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RESEARCH ARTICLE

Efficacy and potency of lichens of Mizoram as antimycotic agents

AMRITESH C. SHUKLA[1*], M. CHINLAMPIANGA[1], ARCHANA VERMA[1], ANUPAM DIKSHIT[2] and D.K. UPRETI[3]
[1]*Department of Horticulture, Aromatic and Medicinal Plants, Mizoram University, Aizawl 796 004*
[2]*Biological Product Laboratory, Botany Department, University of Allahabad 211 002*
[3]*Lichenology Laboratory, National Botanical Research Institute, Lucknow 226 001*

ABSTRACT: Antifungal property of aqueous and acetone extracts of two lichens was investigated against plant pathogenic fungi viz., *Alternaria alternata, Aspergillus flavus* and *Penicillium italicum* shows strong efficacy against the test fungi. Acetone extracts of both the lichen spp. were more effective than aqueous extracts. Aqueous and acetone extracts of *Stereocaulon* sp. were more effective than *Ramalina sp*. The aqueous and acetone extracts of *Ramalina* sp. and *Stereocaulon* sp. at 50 µl/ml concentration contains broad fungi toxic spectrum. Based on these findings as well as after detailed *in vitro* investigations, the active constituents of lichens can be synthesized and used as a potential substitute of synthetic fungicides.

Key words: Antifungal activity, lichens, synthetic fungicides

Lichens produce a diverse range of secondary metabolites/ chemical products, many of which have been found to have antimicrobial activity (Lawrey, 1986; Elix, 1996; Land and Lundstrom, 1998; Shahi *et al*., 2001, Boustie and Grube, 2005). Most of these antimicrobial substances are phenolic derivatives (e.g. usnic acid) and have extremely low solubility's in water. The present finding deals with the evaluation of aqueous as well as acetone soluble substances from *Ramalina* sp. and *Stereocaulon* sp. against some common plant pathogens.

MATERIALS AND METHODS

Maintenance of fungus culture

The test fungi, *Alternaria alternata* (Fr.) Keissler, *Aspergillus flavus* Link, and *Pencillium italicum* Wehmer were maintained on potato dextrose agar medium. Seven day old cultures of each fungus were used for antifungal testing.

Isolation of active constituents

Lichens material (thallus) were collected from sub temperate climates of Mizoram, India; washed with distilled water and dried at room temperature. A stock solution of aqueous and acetone extract were prepared by macerating 10 g of lichen material in 20 ml each of sterile distilled water and acetone respectively, using a pestle and mortar followed by filtering through muslin cloth and a Millipore filter (pore size 0.22 mm).

Antifungal screening

The antifungal activity of the extracts was determined following the modified spore germination inhibition technique (MSGIT) of Shahi *et al* (1997) with a slight modification of Shukla (2010). Potato dextrose broth was prepared and amended with Penicillin G (5 mg/l) and Streptomycin sulphate (5 mg/l) in the medium at 40°C in order to prevent bacterial growth, as suggested by Gupta and Banerjee (1970). Culture discs containing spores (5 mm diameter) cut out from the 7 day old cultures, grown in petri dishes were transferred aseptically in flasks (100ml) containing the broth, and shaken well for even distribution of spores. The numbers of spores were counted per microscopic field using 'Modified Cytometer Technique' (MCT) (Shahi *et al*, 1997). The diameter of Microscopic field was measured by using micrometer and then the area and volume of microscopic field was calculated by the formula:

$$AMF = \pi r^2 \qquad VMF = (AMF)\ h$$

where,

AMF = area of microscopic field;

VMF = volume of microscopic field;

h = thickness of medium (in between slide and cover glass) 0.1 mm.

The number of spores (average count value of 5 microscopic fields) was counted just by eliminating the overlapped spores. The number of spores in the volume of microscopic fields (NSV) was calculated by the formula:

$$NSV = ANS/VMF$$

where,

ANS = average number of spores in microscopic field.

The volume of liquid medium (VLM) per microscopic field was calculated by the formula:

$$VLM = 2rh$$

The total inoculum density (TID) was calculated in the initial volume of medium as per formula:

$$TID = (NSV/VLM)\ IVM$$

where,

IVM = initial volume of medium.

*Corresponding author: amriteshmzu@gmail.com

The effective concentration of water and acetone extracts were determined by dissolving requisite quantity of extracts in water and acetone respectively (2% of the required quantity of the seed medium) and mixed it to the standardized inoculums suspension, in the culture tubes. In controls, sterilized water was used in place of the extracts. Culture tubes thus prepared were incubated at 27°±1°C and the observations were recorded at the interval of 24 hr up to 96 hr, by counting the number of germinated spores. Percent inhibition of spore germination (SGI) was calculated as per formula:

$$SGI\ (\%) = \frac{(Gc - Gt)\ 100}{Gc}$$

where,

Gc = number of spore germination in control;

Gt = number of germinated spore in treatment

Fungitoxic spectrum

The fungitoxic spectrum with the aqueous as well as acetone extracts of both the lichens (*Ramalina* and *Stereocaulon* spp), at 50.0 µl/ml was also determined against some plant pathogenic fungi viz., *Aspergillus parasiticus* Speare, *Cladosporium cladosporioides* (Fresenius) de Vries, *Curvularia lunata* (Wakker) Boedijin, *Colletotrichum capsici* (Syd.) Butler & Bisby, *C. falcatum* Went, *Fusarium oxysporum* Schlecht, *F. udum* de vries, *Helminthosporium maydis* Nisikado & Miyakel, *H. oryzae* Breda de Haan, *Penicillium implicatum* Biourge and *P. miniolluteum* Dierckx; using the MSGIT of Shahi *et al* (1997) with a slight modification of Shukla (2010).

All the experiments were repeated twice and each contained three replicates; the data presented in the tables are the mean values.

RESULTS

In vitro antifungal investigations

The data reveals that the percentage of spore germination inhibition with aqueous extract of *Ramalina* sp. in *A. alternata* ranged from 6.89 to 100.00 at the concentration 0.1 to 50 µl/ml respectively while with acetone extract it ranged from 17.24 to 100.00 at the same concentrations, percentage of spore germination inhibition with aqueous extract in *A. flavus* ranged from 8.33 to 100.00 at 0.1 to 50 µl/ml respectively while in acetone extract it ranges from 12.5 to 100.00 at the same concentrations. Inhibition with aqueous extract in *Pencillium italicum* ranged from 11.53 to 100.00 at 0.1 to 50 µl/ml, while with acetone extract it ranged from 11.53 to 100.00 at the same concentrations. Spore germination inhibition with aqueous extract of *Stereocaulon* sp. in *A. alternata* ranged form 10.34 to 100.00 at 0.1 to 50 µl/ml; for *A. flavus* ranged from 12.50 to 100.00 at the same concentrations and for *P. italicum* percent inhibition ranged from 15.38 to 100.00 at the respective concentrations.

Percent spore germination inhibition with acetone extract in *A.alternata* ranged from 13.79 to 100.00 at 0.1 to 50 µl/ml, in *A. flavus* ranged 16.66 to 100.00 at the same concentration and in *P. italicum* 15.38 to 100.00 at the respective concentrations (Table 1 & 2).

Table 1. Antifungal activity of *Ramalina* sp

Concentration	Spore germination inhibition (%)					
	Aqueous extract			Acetone extracts		
	Alternaria alternata	*Aspergillus flavus*	*Penicillium italicum*	*Alternaria alternata*	*Aspergillus flavus*	*Penicillium italicum*
00.00	7.25	6.00	6.50	7.25	6.00	6.50
00.10	6.89	8.33	11.53	17.24	12.50	11.53
01.00	17.24	20.83	23.07	27.58	25.00	23.07
05.00	31.03	29.16	34.61	55.17	41.66	42.30
10.00	51.72	41.66	46.15	62.06	54.16	53.84
20.00	94.60	82.66	86.25	100.00[s]	88.16	100.00[s]
50.00	100.00[c]	100.00[s]	100.00[c]	100.00[c]	100.00[c]	100.00[c]

[s]indicates static; [c]indicates cidal in nature

Table 2. Antifungal activity of *Stereocaulon* sp

Concentration	Spore germination inhibition (%)					
	Aqueous extract			Acetone extracts		
	Alternaria alternata	*Aspergillus flavus*	*Penicillium italicum*	*Alternaria alternata*	*Aspergillus flavus*	*Penicillium italicum*
00.00	7.25	6.00	6.50	7.25	6.00	6.50
00.10	10.34	12.50	15.38	13.79	16.66	15.38
01.00	34.48	25.00	38.46	34.48	29.16	30.76
05.00	44.82	41.66	46.15	58.62	45.83	50.00
10.00	58.62	50.00	53.84	65.51	58.33	61.53
20.00	98.12	88.60	96.20	100.00[c]	94.66	100.00[s]
50.00	100.00[c]	100.00[s]	100.00[c]	100.00[c]	100.00[c]	100.00[c]

[s]indicates static; [c]indicates cidal in nature

Table 3. Fungitoxic spectrum of *Ramalina* sp with aqueous and acetone extract

Fungi Tested	*Ramalina* sp (50.0 µl/ml)	
	Aqueous Extract	Acetone Extract
Plant Pathogens		
Aspergillus parasiticus	100[s]	100[c]
Cladosporium cladosporioides	100[c]	100[c]
Curvularia lunata	100[s]	100[c]
Colletotrichum capsici	100[c]	100[c]
C. falcatum	100[c]	100[c]
Fusarium oxysporum	100[c]	100[c]
F. udum	100[c]	100[c]
Helminthosporium maydis	100[c]	100[c]
H. oryzae	100[c]	100[c]
Penicillium implicatum	100[s]	100[c]
P. minio-luteum	100[c]	100[c]

[s]indicates static; [c]indicates cidal in nature

Table 4. Fungitoxic spectrum of Stereocaulon sp with aqueous and acetone extract

Fungi Tested	*Stereocaulon* sp (50.0 µl/ml)	
	Aqueous Extract	Acetone Extract
Aspergillus parasiticus	100[s]	100[c]
Cladosporium cladosporioides	100[c]	100[c]
Curvularia lunata	100[c]	100[c]
Colletotrichum capsici	100[c]	100[c]
C. falcatum	100[c]	100[c]
Fusarium oxysporum	100[c]	100[c]
F. udum	100[c]	100[c]
Helminthosporium maydis	100[c]	100[c]
H. oryzae	100[c]	100[c]
Penicillium implicatum	100[c]	100[c]
P. minio-luteum	100[c]	100[c]

[s]indicates static; [c]indicates cidal in nature

Fungitoxic Spectrum

The fungitoxic spectrum with the aqueous as well as acetone extracts of both the lichens (*Ramalina* and *Stereocaulon* spp), at 50.0 µl/ml contains a broad fungi toxic spectrum. Further, the nature of toxicity of the aqueous extract (50.0 µl/ml) of *Ramalina* sp. against the test fungi *Aspergillus parasiticus, Curvularia lunata* and *Penicillium implicatum* was static (a conc which checks the growth but could not kill the organism), however, with the acetone extract (50.0 µl/ml), it was cidal (lethal) against all the test fungi (Table 3). In case of *Stereocaulon* sp. the nature of toxicity with the aqueous extract (50.0 µl/ml), was static only against the *Aspergillus parasiticus*, however, it was cidal against all the test fungi, in aqueous as well as acetone extract (Table 4). The comparative analysis of the aqueous and acetone extracts of the lichens (*Ramalina* and *Stereocaulon* spp), with some synthetic fungicides viz., Benlate, Mancozeb, Thiram were also investigated (Table 5).

DISCUSSION

Antifungal efficacy of 50% ethanolic extract from some macrolichens*, Parmelia tintorum, Ramalina sp., Teloschistes flavicans, Usnea undulate* had been tested by Dikshit (1991) against pathogenic fungi *Aspergillus flavus* only. However, in the present investigation two moulds *A. flavus* and *P. italicum* and a filamentous fungus *A. alternata* have been selected to evaluate the broad spectrum antifungal activity of extracts. Aqueous extracts from *Nephroma articum, Heterodermia leucomela* (Shahi *et al.*, 2001) and *Parmelia cirrhatum* (Dikshit, 1991*)* have been tested for their antifungal efficacy against some plant and human pathogenic fungi and found encouraging results. However, in the present communication, the acetone extracts of *Ramalina* sp. and *Stereocaulon* sp**.** have been found more effective than aqueous extracts. To the best of our knowledge the antifungal activity as well as fungitoxic spectrum of aqueous and acetone extracts of the samples against tested plant pathogenic fungi is reported for the first time. Thus, the

Table 5. Comparative efficacy of the lichen extracts with some synthetic fungicides

Trade Names of Plant/ Synthetic Fungicides	Active Ingredients	Characteristics features	Plant Pathogens (MECs)		
			Alternaria alternata	*Aspergillus flavus*	*Penicillium italicum*
Ramalina sp	Extract *(aqueous & acetone)*	Renewable, biodegradable, non-residual toxicity.	20 µl/ml	50 µl/ml	20 µl/ml
Stereocaulon sp	Extract*(aqueous & acetone)*	Renewable, biodegradable, non-residual toxicity.	20 µl/ml	50 µl/ml	20 µl/ml
Benlate	Benomyl	Non-renewable, non-biodegradable and residual toxicity.	7.0 µl/ml	7.0 µl/ml	6.0 µl/ml
Mancozeb	Zn, Mn, Ethylene bis-dithiocarbamate (Zn 2%, Mn 16%, Ethylene bis-dithiocarbam 62%) (75% ww)	Non-renewable, non-biodegradable and residual toxicity	2.6 µl/ml	2.6 µl/ml	6.0 µl/ml
Thiram	Tetra methyl thiram disulfide (75%ww)	Non-renewable, non-biodegradable and residual toxicity	10.0 µl/ml	10.0 µl/ml	7.0 µl/ml

*MEC=Minimum Effective Concentration

finding will be useful for the conservation of taxa also by isolation, characterization and identification of the active principle(s), which could be used (after detailed *in vitro* and *in vivo* investigations), for the development of harmless and effective antifungal formulations.

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