipolia* at R_f 0.77. *Parmotrema austrosinensis*, *Parmotrema reticulatum* and *Roccella montagnei* did not show inhibition zone on bioautogram. Total activity of a plant is the quantity of material extracted from 10 gms of dried plant material divided by the MIC value obtained (Mdee *et al.*, 2009). It indicates the largest volume to which the biologically active compounds in 10 gm of plant material can be diluted and still inhibits the growth of the test organism. Since *Parmotrema tinctorum* and *Flavoparmelia caperata* showed least MIC value, total activity of these is 414.85 ml and 851.47 ml respectively (Table 3). Which means 10 gms of lichen extracts can be mixed with 414.85 ml and 851.47 ml of ethyl acetate and still inhibits the pathogen *Fusarium solani* which causes rhizome rot in ginger.

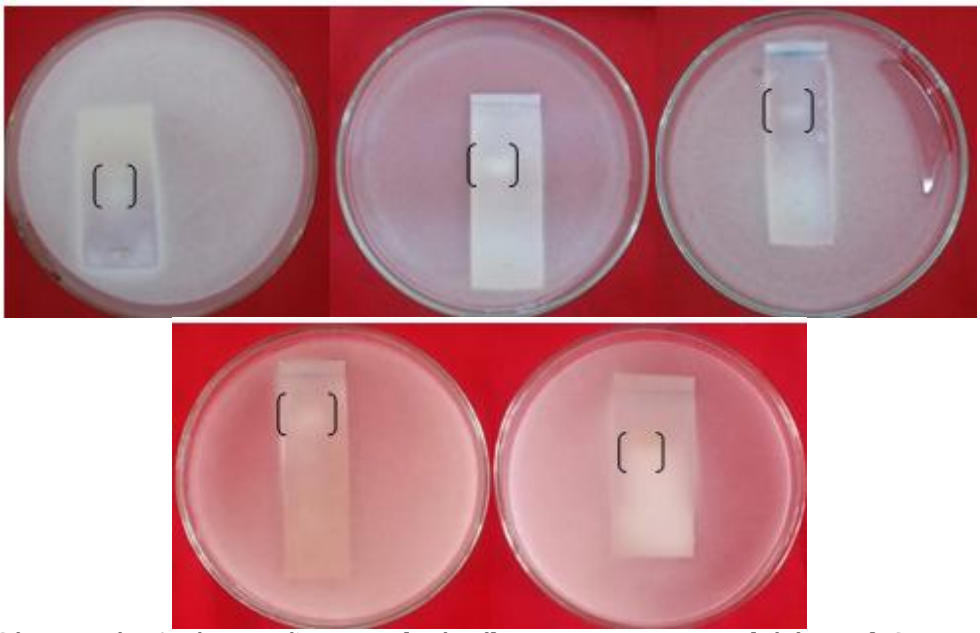

Fig. 1: Results of TLC-bioautography- A. *Flavoparmelia caperata* [Methanol]; B. *Parmotrema grayanum* [Ethyl acetate]; C. *Parmotrema tinctorum* [Ethyl acetate]; D. *Physcia aipolia* [Ethyl acetate]; E. *Teloschistes flavicans* [Ethyl acetate].

Table 1: List of lichens collected and identified.

Sl no.	Lichen samples	Family	Growth form	Spot test	Metabolites in lichens
1	*Flavoparmelia caperata* (L.) Ach.	Parmeliaceae	Foliose	KC+ red	Atranorin, usnic acid, protocetraric acid
2	*Parmotrema austrosinensis* (Zahlbr.) Hale	Parmeliaceae	Foliose	C+ red, KC+ red	Lecanoric acid
3	*Parmotrema grayanum* (Hue) Hale	Parmeliaceae	Foliose	-	Atranorin
4	*Parmotrema reticulatum* (Taylor) Choisy	Parmeliaceae	Foliose	K+yellow, P+ orange	Atranorin, salazinic acid
5	*Parmotrema tinctorum* Nyl.	Parmeliaceae	Foliose	K+ red, C+red	Lecanoric acid, atranorin, chloroatranorin
6	*Physcia aipolia* (Ehrh. ex Humb.) Furnr.	Physciaceae	Foliose	K+yellow, P+yellow	Atranorin, zeorin
7	*Roccella montagnei* Bèl. Emend. Awas.	Roccellaceae	Fruticose	K+red, C+red	Lecanoric acid, erythrin
8	*Teloschistes flavicans* (Swartz) Norm.	Teloschistaceae	Fruticose	K+red	Parietin, teloschistin

Table 2: Inhibitory activity of lichen extracts against *Fusarium solani*.

Sl no.	Lichen samples	Solvent extract	Diffusion assay* in mm	MIC in µg/ml	Bioautography of R_f value
1	*Flavoparmelia caperata* (L.) Ach.	Ethyl acetate	-	-	-
		Methanol	12.6±0.5	1.562	0.46, 0.6
2	*Parmotrema austrosinensis* (Zahlbr.) Hale	Ethyl acetate	12.3±0.5	6.25	-
		Methanol	-	-	-
3	*Parmotrema grayanum* (Hue) Hale	Ethyl acetate	15.3±0.5	6.25	0.64
		Methanol	-	-	-
4	*Parmotrema reticulatum* (Taylor) Choisy	Ethyl acetate	17±1	6.25	-
		Methanol	-	-	-
5	*Parmotrema tinctorum*Nyl.	Ethyl acetate	18.6±1.15	1.562	0.67
		Methanol	-	-	-
6	*Physcia aipolia* (Ehrh. ex Humb.) Furnr.	Ethyl acetate	14±1	6.25	0.77
		Methanol	-	-	-
7	*Roccella montagnei*Bèl. Emend. Awas.	Ethyl acetate	13.3±0.5	6.25	-
		Methanol	11.6±0.5	6.25	-
8	*Teloschistes flavicans* (Swartz) Norm.	Ethyl acetate	18.6±0.5	12.25	0.61
		Methanol	-	-	-
9	Bavistin (positive control)	-	16±0	1.562	-
10	Pure Solvent (negative control)	Methanol	-	-	-
		Ethyl acetate	-	-	-

*Values are in mean ± standard deviation, $n = 3$.

Table 3: Total activity of tested lichens.

Sl no.	Lichen samples	Solvent extract	MIC in µg/ml	Mass in mg	Total activity in ml
1	*Flavoparmelia caperata* (L.) Ach.	Ethyl acetate	-	700	-
		Methanol	1.562	1330	851.47
2	*Parmotrema austrosinensis* (Zahlbr.) Hale	Ethyl acetate	6.25	1270	203.2
		Methanol	-	700	-
3	*Parmotrema grayanum* (Hue) Hale	Ethyl acetate	6.25	440	70.04
		Methanol	-	590	-
4	*Parmotrema reticulatum* (Taylor) Choisy	Ethyl acetate	6.25	520	83.2
		Methanol	-	492	-
5	*Parmotrema tinctorum* Nyl.	Ethyl acetate	1.562	648	414.85
		Methanol	-	580	-
6	*Physcia aipolia* (Ehrh. ex Humb.) Furnr.	Ethyl acetate	6.25	788	126.08
		Methanol	-	1014	-
7	*Roccella montagnei* Bèl. Emend. Awas.	Ethyl acetate	6.25	880	140.8
		Methanol	6.25	1080	172.8
8	*Teloschistes flavicans* (Swartz) Norm.	Ethyl acetate	12.25	322	26.28
		Methanol	-	586	-

DISCUSSION

The results obtained in the present study showed lichens have strong antifungal activity. Evaluation of eight lichen extracts showed to be effective in inhibiting the growth of the fungus. Amongst these, the most promising were *Parmotrema tinctorum* and *Flavoparmelia caperata*, which showed highest zone of inhibition in diffusion assay, least MIC value and good inhibition zones against the growth of *Fusarium solani* due to the fungicidal principle of metabolites present in the lichens. *Flavoparmelia caperata* had showed lowest MIC of 0.097mg/ml when compared to other extracts in inhibiting *Fusarium oxysporum* sp. *Capsici* (Shivanna and Garampalli, 2014), whereas moderate activity was reported against *Fusarium solani* (Babiah *et al.*, 2014b). Antifungal activities of similar results were reported by certain plant extracts to control *Fusarium solani* in ginger crop (Ramteke and Kamble, 2011). Strong inhibitory effects were observed in *Hypogymnia physodes* on *Fusarium solani* (Halama and Haluwin, 2004). Biocontrol potential of a macrolichen *Usnea pictoides* G. Awasthi were evaluated against *Fusarium oxysporum* f. sp. *Zingiberi* and *Pythium aphanidermatum* isolated from rhizome rot of ginger (Vinayaka *et al.*, 2014). *Heterodermia diademata* showed comparative good results against *Fusarium solani* (Tiwari *et al.*, 2011). The antifungal activity of protolichesterinic acid isolated from *Parmelia perlata* was tested against plant pathogenic fungus (Goel *et al.*, 2011). *Parmotrema andinum* was screened against various pathogenic microorganisms in which moderate activity was reported against the pathogen *Fusarium solani* (Devi *et al.*, 2015). Positive control bavistin showed 16±0mm inhibition zone, which is moderate activity, comparatively tested lichens have showed highest activity and which could be a better performer that the synthetic fungicide. Thippeswamy *et al.* (2013) also procured the same results where both crude extracts and isolated compounds were more susceptible than Bavistin. In the present result preliminary phytochemical analysis of *Parmotrema tinctorum* had revealed the presence Tannins, alkaloids, proteins and carbohydrates whereas *Flavoparmelia caperata* showed most of the constituents like Tannins, Flavanoids, Proteins, Triterpenes, Carbohydrates and Steroids (Rashmi and Rajkumar, 2014). Methanolic extract of *Ramalina hossei* showed the presence of

tannins and terpenoids (Kumar *et al.*, 2010). TLC showed lichen metabolites like Atranorin, usnic acid, protocetraric acid in *Flavoparmelia caperata*, and atranorin, chloroatranorin, lecanoric acid in *Parmotrema tinctorum*. Santiago *et al.* (2013) reported usnic acid, norstictic acid, and salazinic acid as bioactive lichen metabolite from *Usnea* sp. by TLC- bioautography against pathogens.

The results of Bioautography showed good results in most of the samples tested, which will be useful in the further isolation and characterization of metabolites.

CONCLUSION

The present work concentrated on finding out the effect of natural products, which are eco-friendly and less harmful than commercial synthetic compounds like fungicides. Consequently the investigated lichens could be used as a natural fungicide in the management of the diseases caused by plant pathogens. Further studies on the fractionation of solvent extracts and characterization may reveal the compounds responsible for the antifungal potentials.

ACKNOWLEDGEMENTS

One of the author (Rashmi S.) is thankful to the University of Mysore for awarding NON-NET fellowship during preparation of this article.

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How to cite this article:

Rashmi Shivanna, Rajkumar H Garampalli. Evaluation of fungistatic potential of lichen extracts against *Fusarium solani* (Mart.) Sacc. causing Rhizome rot disease in Ginger. J App Pharm Sci, 2015; 5 (10): 067-072.