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Inhibitory action on the growth of *Aspergillus flavus* and degradation of Aflatoxin B1 by medicinal herbs

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Abstract

Aflatoxin contamination is a serious food safety issue throughout the World. The present study aims to determine the efficacy of the aqueous extracts of *Catharanthus roseus*, *Andrographis paniculata*, *Syzium aromaticum* and *Ocimum gratissium* for their potential in preventing aflatoxin (AFB1) contamination. The antifungal activities of these medicinal plants were tested against the inhibitory action on the growth of *A. flavus* and control of aflatoxin production (AFB1) and also the degradation of AFB1. Various concentrations of aqueous plants extracts were prepared and tested for their ability to inhibit the growth of *A. flavus* and subsequent aflatoxin production. All medicinal plant extracts effectively reduced the growth of *A. flavus* ranging from 14% to 72% under *in vitro* and subsequently inhibit the AFB1 production ranging from 3.4% to 86.7% at different concentrations. Among the plant extracts tested, *C. roseus* effectively reduced (72%) the growth of *A. flavus* followed by *A. paniculata* (70%), *O. gratissium* (60%) and *S. aromaticum* (50%). Similarly, *C. roseus* inhibited the maximum level (87%) of AFB1 produced by *A. flavus* under *in vitro* condition (liquid medium) when compared to *A. paniculata* (73%), *S. aromaticum* (34%) and *O. gratissium* (64%). *In-vitro* fungal studies showed that higher level of inhibition in the growth of *A. flavus* by *C. roseus* extract (65%) compared to other plant extract. Degradation of AFB1 in the mixture with plant extracts revealed a loss of over 90% of AFB1 suggested that, aqueous extract of *C. roseus* have the ability to degrade the aflatoxin B1 at maximum level followed by *A. paniculata* (85%), *S. aromaticum* (75%) and *O. gratissium* (70%). This study showed the inhibitory action on the growth of aflatoxigenic fungi and their aflatoxin by *C. roseus*.

Keywords: Medicinal plants, *A. flavus*, AFB1, Detoxification, TLC

Introduction

Aflatoxins (AFs) are secondary metabolites which primarily produced by the food-borne fungi *Aspergillus flavus* and *A.parasiticus* in wide variety of natural substrates such as grains, nuts, food products, etc. (Karami-Osboo et al., 2012). Aflatoxins are both acutely and chronically toxic to humans and animals (Elshafie et al., 2011; El-Nagerabi et al., 2012). Aflatoxins are carcinogenic and immunosuppressive metabolites produced by *A. flavus* and

A. parasiticus. These fungi infected the crops before and after harvest there by contaminating food and feed and threatening both human and animal health (Omidbeygi et al., 2007). These toxins have been incriminated as the cause of high mortality in livestock and some cases of death in human being (Egal et al., 2005). Among all classes of aflatoxins, aflatoxin B1 is known to be the most significant in terms of animal and human health risk (Coulombe, 1993). Thus, foods contaminated with these toxigenic fungi and presence of aflatoxin is a major concern which has received worldwide attention due to their deleterious effect on human and animal health as well as their importance in International food trade (Soubra, 2009). The Food and Agriculture Organization (FAO) estimates that at least 25% of world cereal production is contaminated with mycotoxins (Villa and Markaki, 2009). The plant-based, traditional medicine system continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care (Owolabi et al., 2007). In general, medicinal plants are the best source to obtain a variety of drugs (WHO). Therefore, such plants should be thoroughly studied for their properties, safety and efficacy (Nascimento et al., 2000). Earlier studies have demonstrated that the use of local plant products for the control of fungi mostly proving their efficacy *in-vitro* (Hsieh et al., 2001), but these products have not been sufficiently tested for their efficiency in controlling aflatoxin in stored crops. There is need to verify the efficacy of the plant products and their potential in reducing aflatoxin contamination. Over the years, efforts have been devoted to search for new antifungal materials from natural sources for food preservation (Boyraz and Ozcan, 2005; Reddy et al., 2010). Several edible botanical extracts have been reported to have antifungal activity (Reddy et al., 2009). *Trachyspermum ammi* and *zimmu* are having antifungal activity and the degradation capacity of AFB1 *in vitro* condition has been reported (Velazhahanetal., 2010; Karthikeyan et al., 2006). Some plants belong to Lamiaceae, Asteraceae, Apiaceae, Myrtaceae, Lauraceae were found to be active in reduction of AFB1-contamination primarily by inhibition of *A. flavus* growth and AFB1-biosynthesis (Reddy et al., 2009). However, there is a little information available for degrading AFB1-contamination by plants. The fungi *A. flavus* and *A. parasiticus* which often contaminate foods and feeds produce aflatoxin a secondary metabolite and the most potent naturally occurring carcinogen known. Aflatoxins are highly stable molecules which are extremely difficult to remove or destroy once formed in a commodity. Among biological and natural sources, medicinal plants and herbs have received major consideration regard to their relatively safe status and enrichment by a wide range of structurally various useful constituents. In the present study, the aqueous extracts obtained from leaves of medicinal plants viz., *Catharanthus roseus*, *Andrographis paniculata*, *Syzium aromaticum* and *Ocimum gratissium* were evaluated for their potential in preventing aflatoxin contamination. The antifungal activities of medicinal plants were tested against the inhibition of the growth of *A. flavus*, control of aflatoxin production (AFB1) and degradation of AFB1.

Materials and methods

Screening of plant extracts

The apparently healthy medicinal plants viz., *Andrographis paniculata, Catharanthus roseus, Syzygium aromatium* and *Ocimum gratissium* were collected during the year 2010 from in and around Thanjavur, Tamilnadu, India. It was taxonomically identified and authenticated. The plant leaves were washed thoroughly

with running tap water 2 to 3 times and once with sterile distilled water. These plants were then applied for extraction and tested for the ability to degrade the aflatoxin.

Preparation of Plant extract

The leaves from the air dried healthy plant materials (10g) were macerated with 10ml of sterile distilled water in a warning blender (Waring international, new hart-ford, CT, USA) for 5 min. The homogenate was centrifuged at 14,000 ×g for 15 min and the supernatant was made up to 10 ml with distilled water. This extracts were preserved aseptically in a brown bottle at 4ºC until further use.

Test fungi

The fungus, *A. flavus* were previously isolated and identified by PCR technique from an infected groundnut and maintained on Potato dextrose agar (PDA) medium under laboratory conditions.

***A. flavus* strain and culture conditions**

A. flavus strains were screened for their ability to the production of AFB1 was described by Singh et al. (1991). Our primary data for the screening of different strains for AFB1 production suggested that the strain AFG7 produced maximum level of AFB1 at *in vitro* condition (data not shown). This strain was used for further investigations. Spore suspension was prepared by growing the fungi on Petri dishes for 7 days with Potato dextrose agar (PDA) containing 50 mg/L of streptomycin. After incubation at 25°C, spores were harvested by adding sterilized distilled water on plates (10 ml). The spore suspension was filtered using cheese cloth and spores were counted using a haemocytometer and brought to a final concentration of 10^5 conidia/ml.

Efficacy of aqueous extracts on *A. flavus* growth and AFB1 production *in vitro*

Twenty mL aliquots of yeast extract sucrose broth (YES - yeast extract 20g, sucrose 200g, distilled water 1L) were prepared in 100 ml conical flasks and sterilized at 121^{o}C for 15 min. Various concentrations (2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 mg/ml) of aqueous plant extracts were added to liquid broth (Optimum temperature). A 10 µl from suspension contained 10^5 spore/ml of fungus was inoculated in each flask and shaken at 200 rpm for 7 days at 25±2°C. The control contained YES broth and 10 µl of fungal suspension. The fungal mycelium was harvested by filtrating to separate from liquid culture and dried at 60^{o}C for 24 h. The dry weight of mycelium was determined.

Determination of AFB1 production by *A. flavus* in liquid media

The culture filtrates (10 ml) obtained from above experiment was used for estimation of AFB1. Briefly, AFB1 was extracted three times with chloroform (1:1 v/v), pooled and concentrated *in vacuo* at 40^{o}C using a rotary evaporator. The crude extract was diluted in minimum amount of chloroform (2 ml) and AFB1 was estimated by thin layer chromatography (TLC) according to Razak et al. (2009) with minor modifications. Briefly, different volumes (1-5 µl) of sample extracts were applied to precoated TLC plates (TLC Silica gel 60 F254, Merck, Germany) along with standard AFB1 (0.5 µg/ml) obtained from Sigma Chemical Co. (St Louis, MO, USA). The plates were developed in Chloroform: Acetone: Water (88:12:1) in glass tanks covered with aluminum foil. After development, plates were dried and observed under long wavelength (365 nm). The intensity of the sample spots was compared with that of the standard spot. The AFB1 concentration was calculated according to Younis and Malik (2003).

Fungal growth Inhibition assay

Antifungal activity of plant extracts against *A. flavus* was determined by fungal growth inhibition assay as described by Fiori et al. (2000). The plant extract was sterilized using 0.2 µm disposable millipore filters (Millipore, Bedford, MA, USA) for antifungal activity. The sterilized leaf extract was mixed with PDA medium to provide different concentrations of aqueous extracts viz., 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 mg/ml. The sterilized media was poured in to the petriplates and allowed to solidify. A 7 mm diameter disc containing 5-day-old *A. flavus* culture in PDA medium was transferred to the centre of the treated petridish and incubated at room temperature (27±2ºC). PDA medium alone was used as control and the colony diameter was measured after four days.

Growth Degradation and Inhibition Formula

Percentage of loss/gain in mycelial dry weight was calculated by using the formula:

$100 \times (C-T)/C$

Where, C = mycelial dry weight in control, T= mycelial dry weight in treatment.

Degradation of aflatoxin B1 by medicinal plant extract

Five hundred µl of plant extract was mixed with 50 ng of aflatoxin in a microfuge tube and kept at 27ºC for 24 h in an incubator. After incubation, aflatoxin in the mixture was extracted with 300 µl of chloroform. The chloroform fraction was evaporated on a heat block at 60ºC and the residue was dissolved in 10 µl of methanol and analyzed by TLC. Ten µl of the chloroform extract was separated on 0.25 mm silica gel G TLC plate (Merck). The plate was developed using the solvent chloroform: acetone: water (88:12:1). The chromatogram was viewed under UV light (365nm) (Sandosskumar et al., 2007).

Results

Effect of aqueous plant extracts on growth of *A. flavus in vitro*

All plant extracts effectively inhibited the mycelia growth and dry weight of *A. flavus* under *in vitro* condition and subsequently inhibited the AFB1 production up to 87%. Among the plant extracts tested, *C. roseus* effectively reduced the growth of *A. flavus* maximum of 72% followed by *A. paniculata* (70%), *O. gratissium* (60%) and *S. aromaticum* (50%) (Table 1). Among the five different concentrations, 12.5 mg/ml of plant extracts showed maximum inhibition of *A. flavus* growth. Medium level was observed in 7.5 mg/ml and minimum level of *A. flavus* was observed in 2.5 mg/ml. In 10mg/ml concentration 66% of growth inhibition was observed in *C. roseus*, followed by *A. paniculata* (60%), *O. gratissium* (55%) and *S. aromaticum* (40%). Among the four plant extract tested, *C. roseus* effectively inhibited the growth of *A. flavus* in liquid media at *in vitro* conditions.

Effect of aqueous medicinal plant extracts on AFB1 production by *A. flavus in vitro*

All plant extracts potentially reduced AFB1 production ranging from 3%-87% at different concentrations. *C. roseus* at 12.5 mg/ml concentration inhibited the maximum level (87%) of AFB1 production by *A. flavus* under *in vitro* condition when compared to other plant extracts viz., *A. paniculata* (73%), *S. aromaticum* (34%) and *O. gratissium* (64%). Moderate level of inhibition was observed by the extracts of *C. roseus* (72%), followed by *O. gratissium* (54%), *A. paniculata* (52%) and *S. aromaticum* (32%) against AFB1 production by *A. flavus*. In 7.5 mg/ml concentration shows medium level of AFB1 production viz., *C. roseus* (58%), *O. gratissimum* (3%), *A. paniculata* (31%) and *S. aromaticum* (24 %). In 2.5 and 5.0 mg/ml concentrations, low level of AFB1 production was observed in liquid media.

The highest growth inhibition was observed in *C. roseus* leaf extracts in all the concentration tested.

Antifungal activity of Medicinal plants extract

Aqueous extracts of four medicinal plants (*C. roseus, A. paniculata, S. aromatium* and *O. gratissium*) were evaluated for their ability to inhibit *A. flavus* growth by poison food technique described by Reddy et al. (2009). Among the four plant extracts, maximum growth inhibition was observed in *C. roseus* extract (65%) followed by *S. aromaticum* (60%), *A. paniculata* (55%) and *O. gratissium* (50%) against control (Table 3). Among the different concentrations tested, 12.5 mg/ml showed maximum activity for all the plant extracts. The antifungal activity was increased with the increasing concentration of plant extracts.

Table 1: Efficacy of medicinal plant extracts on growth of *A. flavus* in liquid media.

Concentration (mg/ml of media)	*C. roseus*		*A. paniculata*		*S. aromaticum*		*O. gratissium*	
	Mdw (mg/ml)	%INH	Mdw (mg/ml)	%INH	Mdw (mg/ml)	%INH	Mdw (mg/ml)	%INH
2.5	365±2.6	27.0±1.1	430±3.5	14.0±0.9	392±3.5	21.0±0.1	385±3.8	23.0±0.5
5.0	285±2.3	43.0±1.3	350±1.8	30.0±1.2	365±2.9	27.0±0.8	325±4.2	35.0±0.8
7.5	225±2.8	55.3±1.5	280±3.6	44.0±1.6	324±3.2	35.2±1.2	295±2.9	41.0±1.2
10.0	170±1.8	66.0±2.3	200±2.4	60.0±2.3	298±1.4	40.4±1.6	225±2.6	55.1±2.1
12.5	140±1.0	72.0±0.0	150±1.2	70.0±2.1	250±1.2	50.0±0.1	200±1.4	60.0±1.1
Control	500±4.2	0.0±0.0	500±4.2	0.0±0.0	500±4.2	0.0±0.0	500±4.2	0.0±0.0

Mdw= Mycelial dry weight, %INH= % Inhibition over control
Values of results are expressed as Mean ± SD

Table 2: Efficacy of medicinal plant extracts on AFB1 production by *A. flavus* in liquid media.

Concentration (mg/ml of media)	*C. roseus*		*A. paniculata*		*S. aromaticum*		*O. gratissium*	
	AFB1 (µg/ml)	%INH	AFB1 (µg/ml)	%INH	AFB1 (µg/ml)	%INH	AFB1 (µg/ml)	%INH
2.5	13.5±0.5	9.0±0.3	13.8±0.9	8.0±0.4	14.5±0.9	3.4±0.0	14.5±0.6	3.4±0.0
5.0	10.4±0.2	30.7±1.1	12.8±0.5	14.7±1.3	13.6±0.5	9.4±0.3	11.9±0.7	20.7±1.1
7.5	6.2±0.5	58.7±1.3	10.3±0.2	31.4±1.0	11.4±0.7	24.1±1.2	10.2±0.9	32.0±1.5
10.0	4.2±0.2	72.0±1.6	7.2±0.4	52.0±1.2	10.2±0.2	32.0±1.3	6.8±0.2	54.7±1.9
12.5	2.0±0.2	86.7±1.0	4.0±0.1	73.4±1.0	9.8±0.1	34.0±1.6	5.4±0.1	64.0±1.4
Control	15.0±0.3	0.0±0.0	15.0±0.3	0.0±0.0	15.0±0.3	0.0±0.0	15.0±0.3	0.0±0.0

%INH= % Inhibition over control
Values of results are expressed as Mean ± SD

Table 3: Antifungal activity of plant extracts against *A. flavus*.

Concentration (mg/ml of media)	*C. roseus*		*A. paniculata*		*S. aromaticum*		*O. gratissium*	
	Growth	% INH	Growth	% INH	Growth	%INH	Growth	% INH
2.5	35 ± 0.2	56.2±0.3	60± 0.1	25.0±0.1	40± 0.1	50.0±0.2	68±0.6	15.0±0.2
5.0	38 ± 0.4	52.5±0.1	54± 0.8	32.5±0.1	50± 0.3	37.5±0.1	70±0.6	25.0±0.3
7.5	40 ± 0.8	50.0±0.2	55± 0.6	31.2±0.3	48± 0.3	40.0±0.0	62±0.4	22.5±0.4
10.0	34 ± 0.6	57.5±0.0	42± 0.5	47.5±0.1	43± 0.6	46.2±0.3	58±0.6	27.5±0.2
12.5	28 ± 0.2	65.0±0.2	36± 0.2	55.0±0.1	32± 0.2	60.0±0.1	40±0.2	50.0±0.1
Control	80± 0.3	0.0±0.0	80± 0.3	0.0±0.0	80± 0.3	0.0±0.0	80±0.3	0.0±0.0

%INH= % Inhibition over control (Fungal growth inhibition assay)
Values of results are expressed as Mean ± SD

The minimum growth inhibition was observed in 10 mg/ml of the concentration tested and the inhibition ranges are *C. roseus* (65%), *A. paniculata* (47%), S. *aromaticum* (46%) and *O. gratissium* (27%). Low level of growth inhibition was observed in 7.5 mg/ml concentration followed by 5.0 mg/ml and 2.5 mg/ml. Among the four plant extracts, *C. roseus* effectively inhibiting the growth of *A. flavus* compared to the other plant extracts tested.

Degradation of Aflatoxin B1 by medicinal plants

In *in-vitro* screening, four plant extracts were found to be effective against aflatoxin B1 degradation. Aqueous extracts obtained from four medicinal plants were evaluated for their ability to detoxify AFB1 by TLC. Among the four plant extracts, the extracts of *C. roseus*, showed maximum degradation of AFB1 after incubating 24h at 27°C. Figure.1 shows that AFB1 in the mixture revealed a loss of over 90 % of AFB1 suggesting that the aqueous extract *C. roseus* could degrade the toxin at maximum level followed by *A. paniculata* (85%), *S. aromaticum* (75%) and *O. gratissium* (70%). The present work suggested that the degradation potential of these important medicinal plants extract against to aflatoxin B1 and the maximum degradation was observed in *C. roseus* extracts compared to other medicinal plants.

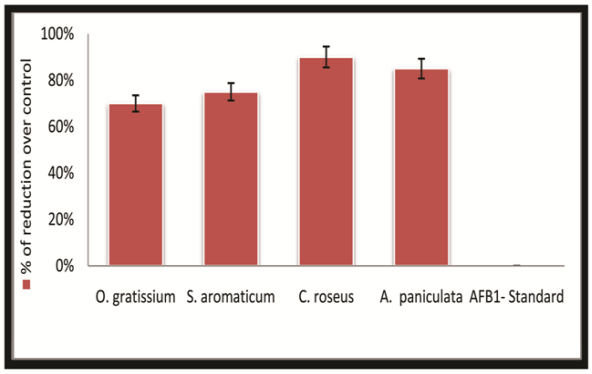

Fig. 1: Effect of leaf extract on AFB1 degradation.

Discussion

Detoxification of aflatoxin by plant products have been reported by several workers (Hajare et al. 2005; Sapcota et al. 2005; Sandosskumar et al. 2007; Velazhahan et al. 2010).The current study demonstrated that the antifungal activity of medicinal plants extract of *C. roseus, A. paniculata, O. gratissium* and *S. aromaticum.* Five different concentrations of plant extracts were used for the antifungal activity and found the maximum inhibition of *A. flavus* growth at 12.5 mg/ml in all the plant extracts. In addition, the extract of *C. roseus* reduced maximum mycelial dry weigh and maximum inhibition of *A. flavus* growth at this concentration. Control by naturally produced agents is becoming increasingly important because of consumers' mistrust of food and feed treatments that involve using synthetic xenobiotic substances. Natural plant compounds have been used traditionally to preserve foods in countries like Japan, India and Russia (Garcia-Cela et al., 2012). Murugan et al. (2007) found that maximum inhibition of *A. flavus* growth at 30 mg/ml concentration of *Euphorbia milli* dry powder. Our results showed that 14% - 72% reduction of mycelial dry weight of *A. flavus* by using different concentration of aqueous extracts. Further, extracts of *C. roseus* inhibited AFB1 production by *A. flavus* at maximum level compared to other plant extracts. Razak et al. (2009) found that the high reduction of aflatoxin B1 by using herbal extracts. It is widely accepted that higher concentrations of plant extracts are required in foods than in liquid media (Dikbas et al., 2008). Extracts and powders of various spices, herbs and essential oils have been reported to have antimicrobial activity against aflatoxin producing fungi and some of them also inhibit aflatoxin formation (Thanaboripat et al., 2004). Four plant extracts were found to be effective against aflatoxin degradation at *in vitro* condition. Aqueous extracts obtained from

four medicinal plants were evaluated for their ability to detoxify the AFB1. Among the four plant extracts, the extracts of *C. roseus,* showed maximum degradation of AFB1 after incubation for 24h at 27ºC.

Hajare et al. (2005) reported that the ajowan extract was effectively inactivated the aflatoxin. They proposed that ajowan extract act on the toxin molecule leading to its structural alteration or bind strongly to it, probably interfering in its detection. Lee et al. (1981) have observed that lactone ring plays an important role in fluorescence as well as toxicity of aflatoxin molecule. On its cleavage, the molecule became non fluorescent and showed remarkable reduction in toxicity. Sandosskumar et al. (2007) reported that *A. flavus* culture filtrate containing AFB1 was incubated with zimmu extract observed a complete degradation of AFB1 after 5 days of incubation. When the roots of whole zimmu plant were incubated in water containing AFB1, a reduction by 58.5% in AFB1 concentration was observed 5 days after incubation. In the absence of zimmu, AFB1 was stable in the solution as shown by the lack of change in the AFB1 concentration in solution. It is possible that the reduction in AFB1 content may be due to detoxification of AFB1 by root exudates of zimmu extract (Sandosskumar et al., 2007). Liu et al. (1998) have also demonstrated the importance of lactone ring in the florescence of toxin molecule. This loss of molecular fluorescence may be used as an indicator in tests of the efficacy of aflatoxin detoxification procedures. Velazhahan et al. (2010) have observed that degradation product of AFG1 suggests the modification of lactone ring structure. However, AFG1 after treatment with *T. ammi* extract failed to induce chromosomal aberration demonstrating the degradation of AFG1 by *T. ammi* extract. They recommended the ajowan extract is a biologically safe to protect poultry or livestock feeds and other agricultural commodities from aflatoxin B1. The present investigation shows the degradation potential of these four medicinally important plants extract against aflatoxin B1 and degradation of AFB1.

Conclusion

Aflatoxins are very important because the contamination of aflatoxins pose serious problems in public health, agricultural and economic aspects. Antifungal chemicals have been used for preservation of stored grains. Because of health and economic considerations, natural plant products may be replaced by toxic chemicals and provide an alternative method to protect from aflatoxin B_1 contamination. These could be better exploited in protection of all other agricultural commodities from growth and aflatoxin production by *A. flavus* as they are very abundant, simple to apply and cost effective. Fungal deterioration of stored seeds and grains is a chronic problem in the Indian storage system because of the tropical hot and humid climate. Harvested grains are colonized by various species of *Aspergillus*, under such conditions leading to deterioration and mycotoxin production. Aspergilli are the most common fungal species that can produce mycotoxins in food and feedstuffs. The overall results of this study showed that aqueous extracts obtained from medicinal plants were effective in reducing *A. flavus* growth and AFB1 production under *in vitro* condition. These plants extract may be used as potential source of sustainable eco-friendly botanical fungicides to protect from toxigenic fungi and their toxins under storage conditions. This study will pave way to facilitate further screening of various plant extracts to find effective novel antifungal compounds to control mycotoxigenic fungi and resultant no mycotoxins on food grains to ensure food safety.

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