International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (3), pp. 001-007, March, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Ephedra alata as biologically-based strategy inhibit aflatoxigenic seedborne mold

Al-Qarawi, A. A.[1], Abd_Allah, E. F.[1]* and Hashem Abeer[2]

[1]Department of Plant Production, College of Food and Agricultural Sciences, King Saud University, Saudi Arabia.
[2]Department of Botany and Microbiology, Faculty of Science, King Saud University, Saudi Arabia.

Accepted 11 January, 2019

Investigation has been carried out to evaluate the effect of range plant *Ephedra alata* as biologically-based strategy inhibit growth characters and aflatoxin production (*in vitro* and *in vivo*) of *Aspergillus flavus*. The aqueous extract of *E. alata* had significant inhibitory potential against growth as well as aflatoxin production by aflatoxigenic seedborne mold (*Aspergillus flavus*). Moreover, it has been found that, the addition of 1 and 2% (w/w) of plant powder material of *E. alata* to corn grains and soybean seeds respectively decreased the aflatoxin contamination and improve their nutritional value (total nitrogen content, fiber content, total lipids content and ash content) under storage conditions. The results observed here suggest employment of *E. alata* as an alternative non-chemical mean to control aflatoxin contamination of fodder in Saudi Arabia.

Key words: Biocontrol, Aflatoxins, *Aspergillus flavus*, *Ephedra alata*, nutritional value, Saudi Arabia.

INTRODUCTION

Aflatoxins are secondary metabolites produced by many strains of *Aspergillus flavus,* known to be potent carcinogens as well as hepatotoxic agents and pose serious hazards to human, animal health and milk production in many countries including Saudi Arabia. *Aspergillus flavus* are widely distributed in agricultural commodities like maize (Giorni et al., 2007) and soybean (Gupta and Venkitasubramanian, 1975). The death of thousands of camels in Saudi Arabia may have been caused by the consumption of these aflatoxins-contaminated fodders (Bokhari, 2010). Bioremediation of mycotoxins has been carried out by bacteria (Abd_allah and Ezzat, 2004), fungi (Abd_allah and Ezzat, 2005) and friendly chemicals such as chitosan (Abd_Allah and Hashem, 2006). Recently, impacts of natural products of plant origin have been introduced as non-chemical mean for biocontrol and bioremediation of mycotoxins in agricultural commodities (Parsaeimehr et al., 2010). Ephedra, a medicinal plant belonging to the Ephedraceae is a genus of non-flowering seed plants belonging to the Gnetales, the closest living relative of the angiosperm (Friedman, 1996). The antimicrobial potential of some Epedhra species such as *E. altissima* Defs (Tricker et al., 1987), *E. transitorai* (Al-Khalil, 1998), *E. nebrodensis* (Cottiglia et al., 2005), *E. major* (Bagheri et al., 2009) and *E. breana* (Feresin et al., 2001) has been recognized. *Ephedra alata* is a range plant with medicinal application, belonging to ephedra native of Asia including Saudi Arabia (Abourashed et al., 2003). Additionally, the foliage of *E. alata* has acceptable aroma and used as food stuff during animal grazing in Saudi Arabia (Al-Taisan et al., 2010).

The objective of the current study was to describe the antifungal activity of *E. alata* (aqueous extract of shoot system) grown in Saudi Arabia, against growth characters and aflatoxin production of local seedborne isolate of *Aspergillus flavus in vitro* and on both maize grains and soybean seeds under storage conditions. Also, the relevance of nutritional value of stored agricultural commodities (maize grains and soybean seeds) was studied in relation to storage treatments.

MATERIALS AND METHODS

Experimental mold

Local aflatoxin producing isolate similar to *Aspergillus flavus* link was isolated from soybean seed samples collected from Derab

*Corresponding author. E-mail: eabdallah@ksu.edu.sa.

Agriculture Research Station, Riyadh, Saudi Arabia. The mold was identified using current universal keys of identification (Domsch et al., 1993) by Dr. Abeer Hashem, Botany and Microbiology Department, Faculty of Science, King Saud University, Riyadh, Saudi Arabia.

Plant extract and antifungal evaluation

Fresh aerial parts (shoot system) of *E*. *alata* Decne were collected from wildlife Research and Development at Thumama, Riyadh, Saudi Arabia. Plant materials were dried in dark at room temperature (25±1°C) and powdered. Known weight of powdered material was extracted with aqueous ethanol (10:90, v/v) at 30°C for overnight and filtered through double layers of Whatman no. 1 filter paper. The extract was completed to known volume using aqueous ethanol as above. The extract was filtered using 0.22 micron cellulose acetate filter units (Whatman, Dassel, Germany) for sterilization. The antifungal activity of plant extract was determined by disc method (Roberts and Selitrenikoff, 1988), agar plate method (Bobbarala et al., 2009) and broth culture flask using glucose-ammonium nitrate salt medium (Brain et al., 1961). The production and germination of conidia were estimated according to the methods described by Wilson et al. (2004) and Ghaly et al. (1998) respectively.

Mycelial dry weight

At the end of incubation period, the culture broths were filtered through pre-weighed filter papers (Whatman no. 1). The filter papers with mycelial growth washed carefully with distilled water followed by drying at 80°C up to two successive constant weights were obtained. The net dry weight of mycelia was then determined.

Aflatoxin analysis

Replicated samples (each 50 ml of culture growth medium or 100 g of both maize grains and soybean seeds) were extracted using chloroform and cleaned up as described by Dutton and Westlake (1985). Column chromatography (AOAC, 1970) was carried out on silica gel G-60 mesh 0.02- 0.2 (BDH Chemicals, Poole, UK). Chemical and biological confirmatory tests to aflatoxin B_1 were carried out according to AOAC (1970) and Madhyastha et al. (1994), respectively. The sensitive strain of *Bacillus subtilis* was used throughout for biological confirmatory test (Abd_Allah and Ezzat (2004). Thin layer chromatography (TLC) plates (20 x 20 cm) coated with 0.5-mm-thick silica gel DG (Kieselgel-DG, Riedel- De Haen, Seelze-Hannover, Germany) were used for chromatographic separation of aflatoxin B_1 followed with elusion of aflatoxin B_1 spots using benzene: acetonitrile (98:2 [v/v]). Quantitative determination of aflatoxin B_1 was done spectrophotometrically according to Nabney and Nesbitt (1965). Standard aflatoxin B_1 (Sigma) was used as reference in all experiments.

Storage experiments

Storage experiment was carried out in polyethylene pouches (200 x 150 mm) each containing 100 g of blend consisted of powdered *E. alata,* mixed well with powdered maize grains or soybean seeds to give final concentration of 0.5, 1.0 and 2.0% (w/w). The pouches were inoculated with *A. flavus* (10^6 spores/pouch). Control pouches were used as reference. The moisture content was expressed as percentage of net weight (Abd_Allah and Hashem, 2006). The storage experiment continued for six months at room temperature (25 ± 1°C) in dark.

Estimation of nutritional values

The nutritional values (total lipid content, ash content, fiber content, total nitrogen content) of all plant materials were estimated according to AOAC (1995).

Statistical analysis

All experiments were repeated at least three times and treatment means were compared using Least Significant Difference (LSD) analysis according to Daniel (1987).

RESULTS

Thin layer chromatographic examinations indicated that, R_f -values and color of spots developed from both chloroform extract of *A. flavus* and standard aflatoxin B_1 were the same before and after chemical confirmatory tests. The first conformity test (addition of HCl) indicated the presence of one blue spot (under UV light 366 mm) with R_f -value 0.66. The second conformity (addition of conc. HCl + Acetanhydride) test indicated the presence of two spots with R_f -values were 0.10 and 0.42 respectively. Bioautographic examination (for both chloroform extract and standard aflatoxin B_1) against *Bacillus subtilis* (Biological indicator) indicated the presence of one inhibitor zone with R_f value was 0.78.

Preliminary experiment was carried out (*in vitro*) to demonstrate the antifungal potential of the plant (*E*. *alata*) extract against *A*. *flavus* using disc diffusion plate bioassay (Figure 1). The results in Table 1 revealed that the gradual concentrations of plant extract caused significant inhibition in both radial growth rate by percent of 41.55, 67.98, 88.83 and conidial production (Figure 2) by percent of 13.82, 50.00 and 90.42 of *A*. *flavus*, respectively .

Data in Table 2 shows the inhibitory effect of plant extract on the number of germinated conidia especially after 6 h (absolutely inhibition was observed). It was noted that, the prolongation of incubation period decrease the inhibitory effect potential of plant extract at all concentrations used (Table 2).

The results in Table 3 indicated clearly that all concentrations of plant extract induced significant inhibition in both mycelial growth (Figure 3) and aflatoxin B_1 production by percent of 33.49, 58.90, 79.76 and 65.75, 83.90, 100 respectively. It is worthy to mention that such inhibitory effect of plant extract was more intense towards aflatoxin B_1 production compared with mycelial growth at the same concentrations.

A storage experiment was carried out to investigate the antagonistic potential of *E*. *alata* against aflatoxin production by *A*. *flavus* on maize grains and soybean seeds (*in vivo*) with special reference to their nutritional value. The obtained data indicated that employment of *E*. *alata* at concentrations 0.5 and 1.0% caused significant inhibition in the accumulation of aflatoxin B_1 on maize grains and soybean seeds (Table 4) by percent of 63.81,

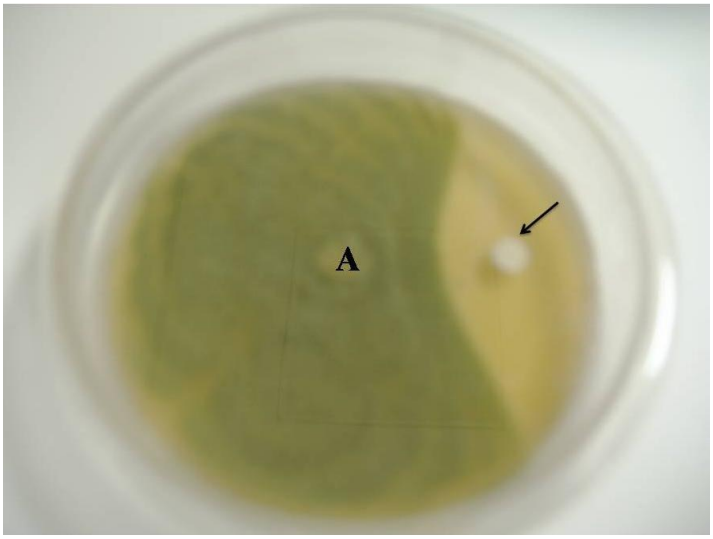

Figure 1. The antifungal activity of plant (*E*. *alata*) extract (arraw) against radial growth of *A*. *flavus* (A) using disc assay method.

Table 1. Effect of different concentrations of *E. alata* (w/v) on radial growth rate (cm day^{-1}) and conidial production ([conidia mm^{-2}] X 10^{4}) of *A. flavus* grown at 28±°C for 96 h in dark.

Treatment (*E. alata* w/v)	**Radial growth rate (cm day^{-1})**	**Conidial production (conidia mm^{-2} X 10^{4})**
Control	1.362	9.382
E. alata 0.5%	0.796	8.102
E. alata 1.0%	0.436	4.708
E. alata 2.0%	0.152	0.584
LSD at: 05	0.066	0.475

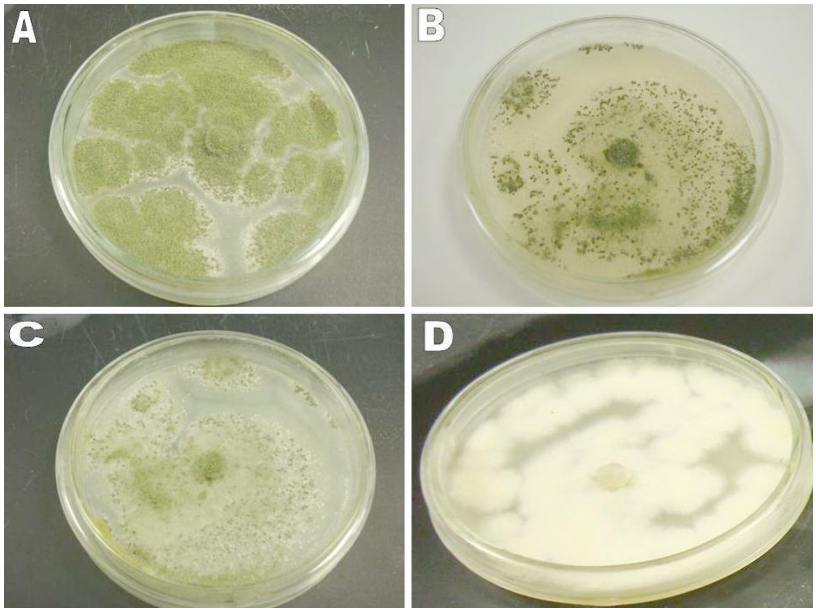

Figure 2. Effect of different concentrations (A: control; B: 0.5%; C: 1.0%; D: 2.0%) of plant (*E*. *alata*) extract on conidial production of *A. flavus*.

Table 2. Effect of different concentrations of *E. alata* (w/v) on number of germinated conidia (out of 50 conidia) after different incubation periods (hours).

Treatment	Number of germinated conidia (out of 50 conidia) after different incubation periods (h)		
(*E. alata* w/v)	6 h	12 h	24 h
Control	20.67	49.33	50.00
E. alata 0.5%	11.00	42.83	49.17
E. alata 1.0%	6.17	36.67	48.67
E. alata 2.0%	0.00	32.17	47.17
LSD at: 05	4.7884	3.4099	2.4237

Table 3. Effect of different concentrations of *E. alata* (w/v) on mycelial dry weight (g 50 ml^{-1} culture medium) and aflatoxin B_1 production (µg 50 ml^{-1} culture medium).

Treatment	Mycelial dry weight	Aflatoxin B_1 production	
(*E. alata* w/v)	(g 50 ml^{-1} culture medium)	µg 50 ml^{-1} culture medium	µg g^{-1} dry weight
Control	0.50154	351.6	701.04
E. alata 0.5%	0.33354	120.4	360.97
E. alata 1.0%	0.21616	56.6	261.84
E. alata 2.0%	0.1015	0.0	0.0
LSD at: 05	0.0331	22.192	

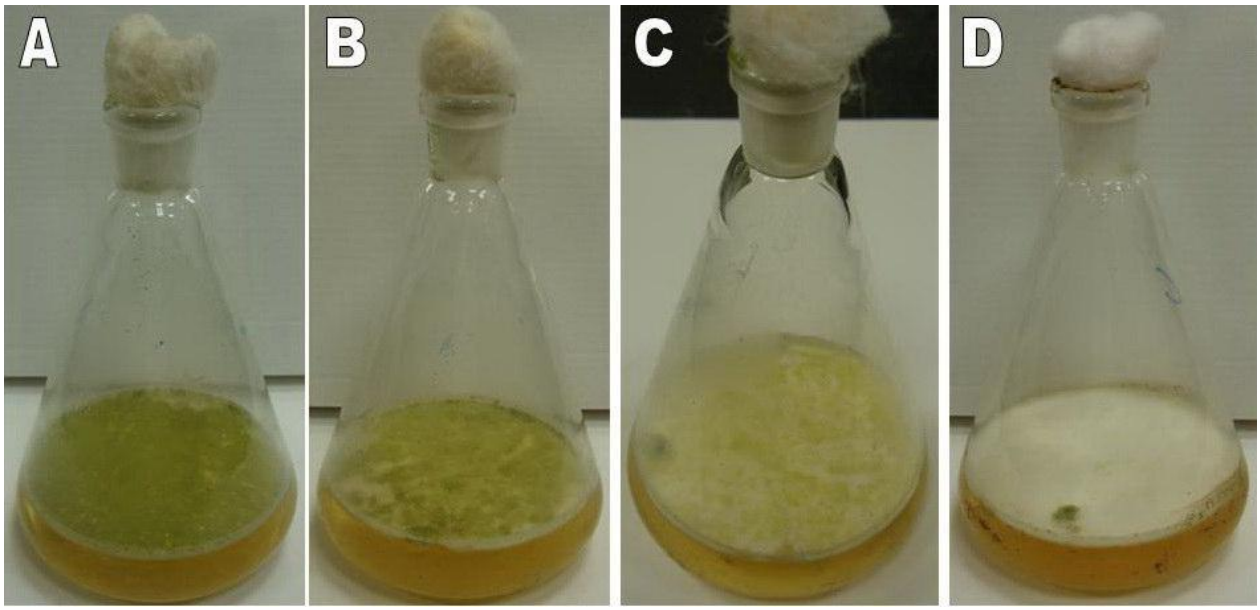

Figure 3. Effect of different concentrations [w/v] (A, control, B, 0.5%, C, 0.1%, D, 0.2%) of plant (*E. alata*) extract on mycelial growth of *A. flavus*.

84.89 and 59.57, 89.72, respectively. Absolute inhibition of aflatoxin B_1 accumulated in both maize and soybean grains was observed accompany with employment of 2.0% (w/w) *E. alata* (Table 4).

The results show that *A. flavus* caused significant deterioration in the nutritional value of maize grains (Table 5) and soybean seeds (Table 6). Such deterioration in the nutritional value of agricultural commodities was aware as significant decrease in their total lipids, crude fibre and crude protein accompanied with a significant increase in ash content (Table 5 and 6). The employment of powdered *E. alata* succeeded to

Table 4. Effect of different concentrations of *E. alata* (w/w) on aflatoxin B_1 production (µg kg^{-1}) on both maize grains and soyabean seeds stored under biological stress of *A. flavus*.

Treatment (*E. alata* w/w)	Aflatoxin B_1 production (µg /kg^{-1})	
	Maize grains	Soyabean seeds
control	212.22	131.20
E. alata 0.5%	76.79	53.04
E. alata 1.0%	32.06	13.48
E. alata 2.0%	0.0	0.0
LSD at: 05	23.096	17.137

Table 5. Effect of different concentrations of *E. alata* (w/w) on nutritional value of maize grains stored under biological stress of *A. flavus*.

Treatment (*E. alata* w/w)	Nutritional value of maize grains[Z]			
	Total lipids*	Crude fiber*	Ash content*	Crude protein**
Control (maize before storage)	4.7752	6.3021	4.2205	2.8240
Maize + *A. flavus*	2.2577	1.0217	6.3914	1.8387
E. alata 0.5%	3.6204	2.4314	5.3093	2.1400
E. alata 1.0%	4.1111	4.7984	5.4991	2.7304
E. alata 2.0%	4.1139	5.3666	5.6747	3.0224
LSD at: 05	0.3725	0.5438	0.1567	0.3331

*= Total lipids, crude fiber and ash content expressed as percentage (%).
**= Crude protein = total nitrogen (mg/g dry wieght) X 6.25.
Z= Total lipids, crude fiber, ash content and crude protein of *E. alata* (shoot system) were 1.0425, 4.2463, 7.7599 and 3.1341, respectively.

Table 6. Effect of different concentrations of *E. alata* (w/w) on nutritional value of soyabean seeds stored under biological stress of *A. flavus*.

Treatment (*E. alata* w/w)	Nutritional value of maize grains[Z]			
	Total lipids*	Crude fiber*	Ash content*	Crude protein**
Control (maize before storage)	18.5986	4.3731	6.4366	34.1012
Maize + *A. flavus*	15.4252	1.9727	7.5799	12.2231
E. alata 0.5%	16.3723	2.5918	6.8635	18.0028
E. alata 1.0%	17.7983	3.5118	6.4536	24.6835
E. alata 2.0%	18.2606	4.0598	6.3337	30.7081
LSD at: 05	0.7713	0.2725	0.6477	11.735

*= Total lipids, crude fiber and ash content expressed as percentage (%).
**= Crude protein = total nitrogen (mg/g dry wieght) X 6.25.
Z= Total lipids, crude fiber, ash content and crude protein of *E. alata* (shoot system) were 1.0425, 4.2463, 7.7599 and 3.1341, respectively.

protect the maize grains (Table 5) and soybean seeds (Table 6) against the destroyer effect of *A. flavus*. The constrictive role of *E. alata* was directly proportional with its employed concentrations.

DISCUSSION

Aflatoxins are highly toxic secondary metabolites produced by certain strains of *Aspergillus flavus* caused many diseases for animals as well as humans. In our investigation, the chemical and biological confirmatory tests demonstrated the production of aflatoxins B_1 by our experimental mold (*A. flavus*). Aflatoxins contamination of foodstuffs and agricultural commodities is a worldwide problem in tropical and subtropical regions where climatic conditions and storage practices are conducive to fungal growth and mycotoxin production (Thompson and Henke, 2000). Many strategies including employment of biological products from plant origin are being investigated to

manage deterioration of agricultural commodities including animal fodder. In the same connection, investigations of antifungal potential of Ephedra were carried out recently (Bagheri et al., 2009; Parsaeimehr et al., 2010). In our results, the aqueous extract of *E. alata* was found to be effective in controlling the growth rate and conidial production by *A. flavus*. The antifungal potential of *E. alata* (aqueous extract) was extended to inhibit number of germinated conidia, mycelial growth (mycelial dry weight) and aflatoxin B_1 production by *A. flavus* in variable percentages. In the same concept, the results of storage experiment indicated that application of plant (*E. alata*) powder was accompanied with significant decrease in contamination of both maize grains and soybean seeds with aflatoxin. The antimicrobial potential of *E. alata* noted here in the present study has been attributed to presence of Cis- 314-methanoproline (Caveney et al., 2001), Citronellol (Rosato et al., 2007) and Heptadecane (Bagheri et al., 2009) which are recorded as photochemicals possess antimicrobial activity found in Ephedra.

In geneal, oil seeds introduced into animal feeds as source of fiber, lipids and fat soluble vitamins, however the fungal contamination lead to significant deterioration in their nutritional value due to substrate utilization by molds. Previous studies indicated the decrease in fat, protein and fiber contents of many seeds and grains involved sorghum and soybean infected with different seedborne fungi and this was accompanied with an increase in ash content (Fapohunda and Olajuyiabe, 2006; Ezekiel et al., 2010). In this regard, fiber content with available protein and fat formed ready substrate for fungal activities including production of hydrolytic enzymes (Mellon et al., 2007). The results of our study recorded similar significant decrease in protein, fiber, and lipids accompanied with an increase in ash content of both maize grains and soybean seeds due to the inoculation with *A. flavus*. The application of plant (*E. alata*) powder succeeded to decrease the deterioration of the nutritional value (fat, protein, fiber, ash contents) of both maize grains and soybean seeds due to infection with *A. flavus*. Regarding the justification manner, our results showed *E. alata* possess highly antagonistic potential against growth and aflatoxin production of *A. flavus* in agree with Bagheri et al. (2009) and Parsacimehr et al. (2010).

Furthermore, with respect to *E. alata*, it has been shown that range plant contain suitable contents of protein and fiber (Nawwar et al., 1985) able to compensate the decrease in nutritional value of maize grain and soybean seeds due to mold contamination. Our results provide evidence for the first time to application of *E. alata* as naturally additive control aflatoxin contamination of fodder in addition to improve its nutritional value. This may be a novel non-chemical strategy to protect thousands of camels in Saudi Arabia against the mystery of fodder contamination with aflatoxins, the main reason for camels' death in our country.

ACKNOWLEDGMENT

The authors wish to acknowledge Research Centers in both college of Science and college of food sciences and agriculture, King Saud University, Riyadh, Saudi Arabia. At the same time, we acknowledge Deanship of Scientific Research, King Saud University, Riyadh, Saudi Arabia.

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