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Synergistic effect of *Pongamia pinnata* bark and *Tamarindus indica* fruit extract against aflatoxin producing fungi i.e. *Aspergillus flavus*

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Abstract

Aflatoxin is the most toxic of many naturally occuring toxins produced by fungi. *Aspergillus flavus* and *Aspergillus parasiticus* are the major casual organisms. These co-exist and grow on almost all crops. *Pongamia pinnata* (Karanj) and *Tamarindus indica* (Imli) and other tree species producing non edible oil were screened for their possible antifungal activity. Methanolic fraction were assayed to control the fungi and significant reduction in fungus growth was observed when applied synergistically than the individual plant extract.It was found that Combination of both extract were more effective than the individual extract when tested alone i.e 62% inhibition of fungal growth as comapared to *Pongamia* bark alone (33.20%) and *Tamarindus indica* alone (34.12%).

Keyword: Aflatoxin, *Tamarindus indica*, *Pongamia pinnata*.

Introduction

Aflatoxin are the secondary metabolite i.e. the metabolite not required during the growth of microorganism and it was mainly produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* and in some cases by *Aspergillus nomius*. The most important group of toxigenic Aspergilli are the Aflatoxigenic molds, *A. flavus* , *A. parasiticus* and the recently described but much less common species *A. nomius* all of which are classified in *Aspergillus* section Flavi (Gams *et al.*, 1985). Although these three species are closely related and shares many similarities a number of characteristics may be used in their differentiation. *A. flavus* is widely distributed in nature but *A. parasiticus* is less wide spread, the actual extent of its occurrence being complicated by the tendency for both species to be reported indiscriminately as *A. flavus*. In a wide ranging survey of the mycoflora of commodities in Thailand. *A. flavus* was the most common species in the peanut and second most common (after *Fusarium moniliforme*) in corn. *A. nomius* was reported from both commodities. Soyabeans, mung bean, sorghum and other commodities also contained considerable population of *A. flavus* but *A. parasiticus*.

A. flavus and *A. parasiticus* have strong affinity with nuts and oil seeds, corn, peanuts and cotton seed are the most important crops invaded by these mold and in many instances, invasion takes place before harvest not during storage. Peanuts are invaded while still in the ground if the crops suffer drought stress or related factor (Cole *et al.*, 1982; Pitt *et al.*, 1991; Sanders *et al.*, 1981). In corn insect damage to developing kernels allow entry of Aflatoxigenic molds but invasion can also occur through the silks of developing

ears (Lilehoj *et al.*, 1980) cotton seeds invaded through nectaries. (Klich *et al*, 1984). Cereals and spices are common substrate for *A. flavus* (Pitt *et al*, 1991), but aflatoxin production in these commodities is almost always a result of poor drying, handling or storage and aflatoxin levels are rarely significant .

Significant amount of aflatoxin can occur in peanuts, corn and other nuts and oil seeds particularly in some tropical countries where crops may be grown under marginal condition and where drying and storage facilities are limited.

A. flavus can produce Aflatoxin B_1, B_2 and cyclopiazonic acid, but only a proportion of isolates are toxigenic. *A. parasiticus* produces Aflatoxin B_1, B_2, G_1 and G_2 but not cyclopiazonic acid, and almost all the isolate are toxigenic. *A. nomius* is morphologically similar to *A. flavus*, but like *A. parasiticus* produces B and G aflatoxin without cyclopiazonic acid. Because these species appear to uncommon, it has been little studied , so the potential toxigenicity of isolates is not known and practical importance of this species is hard to access.

There is an immense potential of active fractions from many biodiversity resources available in the country. The proposed study focuses on a systematic study with respect to antifungal potential of bioactive compounds of these resources in relation to fungal infestation and aflatoxin production in high risk groundnut, its oil and cake. *Pongamia pinnata Pierre* (Leguminosae) is commonly known as Karanja. It is distributed throughout Western Ghats and chiefly found in tidal forests of India (Krishnamurthi, 1969). Different parts of the plant have been used in traditional medicines for bronchitis, whooping cough, rheumatic joints and to quench dipsia in diabetes (Kirtikar *et al.*, 1995). Previous phytochemical examination of this plant indicated the presence of furanoavones, furanoavonols, chromenoavones, avones, and furanodiketones (Talapatra *et al.*, 1980,1982; Murty *et al.*, 1944; Rangaswami *et al.*, 1942; Sharma *et al.*, 1973; Pathak *et al.*, 1983; Toshiyuki *et al.*,1992). In the present communication, we describe the isolation and characterization of three new furano-.avonoid glucosides, pongamosides A-C (1–3), and anew .avonol glucoside pongamoside D (4). Tamarind (*Tamarindus indica L.*) belongs to the family Leguminaceae and grows naturally in many tropical and sub-tropical regions. In Thailand, two types of Tamarind are found in abundance, the so-called sweet and sour varieties. Tamarind is an important food resource for the Thai population. The flower and leaf are eaten as vegetables, while the germ obtained from the seed is used for manufacturing Tamarind gum, which is well known as a component of jelly (Phakruschaphan, 1982). Tamarind seeds are also reported to contain phenolic antioxidants, such as 2-hydroxy-30, 40-dihydroxyacetophenone,methyl 3,4-dihydroxybenzoate, 3,4-dihydroxyphenyl acetate and epicatechin (Tsuda *et al.*,1994). Extracts exhibit antioxidant potential by reducing lipid peroxidation *in vitro* (Tsuda *et al.*, 1993, 1994) and anti-microbial activity (De *et al.*, 1999). Pumthong (1999) described the antioxidant activity of extracts of Tamarind pericarp, and reported the presence of mainly polymeric tannins and oligomeric procyanidins but these were not identified or quantitated. From this stand point it was of interest to compare the polyphenolic content in methanolic extracts of Tamarind pericarp and seeds, utilizing the methods of Owen *et al.* (2000a, 2003b).

Material and Methods

Collection and extraction of plant materials

Pongamia bark were collected from the campus of Banthara Research Station of National Botanical Research Institute (CSIR), Lucknow and Fruits of *Tamarindus* were purchased from the local market of

Lucknow. Pongamia bark were then air dried to a uniform moisture level. Fruits of *T. indica* obtained were extracted in methanol using Polytron homogenizer (PT 6100 KINEMETICA). Methanolic extracts collected by filtration (Whatman No. 1) were concentrated under vacuum at low temperature using Rotary Evaporator of Heidolph, Switzerland. The residue then dissolved in 15% ethyl alcohol to get the desired concentration for the activities.

Maintenance of fungal strains

The strain of *Aspergillus flavus* MTCC2799 were obtained from Microbial Type Culture Collection from IMTECH, Chandigarh. The culture was maintained at 4 ± 1°C on Slants of Potato Dextrose Agar (PDA)

Antifungal assay

Antifungal activity of *P.pinnata* and *T. indica* was tested against aflatoxin producing fungal strains of *A. flavus* obtained from IMTECH, Chandigarh, India.

Preparation of Inoculum

The spore suspension was prepared as described by Fan & Chen (1999). *A. flavus* was grown on PDA (HiMedia) slant for 5 - 7 days at 25 ± 3°C and the spore were harvested by adding 10 ml of sterile water and aseptically dislodging the spore with a sterile inoculating loop. This was diluted to obtain desired concentration of spore suspension.

Agar Well Diffusion Method

The Potato Dextrose Agar media (HiMedia) was cooled down up to 40-45 °C after autoclaving and added desired amount or concentration of plant extract. Shaked it very well and poured the media in Petriplates. After solidifying the media, three wells of 8mm diameter were made in each Petriplates. Without addition of plant extract were used as controls. 40 µL of spore suspension contained 18 x 10^4 spores mL^{-1} of *A. flavus* were added to each wells and incubated at 28°C for 5 days. Fungal growth of both the treated and untreated control plates was measured at every 24 hrs for 5 days (Perea *et al.*, 1990).

The percentage of inhibition was calculated using the following formula (Rasooli *et al.*, 2004)

$$I = C - T / C * 100$$

Where **I** = percentage inhibition

C = radial growth in control

T = radial growth in treatment

Result and Discussion

Production of toxin are linked to fungal growth and the environment in which the grains/ cereals are stored (especially Relative Humidity and Temperature). Fungal growth and subsequent mycotoxin product in stored grain can be inhibited by physical method (aeration, modified atmosphere, etc.) or by fungistatic of which is propionic acid, acetic acid and sorbic acid are the most common used (Paster *et al.*, 1988). Report by several authors (Monzumi, 1978; Azzouz and Bullerman, 1982; Bahk and Marth, 1983; Bullerman *et al.*, 1980; Yin & Cheng, 1998; Hitokoto *et al.*, 1980) supports the fact that the extract of certain spices and herbs

of medicinal importance exhibit antifungal property. These natural antifungal agents can be potential exploited in controlling the growth of fungi consequently inhibiting aflatoxin formation (Yin and Cheng, 1998; Grayer & Harborne, 1994).

*M*ethanolic extract of *Pongamia* bark were tested for their antifungal activity against aflatoxin producing fungi. Fungal growth was significantly reduced in all the treatments as compared to that of control. Antifungal activity varied significantly among different treatments (Table 1). Methanolic extract of bark inhibited fungal growth from 26.44% to 33.20%.

Different concentrations i.e 500ppm, 1000ppm, 1500ppm and 2000ppm of methanolic extract of tamarind fruits were tested for their efficacy against aflatoxin producing fungi. All the concentrations tested were found to decrease fungal growth as compared to that of control. Percentage inhibition of fungal growth ranged from 11.60 to 34.12% at 500ppm and 2000ppm concentration, respectively (Table2). Another experiment was also set to study the synergistic effect of both extract. Percentage inhibition of fungal growth ranged from 39.75 to 62.72% at 500ppm and 2000ppm concentration, respectively it was found that at 2000 ppm i.e percentage inhibition increased approximately 50 times higher than individual extract.

Table 1. Efficacy of bark of *P. pinnata* against aflatoxin producing fungal strain *A. flavus*

Plant parts used	Concentration (in ppm)	Radial dia in mm after 72 hrs	Percentage inhibition in %
Bark	Control	30.21 ± 1.436	--
	500	22.22 ± 0.780	26.44
	1000	21.78 ± 1.080	27.9
	1500	20.35 ± 0.800	32.6
	2000	20.18 ± 0.980	33.20

Table 2. Efficacy of polar fraction of *Tamarindus indica* against *A. flavus*.

Plant parts used	Concentration (ppm)	Radial dia in mm after 72 hrs	Percentage inhibition
Fruit	Control	30.21± 1.436	0
	500	26.70 ± 0.025	11.6
	1000	25.03 ± 0.095	17
	1500	23.13 ± 0.145	23.4
	2000	19.9 ± 0.295	34.12

Table.3 Combined effect of methanolic extract *Tamarindus* Fruit and *Pongamia* bark against aflatoxin producing *A. flavus*.

Plant parts used	Concentration (ppm)	Radial dia in mm after 72 hrs	Percentage inhibition in %
Fruit + Bark	Control	30.21± 1.436	0
	500	18.20 ± 0.010	39.75
	1000	16.13 ± 0.085	46.6
	1500	12.45 ± 1.055	58.78
	2000	9.75 ± 0.470	62.72

In conclusion, the present study reports the antifungal properties of *P. pinnata* and *T.indica* extracts, which can be commercially exploited and applied to foodsystems. Further studies are needed on the isolation and characterization of individual compounds to elucidate their different antifungal components.

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