Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

Advances in Applied Science Research, 2014, 5(5):273-277

ISSN: 0976-8610
CODEN (USA): AASRFC

Efficacy of lichen extracts as biocontrol agents against *Fusarium oxysporum* F. Sp. Capsici

Rashmi Shivanna and Rajkumar H. Garampalli*

Department of Studies in Botany, Manasagangotri, University of Mysore, Mysore, Karnataka, India

---

ABSTRACT

In vitro antifungal activity of ten lichens was evaluated against phytopathogenic fungi Fusarium oxysporum using Agar well diffusion, Microdilution assay and Direct Bioautographic technique. Methanol, Ethyl acetate and Acetone extracts were used to determine antifungal properties of lichens. Highest yield was obtained from Flavoparmelia caperata with 1330 mg/ml from methanol. Well diffusion assay showed maximum inhibition with 21.3±1.5 mm in the methanol extract of Parmotrema austrosinensis. The MIC of the all tested extracts ranged from 0.097 – 6.25 mg/ml. The lowest concentration of 0.097 mg/ml was inhibited in the methanolic extract of Flavoparmelia caperata. The TLC bioautographic method showed zone of inhibition with 3 spots at an Rf value of 0.38, 0.64, 0.96 and 0.2, 0.41, 0.62 in Ethyl acetate extracts of Parmotrema austrosinensis and Parmotrema grayanum respectively. Sticta sp. and Parmotrema reticulatum did not show any activities.

Key words: Lichens, antifungal, bioautography, *Fusarium oxysporum*.

INTRODUCTION

Lichen is a symbiotic organism consisting of a fungus (mycobiont) and a photosynthetic partner (photobiont) which can be either an alga or a cyanobacterium [1]. The challenge for today's pharmaceutical industry lies in the discovery and development of new pharmacological active molecules due to microbial resistance to available antibiotics [2]. Since long back, plants have provided a source of inspiration for novel drug compounds, as plant derived medicine have made large contributions to human health and well being by becoming the natural blue print for drug discovery and the development of phytomedicines to cure diseases. Similar to higher plants, lichens were used since antiquity as natural drugs [3].

Bioactive secondary metabolites have been isolated from lichens and some of them are used in pharmaceutical sciences [4]. Screening the lichens has revealed the frequent occurrence of metabolites with antibiotics, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic properties [5]. Even though these manifold activities of lichen metabolites have now been recognized, their therapeutic potential has not yet been fully explored and thus remains pharmaceutically unexploited [6]. Lichen forming fungi produce antibiotic secondary metabolites that protect many animals from pathogenic microorganisms [7]. The development and spread of microbial resistance to available antibiotics has prompted resistance to study antimicrobial substances from other sources like lichens which attract much attention of researchers as significant new sources for novel bioactive substances.

A large number of synthetic chemicals used to control these pathogenic fungi are detrimental to the environment and human health and need to be replaced by safe, biodegradable products. Among the various alternatives, natural plant products are gaining momentum and catching the attention of scientists worldwide [8]. Such products are relatively broad-spectrum, bioefficacious, economical, biodegradable and environmentally safe and can be ideal candidates for use as Agrochemicals [9]. Natural products with diverse bioactivities and structures are an important source of novel

chemicals with pharmaceutical potentials [7]. Lichens are inherently resistant to microbial infection due to the production of large numbers of unique secondary metabolites. Their flexibility in habitat enables them to synthesize unique, naturally occurring secondary metabolites, which not only are different in their chemical structures but also show differences in their biological activity. However, studies related to their potential use as Agrochemicals are very few and the usage of lichens as an Agrochemicals has been proved by many researchers [10, 11]. Therefore, the aim of the present study is to evaluate the antifungal activity of the Methanol, Ethyl acetate and Acetone extract of 10 different lichens against *Fusarium oxysporum* f.sp.capsici which is an important plant pathogenic fungus.

MATERIALS AND METHODS

Collection of lichens: Field survey was carried out in order to collect the lichen samples from Mysore and Madikeri districts in Karnataka state, India. The lichen specimen was identified by morphological, anatomical and chemical tests [12]. The lichens used in the present investigation are *Flavoparmelia caperata* (L.) Ach., *Parmotrema austrosinensis* (Zahlbr.) Hale, *Roccella montagnei* Bèl. Emend. Awas., *Teloschistes flavicans* (Swartz) Norm., *Physcia aipolia* (Ehrh. ex Humb.) Furnr., *Parmotrema grayanum* (Hue) Hale, *Parmotrema tinctorum* Nyl., *Parmotrema reticulatum* (Taylor) Choisy, *Usnea* sp. and *Sticta* sp. Lichens were thoroughly washed 2-3 times with water and shade dried at room temperature. The dried plants were milled to a fine power with the help of a blender and stored at room temperature in closed containers in the dark until used.

Extraction Procedure: Lichen samples from each species were individually extracted by soaking 1g of finely ground lichen material with 10 ml of Ethyl acetate, Acetone and Methanol solvents separately in conical flasks, plugged with cotton and kept on a rotary shaker at 180-200rpm for 24 hrs. Later it was filtered through 3 layered muslin cloths and the supernatant was filtered through Whatman No.1 filter paper. Filtered extracts were concentrated by air-drying for 4 –5 days or until the extracts crystallized, and the weight/yield of the crude extracts were determined and preserved at 5℃ in airtight bottles until further use.

Fungal Test Organism: The phytopathogenic fungus selected for this study was *Fusarium oxysporum* which is a causal organism of *Fusarium* wilt in chilly. The fungus was isolated from the diseased chilly samples and 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 [13].

Antifungal Activity Assay: Antifungal activity assays were carried out by Agar well diffusion, Microdilution assay and Direct bioautographic method.

Agar well diffusion was carried out as follows: 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 control 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 [14]. 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 [15]. 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.

Direct Bioautography method: Bioautographic method was developed to determine active compounds. Aluminium-backed TLC plates (ALU-GRAM® SIL G/UV254, MACHERY-NAGEL) were loaded with 20µl of 100µg extracts. The TLC plate was developed in solvent system A (180 ml toluene: 60 ml 1-4, dioxine: 8 ml acetic acid) [16]. The chromatogram was dried for complete removal of solvents. About 25-50ml of inoculum spray solution was prepared containing approximately 3 x 10^4 spores/ml of actively growing fungi. The plates were sprayed lightly 3 times with spore suspension and incubated for 24 hr in darkness in a moist chamber at 26℃ and

then sprayed with 20mg/ml of TTC in boiling water and further incubated overnight. Fungal growth inhibition appeared as clear zones against a dark background [17]. Rf value of the zone of inhibition are recorded.

RESULTS AND DISCUSSION

A total of 10 lichens was screened for *in vitro* antifungal activity against *Fusarium oxysporum*. The total yield of the extract varied from 22- 1330 mg/ml (Figure 1). Highest yield was obtained from *Flavoparmelia caperata* with 1330 mg/ml of methanol, *Roccella montagnei* yielded 880 mg/ml of ethyl acetate extract, lowest yield was from *Usnea* Sp. with only 22mg/ml.

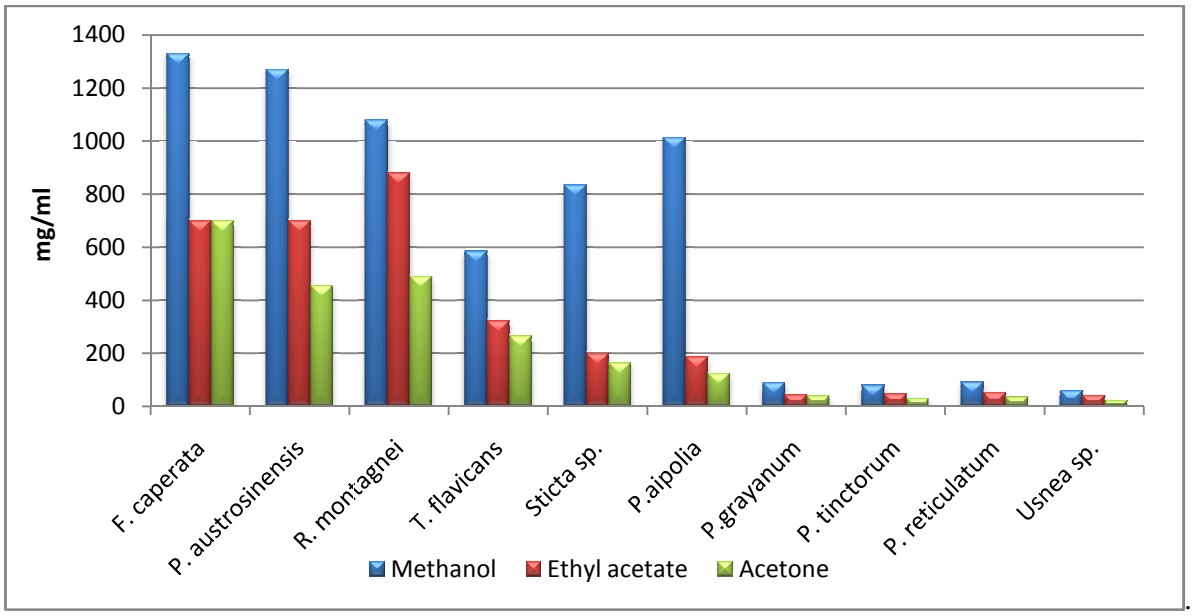

Fig 1: Total yield of the extracts obtained by different solvents in mg/ml

Diffusion assay was carried out by agar well diffusion method. Methanol and Ethyl acetate extracts of most of the lichen species tested in the present study showed strong inhibitory activity. The largest zone of inhibition (21.3±1.5mm) was measured in the methanol extract of *Parmotrema austrosinensis*, 18.3±1.5 of Ethyl acetate extract of *Flavoparmelia caperata* and *Teloschistes flavicans* which showed 16.6±1.5 mm zone of inhibition in ethyl acetate. Ethyl acetate extract of Roccella montagnei, and Mehtanolic extract of Usnea sp. showed poor zone of inhibition with 11.3±0.5 mm. The 14.3±0.5 mm inhibition zone was observed in the Ethyl acetate extract of *Parmotrema tinctorum*. 13.3±0.5 mm inhibition zone in Methanol extract and Acetone extract with 13.0±0.0 in *Flavoparmelia caperata*, Methanol extract of *Roccella montagnei* showed 13.0±0.0 mm zone of inhibition. Extracts from *Sticta* sp. and *Parmotrema reticulatum* did not show any result (Table 1).

Table 1: Results of Zone of inhibition (mm) of lichen extracts against *Fusarium oxysporum*.

Sl no.	Lichen samples	Methanol extract	Ethyl acetate extract	Acetone extract
1	*Flavoparmelia caperata* (L.) Ach.	13.3±0.5	18.3±1.5	13.0±0.0
2	*Parmotrema austrosinensis* (Zahlbr.) Hale	21.3±1.5	14.6±0.5	12.3±1.1
3	*Roccella montagnei* Bèl. Emend. Awas.	13.0±0.0	11.3±0.5	12.6±0.5
4	*Teloschistes flavicans* (Swartz) Norm.	0.0±0.0	16.6±1.5	0.0±0.0
5	*Sticta* sp.	0.0±0.0	0.0±0.0	0.0±0.0
6	*Physcia aipolia* (Ehrh. ex Humb.) Furnr.	12.6±0.5	12.3±1.1	0.0±0.0
7	*Parmotrema grayanum* (Hue) Hale	0.0±0.0	11.6±0.5	0.0±0.0
8	*Parmotrema tinctorum* Nyl.	11.3±1.1	14.3±0.5	0.0±0.0
9	*Parmotrema reticulatum* (Taylor) Choisy	0.0±0.0	0.0±0.0	0.0±0.0
10	*Usnea* sp.	11.3±0.5	0.0±0.0	12.6±0.5
11	Bavistin (positive control)	16.5±0.3	16.5±0.3	16.5±0.3
12	Pure Solvent (negative control)	0.0±0.0	0.0±0.0	0.0±0.0

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

The micro dilution assay was carried out in 96 well flat bottom microtitre plates to record the minimum inhibitory concentration of extract at which the activity of the pathogen will be inhibited. The lower the MIC value is, the more active the extract is. The MIC of the all tested extracts ranged from 0.097 – 6.25 mg/ml. At the lowest concentration of 0.097 mg/ml only one lichen extract obtained from *Flavoparmelia caperata* in methanolic extract was able to

inhibit the growth of the fungus. The next low concentration was 0.390mg/ml at which most of the sample extract showed growth inhibition of the fungus viz. methanolic extract of *Parmotrema austrosinensis*, methanol and ethyl acetate extracts of *Roccella montagnei* and *Physcia aipolia* (Table 2).

Table 2: Results of Minimum inhibitory concentration (MIC) in µg/ml of lichen extracts against *Fusarium oxysporum*

Sl no.	Lichen samples	Methanol extract	Ethyl acetate extract	Acetone extract
1	*Flavoparmelia caperata* (L.) Ach.	0.097	1.875	1.562
2	*Parmotrema austrosinensis* (Zahlbr.) Hale	0.390	3.125	1.562
3	*Roccella montagnei* Bèl. Emend. Awas.	0.390	0.390	3.125
4	*Teloschistes flavicans* (Swartz) Norm.	NA	6.25	NA
5	*Physcia aipolia* (Ehrh. ex Humb.) Furnr.	0.390	0.390	NA
6	*Parmotrema grayanum* (Hue) Hale	NA	3.125	NA
7	*Parmotrema tinctorum* Nyl.	6.250	3.125	NA
8	*Usnea* sp.	3.125	NA	6.25

NA: No Activity

The tested extracts which showed results in the MIC were further analyzed by the TLC bioautography method. This method was basically used to localize the antifungal compound from the crude extract into the chromatogram. Fungal growth inhibition was observed as clear zones against a pink background, 3 spots of zone of inhibition were observed in R_f value 0.38, 0.64, 0.96 and 0.2, 0.41, 0.62 in Ethyl acetate extracts of *Parmotrema austrosinensis* and *Parmotrema grayanum* respectively. 2 spots of zone of inhibition were observed each in R_f value of 0.27 and 0.67 of ethyl acetate extract of *Flavoparmelia caperata*, R_f value 0.77 and 0.89 of the ethyl acetate extract of *Physcia aipolia* and R_f value 0.7 and 0.86 of methanol extract of *Parmotrema tinctorum*. The rest showed zone of inhibition by one zone each with R_f value 0.67 and 0.47 in methanol and ethyl acetate extract of *Roccella montagnei*, R_f value 0.47 in the ethyl acetate extract of *Teloschistes flavicans*, and R_f value 0.65 in *Usnea* Sp (Table 3).

Table 3: Results of TLC Bioautography of lichen extracts against *Fusarium oxysporum* by recording the Rf value

Sl no.	Lichen samples	Methanol extract	Ethyl acetate extract	Acetone extract
1	*Flavoparmelia caperata* (L.) Ach.	0.6	0.27 0.67	-
2	*Parmotrema austrosinensis* (Zahlbr.) Hale	-	0.38 0.64 0.96	1.562
3	*Roccella montagnei* Bèl. Emend. Awas.	0.67	0.47	-
4	*Teloschistes flavicans* (Swartz) Norm.	-	0.47	-
5	*Physcia aipolia* (Ehrh. ex Humb.) Furnr.	0.72	0.77 0.89	-
6	*Parmotrema grayanum* (Hue) Hale	-	0.2 0.41 0.62	-
7	*Parmotrema tinctorum* Nyl.	0.7 0.86	0.67	-
8	*Usnea* sp.	0.65	-	-

Extracts of lichen thalli proved to have strong antifungal activity against various plant pathogenic fungi [11, 18].In this study, the extracts from the lichens viz. *Flavoparmelia caperata, Parmotrema austrosinensis, Roccella montagnei, Teloschistis flavicans, Physcia aipolia, Parmotrema grayanum Parmotrema tinctorum, Parmotrema reticulatum, Usnea* sp. and *Sticta* sp. were obtained in three solvents viz. methanol, ethyl acetate, and acetone and differential activities were observed. Amongst the three, methanol extract was found to be more effective and showed strong inhibitory activity of the tested fungi than the ethyl acetate and the acetone extracts, which showed comparatively inferior activity. The results corroborate with previous work, which showed significant bioactive characteristics of similar lichens [19]. However, there was a variation in the extract yield of the different lichens in the various solvents. The yield percentage of the various fractions of the lichen extracts (*Usnea longifolia, Cetraria* spp, *Palmelia milghenensis* and *Evernastrium nepalense*) were evaluated by Baral et al. [20] which varied from 0.07- 29.4%, which deviates from the present investigation results.

Antifungal activities eight lichens showed to be effective in inhibiting the growth of the fungus. Amongst these, the most promising were *Parmotrema austrosinensis* and *Teloschistes flavicans*, which showed highest zone of inhibition, followed by *Parmotrema tinctorum, Flavoparmelia caperata, Roccella montagnei* in different solvent extracts (Table 1) which confirms earlier reports [21, 22]. Least measured MIC value was observed at 0.0975 mg/ml, which is the least inhibitory value when compared to earlier report [23]. 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 and which is in confirmation of previous reports [24, 25].

CONCLUSION

The present results showed that the tested lichen extracts proved to be a significant biocontrol agent with the tested fungi. Further investigations on the antimicrobial activity as well as the economical and fast isolation of the metabolite from the lichens are needed. Consequently, the antimicrobial effect of lichens tested can be explained with new studies by testing other microbes and conducting the pharmacological tests. Further, research in isolation of the lichen metabolites, and their detail investigation in the mode of action of lichen extract have to be investigated which will help with a specific application in the field of disease management.

REFERENCES

[1] Ahmadian V. The lichen symbiosis, John Wiley and sons, **1993**.
[2] Bahera BC, Verma N, Sonone A, Makhija U. *Biotech. Lett*., **2005**, 27: 991-995.
[3] Kingkorn AD. *Journal of Pharmacy and Pharmacology*, **2000,** 264: 701-703.
[4] Huneck S. *Naturwissenschaften*, **1999,** 86: 559–570.
[5] Boustie J, Grube M. *Plant genetic resources: Characterization and utilization*, **2005**, 3(2):273-287.
[6] Muller K. *Applied Microbiology* and *Biotechnology*, **2002**, 56**:** 9-16.
[7] Lawrey JD. *Bryologist*, **1986,** 89: 111–122.
[8] McLaren JSM. *Pesticide Science*, **1986**, 17: 559–578.
[9] Richardson DHS. *Mycol*, **1991**, 187–210.
[10] Goel M, Dureja P, Rani A, Uniyal PL, Laatsch H. *Journal of Agricultural and Food Chemistry*, **2011**, 59: 2299–2307.
[11] Halama P, Haluwin VC. *Biocontrol*, **2004,** 49**:** 95-107.
[12] Awasthi DD. *J. Hattori Bot. Lab*, **1988**, 65: 207–302.
[13] Aberkene A, et al.,. *J. Antim. Chem.*, **2002**, 50: 719-722.
[14] Andrews JN. *Journal of Antimicrobial chemotherapy*, **2001,** 48**:** 5-16.
[15] Eloff JN. *PlantaMedica*, **1998,** 64:711-713.
[16] Culberson CF. *Journal of Chromatography*, **1972,** 72: 113-125.
[17] Guleria S, Kumar A. *Journal of Cell and Molecular Biology*, **2006,** 5: 95-98.
[18] Gulluce M, Aslan A, Sokmen M, Sahin F, Adigu- zel A, Agar G, Sokmen A. *Phytomedicine*, **2006,** 13**:** 515-521.
[19] Tiwari P, Rai H, Upreti DK, Trivedi S, Shukla P. *American Journal of Plant Sciences*, **2011**, 2: 841-846.
[20] Baral B, Mahajan BL. *Journal of Microbiology, Biotechnology and Food Sciences*, **2011**, 1: 98-112.
[21] Ramamoorthy PKT, Lakshmanashetty RH, Devidas S, Mudduraj VT, Vinayaka KS. *Chiang Mai Journal* of *Science*, **2012,** 39**:** 76-83.
[22] Kumar SVP, Kekuda TRP, Vinayaka KS, Swathi D, Mallikarjun N, Nishanth BC. *International Journal of Biotechnology and Biochemistry*, **2010,** 6**:** 193–203.
[23] Ranković B, Kasović M. *Pakistan Journal of Botany*, **2012,** 44**:** 429-433.
[24] Tay T, Türk AÖ, Yilmaz M, Türk H, Kivanc M, *Z. Naturforsch*., **2004**, 59: 384-388.
[25] Yilmaz M, Türk AÖ, Tay T, Kivanc M. *Z. Naturforsch*., **2004**, 59: 249-254.