JOURNAL OF PURE AND APPLIED MICROBIOLOGY, September 2013. Vol. **7**(3), p. 1943-1950

Inhibitory Effect of Cardamom Essential Oil on Aflatoxin B Production by *Aspergillus* spp. in Arabic Coffee

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(Received: 26 March 2013; accepted: 25 May 2013)

Coffee bean contamination by aflatoxigenic fungi, ten samples were collected from local markets of the Saudi Arabia, Riyadh. Out of these samples, seven species of Aspergillus were obtained from the tested samples. Examination of coffee bean samples for the presence of toxigenic fungi was carried out. It has been found that thirteen isolates of *A. flavus*, twenty of *A. niger* , ten of *A. ochraeceous,* four isolates for both *A. fumigates* and *A. parasiticus* and two isolates of *A. tamari* were tested for their production of Aflatoxin B (AFB) by detection under UV ligh.. Antiaflatoxigenic effect of Cardamom essential oil was studied by HPLC, where Cardamom oil at 4 % lead to the highest level on% inhibition aflatoxins B1 and aflatoxins B2 were ranged from 48.6 to 80.1% and 57.4 to 100%, respectively. As well as, natural occurrence of Aflatoxin B (μg/kg) in ten coffee samples were determining in this study and obtained that five samples were produced both aflatoxins (B1 and B2) in varying amounts. The chemical constituents of Cardamom essential oil was analyzed by gas chromatography–mass spectrometry. The major constituents were identified as 1,8-cineol (42.53%), 4- terpineol (22.6%), Beta cymene (5.22%), pinnen (5.12%), Alpha –terpinene (4.15%), Alpha terpinene (3.36%), linalool (2.68%), Gamma terpinene (1.14%), caryophyllen (1.09%),.Alpha Selinene (0.62%), Cis ocimene (0.56%), Farnesol (0.24%) and Alpha terpinol (0.17%).

Key words: Coffee beans, *Aspergillus*, antiflatoxins B, cardamom essential oil, GC Mass

Aspergillus spp. are a filamentous fungi that produces mycotoxins in many food coffee such as beans. Aflatoxins are secondary metabolites, highly toxic, mutagenic and carcinogenic and cause crop losses and represent a significant hazard to the food chain (Magan and Aldred, 2007). Aflatoxins are stable under normal food processing conditions as well as in processed products (Sidhu *et al.,* 2009). *A. flavus, A. parasiticus, A. tamarii*, *A. pseudotamarii, A. bombycis, A. parvisclerotigenus, A. nomius, A. minisclerotigenes, A. oryzae, A. toxicarius, A. versicolor, A. rambellii, A. arachidicola, A. ochraceoroseus, astellata, E. venezuelensis* are produced aflatoxin (Pildain *et al.,* 2008, Murphy *et al.*, 2006, *Samson et al*., 2006), Aflatoxin production in coffee *Aspergillus* species was suggested by many authors (Bokhari 2007, Bokhari and Aly, 2009). Arabic coffee is considered one of soft drinks in Saudi Arabia, total yield reached in the world and is 8.2 million tonnes (FAO, 2010. Studies on the microbiology of coffee beans were shown that the main toxigenic fungal genera (*Aspergillus*, *Penicillium* and *Fusarium*) are natural coffee contaminants (Bokhari and Jee, 2002). The climatic conditions of some areas of Saudi as well as the badr conditions of coffee storing could encourage toxigenic (Bokhari, 2007).

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Recently, a natural product has become interesting as an alternative to pesticides that have hazard effects. Many essential oils have been reported as effective inhibitors of aflatoxin production (Razzaghi *et al,* 2008). Great results has been achieved to reduce mycotoxigenic fungi and mycotoxins in foods using plant products such as plant extracts and plant essential oils (Reddy *et al.,* 2010). Cardamom is an important economic crop and is a highly valued spice, essential oil from cardamom, (*Elettaria cardamomum* L) has inhibitory effects against fungus growth (Utta-U´r *et al.* 2000). In the present study, was to investigate the effects of Cardamom oil on aflatoxin production by *Aspergillus* spp were isolated from Arabic caffee in Saudi Arabia and determination of AFs using HPLC and Chemical composition and Relative concentration of Cardamom oil by GC-Mass.

MATERIALS AND METHODS

Collection of samples

Ten samples of coffee beans were collected randomly from different markets in Riyadh, Saudi Arabia during 2012, They were stored at 2°C to avoid any microbial contamination before analysis (Czerwiecki *et al*., 2002).

Isolation, purification *Aspergillus* spp. associated with coffee sample

Samples were surface sterilized with 5% sodium hypochlorite solution for one minute, before they were rinsed three times with sterilized distilled water. Five pieces were placed on the surface of Petri dishes containing potato dextrose agar (PDA), and each entry replicated three times. Petri dishes were incubated at 25º C. Isolates were purified by single spore methods and then transferred to PDA slants. All the isolates of Aspergillus species were identified by Regional Center of the Fungi and their Applications, Al-Azhar University, Cairo, Egypt.

The frequency of *Aspergillus* species with in a genus of fungi was calculated using the formula of Ghiasian *et al.* (2004).

$$\text{Frequency} = \frac{\text{Number of fungal species isolated}}{\text{Total Number of fungi isolated}} \times 100$$

Extraction of aflatoxin B from coffee samples

Twenty grams of each tested coffee samples were mixed with 100 ml of 4% acetonitrile aqueous solution of potassium chloride (9:1) then shaking for 20 min and filtered through filter paper Whatman No.4. For purification, 100 ml of n-hexane were added to the filtrate and shaken for 10 min. After separating, the upper phase (n hexane) was discarded., 50 ml deionized water and 50 ml chloroform were added to the lower phase and this solution was shaken for 10 min. then the upper phase was re-extracted twice with 25 ml of chloroform. Then the chloroform was evaporator at 40°C in water bath at low speed after that add two ml methanol then the solution filtered through a 0.45 µl filter. (Zaboli *et al*, 2011).

Three different concentrations (1, 2 and 4%) of Cardamom oil was obtained as follow: 4ml of essential oil and 0.4mL of Tween 80 were taken in sterile tubes and the volume was completed to 5mL using distilled sterile water. This mixture was shaken for 5 minutes using Vortex and serial dilutions were made to obtain solutions with final concentrations. Control was carried out with Tween 80 (Souza *et al*., 2005).

Detection of aflatoxigenic isolates under UV light

The culture media used was potato dextrose agar. The presence or absence of fluorescence surrounding the colonies under UV light (365 nm) and expressed as positive or negative according to Franco *et al*., (1998).

Extraction of aflatoxins from different isolates

Isolates were grown on sterilized SMKY liquid medium (sucrose, 20 g; magnesium sulfate, 0.5 g; potassium nitrate, 3 g; yeast extract, 7 g and distilled water, 1000 ml). The flasks were inoculated with discs of 6 mm diameter of *Aspergillus* spp. at 25 ± 2°C for 7 days, the filtrates of each flask were treated three times with 50 ml of chloroform in a separating funnel. The chloroform extract was evaporated till dryness on water bath at 50°C under vacuum. The residues were dissolved in methanol (Dos Santos *et al*., 2003).

Antiaflatoxigenic efficacy of Cardamom essential oil on *Aspergillus* spp.

Antiaflatoxigenic efficacy of Cardamom oil was 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) **(**Abd El-Aziz *et al*.,2012**)**. Three different concentrations of essential oils (1,2 and 4 %) were prepared and added to flasks then inoculated with

discs of 6 mm diameter of *Aspergillus* spp. at 25 ± 2°C for 7 days and the control set was kept parallel to the treatment without Cardamom oil. After incubation, content of each flask was filtered (Whatman, No.1). The filtrates of each flask were with 50 ml of chloroform in a separating funnel. The chloroform extract was evaporated on water bath at 50°C. The residues were dissolved in methanol (Dos Santos *et al.,* 2003).

High -performance liquid chromatography (HPLC)

The aflatoxins were measured using high-performance liquid chromatography (HPLC) (model PerkinElmer series 200 UV/VIS) with a C18 column with an internal diameter of 300 mm x 3.9 mm. The HPLC was equipped with an UV detector and fluorescence with 365 nm excitation and 430 emission wavelengths. The mobile phase consisting of methanol: acetic acid: water (20:20:60 v/v/v). The total run time for the separation was approximately 30 min at a flow rate of 1ml/min. (Christian, 1990).

GC/GC-MS analysis of Cardamom oil

Cardamom oil was analyzed according to the method described by Abbasipour *et al.* (2011) The sample was diluted 25 times with acetone, and 1 µL was injected through gas chromatography (model PerkinElmer clarus 500) equipped with a flame ionization detector, and quantitation was carried out by the area normalization method neglecting response factors. The analysis was carried out using a VF-5MS capillary column (30 m x 0.25 mm; 0.25 µm film thickness). The operating conditions were as follow: injection and detector temperature, 250 and 300°C respectively; split ratio, 1:25; Helium was the carrier gas at a flow rate of 1 mL/min. The injector port temperature was 225 °C, the detector temperature was 250 °C, and the oven temperature was maintained at 60 °C for 1 min and then increased to 225 °C at the rate of 2 °C/min, at which temperature the column was maintained for 5 min. Mass spectrometer conditions were: ionization potential, 70 eV; mass range from, 40 to 400 m/z; electron multiplier energy, 2000 V. The components of plant extracts were identified by comparison of their relative retention times and the mass spectra with those authentic reference compound shown in the literature and by computer matching of their MS spectra with Nist mass spectral library.

RESULTS AND DISCUSSION

Isolation and purification *Aspergillus* spp. associated with coffee sample

Data in (Table 1) show that seven species of *Aspergillus* were obtained from the ten tested samples. Examination of coffee bean samples for the presence of toxigenic fungi was carried out. It has been found that thirteen isolates of *A. flavus*, twenty of *A. niger* ,ten of *A. ochraeceous,* four isolates for both *A. fumigates* and *A. parasiticus* and two isolates of *A. tamari.* The same findings were obtained by many other (Nunnes *et al.,* 2001; Panneerselvam *et al.* ,2001; Bokhari, 2007, Bokhari and Aly,2009). The highest contamination was estimated in occurring in coffee bean sample No. (1). It was represented by 7 species of which *A. flavus* and *A. niger* were the most common and were isolated in high frequency of all species identified . Whereas, no show any fungus during isolation from sample No. (3).

Natural occurrence of Aflatoxin B (µg/kg) in ten coffee samples

Table (2) shows the determination of aflatoxin B in coffee beans. The highest contamination levels of aflatoxins B were found in samples No. 5 were contaminated with aflatoxins B1 and B2,(54.3 and 9.2µg/kg) respectively. Two samples don't produced any aflatoxins B1 or B2 (sample No. 3 and No. 6), three isolates don't produced aflatoxins B2 (sample No. 4, 7 and 9). Five samples were produced both aflatoxins (B1 and B2) in varying amounts. Differences in aflatoxin contamination may be attributable to climatic conditions and to agricultural practices and relative humidity and pre-harvest and storage conditions (Nakai *et al.,* 2008).

Percentage of toxigenic isolates of *Aspergillus* spp under UV light

The contamination of tested ten coffee samples with aflatoxin B was determined by detection under UV light qualitatively as shown in Table (3). The percentage of toxigenic isolates of *Aspergillus* spp were 50, 46.2,30 and 25% in four species *(A. tamarii, A. flavus, A. ochraeceous, A. parasiticus,* respectively), whereas, *A. niger, A. terreus* and *A. fumigates* gave negative detect for aflatoxin under UV light. These results were similar to a great extent to results obtained by Pildain *et al.* (2008), Murphy *et al.* (2006)

Table 1 . Isolation and %frequency of Aspergillus spp. from ten samples of coffee bean at 28ºC for 7 days at PDA medium

No. of	Aspergillus spp.														
	A. flavus		*A. niger*		*A. terreus*		*A. ochraeceous*		*A. fumigatus*		*A. parasiticus*		*A. tamarii*		Total of
samples	No.of isolates	% Frequency	No.of isolates	% Frequency	No. of isolates	% Frequency	No.of isolates	% Frequency	No.of isolates	% Frequency	No.of isolates	% Frequency	No.of isolates	% Frequency	isolates
1	3	27.3	4	36.4	0	0	2	18.2	0	0	1	9.1	1	9.1	11
2	2	33.3	2	33.3	0	0	1	16.7	0	0	1	16.7	0	0	6
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	2	40.0	1	20.0	1	20.0	0	0	1	20.0	0	0	0	0	5
5	0	0	2	33.3	1	16.7	2	33.3	1	16.7	0	0	0	0	6
6	0	0	3	75.0	0	0	1	25.0	0	0	0	0	0	0	4
7	1	16.7	1	16.7	0	0	3	50.0	0	0	0	0	1	16.7	6
8	3	42.9	2	28.6	1	14.3	0	0	0	0	1	14.3	0	0	7
9	0	0	2	28.6	2	28.6	1	14.3	1	14.3	1	14.3	0	0	7
10	2	28.6	3	42.9	1	14.3	0	0	1	14.3	0	0	0	0	7
Total	13	20	6	10	4	4	2	59							

Effect of treatment with Cardamom oil on aflatoxin B (µg/ml) produced by *Aspergillus* spp isolated from coffee

Data in table (4) obtained that eleven isolates were capable of producing detectable levels of aflatoxins B, although three isolates (No.1, 7 and 45) failed to produce any detectable amount aflatoxins B2. Antiaflatoxigenic effect of Cardamom essential oil was studied where Cardamom oil at 4 % lead to the highest level on% inhibition aflatoxins B1 and aflatoxins B2 were ranged from 48.6 to 80.1% and 57.4 to 100%, respectively. In addition, treatment with Cardamom oil leads to complete inhibition on aflatoxins B2 produced by *A. tamarii* (isolate No. 58) and *A. ochraeceous* (isolate No. 47). As well as Cardamom oil more effective on aflatoxins B1 produced by *A. parasiticus* (isolate No. 55). This result is confirmed by Alpsoy (2010) reported that Essential oils from common spices such as cardamom was tested for their ability to suppress the formation of DNA adducts by AFB1 *in vitro* in a microsomal enzyme-mediated reaction.

Table 2. Natural occurrence of Aflatoxin B (µg/kg) in ten coffee samples

No. of coffee samples	B 1	B2
1	21.8	11.3
2	25.2	17.1
3	0	0
4	33.1	0
5	54.3	9.5
6	0	0
7	22.4	0
8	15.9	12.8
9	23.2	0
10	47.7	14.3

Table 3. Percentage of toxigenic isolates of *Aspergillus* spp under UV light.

Aspergillus spp	No.of isolates	% Toxigenic isolates (expressed as positive)	Isolates No.
A.flavus	13	46.2	1,3,6,7,10,11
A.niger	20	0	0
A.terreus	6	0	0
A. ochraeceous	10	30	40,45,47
A. fumigatus	4	0	0
A. parasiticus	4	25	55
A.tamarii	2	50	58

Table 4. Effect of treatment with Cardamom oil on aflatoxin B (µg/ml) produced by Aspergillus spp isolated from coffee

Isolates No.	Control		Concentration of Cardamom oil					
	B1	B2	B 1			B2		
			1%	2%	4%	1%	2%	4%
1	22.3	0	13.2	9.1	7.05	0	0	0
3	35.3	3.4	21.3	14.3	11.4	2.9	2.3	1.1
6	28.2	6.1	19.2	10.1	6.3	5.0	3.7	2.6
7	12.1	0	8.3	7.3	5.2	0	0	0
10	14.3	2.1	10.2	8.3	5.6	1.8	1.4	0.89
11	9.8	5.0	6.3	3.5	2.9	4.1	3.2	1.7
40	17.4	4.3	11.6	8.6	7.2	3.1	2.4	0.36
45	20.1	0	13.4	9.2	9.1	0	0	0
47	16.2	1.6	12.1	10.3	8.3	1.3	1.1	0
55	11.7	1.3	9.4	7.1	2.1	1.0	0. 41	0.21
58	16.3	3.6	10.6	6.7	4.4	2.4	1.3	0

Table 5. Chemical composition and Relative concentration of Cardamom oil by GC-Mass

Peak Number	Compounds	Rt (min)	Relative concentration
1	pinnen	5.12	2.19
2	linalool	15.11	2.68
3	Alpha terpinol	15.36	0.17
4	1,8 cineol	10.31	42.53
5	Alpha Selinene	15.51	0.62
6	Farnesol	16.42	0.24
7	Alpha terpinene	6.32	3.36
8	Gamma terpinene	2.62	1.14
9	Cis ocimene	5.29	0.56
10	Beta cymene	23.64	5.22
11	Alpha -terpinene	8.62	4.15
12	4- terpineol	7.83	22.6
13	caryophyllen	23.21	1.09

The extent of inhibition of fungal growth and mycotoxin production was dependent on the concentration of essential oils used (Soliman & Badeaa, 2002).

Their antimicrobial properties of essential oils are mostly due to the presence of alkaloids, phenols, glycosides, steroids, terpenes and tannins which penetrate into the fungal cell and interacting with critical intracellular sites (Ebana *et al.,* 1991, Cristani *et al*., 2007). Moreover, lysis of the hyphae and spores of the toxigenic fungi are characteristics of aflatoxin deactivation process (Namazi *et al*., 2002).

Essential oils would act on the hyphae of mycelium, lead to exit of components from the cytoplasm lead to loss of rigidity and integrity of hypha cell wall and cause damage to the membrane and the loss of homeostasis and effect in the specific enzymatic systems. (Sharma &Tripathi, 2008). Syntesis of aflatoxins is control by specific enzymes which are expressed by DNA through many steps may be this steps inhibited by natural plant (Trail *et al*., 1995)

Chemical constituents of Cardamom essential oil

The results of the chemical analysis are presented in Table (5). Thirteen compounds in the Cardamom essential oil were positively identified, pinnen (5.12%),linalool (2.68%), Alpha terpinol (0.17%),1,8-cineol (42.53%), Alpha Selinene (0.62%), Farnesol (0.24%), Alpha terpinene (3.36%), Gamma terpinene (1.14%), Cis ocimene (0.56%), Beta cymene (5.22%), Alpha –terpinene (4.15%) , 4-terpineol (22.6%) and caryophyllen (1.09%).This result is confirmed by **(**Ahn *et al.* 1998; Abbasipour *et al.,* 2011 and Gochev *et al*., 2012)

ACKNOWLEDGMENTS

The authors would like to extend their appreciation to the Deanship of the Scientific Research at King Saud University for its funding of this research through the Research Group Project no. RGP-VPP-269

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