Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Suppl 3, 2012

Research Article

IN VITRO AND *IN VIVO* ANTIFUNGAL ACTIVITY OF *CASSIA LAEVIGATA*: A LESSER KNOWN LEGUME

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Received: 29 Dec 2011, Revised and Accepted: 3 Mar 2012

ABSTRACT

Cassia spp of caesalpiniodeae (*C. fistula*, *C. laevigata, C. renigera, C. biflora and C. siamea,*) have been of special interest due to their good therapeutic value in folk medicine. The present study was designed to evaluate the antifungal activity of some well and lesser known above mentioned *Cassia spp*. against five economically important fungal phytopathogens (*Aspergillus niger, Rhizoctonia solani*, *Macrophomina phaseolina*, *Fusarium oxysporum* and *Colletotricum falcatum).* Hexane, ethyl acetate, methanol and water extracts of five *Cassia* spp leaves were prepared and tested for their *in vitro* antifungal activity against above phytopathogenic fungi by agar well diffusion method. Among all the extracts tested, methanol extracts of *C. laevigata* and *C. fistula* leaf significantly inhibit the growth of *F. oxysporium* and *A. niger* on agar plate showing mean diameter of inhibition zone (MDIZ) of 2.6 cm *(F. oxysporium)* and 1.8cm *(A. niger)* against *C. laevigata* and MDIZ of 1.9 cm *(F. oxysporium)* and 1.4cm *(A. niger)* against *C. fistula.* The minimum inhibitory concentration (MIC) for the *C. laevigata* methanol extract was found to be 5 % against *F. oxysporium* and 7.5 % against *A. niger.* While methanol extract of *C. fistula* was found to have MIC of 7.5% against *F. oxysporium* and 10% against *A. niger*. Application of *C. laevigata* methanol extract on the 3week old *F. oxysporum* infected *Vigna ungiculata* plants decreases the defense related enzymes e.g. peroxidase, catalase and pectinase activity by 50 % compared to infected untreated plants. To the best of our knowledge there was no earlier reports regarding the antifungal properties of *C. laevigata* against different phytopathogenic fungi. Thus this report may be the first study suggesting its potent antifungal activity, which may serve as a new cost effective botanical fungicide to control pathogenic fungi in agricultural fields.

Keyword: *Cassia laevigata*, Antifungal, *Fusarium oxysporum*

INTRODUCTION

Past few years have been witnessed for a growing trend all over the world to shift from synthetic to natural products. Now its time to consider the neglected and little known botanicals to fight against various plant diseases, which create challenging problems in agriculture and cause real economic and environmental threats. The presence of inherent antimicrobial compounds in several higher plants has long been recognized for controlling various plant diseases caused by viruses, bacteria and fungi[1-5]. Pathogenic fungi alone causes nearly 20 per cent reduction in the yield of major food and cash crops[6]. Among the phytopathogenic fungi *Macrophomina phaseolina*, *Rhizoctonia solani, Colletotrichum falcatum, Fusarium oxysporum and Aspergillus niger* are the major pathogens causing root rot in groundnut, sheath blight in rice, red rot in sugarcane, wilt in pulses and crown rot in groundnut respectively[7-9].

Cassia spp of caesalpiniodeae have drawn attention due to their significant therapeutic value in traditional medicine. These plants are native to Southeast Asia, Africa, northern Australia and Latin America[10]. Members of *Cassia spp*. like *C. fistula*, *C. siamea, C. tora, C. senna* and *C. auriculata* are rich in bioactive molecules which are responsible for their antioxidant[11], antimicrobial[12] and antidiabetic activities[13], but the other lesser known spp like *C. renigera*, *C. biflora and C. laevigata* have not been studied well for their biological activity. Comparative antimicrobial potential of various *Cassia spp* also yet to be Examined. Keeping the above facts in consideration, the present study was designed to evaluate the antimicrobial activity with special reference to antifungal properties of some well and lesser known *Cassia spp.* against above mentioned five economically important fungal spp.

MATERIALS AND METHODS

Plant material

Leaves of five Cassia spp. (C. fistula, C. siamea, C. renigera, C. biflora, C. laevigata) were collected from the Botanical garden of Tamilnadu Agricultural University (TNAU), Coimbatore, India and authenticated by Professor J. Prabakaran, Biologist, TNAU. Five voucher specimens (CF001, CS001, CR001, CB001 and CL001 respectively) were kept in Centre for plant molecular biology biotechnology, TNAU, India.

Preparation of various solvent extracts

About 30 g of each leaf powder was percolated with 150 ml of various solvents (in the order of increasing polarity) e.g., hexane, ethyl acetate, methanol, and water separately (at the ratio of 1:5) and kept for overnight for digestion. The extracts were then filtered using Whatman No. 4 filter paper. The procedure was repeated 3 times and the combined filtrates were concentrated using rotary vacuum evaporator (Rota vapor® R-210/R-215 equipped with Vacuum controller V-850, Buchi, Switzerland) at 40° C to obtain dried hexane, ethyl acetate and methanol extracts. The water extract was freeze dried by a Flexi-Dry MP™ Freeze-Dryer (Model- VP100D) which was then stored at refrigerated condition for further use. 300 mg of dried extract of each plant sample were dissolved in 3 ml of ethanol (10%) and used for the antifungal assays.

Test organisms and culture media

The fungal cultures of Aspergillus niger, Rhizoctonia solani, Macrophomina phaseolina, Fusarium oxysporum and Colletotricum falcatum used in the study were obtained from Department of Plant Pathology, TNAU, Coimbatore, Tamil nadu, India. The fungi were cultured and maintained on potato dextrose agar (PDA) medium (Potato - 250 gm, Dextrose- 20gm, Agar -20gm, Distilled water-1 litre). For the bioassay, a loopful of the organism was inoculated into 100 ml of the respective broth as the medium. The conical flasks were incubated at a temperature of 37° C in shaker.

In vitro antifungal activity by Agar well diffusion assay

The *In vitro* antifungal activity of various leaf extracts were evaluated by Agar well diffusion method[14]. Briefly 24 hrs old culture

(broth) was added to the autoclaved PDA media (1 ml culture/100 ml of media). Sterilized medium containing the fungal culture was mix properly and poured into the petri plates (15 ml media/Petri plate) and allowed to solidify. Each Petri plates were divided into four equal quarters using a marker pen. Using a sterile cork borer, wells of 9 mm in diameter were punctured in the plates containing the media @ one well in each quadrate. For each organism, 50 µl of the prepared plant sample was loaded in each well using sterilized dropping pipette. Three replications were taken for each microorganism; the negative control (ethanol) was also loaded in the same plate. The plates were incubated for 48 hrs and the observations were made by measuring the inhibition zone (halo like area), which indicates the absence of fungal growth around the well. The diameter of inhibition zone was measured and the mean diameter of inhibition zone (MDIZ) was calculated.

Determination of Minimum Inhibitory Concentration (MIC)

MIC is defined as the lowest concentration of the plant extracts at which no or minimal signs of mycelia growth were detectable visually. As an effective inhibition was found in C. laevigata and C. fistula methanol extracts, the MIC was determined only in those extracts. Different concentrations (2.5, 5.0, 7.5 and 10.0 per cent) of methanol extract were tested for MIC by the method explained in above section.

In vivo* antifungal activity of methanol extract of *C. laevigata* against *F. oxysporum

As methanol extract of *C. laevigata* exhibited maximum antifungal activity against *F. oxysporum*, only this extract was tested for its *in vivo* efficacy. Clay loam soil collected from fields was dispensed in plastic pots (4 cm diameter, 8 cm depth, 250 gm soil/pot). Pots were divided into 3 groups. The first consisted of healthy (control) cow pea (*Vigna ungiculata*) plants, second Infected with *F. oxysporum* and untreated, third infected and treated with MeOH extract of *C. laevigata*, Cow pea plants were infected by spraying 10 ml of *F. oxysporum* spore suspension, on to the 2 week old shoots. After one week infected plants of each pot were sprayed with 10 ml of MeOH extract. Thereafter, plants in each pot were left to be air-dried, sprayed with 15 ml of distilled water and covered with plastic bags for 2 h to maintain the high humidity atmosphere around the leaves. After four week of sowing, leaves were harvested and prepared for the assay of various defense related enzymes.

Biochemical analysis of defense related enzymes.

Extraction of enzyme

The cow pea (*Vigna ungiculata*) leaves from different treatments were collected and homogenized immediately with 2 ml of 0.05 M Sodium acetate buffer (pH 5.0) at 4° C. The homogenate was centrifuged for 20 min at 10,000 rpm. The supernatant was used as crude enzyme extract for enzyme assay.

Assay of peroxidase (EC 1. 11. 1.7)

In case of peroxidase, the assay mixture contained 0.1 M sodium phosphate buffer (pH 5.8), 7.2 mM tetraguaiacol, 11.8 mM H_2O_2 and 0.1 ml of crude extractin a final assay volume of 3 ml[15]. The reaction was initiated by adding H_2O_2 and the change of absorbance was recorded at 470 nm. Peroxidase activity was calculated using the extinction coefficient (26.6 mM/cm at 470 nm) for tetraguaiacol.

Assay of catalase (EC 1. 11. 1. 6)

Catalase was assayed according to the previously described method[15] by measuring the initial rate of H_2O_2 disappearance. A sample of 0.1 ml of crude extract was added to 3 ml of the reaction mixture containing 0.1 M sodium phosphate buffer (pH 7), and 2 mM H_2O_2. The breakdown of H_2O_2 was followed by measuring the absorbance change at 240 nm and the enzyme activity was calculated using the extinction coefficient (40 mM /cm at 240 nm) for H_2O_2.

Assay of Pectinase (EC 3. 2. 1. 15)

Pectinase activity was assayed as previously described method[16]. The reaction mixture contained 0.8 ml of 0.5% sodium polypectate in 0.2 M sodium acetate buffer (pH 4.8) and 0.2 ml of crude extract. After 1 hr incubation at 30^0 C, pectinase activity was determined by measurement of the release of reducing groups.

RESULTS

***In vitro* antifungal activity screening**

In this study five *Cassia spp*. were screened against five phytopathogenic fungi. It was found that among four different solvent tested, only methanol extract exhibit maximum antifungal activity. Among the five plant species used only *C. laevigata* and *C. fistula* exhibited maximum antifungal activity against *Fusarium oxysporum* and *Aspergillus niger,* whereas other plant extracts exhibited either no inhibition or very minimum inhibition (Table.1). So the methanol extract of the above two plant species were tested for minimum inhibitory concentration.

The methanol extract of *C. laevigata* leaves were found to be effective against *Fusarium oxysporum* (Fig. 1A) and *Aspergillus niger* (Fig. 1B) with mean diameter of inhibition zone (MDIZ) 2.6 cm and 1.6 cm respectively. The results were also compared with the control (ethanol) taken in the same plates. It was also found that the inhibition against *F. oxysporum* was more (MDIZ-2.6 cm) than inhibition to A. niger (MDIZ-1.6 cm). The methanol extract of *C. laevigata* was found to be ineffective against *R. solani, M. phaseolina* and *C. falcatum*. The results obtained from the the study of *C. fistula* leaf extract was similar that of like *C. laevigata*, but the inhibitory effect against the above two fungi was less (MDIZ for *F. oxysporum* and *A. niger* were 1.9cm and 1.4cm respectively, Fig.1C , Fig. 1D) as compared to *C. laevigata*. *C. fistula* methanol extract was also found to be ineffective against *R. solani, M. phaseolina* and *C. falcatum*..

MIC of *C. laevigata* and *C. fistula* methanol extract

Methanol extract of *C. laevigata* leaf exhibited MIC at 5 % (w/v) of the extract against *F. oxysporum* and 7.5 % (w/v) against *A. niger*,. Whereas the same extract of C. fistula leaf showed MIC at 7.5%(w/v) against *F. oxysporum* and 10%(w/v) against *A. niger*, Since, *C. laevigata* methanol extract exhibited minimum MIC (Table.2), the in vivo efficacy of this plant extract was examined in green house condition against *F. oxysporum*.

Table 1: Antifungal activity of various solvent extracts of *Cassia spp*. against phytopathogenic fungi

	C. fistula				*C. siamea*				*C. renigera*				*C. biflora*				*C. laevigata*			
	W	M	E	H	W	M	E	H	W	M	E	H	W	M	E	H	W	M	E	H
A. niger	+	++	+	-	+	+	-	-	+	+	-	-	+	+	-	-	++	+++	++	-
R. solani	-	+	-	-	-	+	-	-	-	-	-	-	+	+	-	-	+	++	+	-
M. phaseolina	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	-
F. oxysporum	+	+++	+	-	+	+	-	-	+	+	-	-	+	++	-	-	+++	++++	++	-
C. falcatum	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	++	+	-

- = No inhibition, + = < 5mm mean diameter of inhibition zone, ++ =5-10mm mean diameter of inhibition zone, +++ = 10-20mm mean diameter of inhibition zone,++++ = 20-30 mm mean diameter of inhibition zone

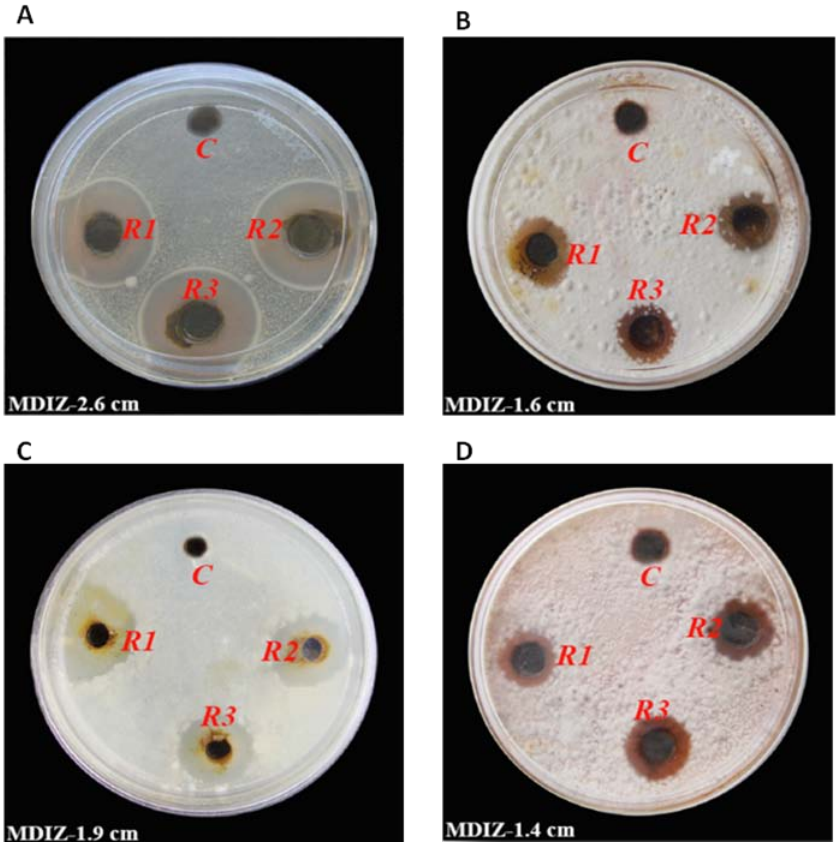

Fig. 1: **Inhibition of *F. oxysporum* and *A. niger* by methanol extracts of *C. laevigata* and *C. fistula* leaves**

A - Inhibition of *F. oxysporum* by *C. laevigata* methanol extract

B - Inhibition of *A. niger* by *C. laevigata* methanol extract

C - Inhibition of *F. oxysporum* by *C. fistula* methanol extract

D - Inhibition of *A. niger* by *C. fistula* methanol extract

C - Negative control (Ethanol)

R1, R2, R3 - Replication one, two and three respectively; MDIZ - Mean diameter of the inhibition zone

Table 2: MIC of methanol extracts of *C. laevigata* and *C. fistula*

	Methanol extract of *Cassia laevigata*				**Methanol extract of *Cassia fistula***			
	Conc.1 (10%)	**Conc. 2 (7.5%)**	**Conc. 3 (5%)**	**Conc. 4 (2.5%)**	**Conc. 1 (10%)**	**Conc. 2 (7.5%)**	**Conc.3 (5%)**	**Conc.4 (2.5%)**
Fusarium oxysporum	N	N	N	G	N	N	G	G
Aspergillus niger	N	N	G	G	N	G	G	G
Control(ethanol)	G	G	G	G	G	G	G	G

N- Inhibition, G-No inhibition, Conc.-concentration

In vivo* efficacy of *C. laevigata* methanol extract against *F. oxysporum

Since *C. laevigata* methanol extract showed highest antifungal activity against *F. oxysporum* and Fusarium wilt is an important fungal disease in pulses e.g. *Vigna* spp., methanol extract of *C. laevigata* was tested for their inhibitory effect on *F. oxysporum in vivo* considering *Vigna ungiculata* as a model plant. The level of plant defense related enzymes, e.g. peroxidase, catalase and pectinase against *F. oxysporum* pathogenicity were investigated. The activities of above enzymes were lowest in the healthy plants (3.72, 3.2 and 3.4units /mg of protein respectively) and they reached the highest levels (8.72, 5.8 and 7.35 units /mg of protein respectively) in infected untreated *Vigna ungiculata* leaves.

Moreover, activities of all the enzymes, in leaves of infected plants, decreased significantly (3.9, 3.3 and 3.53 units /mg of protein respectively) by methanol extract treatment (Table 3).

Table 3: Levels of plant defense related enzymes [unit /mg of protein] in leaves of cow pea plants in various treated groups

Plant treatment	**Peroxidase**	**Catalase**	**Pectinase**
Healthy (control)	3.72a	3.20a	3.41b
Infected untreated	8.72b	5.80c	7.35c
Infected and treated with MeOH extract	3.90c	3.30b	3.53a

Means in the same column followed by the same letter are not significantly different at the 0.05 level according to LSD

DISCUSSION

Plant diseases are the major biotic constraints to crop growth and causes variety of damage and significant yield loss. The disease management requires effective integration of approaches to reduce the crop loss effectively. Several strategies have been developed based on genetic, chemical, biological, cultural methods and also combined with integrated diseases management framework[17]. New approaches involving botanical fungicides are considered as an alternative to synthetic fungicides as they maintain low mammalian toxicity, target specificity and biodegradability[18].

In the present study, among all the solvent extracts tested, only methanol extracts of two *Cassia* spp. (*C. laevigata* and *C. fistula*) were found to be more effective to inhibit fungal growth than hexane, ethyl acetate and water extract, which may be due to the wide range of solubility of various polar compounds present within plant in methanol. Several other studies also showed that methanol extract of various plant sample are rich in antimicrobial agents[19-22], which is having some degree of similarity with our study.

There are several earlier reports on the antifungal activity of genus *Cassia*. Methanol extract of *C. fistula*, *C. alata* and *C. tora* leaf extract are inhibitory towards pathogenic fungi *Trichophyton rubrum*, *Microsporum gyseum* and *Penicillum marneffei*[23]. *C. tora* methanol extracts also having antifungal activity against *Pyricularia grisea*, *Phytophthora infestans* and *Erysiphe graminis*[24]. But there was no earlier report on the antifungal activity of *C. laevigata* in current literature, though few reports are there for antifungal activity of *C. fistula*[23]. But in this study it was observed that *C. laevigata* is having more antifungal activity than that of *C. fistula* probably due to presence of various flavonoid aglycones or their derivatives[25].

Methanol extract of *C. laevigata* leaf was found to be more effective than that of *C. fistula* against *F. oxysporum* and *A. niger*. Among the two fungi *F. oxysporum* was inhibited more effectively than *A. niger* by the methanol extract of *C. laevigata*. So *C. laevigata* methanol extract having MIC 5%w/v against *F. oxysporum* can be effectively used to control this fungi. To verify this hypothesis we design the *in vivo* experiment where we found, there is significant reduction in the level of defense related enzymes in *Fusarium* infected and subsequently treated *Vigna ungiculata* plants with methanol extract (10%) of *C. laevigata*. The rationale behind choosing this crop was, it mostly grown as a kharif crop, but can be grown as a rabi crop in peninsular India and *Fusarium* wilt is an important fungal disease of this crop[26]. A similar experiment was done by Mahmoud *et al.* (2004) to find out the efficacy of *Eucalyptus citriodora* methanol extract on *Botrytis fabae* infected Faba bean (*Vigna faba*) plants, where they observed a decrease in peroxidase and catalase activity to 50% as that of infected untreated Faba bean plant[27].

To best of our current understanding there is no earlier studies regarding the antifungal properties and uses of *C. laevigata* against different phytopathogenic fungi. Therefore this may be the first report showing that the methanol extracts of *C. laevigata* possesses higher antifungal activity especially with respect to *F. oxysporum*. Based on these results it may be concluded that the leaf extract of this lesser known legume can be used as a potent antifungal agent. Further study is required to investigate the chemical ingredients of the methanol extract of *C. laevigata* leaf that is responsible for the above mention activity.

Conflict of Interest

The authors declare that there is no conflict of interest involved in this study.

ACKNOWLEDGEMENT

We are grateful to the director of Centre for Plant Molecular Biology, Tamil nadu Agricultural University, for his keen interest in this study. This work was financially supported by Centre for Plant Molecular Biology, Tamil Nadu Agricultural University. GKP is thankful to Department of Biotechnology, New Delhi for his fellowship during this study.

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