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Antifungal activity of some plant extracts against important seed borne pathogens of *Aspergillus* sp.

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Aqueous extract of fifty-two plants from different families were tested for their antifungal potential against eight important species of *Aspergillus* such as *A. candidus*, *A. columnaris*, *A. flavipes*, *A. flavus*, *A. fumigatus*, *A. niger*, *A. ochraceus*, and *A. tamarii* which isolated from sorghum, maize and paddy seed samples. The test fungi were mainly associated with seed biodeterioration during storage. Among fifty-two plants tested, aqueous extract of *Acacia nilotica*, *Achras zapota*, *Datura stramonium*, *Emblica officinalis*, *Eucalyptus globules*, *Lawsonia inermis*, *Mimusops elengi*, *Peltophorum pterocarpum*, *Polyalthia longifolia*, *Prosopis juliflora*, *Punica granatum* and *Sygigium cumini* have recorded significant antifungal activity against one or the other *Aspergillus* species tested. *A. flavus* recorded high susceptibility and hence solvent extracts viz., petroleum ether, benzene, chloroform, methanol and ethanol extracts of all the twelve plants were tested for their antifungal activity against it. Among the solvent extracts tested, methanol gave more effective than ethanol, chloroform, benzene and petroleum ether, except for *Polyalthia longifolia*, where petroleum ether extract recorded highly significant antifungal activity than other solvent extracts.

Key words: Antifungal activity, *Aspergillus*, *Prosopis juliflora*, *Mimusops elengi*

Introduction

Fungi are significant destroyers of foodstuffs and grains during storage, rendering them unfit for human consumption by retarding their nutritive value and often by producing mycotoxins (Marin *et al*., 1999; Janardhana *et al*., 1998). A significant portion of the agricultural produce in the country and the world over become unfit for human consumption due to mycotoxins contamination of grains, especially those produced by species of *Aspergillus* (Janardhana *et al*., 1999; Chandra and Sarbhoy, 1997; Devi *et al*., 2001). More than 25% of the world cereals are contaminated with known mycotoxins and more than 300 fungal metabolites are reported to be toxic to man and animals

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(Galvano *et al*., 2001). The main toxic effects are carcinogenicity, genotoxicity, terratogenicity, nephrotoxicity, hepatotoxicity, reproductive disorders and immunosuppression (Lacey, 1988; Desjardins *et al*., 2000). A sizeable portion of the world population living below poverty line in the developing and underdeveloped countries of Asia and Africa are suffering from health problems associated with consuming mycotoxin contaminated grains and cereals (Majumder *et al*., 1997). Eventhough effective and efficient control of seed borne fungi of seeds can be achieved by the use of synthetic chemical fungicides, the same cannot be applied to grains for reasons of pesticide toxicity (Ferrer and Cabral, 1991; Harris *et al*., 2001; Dukic *et al*., 2004). Thus, there is a need to search for alternative approaches to store grains/cereals for human consumption without toxicity problems that are ecofriendly and not capital intensive. Plant extracts of many higher plants have been reported to exhibit antibacterial, antifungal and insecticidal properties under laboratory trails (Satish *et al*., 1999; Okigbo and Ogbonnaya, 2006; Shariff *et al*., 2006; Bouamama *et al*., 2006; Ergene *et al*., 2006; Kiran and Raveesha, 2006; Mohana and Raveesha, 2006). Plant metabolites and plant-based pesticides appear to be one of the better alternatives as they are known to have minimal environmental impact and danger to consumers in contrast to the synthetic pesticides (Varma and Dubey, 1999). This led the authors to screen *in vitro* a large number of plants for antifungal activity against important seed borne *Aspergillus* species with the ultimate aim of developing plant based formulations for plant disease management and safe storage of grains.

Materials and methods

Plant material

Fresh disease free leaves of Fifty-two plant species were collected from Mysore, Karnataka, India (Table 1). A voucher specimen of all plants has been deposited in the herbarium of Department of Studies in Botany, University of Mysore, Mysore.

Table 1. List of plant species tested for antifungal activity.

Sl. No.	Name of the Plant	Family
1	*Acacia nilotica* (L.) Del.	Mimosaceae
2	*Aegle marmelos* Corr.	Rutaceae
3	*Aloe vera* Linn.	Liliaceae
4	*Anacardium occidentale* L.	Anacardiaceae
5	*Argemone mexicana* L	Papaveraceae
6	*Artocarpus heterophyllus* Lamb.	Moraceae
7	*Azadirachta indica* A. Juss.	Meliaceae
8	*Boerhaavia rependa* Willd.	Nyctanginaceae
9	*Caesalpinia coriaria* (Jacq.) Willd.	Caesalpinaceae
10	*Calotropis gigantea* R. Br.	Asclepidaceae
11	*Catharanthus roseus* (L.) G. Don.	Apocyanaceae
12	*Clerodendron inerme* Gaertn.	Verbenaceae
13	*Coleus aromaticus* Benth.	Lamiaceae
14	*Cuscuta chinensis* Lam.	Cuscutaceae
15	*Datura stramonium* L.	Solanaceae
16	*Delonix regia* Raf.	Caesalpinaceae
17	*Derris indica* (Lawk.) Bennet	Fabaceae
18	*Dolichos lablab* L.	Fabaceae
19	*Emblica officinalis* Gaertn.	Euphorbiaceae
20	*Eucalyptus globulis* Labill.	Myrtaceae
21	*Euphorbia tirucalli* L.	Euphorbiaceae
22	*Euphorbia pulcherrima* Willd.	Euphorbiaceae
23	*Hibiscus vitifolius* L.	Malvaceae
24	*Jacaranda acutifolia* Humb and Bonpl.	Bignoniaceae
25	*Lantana camara* L.	Verbenaceae
26	*Lawsonia inermis* L.	Lythraceae
27	*Leucas aspera* L.	Lamiaceae
28	*Macroslen parasiticus* (L.) Danser.	Loranthaceae
29	*Manilkara zapota* L.	Sapotaceae
30	*Mimosa pudica* L.	Mimosaceae
31	*Mimusops elengi* L.	Sapotaceae
32	*Morinda tinctoria* Roxb.	Rubiaceae
33	*Moringa oleifera* Lam.	Moringaceae
34	*Murraya koenigii* (L.) Spreng.	Rutaceae
35	*Oxalis corniculata* L.	Oxalidaceae
36	*Peltophorum pterocarpum* (DC.) Baker ex Heyne.	Caesalpinaceae
37	*Phyllanthus acidus* Linn.	Euphorbiaceae
38	*Phyllanthus amarus* L.	Euphorbiaceae
39	*Plumbago zeylanica* L.	Plumbaginaceae
40	*Polyanthia longifolia* HK. F & T.	Annonaceae
41	*Psidium guajuva* L.	Myrtaceae
42	*Punica granatum* L.	Punicaceae
43	*Salvia offinalis* L.	Lamiaceae
44	*Samanea saman* Prain.	Mimosaceae
45	*Sapindus laurifolius* Vahl.	Sapindaceae

Table 1. Continued...

Sl. No.	Name of the Plant	Family
46	*Spathodea campanulata* Beaur.	Bignoniaceae
47	*Syzygium cumini* (L.) Skeels	Myrtaceae
48	*Tabebuia argentia* Britt.	Bignoniaceae
49	*Tamarindus indica* L.	Caesalpinaceae
50	*Tinospora cordifolia* Miers.	Menispermaceae
51	*Tribulus terrestris* L.	Zygophyllaceae
52	*Viscum orientale* Willd.	Viscaceae

Preparation of extracts

Aqueous extract

Leaf samples (100 gm) of all plants were thoroughly washed, blot dried and macerated with 100 ml sterile distilled water in a blender (Waring international, New Hartford, CT, USA) for 10 min. The macerate was first filtered through double layered muslin cloth and then centrifuged at 4000 *g* for 30 min. The supernatant was filtered through Whatmann No. 1 filter paper and sterilized at 120 ^{0}C for 30 min., which served as the mother extract.

Solvent extract

Thoroughly washed mature leaves of all the test plants were shade dried and then powdered with the help of a blender. Twenty-five grams of the powder was filled in the thimble and extracted successively with petroleum ether, benzene, chloroform, methanol and ethanol using a Soxhlet extractor for 48 h. All extracts were concentrated using rotary flash evaporator and preserved at 5 ^{0}C in airtight brown bottle until further use. All the extracts were subjected to antifungal activity against the test fungi.

Test fungi

Seed samples (sorghum, maize and paddy) were plated on Czapeck-Dox-Agar (CDA), Malt extract-Salt-Agar (MESA) and subjected to Standard Blotter Method (SBM) to isolate the frequently occurring important seed-borne phytopathogenic fungi and storage fungi associated with these seeds. Eight species of Aspergillus viz., A. candidus, A. columnaris, A. flavipes, A. flavus, A. fumigatus, A. niger, A. ochraceus and A. tamari were isolated. The cultures of Aspergillus were maintained on CDA medium, which served as the test fungi for antifungal activity assay.

Antifungal activity assay

Aqueous extract

CDA medium with 25% concentration of the aqueous extracts of the test plants were prepared. About 15 ml of the medium was poured into each petriplate and allowed to solidify. Five mm disc of 7-day-old culture of the test fungi were placed at the center of the petriplates and incubated at 25±2 ºC for seven days. After incubation the colony diameter was measured in millimeter. For each treatment four replicates were maintained. CDA medium without the aqueous extract served as control. The fungitoxicity of the extracts in terms of percentage inhibition of mycelial growth was calculated by using the formula

$$\% \text{ inhibition} = dc - dt/dc \times 100$$

Where dc = Average increase in mycelial growth in control, dt = Average increase in mycelial growth in treatment (Singh and Tripathi, 1999).

Synthetic fungicides, viz., Blitox (copper oxychloride), Captan (Phthalimide), Dithane M-45 (Mancozeb) and Thiram (Tetramethyl thiuramidisulphide) were also tested at their recommended dosage (2gm l^{-1}) for antifungal activity by poisoned food technique.

Solvent extracts

One gram of each of the dried evaporated solvent extract of all the test plants was dissolved in 10 ml of methanol. 500 µl of each of the solvent extract was amended with 15 ml of CDA medium before solidification of the medium. The medium amended only with 500 µl of methanol served as a control. *A. flavus* was inoculated and percent inhibition of the mycelial growth was determined as described earlier.

Results

Aqueous extract

Among the fifty two plants screened, aqueous extract of *Acacia nilotica*, *Achras zapota*, *Datura stramonium*, *Emblica officinalis*, *Eucalyptus globules*, *Lawsonia inermis*, *Mimusops elengi*, *Peltophorum pterocarpum*, *Polyalthia longifolia*, *Prosopis juliflora*, *Punica granatum* and *Sygigium cumini* (Table 2) have recorded significant antifungal activity against one or the other *Aspergillus* species tested.

The percent of inhibition of aqueous extract of the twelve plants were more than 50% against all the test fungi except *Manilkara zapota, Polyanthia longifolia* and *Eucalyptus globules* against *A. ochraceus* and *A. tamarii*. A clear

difference in antifungal activity of aqueous extracts of other plants against all the eight test fungi was observed. The most resistant was being found in *A. flavipes* for aqueous extracts of *Acacia nilotica, Emblica officinalis, Lawsonia inermis. Prosopis juliflora, Punica granatum* and *Syzygium cumini* and *A. fumigatus* was found resistant to *A. flavipes*.

The resistance is also observed in all the test fungi against aqueous extracts of *M. zapota* and *Polyalthia longifolia* and in *A. ochraceus* and *A. tamarii* against *Eucalyptus globulus*. Among eight species of *Aspergillus* tested *A. niger* had recorded high susceptibility to aqueous extract of *A. nilotica, M. zapota, E. officinalis, Eu. globulis, L. inermis, M. elengi, P. juliflora* and *P. granatum*. The susceptibility of it was the highest compared to other species of *Aspergillus. A. candidus* being more susceptible next to *A. niger* for aqueous extract of *D. stramonium* and *Pelto. pterocarpum*.

Comparative efficacy of the aqueous extracts of yhe twelve plants with four synthetic fungicides such as Blitox, Capton, Dithane M-45 and Thiram revealed that, completer inhibition of mycelial growth of all the test fungi were observed only in Thiram.

Highly significant inhibition of mycelial growth of *A. niger* was observed in aqueous extracts of *A. nilotica, M. elengi* and *P. juliflora* compared to Blitox and Dithane M-45. Dithane M-45 recorded least activity compared to test plants extracts and other chemical fungicides.

Solvent extracts.

Aspergillus flavus, which recorded more susceptibility to aqueous extracts of test plants also recorded high susceptibility to methanol and ethanol extracts of all tested plants, excepted for *Polyalthia longifolia* extracts, methanol was being more effective followed by ethanol, chloroform benzene and petroleum ether (Table 3).

It is observed in case of *Polyalthia logifolia* that petroleum ether extracts is highly effective than other solvent extract and the activity is highly pronounced compared to aqueous extract. The percentage of inhibition was more than 90% in methanol extract of *A. nilotica, M. elengi* and *P. juliflora*.

Discussion

Pre and post harvest bio-deterioration and spoilage of grains, vegetables, fruits and agricultural produce due to infestation by insects and microorganisms may cause losses of up to 100%. Association of variety of

fungi including species of *Aspergillus* causing significant loss in seed quality and nutritional quality of grains have been reported (Koirala *et al*., 2005). World Health Organization (WHO) banned many agriculturally important pesticides due to wide range of toxicity against non-target organisms including humans, which are known to cause pollution problem (Barnard *et al*., 1997). Some of the developing countries are still using these pesticides despite their harmful effects. Excessive usage of pesticides in agriculture to over come the pre-harvest and post-harvest problem was resulted in many toxic epidemics. Generally, toxic synthetic fungicides are not exploited to prevent biodeterioration of grains during storage (Harris *et al*., 2001) even though they are exploited for improving seed quality. Thus, there is an urgent need to search for alternative method for prevention of biodeterioration of grains during storage without any toxicity to the consumer. Many higher plants produce economically important organic compounds, pharmaceuticals and pesticides. Hamburger and Hostettmann (1991) reported that the total number of plant chemicals may exceed 400,000 and out of it more than 10,000 are secondary metabolites whose major role in plant is defensive in nature. Thus, plant based secondary metabolites, which have defensive role may be exploited for the management of storage pest. However, the most species of higher plants have never been described surveyed. Their chemical or biologically active constituent which is potential to be used as new sources of commercially valuable pesticides remain to be discovered (Balandrin *et al*., 1985). This is mainly due to the lack of information on the screening/evaluation of diverse plants for their antifungal potential. Biologically active plant derived pesticides are expected to play an increasingly significant role in crop protection strategies. Exploitation of naturally available chemicals from plants, which retards the reproduction of undesirable microorganisms, would be a more realistic and ecologically sound method for plant protection and will have a prominent role in the development of future commercial pesticides for crop protection strategies, with special reference to the management of plant diseases (Varma and Dubey, 1999; Gottlieb *et al*., 2002).

Considering these as a first step, in the present investigation fifty-two plants were screened *in vitro* for antifungal activity against important seed borne phytopathogenic *Aspergillus* species. These plants were selected based on traditional medicine knowledge and random choosing from the local flora. The screening revealed that only eight plants were effective in inhibiting the mycelial growth of test fungi by poisoned food technique at 25% concentration. The finding of the present investigation is an important step towards crop protection strategies for antifungal activity against important seed borne species of *Aspergillus*. Among the plants *Mimuspos elengi, Punica*

granatum, *Prosopis juliflora*, *Lawsonia inermis*, *Datura stramonium* and *Emblica officinalis* would probably be an important candidate plants for prevention of biodeterioration of grains during storage.

The present investigation is an important step in developing plant based pesticides which are ecofriendly for the management of the seed borne fungi and development of commercial formulation of botanicals. Further investigation will be done for developing commercial formulation based on field trail and toxicological experiment. It is important observation that all the biomolecules are polar in nature with their solubility more to water, methanol and ethanol.

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Table 2. Antifungal activity of different plant extracts at 25% (v/v) concentration against *Aspergillus* sp.

Sl. No.	Plant species	Pathogen							
		Aspergillus candidus	***Aspergillus columnaris***	***Aspergillus flavipes***	***Aspergillus flavus***	***Aspergillus fumigatus***	***Aspergillus niger***	***Aspergillus ochraceus***	***Aspergillus tamarii***
1	*Acacia nilotica* (L.) Del.	67.00±1.29	80.00±1.82	64.75±0.85	75.25±1.31	82.00±1.29	91.00±1.29	85.00±1.29	84.25±1.79
2	*Datura stramonium* L.	87.25±0.85	62.00±0.91	66.75±1.11	75.00±1.08	67.50±1.19	77.00±1.29	68.25±1.11	70.00±0.91
3	*Emblica officinalis* Gaertn.	75.75±1.11	71.50±1.19	64.25±1.31	69.50±1.10	79.50±1.32	86.75±1.49	80.50±1.04	76.75±1.88
4	*Eucalyptus globulus* Labill.	62.00±1.29	68.75±0.85	75.75±1.11	66.00±1.29	59.25±0.85	81.25±1.75	22.50±1.71	25.25±1.55
5	*Lawsonia inermis* L.	78.25±1.49	78.75±1.65	72.25±1.49	86.00±1.58	82.00±1.29	88.00±1.29	80.75±1.55	85.00±1.78
6	*Manilkara zapota* L.	44.75±0.48	44.75±0.85	35.75±0.85	31.00±0.41	34.75±0.85	48.25±0.85	45.75±1.11	45.75±1.11
7	Mimusops elengi	85.75±1.11	70.00±1.20	78.00±1.29	79.25±0.85	86.00±0.91	93.00±2.16	84.25±1.11	80.50±1.71
8	*Peltophorum pterocarpum* (D) Baker ex Heyne	74.25±1.25	69.50±1.04	57.25±0.85	57.50±0.64	53.25±0.85	60.25±1.11	60.25±0.75	55.25±1.70
9	*Polyalthia longifolia* HK.f & T.	40.50±2.72	41.00±0.41	38.75±0.63	36.50±0.64	36.25±1.11	41.50±0.64	32.75±0.85	48.50±1.19
10	*Prosopis juliflora* Swartz.	76.75±0.85	72.00±1.29	70.25±1.11	89.75±1.37	80.25±1.25	91.25±1.11	80.75±1.37	80.00±1.47
11	*Punica granatum* L	77.50±1.04	71.00±1.08	62.25±0.85	70.75±0.85	73.75±1.37	87.50±064	86.75±4.21	65.75±1.11
12	*Syzygium cumini* (L.) Skeels	68.75±0.85	74.00±1.29	55.25±0.85	62.25±1.11	69.50±0.64	70.25±1.37	88.75±0.85	76.50±0.64
13	Blitox	100±0.00	92.28±0.14	86.86±0.22	92.61±0.32	96.03±0.37	75.53±0.2	94.73±0.27	91.74±0.20
14	Captan	100±0.00	92.65±0.67	87.67±0.31	89.32±0.19	91.98±0.14	88.67±0.13	87.16±0.51	82.87±0.43
15	Dithane M-45	48.87±0.3	87.32±0.44	42.96±0.40	25.16±0.13	53.18±0.15	23.62±0.50	40.81±0.35	12.71±0.3
16	Thiram	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00

Data given are mean of four replicates ± Standard error

Table 3. Antifungal activity of different solvent extracts against *Aspergillus flavus*.

Sl.No.	Plant species	Petroleum ether	Benzene	Chloroform	Methanol	Ethanol
1	*Acacia nilotica* (L.) Del.	34.27±1.47	44.58±1.82	55.41±0.91	93.35±1.99	89.07±1.23
2	*Datura stramonium* L.	35.79±1.07	40.45±1.31	66.08±1.08	83.09±1.26	67.17±0.86
3	*Emblica officinalis* Gaertn	24.78±1.40	35.29±0.79	46.94±1.02	88.42±1.21	78.12±2.10
4	*Eucalyptus globulus* Labill.	35.28±0.82	47.74±0.85	56.38±1.83	82.48±1.32	72.38±1.66
5	*Lawsonia inermis* L.	35.67±1.60	38.06±4.37	58.14±4.13	89.32±2.70	78.76±3.14
6	*Manilkara zapota* L.	32.52±0.62	43.05±1.67	55.75±0.47	67.30±1.02	72.17±0.83
7	Mimusops elengi *L.*	31.95±1.47	55.71±0.78	60.61±0.64	93.42±2.23	80.22±2.45
8	*Peltophorum pterocarpum* (D) Baker ex Heyne	38.20±1.10	58.32±1.35	66.14±1.18	70.22±1.51	74.37±1.14
9	*Polyalthia longifolia* HK.f & T.	95.60±0.80	83.97±0.76	72.61±3.72	83.16±2.97	78.64±5.19
10	*Prosopis juliflora* Swartz.	33.24±0.62	50.38±3.58	67.05±8.68	97.74±1.15	88.48±0.98
11	*Punica granatum* L	43.99±1.49	50.06±0.76	65.22±1.12	87.34±1.04	74.79±1.45
12	*Syzygium cumini* (L.) Skeels	35.16±1.08	57.32±1.12	65.37±1.81	73.40±1.01	76.25±1.10

Data given are mean of four replicates ± Standard error