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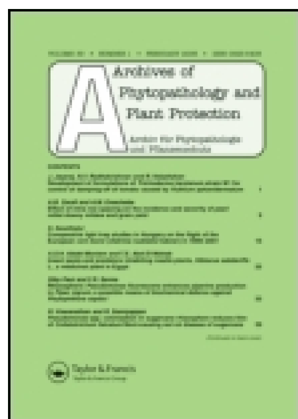

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I. M. Singha [a] [b] , B. G. Unni [b] , Y. Kakoty [b] , J. Das [a] , S. B. Wann [b] , L. Singh [a] & M. C. Kalita [c]

[a] Biotechnology Division, Defence Research Laboratory (DRDO), Tezpur, 784 001, Assam, India

[b] Biotechnology Division, North-East Institute of Science & Technology (CSIR), Jorhat, 785 006, Assam, India

[c] Department of Biotechnology, Gauhati University, Guwahati, 781 014, Assam, India

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Evaluation of *in vitro* antifungal activity of medicinal plants against phytopathogenic fungi

I.M. Singha[a,b], B.G. Unni[b]*, Y. Kakoty[b], J. Das[a], S.B. Wann[b], L. Singh[a] and M.C. Kalita[c]

[a]*Biotechnology Division, Defence Research Laboratory (DRDO), Tezpur 784 001, Assam, India;* [b]*Biotechnology Division, North-East Institute of Science & Technology (CSIR), Jorhat, 785 006, Assam, India;* [c]*Department of Biotechnology, Gauhati University, Guwahati 781 014, Assam, India*

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Fourteen medicinal plants belonging to 13 families were collected and extracted with petroleum ether (PE), chloroform, methanol and water to yield 60 crude extracts. Using agar diffusion method, these extracts were evaluated for antifungal activity on the growth of five phytopathogenic fungi. Among all the extracts tested, PE, chloroform and methanol extracts of *Piper betle* L. and PE and chloroform extracts of *Allamanda cathartica* exhibited promising antifungal activity. Minimum inhibitory concentration (MIC) values of the above promising extracts were determined using broth dilution technique and observed that chloroform extract of *P. betle* L. exhibited the least MIC value ranging from 280 to 1130 $\mu g\ ml^{-1}$. In this study, we report chloroform extract of *P. betle* L. to be thermally stable even when steam sterilised for the first time and that it could be stored at 4°C with almost no change in its activity for a period of 180 days.

Keywords: plant extracts; antifungal activity; MIC

1. Introduction

Outbreak of fungal diseases causes significant loss in many important vegetable crops and plants. Many fungi are harmful as they are pathogens of plants, animals and human beings or produce metabolites that are toxic to plants and animals (Richard et al. 1993; Bowers and Locke 2000). Generally, fungicides are used for control but despite their success, the use has not resulted in the complete eradication of pathogens. Moreover, indiscriminate use of fungicides has resulted in several adverse effects like development of resistance, resurgence of pathogens, toxic effects on beneficial microflora of the soil, residual toxicity to human beings, domestic animals, etc. and takes long time to degrade completely (Fawcett and Spencer 1970). Therefore, there is a need for more effective and less toxic new antifungal agents (Himejima and Kubo 1992; McCutcheon et al. 1992; Moossavi et al. 2001). Searching of plant derived fungicides is one of the novel approaches for replacement of harmful synthetics with safer botanicals. Many plants have been traditionally used

*Corresponding author. Email: bgunni@rrljorhat.res.in or bgunni@yahoo.com

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for the control of phytopathogenic fungus. Plants produce several secondary metabolite compounds including alkaloids, glycosides, flavonoids, saponins, steroids and terpenoids to protect themselves from the continuous attack of naturally occurring pathogens, insect pests and environmental stress (Ebel 1986). These compounds with antimicrobial activity can be explored and used for the control of fungal diseases and as antimicrobial agents. The objective of this study was to evaluate *in vitro* antifungal property of some medicinal plants found commonly in North East India against some selected phytopathogenic fungi.

2. Materials and methods

2.1. Plant materials

Fresh plant materials were collected from parts of North East India during September 2007 to April 2008. The botanical name, local name, family, parts used, moisture content, month as well as their site of collection are listed in Table 1. All samples were identified and specimens were deposited in the herbarium of Agriculture Cell of Defence Research Laboratory, Tezpur, Assam.

2.2. Preparation of extracts

Plant materials were shade dried, powdered and soaked with petroleum ether (PE), chloroform (C) and methanol (M) for 48 h at room temperature and filtered using Whatman filter paper No. 1. The filtrate was concentrated under reduced pressure using a rotary evaporator (Heidolph, Germany). In case of aqueous extract (W), the powdered plant material was soaked with water and heated to 60°C for 2 h, filtered and dried. Crude extract was reconstituted in an appropriate amount of 10% dimethyl sulfoxide (DMSO) and then sterilised with 0.22 μm filter membrane (Millipore^{TM}).

2.3. Test fungus

The fungus used in this study were *Fusarium oxysporum* (MTCC 8608); *F. oxysporum* f. sp. *conglutinans* (MTCC 8610); (MTCC 8474); *Curvularia lunata* (MTCC 8463) and *Rhizoctonia solani*. The fungal cultures were maintained on potato dextrose agar (PDA) medium. The test inoculum was adjusted at 1.1×10^6 spores ml^{-1} using a haemocytometer.

2.4. Antifungal activity

Preliminary antifungal activity of the plant extracts was tested using agar well diffusion method (NCCLS 1997; Garcia et al. 2002). Minimum inhibitory concentration (MIC) was determined using broth dilution technique (Kuzucu et al. 2004; Muschietti et al. 2005; Pawar and Puranik 2008) for plant extracts which had a good zone of inhibition (ZI). In broth dilution technique, serial dilutions of extracts were prepared in potato dextrose broth. An equal volume of fungal inoculum (50 μl) was added in all the tubes. The tubes were incubated at 28 ± 2°C for 72 h and observed for appearance of turbidity in the broth. The MIC value was interpreted as the highest dilution at which there was no turbidity or growth of the fungus in the broth when observed visually. These broths were re-inoculated on PDA

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Table 1. List of medicinal plants evaluated for antifungal activity.

Plant species	Local name[a]	Family	Part used	% moisture content	Collection site	Month gathered
Lawsonia innermis	Jetuka	Lythraceae	Leaf	79.10 ± 0.30	Tezpur, Assam	September
Dodonea viscose	Hege	Sapindaceae	Leaf	83.16 ± 0.60	Bhalukpung, Aru. Pra.	September
Dodonea viscose	Hege	Sapindaceae	Bark	72.60 ± 1.50	Bhalukpung, Aru. Pra.	September
A. calamus	Bosch	Araceae	Leaf	86.80 ± 1.21	Haleshwar, Assam	March
C. dactylon	Dubori bon	Poaceae	Leaf	82.73 ± 0.65	DRLT, Assam	January
M. oleifera	Sojina	Moringaceae	Flower	89.96 ± 1.07	DRLT, Assam	March
Cyclosorus extensus	Bihlongoni	Thelypteridaceae	Leaf	72.26 ± 0.70	Sonitpur, Assam	November
S. melongana	Bengena	Solanaceae	Leaf	69.66 ± 0.83	Haleshwar, Assam	December
P. betle L.	Paan	Piperaceae	Leaf	84.53 ± 0.55	Bhalukpung, Aru. Pra.	February
Mykenia scandens	Mykania lota	Asteraceae	Leaf	85.00 ± 1.10	DRLT, Assam	October
Eupatorium odoratum	Germany bon	Asteraceae	Leaf	69.66 ± 0.45	Bhalukpung, Aru. Pra.	December
Cassia sophera	Medeluwa	Cesalpinaceae	Leaf	81.83 ± 1.06	Sonitpur, Assam	December
Camellia sinensis	Chah	Theaceae	Leaf	77.60 ± 1.02	Sonitpur, Assam	November
Diplezium esculentum	Dhekia	Athyriaceae	Leaf	80.06 ± 1.20	Dalgaon, Assam	April
A. cathartica	Ghonta phool	Apocynaceae	Leaf	79.70 ± 0.75	DRLT, Assam	March

DRLT, Defence Research Laboratory, Tezpur; Aru. Pra., Arunachal Pradesh.
[a]Local names of the plant species given in Assamese language.

plates to confirm the fungistatic and fungicidal properties (Thompson 1989). Systemic fungicide carbendazim was used as positive control whereas sterile distilled water and DMSO were used as the negative control. Each treatment was replicated thrice and the experiment was conducted at least twice.

2.5. *Thermal stability and longevity of plant extracts*

Aliquots of plant extracts were stored in glass vials at 4°C and room temperature (26–36°C). Another vial containing steam sterilised plant extracts were stored at room temperature. Vials of each extract were taken out periodically (30 days) upto180 days and evaluated for their antifungal activity using the poisoned food technique (Perrucci et al. 1994). In this technique, appropriate amount of plant extract was incorporated in 15 ml of PDA and plated in 90 mm plates. Five millimetre fungal discs (7 days old) were inoculated in the centre of the PDA plates and incubated at 28 $\pm$ 2°C for 7 days. Percentage inhibition was calculated using the formula $I = (C - T)/C \times 100$, where I = percent mycelial inhibition of the fungus; C = growth of the fungus in control and T = growth of the fungus in treatment. Each treatment was replicated thrice.

3. Results

"W" extract of *Lawsonia inermis*, methanol extract from bark of *Dodonea viscosa*, "M" extract of *Acorus calamus*, "PE" extract of *Cynodon dactylon*, "M" extract of *Moringa oleifera*, "PE" extract of *Solanum melongana* exhibited a weak ZI (Table 2). "PE", "C" and "M" extracts of *Piper betle* L. and "PE" and "C" extracts of *Allamanda cathartica* exhibited promising antifungal activity with good ZI. Amongst all the plant extracts tested for the presence of antifungal activity, the "C" extract of *P. betle* L. had the maximum ZI against all phytopathogens under investigation. "C" extract of *P. betle* L. exhibited the least MIC value of 280 μg ml^{-1} against *Colletotrichum lindemuthianum*, 840 μg ml^{-1} against *C. lunata* and *R. solani*, 1130 μg ml^{-1} against *F. oxysporum* (F1) and *F. oxysporum* f. sp. *conglutinans* (F3). The extracts were observed to be fungicidal at their MIC values (Table 3). It was observed that, "PE" and "M" extracts of *P. betle* L. and "PE" and "C" extracts of *A. cathartica* lost their antifungal property when subjected to steam sterilisation. Interestingly, it was observed that "C" extract of *P. betle* L. was thermally stable even when steam sterilised and exhibited the same antifungal activity. After 180 days of storage at 4°C (Figure 1a), "C" extract of *P. betle* L. exhibited a lowest inhibition of 90% in case of "F3". When this extract was stored at room temperature (Figure 1b), a lowest inhibition of 88% was recorded against "F3" and when steam sterilised "C" extract of *P. betle* L. was stored at room temperature (Figure 1c), it exhibited lowest activity of 75% inhibition against "F3" at the end of 180 days.

4. Discussion

In this study, leaf extracts of *D. viscosa* exhibited no antifungal activity while methanol extract from barks of *D. viscosa* had a weak ZI against *C. lunata* whereas acetone extract of *D. viscosa* var *angustifolia* was reported to possess antifungal activity against *Candida albicans* (Patel and Coogan 2008). It was reported that "C" extract of *P. betle* L. exhibited significant antifungal activity against the fungus

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Table 2. Antifungal activity of plant extracts against phytopathogens.

Plant species	Solvent extract	% Yield	Antifungal activity				
			F1	Clin	Cluna	F3	Rs
L. inermis	PE	0.41	–	–	–	–	–
	C	0.76	–	–	–	–	–
	M	0.81	–	–	–	–	–
	W	1.64	+	+	+	+	+
Dodonea viscosa (leaf)	PE	0.75	–	–	–	–	–
	C	1.20	–	–	–	–	–
	M	1.80	–	–	–	–	–
	W	2.70	–	–	–	–	–
D. viscosa (bark)	PE	1.10	–	–	–	–	–
	C	2.15	–	–	–	–	–
	M	3.62	+	+	+	+	+
	W	5.42	–	–	–	–	–
A. calamus	PE	0.14	–	–	–	–	–
	C	1.17	–	–	–	–	–
	M	0.42	+	+	+	+	+
	W	0.48	–	–	–	–	–
C. dactylon	PE	1.31	+	+	+	+	+
	C	1.57	–	–	–	–	–
	M	2.10	–	–	–	–	–
	W	2.40	–	–	–	–	–
M. oleifera	P	0.57	–	–	–	–	–
	C	1.83	–	–	–	–	–
	M	0.92	+	+	+	+	+
	W	0.70	–	–	–	–	–
Cyclosorus extensus	PE	0.86	–	–	–	–	–
	C	1.20	–	–	–	–	–
	M	2.95	–	–	–	–	–
	W	4.60	–	–	–	–	–
Solanum melongona	PE	0.11	–	–	–	–	–
	C	0.33	–	–	–	–	–
	M	1.77	–	–	–	–	–
	W	2.15	–	–	–	–	–
P. betle L.	PE	0.18	++	++	++	++	++
	C	0.36	++	++	++	++	++
	M	0.70	++	++	++	++	++
	W	1.52	–	–	–	–	–
Mykenia scandens	P	0.91	–	–	–	–	–
	C	3.56	–	–	–	–	–
	M	1.87	–	–	–	–	–
	W	1.21	–	–	–	–	–
Eupatorium odoratum	P	0.64	–	–	–	–	–
	C	0.92	–	–	–	–	–
	M	0.73	–	–	–	–	–
	W	1.91	–	–	–	–	–
Cassia sophera	PE	0.84	–	–	–	–	–
	C	1.10	–	–	–	–	–
	M	1.61	–	–	–	–	–
	W	3.20	–	–	–	–	–
Camellia sinensis	PE	1.26	–	–	–	–	–
	C	1.80	–	–	–	–	–
	M	2.89	–	–	–	–	–
	W	6.60	–	–	–	–	–

(*continued*)

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Table 2. (*Continued*).

Plant species	Solvent extract	% Yield	Antifungal activity				
			F1	Clin	Cluna	F3	Rs
A. cathartica	P	1.10	++	++	++	++	++
	C	1.70	++	++	++	++	++
	M	2.80	–	–	–	–	–
	W	4.31	–	–	–	–	–
Carbendazim	Nil	Nil	++	++	++	++	++

–, implies absence of zone of inhibition; +, implies weak zone of inhibition; ++, implies good zone of inhibition; F1, *Fusarium oxysporum*; Clin, *Colletotrichum lidemuthianum*; Cluna, *Carvularia lunata*; F3, *Fusarium oxysporum* f. sp. *conglutinans*; Rs, *Rhizoctonia solani*; PE, petroleum ether; C, chloroform; M, methanol; W, water.

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Table 3. Minimum inhibitory concentration (MIC) of potent plant extracts against phytopathogens.

Test fungus	Minimum inhibitory concentration (μg ml^{-1})					
	P. betle L.			*A. cathartica*		
	PE	C	M	PE	C	Carbendazim
F1	3390	1130	9040	3500	7000	45
Clin	840	280	2800	2000	4000	15
Cluna	1680	560	4480	2250	4500	30
F3	3390	1130	9040	3000	6000	45
Rs	840	280	2800	2250	4500	40

PE, petroleum ether extract; C, chloroform extract; M, methanol extract; F1, *Fusarium oxysporum*; Clin, *Colletotrichum lidemuthianum*; Cluna, *Carvularia lunata*; F3, *Fusarium oxysporum* f. sp. *conglutinans*; Rs, *Rhizoctonia solani*.

Cladosporium cucumerinum, and five propenylphenols, viz., chavicol, chavibetol, allylpyrocatechol, chavibetol acetate and allylpyrocatechol acetate were reported to be the active compounds responsible for the antifungal activity (Evans et al. 1984). Leaf extracts of *P. betle* L. were also reported to completely inhibit spore germination of *Ustilago tritici* and *Ustilago hordei* (Mishra and Dixit 1979) and was found to be the best in reducing the growth of pathogens completely *in vitro* and *in vivo* against blast, brown spot and sheath blight diseases of rice (Tewari and Nayak 1991). Alcohol and other organic solvents tend to provide a more complete extraction of compounds with a variety of polarity (Evans 1996). Crude extracts are generally a mixture of active and non-active compounds (crude fusions) and therefore higher MICs are expected (Webster et al. 2008). Different extraction procedures employed may also result in the differences between studies (Rios and Recio 2005). Isolation and identification of active compounds associated for antifungal activity from "C" extract of *P. betle* L. may serve as a promising alternative for synthetic fungicides and may address the problem of fungal plant pathogens and pollution as well.

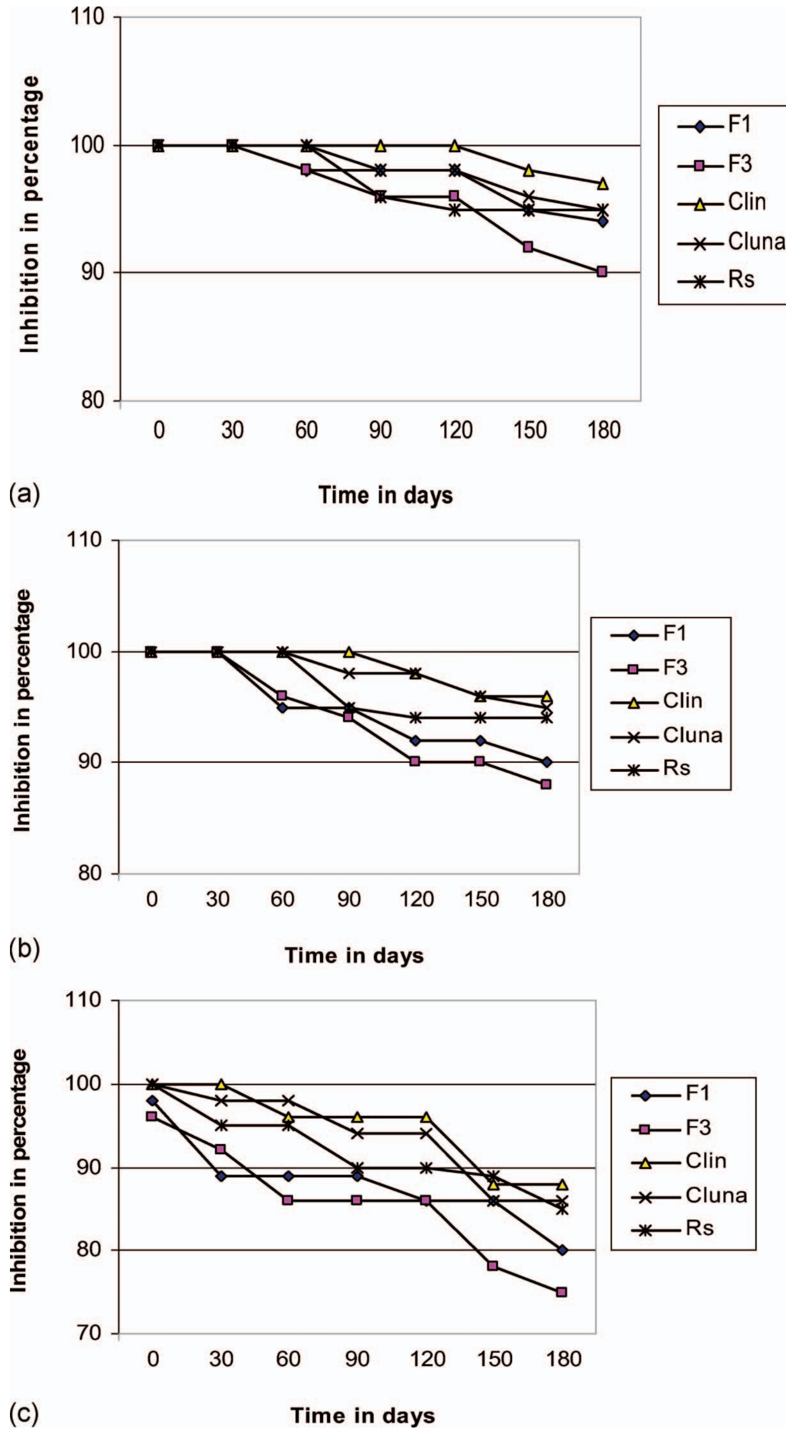

Figure 1. Thermal stability and longevity of chloroform extract of *Piper betle* L. (a) 4°C. (b) Room temperature. (c) Steam sterilised and stored at room temperature.

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