African Journal of Microbiology Research Vol. 6(41), pp. 6931-6935, 27 October, 2012
Available online at http://www.academicjournals.org/ajmr
DOI: 10.5897/AJMR12.1455
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Prevention of aflatoxin contamination of maize by *Aspergillus flavus* through aqueous plant extracts in Saudi Arabia

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Accepted 10 September, 2012

The efficacy of four concentrations of aqueous extracts of 11 local plants in the management of *Aspergillus flavus* and aflatoxin contamination was investigated by measuring the dry weight of *A. flavus.* The extracts of *Allium sativum* gave the best results, decreasing the dry weight of the fungus, followed by *Aloe vera*, whereas 20% *Coriandrum sativum* extract had no significant effect on the fungal dry weight. Aqueous 20% extracts of the herb *Thymus vulgaris* and the rhizome of *Zingiber officinalis* most strongly inhibited aflatoxin production for B1 (79.1%), followed by the leaf extracts of *Olea europaea* and *Eucalyptus globulus* (75.0%), although the effect on *A. flavus* growth was moderate. The herb *T. vulgaris* and extract of *Ocimum basilicum* leaf showed the strongest inhibition of B2 (76.2%). Conversely, the leaf extracts of *Zizyphus spina* and *Cassia italica* produced only marginal effects on the percentage of inhibition of aflatoxins B1 and B2. No positive correlation was observed between mycelial growth and aflatoxin production in *A. flavus.*

Key words: Maize, *Aspergillus flavus*, aflatoxin, aqueous plant extracts.

INTRODUCTION

Aflatoxins are secondary metabolites of *Aspergillus spp.* molds, which can grow on a wide variety of agricultural commodities. Aflatoxins are toxic and carcinogenic, cause crop losses and represent a significant hazard to the food chain (Magan and Aldred, 2007). The use of plant extracts with anti-microbial and antifungal properties has been of wide interest in efforts to remove these aflatoxins from food and feed. *Aspergillus flavus* can produce the aflatoxins B1, B2, G1 and G2 in sorghum (Yassin et al., 2010). *Aspergillus spp* produce aflatoxins in starchy cereal grains, such as maize, wheat, sorghum, barley, millet and rice, and contamination can occur in the field, at harvest, during post-harvest operations and during storage. Additionally, the rate and degree of aflatoxin contamination are dependent on the temperature, humidity, soil and storage conditions (Mojatahedi et al 1974).

Maize is one of the most important cereal crops in the world, with a global harvested area of 159.53 million ha, of which 20.907 million ha are harvested in Saudi Arabia, and a total worldwide yield of 817.1 million tons (FAO, 2009). Aflatoxin contamination of maize is an important problem in warm, humid regions in which *Aspergillus* may infect the crop prior to harvest and remain viable during storage. Indeed, thousands of camels in Saudi Arabia may have been killed by the consumption of aflatoxin-contaminated fodder (Bokhari, 2010). Growing concerns about food safety have led to the development of natural antimicrobials for food preservation.

Plant extracts have demonstrated antimicrobial effects mediated by several compounds, such as phenolics, flavonoids, allicin, thiosulfinates, betalain and phytoalexins (Harris et al., 2001; Beevi et al., 2009), and there is an ongoing and urgent need to discover new antimicro-

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Table 1. Plant species, family, common name and plant parts used in the study.

Plant specie	Family	Common name	Plant part used
Allium cepa	Alliaceae	onion	B
Allium sativum	Alliaceae	Garlic	B
Aloe vera	Xanthorrhoeaceae	Aloe	L
Cassia italica	Fabaceae	*Cassia*	L
Coriandrum sativum	Apiaceae	Coriander	L
Eucalyptus globolus	Myrtaceae	Eucalyptus	L
Ocimum basilicum	Lamiaceae	Basil	L
Olea europaea	Oleaceae	olive	L
Thymus vulgaris	Lamiaceae	Thyme	L
Zingiber officinalis	Zingiberaceae	Ginger	R
Zizyphus spina	Rhamnaceae	Seder	L

L= Leaves, B= Bulbs, R= Rhizomes.

bial compounds with diverse chemical structures and novel mechanisms of action (Parekh and Chanda, 2010). Therefore, the goal of the present study was to investigate the antiaflatoxigenic activities of the aqueous extracts *Aloe vera, Cassia italica, Datura stramonium, Eucalyptus globulus, Lavandula vera, Lawsonia inermis, Olea europaea, Prosopis juliflora, Ricinus communis* and *Zizyphus spina*, as certain medicinal plants and spices have been reported to be useful in inhibiting aflatoxin production (El Shayeb and Mabrouk 1984; Madhyastha and Bhat 1985).

In the present study, the effects of each plant extract on *A. flavus* aflatoxin production were evaluated with the goal of developing a cost-effective method for preventing aflatoxin contamination of maize in Saudi Arabia.

MATERIALS AND METHODS

Fungal isolation

Ten samples of maize were collected from different localities of Riyadh, Kingdom of Saudi Arabia. The maize seeds were disinfected using 2% sodium hypochlorite for two minutes, rinsed three times in sterile distilled water and dried between layers of sterile filter paper (Whatman No. 1). Then, five grains were placed randomly onto potato dextrose agar (PDA) and incubated at 25 ± 2°C for 7 days. The isolates obtained were purified by the single-spore method and then transferred to PDA slants. The fungal isolates were identified based on their morphological and microscopic characteristics according to the method proposed by Dugan (2006).

Preparation of the plant extracts

Materials from eleven plant species are shown in Table 1 (*A. vera, C. italica, D. stramonium, E. globulus, L. vera, L. inermis, O. europaea, P. juliflora, Ricinus communis* and *Z. spina*) belonging to nine botanical families (Table 1) were washed with tap water, disinfected by immersion in 2% sodium hypochlorite solution for 30 min, rinsed with sterile distilled water to eliminate residual hypochlorite and dried in the shade.

The shade - dried materials of each plant species were then ground into a powder using a blender in distilled water at rate of 100 gm/100 ml water (For preparation concentration 100%). The macerated materials were squeezed through double cheese cloth sheets then filtered through filter paper whattman No.(1). The filtrates were centrifuged at 5000 rpm for 30 min and sterilized by Seitz's filter according to the method proposed by Ismail et al. (1989). Different concentrations were added to media before inoculation with the fungus.

Antifungal activity of the plant extracts

The antifungal activity was evaluated using the toxigenic *A. flavus* strain with Czapek Dox broth medium (sucrose, 30 g; sodium nitrate, 3 g; dipotassium phosphate, 0.5 g; magnesium sulfate, 0.5 g; potassium chloride, 0.5 g; ferrous sulfate, 0.01 g and distilled water, 1000 ml, pH, 6.5). The plant extracts were added to the medium at four concentrations (5.0, 10.0, 15.0 and 20.0%) before inoculation with the fungus. A control without plant extracts was included. Flasks were inoculated with 5 mm diameter discs of the toxigenic *A. flavus* isolate and incubated at 25 ± 2°C for 7 days. After incubation, the contents of each flask were filtered (Whatman No. 1), and the biomass of the filtered mycelia was determined after being dried at 70°C for 4 days until the weights stabilized. The percent mycelial inhibition was calculated using the following formula according to Mostafa et al. (2011).

Percent mycelial inhibition = [C – T / C] x 100

Where, C is the dry weight of the control (g), and (T) is the dry weight of the treatment with the tested extract.

Aflatoxin inhibition

The minimal inhibitory concentration (MIC) and antiaflatoxigenic efficacy values of each plant extract were determined using SMKY liquid medium (sucrose, 20 g; magnesium sulfate, 0.5 g; potassium nitrate, 3 g; yeast extract, 7 g and distilled water, 1000 ml) (El-Samawaty, et al., 2011). Different concentrations of each plant extract (5, 10, 15 and 20%) were prepared separately and added to 100 ml flasks, which were incubated at 25 ± 2°C for 7 days. After incubation, the cultures were blended using a high-speed homogenizer and filtered through Whatman No. 1 filter paper. The aflatoxins were extracted from the filtrates using a mixture of chloroform-acetone (9/1 v/v). The dried residues were dissolved in

Table 2. Antifungal screening of eleven aqueous plant extracts at five concentrations against the mycelial dry weight (g) of *Aspergillus flavus* isolated from maize.

Plant species	0%	5%	10%	15%	20%	LSD
Allium cepa	$^{A}3.83^{a}$*±0.19	$^{B}3.20^{c}$*±0.18	$^{B}3.15^{c}$*±0.04	$^{C}2.55^{b}$*±0.13	$^{C}2.72^{b}$*±0.03	0.417
Allium sativum	$^{A}3.83^{a}$*±0.19	$^{C}2.17^{e}$*±0.08	$^{B}2.80^{d}$*±0.07	$^{D}1.20^{d}$*±0.15	$^{E}0.60^{h}$*±0.03	0.374
Aloe vera	$^{A}3.83^{a}$*±0.19	$^{B}2.17^{e}$*±0.04	$^{C}1.54^{g}$*±0.06	$^{D}1.04^{d}$*±0.10	$^{D}1.00^{g}$*±0.00	0.314
Cassia italica	$^{A}3.83^{a}$*±0.19	$^{A}3.61^{ab}$*±0.08	$^{A}3.43^{b}$*±0.06	$^{B}1.80^{c}$*±0.16	$^{C}1.32^{f}$*±0.06	0.379
Coriandrum sativum	$^{A}3.83^{a}$*±0.19	$^{A}3.79^{a}$*±0.05	$^{A}3.84^{a}$*±0.05	$^{AB}3.34^{a}$*±0.19	$^{B}3.11^{a}$*±0.20	0.473
Eucalyptusgobuglobolus	$^{A}3.83^{a}$*±0.19	$^{B}3.29^{c}$*±0.07	$^{B}3.25^{bc}$*±0.10	$^{C}2.63^{b}$*±0.10	$^{C}2.27^{c}$*±0.16	0.406
Ocimum basilicum	$^{A}3.83^{a}$*±0.19	$^{B}2.64^{d}$*±0.04	$^{C}2.14^{f}$*±0.06	$^{D}1.78^{c}$*±0.07	$^{D}1.77^{de}$*±0.06	0.309
Olea europaea	$^{A}3.83^{a}$*±0.19	$^{B}2.77^{d}$*±0.05	$^{B}2.78^{d}$*±0.06	$^{C}2.15^{c}$*±0.09	$^{D}1.60^{ef}$*±0.05	0.318
Thymus vulgaris	$^{A}3.83^{a}$*±0.19	$^{B}3.19^{c}$*±0.07	$^{C}2.83^{d}$*±0.06	$^{D}1.88^{c}$*±0.06	$^{D}1.91^{d}$*±0.10	0.336
Zingiber officinalis	$^{A}3.83^{a}$*±0.19	$^{B}2.83^{d}$*±0.04	$^{B}2.42^{e}$*±0.16	$^{C}1.90^{c}$*±0.17	$^{C}1.80^{de}$*±0.10	0.443
Zizyphus spina	$^{A}3.83^{a}$*±0.19	$^{A}3.54^{b}$*±0.03	$^{B}2.47^{e}$*±0.14	$^{C}1.96^{c}$*±0.09	$^{C}1.93^{d}$*±0.08	0.374
LSD	0.543	0.228	0.254	0.369	0.281	

Values in the same column followed by an asterisk (*) are significantly different (P = 0.05). The data shown are the means (n = 3) ± standard error of three replicates , data followed by the same letter are not significant at P≤0.05, but followed by different letters are significant at P≤0.05).

a mixture of methanol: acetic acid: water (20:20:60 v/v/v), and the toxins were measured using high-performance liquid chromatography (HPLC) (model PerkinElmer series 200 UV/VIS) with a C18 column with an internal diameter of 100 x 4.6 mm. The total run time for the separation was approximately 25 min at a flow rate of 1 ml/min.

The aflatoxin inhibition was calculated according to Mostafa et al. (2011) as follows:

Percentage of inhibition toxin = [A – a / A] x 100,

where "A" is the concentration of aflatoxin in the treated sample and "a" is the concentration of aflatoxin in the control.

Statistical analysis

All measurements were replicated three times for each treatment, and the data were reported as the mean ± SE (standard error). The data were also analyzed statically using a one-way analysis of variance (ANOVA), and the differences among the means were evaluated for significance at P ≤ 0.05 using Duncan's multiple range test in SPSS, 16.1 (SPSS, Chicago, USA).

RESULTS

The efficacy of selected local plants in the management of *A. flavus* and aflatoxin contamination was investigated. The botanical and common names of the eleven plants belonging to nine families and the different parts are used in this study. The aqueous extracts of the plants were screened for their ability to reduce the dry weight and aflatoxin production of *A. flavus*. The effects of the 11 aqueous plant extracts on dry weight of *A. flavus* at four concentrations are shown in Table 2. The plant extracts show varying abilities to suppress *A. flavus* grown in a broth medium, and the inhibitory effect of the extracts increased in proportion to their concentrations, reaching a maximum at the highest concentration. The analysis of variance of the dry weight of *A. flavus* revealed interactions between the plant extract and extract concentration, and the interactions were highly significant (P ≤ 0.05) (Table 1). All the tested aqueous plant extracts caused a significant decrease in the dry weight at a high concentration. The aqueous extract of *A. sativum* was the most effective in inhibiting the fungal dry weight (0.60 gm), followed by 20% *A. vera* (1.0 gm) and 20% *C. italica* (1.32 gm), whereas 20% *C. sativum* (3.11 gm) was only marginally effective in reducing the fungal dry weight.

Table 3 reveals that all 11 aqueous plant extracts were effective inhibitors of *A. flavus* aflatoxin production at the four concentrations tested. The aqueous extracts of the herb *Thymus vulgaris* and the rhizome of *Zingiber officinalis* at 20% concentrations strongly inhibited B1 aflatoxin production inhibition (79.1%), followed by the aqueous leaf extracts of *O. europaea* and *E. globulus* (75.0%), although the *A. flavus* growth inhibition was moderate.

In contrast, the extracts of the herb *T. vulgaris* and the leaf of *Ocimum basilicum* were the most effective in inhibiting B2 (76.2%). Conversely, the leaf extracts of *Z. spina* and *C. italica* had only marginal effects on aflatoxin B1 and B2 production (16.6 and 9.5, and 25.0 and 23.8%, respectively).

The results of the present investigation suggest that plant extracts can be exploited for the management of mold infestation and the mycotoxin contamination of food. However, no correlation between the growth of the fungus and aflatoxin synthesis was found.

DISCUSSION

As alternative control measures to replace conventional synthetic pesticides, we screened eleven plant species (*Allium cepa, A. sativum, A. vera, C. italica, C. sativum, E. globulus, O. basilicum, O. europaea, T. vulgaris, Z. officinalis* and *Z. spina*) for their inhibitory effects on the toxin

Table 3. The efficacy of selected plant extracts as antiaflatoxins at 20%.

Plant specie	B1 (ppb)	Inhibition of aflatoxin B1 (%)	B2 (ppb)	Inhibition of aflatoxin B2 (%)
Allium cepa	7	70.8	8	61.9
Allium sativum	11	54.2	13	38.1
Aloe vera	10	58.3	12	42.8
Cassia italica	18	25.0	16	23.8
Coriandrum sativum	12	50.0	9	57.1
Eucalyptus globulus	6	75.0	7	66.7
Ocimum basilicum	7	70.8	5	76.2
Olea europaea	6	75.0	8	61.9
Thymus vulgaris	5	79.1	5	76.2
Zingiber officinalis	5	79.1	7	66.7
Zizyphus spina	20	16.6	19	9.5
Control	24	0.0	21	0.0

production by *A. flavus*. Aflatoxin contamination has been reported in many foods. In addition, plants such as *E. globulus, O. europaea, Salvadora persica, T. vulgaris* and *Z. spina-christi* have been studied for antifungal activities against *A. flavus* (Al-Rahmah et al., 2011). *A. sativum, Aframomum melegueta* and *Z. officinale* have been reported to inhibit aflatoxin production by *A. flavus* (Ayodele et al., 2009). Salim (2011) found that aqueous extracts of beet root, garlic, leek, radish and turnip could be applied as natural food preservatives against fungal mycotoxin production and that plant extracts can suppress aflatoxin biosynthesis.

The variation in the efficacies of the tested plant extracts against the toxigenic *A. flavus* may be due to the considerable variation in their constituents (Cavaleiro et al., 2006; Rosca- Casian et al., 2007). The anti-fungal properties of these extracts may also be attributed to their distinct phytochemical contents; these molecules, including alkaloids, phenols, glycosides, steroids, essential oils and tannins, may each cause different inhibitory effects (Mostei et al., 2003; Rasoolil et al., 2009).

Other researchers have suggested that the antimicrobial components of the plant extracts cross the cell membrane and interact with the resident membrane proteins, producing a flux of protons toward the cell exterior that induces changes in the cell and, ultimately, cell death (Omidbeygi et al., 2007). However, the inhibitory potential may depend on the mode of extraction and the concentration of the extracts. Plant extract production can be standardized for the effective use as alternatives to chemicals in the preservation of agricultural products against fungal rot during storage (Ayodele et al., 2009). However, according to Masood and Ranjan (1991) no correlation between fungal growth and aflatoxin synthesis was observed.

ACKNOWLEDGEMENT

The project was supported by the Research Center, College of Science, King Saud University.

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