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Spices Mycobiota and Mycotoxins Available in Saudi Arabia and Their Abilities to Inhibit Growth of Some Toxigenic Fungi

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The prevalence and population density of the mycobiota of 50 samples belonging to 10 kinds of spices (anise, black pepper, red pepper, black cumin, peppermint, cardamom, clove, cumin, ginger and marjoram) which collected from different places in Jeddah Governorate were studied. The natural occurrence of mycotoxins in those samples was also investigated. Fifteen genera and thirty - one species of fungi in addition to one species variety were isolated and identified during this study. The most common genera were *Aspergillus*, *Penicillium* and *Fusarium*. Aflatoxins (12~40 μg/kg) were detected in the extract of 5 samples of each of anise seeds and black pepper fruits; three samples of black cumin seeds and on sample only of each of peppermint and marjoram leaves out of 5 samples tested of each. Sterigmatocystin (15~20 μg/kg) was detected in some samples of red pepper, cumin and marjoram. The inhibitory effects of 10 kinds of powdered spices were tested against 3 toxigenic isolates of fungi (*Aspergillus flavus*, *A. versicolor* and *Penicillium citrinum*). Clove proved to be antimycotic compounds. It inhibited the growth of the tested toxigenic fungi. Black pepper, peppermint, cardamom, cumin and marjoram completely inhibited aflatoxins production, while black pepper and cardamom also completely inhibited sterigmatocystin production.

KEYWORDS: Aflatoxin, Clove, Marjoram, Pepper, Sterigmatocystin

Spices are defined as aromatic or pungent fragrance vegetable substances used in minute quantities to enrich, alter, or mask the flavor of foods (Ayres *et al*., 1980). Spices such as pepper, paprika, cumin, ginger, saffron and clove are extensively used in Saudi Arabia to flavoring foods as well as for medication and are highly valuable due to their preservative and antioxidant properties. Few investigations have been carried out on the mycobiota (distribution, composition, density and frequency of occurrence) of the various kinds of spices. Spices are very heavily contaminated at import (Yasair and Williams, 1942; Bokhari, 2002; Julseth and Deibel, 1974; Qaher, 2005) but few published data are available on the microbiology of spices at retail (El-Kady *et al*., 1992; Martins *et al*., 2001; Abdulkadir *et al*., 2003; Fazekas *et al*., 2005). Srivastava and Chandra (1985) studied the mycobiota of 4 kinds of spices (coriander, cumin, fennel and fenugreek) in India and reported that *Aspergillus* followed by *Fusarium* were the most frequent members. Bhat *et al*. (1987) tested the microbial profile of cumin seeds and chilli powder sold in retail shops in the city of Bombay and reported that *Aspergillus* was the predominant genus in chilli powder samples. Also, they reported that no fungi were found in the cumin seed samples examined. Garrido *et al.* (1988) evaluated the fungi contaminated of 33 different commercial spices and reported that only nutmeg, anise, tarragon and cumin showed very low fungal contamination and in all cases *Aspergillus flavus* was found to be a predominant component of the mycobiota. In Egypt, Moharram *et al.* (1989) isolated 25 species of *Aspergillus* in addition to 5 species varieties from anise and fennel seeds, of which *A. niger*, *A. flavus*, *A. ochraceus* and *A. flavus* var. *columnaris* were the most prevalent on both anise and fennel seeds, but *A. fumigatus* and *A. sydowii* were more frequent on anise than on fennel. El-Kady *et al*. (1992) isolated 38 genera and 81 species from 120 samples of 24 kinds of spices collected from different places at Assiut Governorate, Egypt. They reported that the predominant genera were *Aspergillus* (25 species) and *Penicillium* (7 species) of which *A. flavus*, *A. niger*, *A. ochraceus*, *A. fumigatus*, *A. flavus* var. *columnaris*, *A. terreus*, *P. chrysogenum* and *P. corylophilum* were the most commonly occurring.

Spices are largely produced in countries where tropical climates (high ranges of temperature, humidity and rainfall) are favorable to mycotoxin contamination. Furthermore they are usually dried on the ground in the open air in poor hygienic conditions that even more promote growth of moulds and production of mycotoxins (Martins *et al.*, 2001). On a global scale, the contamination of spices by mycotoxins was especially reported in Ethiopia (Fufa and Urga, 1996), Egypt (El-Kady *et al*., 1995; Selim *et al*., 1996; Aziz *et al*., 1998) Turkey (Gurbuz *et al*., 2000), and Portugal (Martins *et al*., 2001) Italy (Romagnoli *et al*.,

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2006) and Morocco (Zinedine *et al.*, 2006). Nonetheless, few countries have effectively established regulations as for aflatoxins AFs in spices. For instance, the maximum tolerable limit for (AFs) allowed in spices in EU member state has been set at 10 μg/kg for total AFs and 5 μg/kg for AFB_1 (Commission Regulation EC, 2006).

The present investigation reports the association of mycobiota with spices, their screening for mycotoxin producing ability, and mycotoxin occurrence in spices samples under Saudi environmental conditions. Also, the potential of the tested spices in the control of some toxigenic fungi and toxin production in culture medium and also for their ability to cause fungal inhibition.

Materials and Methods

Collection of spices samples. Fifty samples belonging to 10 kinds of spices (Table 1) were collected from different places at Jeddah Governorate. Each sample (200 g) was put in a sterile polyethylene bag, sealed and put in another bag which was also sealed. Storage of spices in a double-bag container minimize the loss of water content and gives sufficient aeration. Spices samples were transferred immediately to the laboratory and

Table 1. Average (5 samples of each kind of spices) total counts calculated per gm fresh weight and number of cases of isolation of fungl genera and species on glucose-Czapek's agar at 28°C

	Anis		Black cumin		Black pepper		Red pepper		Pepper-mint		Cardamon		Clove		Cumin		Ginger		Marjoram	
Genera & species	ATC	NCI	ATC	NCI	ATC	NCI	ATC	NCI	ATC	NCI	ATC	NCI	ATC	NCI	ATC	NCI	ATC	NCI	ATC	NCI
Aspergillus	625.3	5	530.3	5	623.5	5	2587.4	5	625.7	5	430.6	5	488.8	5	1219.8	5	1078	5	181.8	5
A. flavus	265.2	5	142.5	5	142.2	5	780.2	5	168.3	5	115.4	5	214.8	5	413.2	5	687.6	5	75.0	5
A. niger	332.4	5	355.0	5	398.6	4	1460.2	5	370.4	5	285.4	5	242.4	4	481.2	5	365.6	5	40.5	5
A. ochraceus	15.2	5	1.6	1	12.4	4	220.6	3	8.6	2	–	–	1.8	1	96.4	4	18.2	4	8.3	1
A. fumigatus	3.4	2	4.6	2	33.2	5	4.6	1	29.6	2	3.4	1	16.4	1	15.4	1	–	–	–	–
A. flavus var. *columnaris*	0.6	1	3.1	2	27.6	3	59.0	1	–	–	14.8	2	–	–	–	–	–	–	–	–
A. nidulans	4.6	2	2.3	1	1.8	2	–	–	–	–	–	–	1.8	1	53.2	3	3.3	2	–	–
A. terreus	0.6	1	14.5	1	3.5	1	–	–	26.0	2	9.8	2	3.3	1	21.2	3	–	–	–	–
A. versicolor	–	–	3.6	2	0.6	1	–	–	–	–	1.8	1	–	–	6.6	2	–	–	–	–
A. sydowii	–	–	–	–	3.6	1	62.8	1	22.8	1	–	–	8.3	2	132.6	1	–	–	–	–
A. tamarii	3.3	2	3.1	1	–	–	–	–	–	–	–	–	–	–	–	–	3.3	2	58.0	1
Penicillium	74.6	5	143.8	5	91.2	5	26.6	1	33.8	3	88	5	288.4	5	283.4	5	40.1	3	–	–
P. chrysogenum	16.4	1	30.5	4	46.8	4	26.6	1	18.2	2	39.4	5	20.6	4	186.8	4	36.6	2	–	–
P. corylophilum	58.2	4	112	5	12.4	1	–	–	15.6	2	7.4	2	267.8	5	96.4	1	3.5	1	–	–
P. funiculosum	–	–	–	–	13.2	1	–	–	–	–	29.2	3	–	–	–	–	–	–	–	–
P. citrinum	–	–	1.3	1	18.8	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. purpurogenum	–	–	–	–	–	–	–	–	–	–	12.0	1	–	–	–	–	–	–	–	–
Fusarium	18.4	2	–	–	3.6	3	–	–	89.8	4	–	–	–	–	1.8	1	11.3	2	195	2
F. oxysporum	0.6	1	–	–	–	–	–	–	4.6	1	–	–	–	–	1.8	1	–	–	35.0	1
F. moniliforme	2.6	1	–	–	1.8	1	–	–	1.8	1	–	–	–	–	–	–	8.0	1	–	–
F. equiseti	–	–	–	–	1.8	2	–	–	78.8	2	–	–	–	–	–	–	3.3	1	160.0	1
F. solani	–	–	–	–	–	–	–	–	4.6	1	–	–	–	–	–	–	–	–	–	–
F. subglutinans	15.2	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Alernaria alternata	16.4	4	–	–	0.6	1	28.4	2	210.9	5	1.8	1	–	–	6.6	2	1.8	1	315.0	2
Rhizopus stolonifer	36.6	5	4.6	2	0.6	1	32.4	3	8.4	2	4.6	2	3.3	1	16.2	4	19.5	3	–	–
Mucor hiemalis	0.6	1	1.6	1	8.6	4	–	–	66.0	5	29.8	1	–	–	17.2	3	36.6	2	–	–
Cladosporium herbarum	0.6	1	–	–	0.6	1	5.8	1	58.0	3	–	–	–	–	1.8	1	3.5	2	–	–
Drechslera spicifera	5.8	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Botryotrichum piluliferum	5.8	2	–	–	–	–	–	–	–	–	–	–	–	–	31.0	4	–	–	–	–
Trichoderma hamatum	6.8	3	–	–	5.8	1	1.8	1	–	–	3.5	1	–	–	26.0	3	1.8	1	–	–
Scopulariopsis brevicaulis	–	–	–	–	–	–	–	–	1.8	1	3.5	1	–	–	23.4	2	–	–	–	–
Paecilomyces variotii	1.8	2	–	–	–	–	–	–	–	–	3.5	1	3.3	1	–	–	–	–	–	–
Stachybotrys chartarum	–	–	–	–	–	–	–	–	7.4	1	–	–	–	–	3.5	1	–	–	–	–
Myrothecium verrucaria	–	–	–	–	–	–	–	–	111.6	2	–	–	–	–	–	–	–	–	–	–
Curvularia lunata	0.6	1	–	–	–	–	–	–	–	–	–	–	–	–	3.5	1	–	–	–	–
Gross total count	793.3		680.3		734.5		2682.4		1213.4		565.3		783.8		1634.6		1192.6		691.8	
Number of genera	11		4		8		6		10		8		4		12		8		3	
Number of species	21+1		12+1		19+1		11+1		19		16+1		11		20		14		7	

ATC = Average total count.

NCI = Number of cases of isolation out of five samples tested.

kept in a cool place for fungal determination or mycotoxins analysis.

Determination of fungi. Ten grams of each sample were added to a 90 *ml* portion sterile 0.85% saline solution in 500 *ml* Erlenmeyer flasks and homogenized thoroughly on an electric shaker at constant speed for 15 min. Tenfold serial dilutions were then prepared (Aziz and Youssef, 1991). One ml portions of three suitable dilutions of the resulting medicinal plant suspension were used to inoculate Petri dishes each containing 15 *ml* glucose-Czapek's agar containing 0.5 mg chloramphenicol/ml medium to suppress bacterial growth. Plates were then incubated for 7 days at 28°C and examined visually and microscopically for the growth of molds. Five replicates were performed for each sample and the developing fungi were counted and the number per mg dry soil was determined and identified according to several key processes (Pitt, 1988; Burgess *et al.*, 1988; Klich and Pitt, 1988; Pitt and Hocking, 1997).

Detection of natural occurrence of mycotoxin. The chloroform extracts were analyzed on precoated silica gel plate type 60F 254 (Merck) for the presence of different mycotoxins: aflatoxins (B_1, B_2, G_1 and G_2), citrinin, sterigmatocystin, using thin layer chromatographic technique according to standard procedures (Scott *et al*., 1970; Thrane, 1986; Van Egmond, 1995).

Quantitative determination of aflatoxins. The spots of aflatoxin B_1 including the standards were removed from the plates, eluded with methanol and estimated spectrophoto-metrically using Spectrophotometer (UNICAM Helios Gamma, Helios Delta) according to the methods described by Scott (1995). The identity and quantity of sterigmatocystin in the extracts were determined by method described by Schroeder and Kelton (1975).

Effect of spices on the growth and mycotoxins production of some toxigenic fungi. The organisms *Aspergillus flavus*, *A. versicolor* and *Penicillium citrinum* were used as indicator organisms. These spices well known as alflatoxin, sterigmatocystin and citrinin producing fungi, respectively. The effect of the tested spices on the growth and mycotoxins production by these fungi was studied as previously described by El-Kady *et al*. (1995).

Results and Discussion

Spices mycobiota. The prevalence and population density of the fungal flora in 50 samples belonging to 10 kinds of spices collected from different places at Jeddah governorate using dilution plate method on glucose-Czapek's agar medium and incubation at 28°C for eight days (Table 1). Samples of red pepper were relatively the richest in the total counts of fungi (average of 5 samples was 2682.4 colonies per g fresh weight). The poorest substrate was black cumin (average of 5 samples was 680.3 colonies perg). Although the extent of contamination of red pepper was slightly greater than other kinds of spices, however this count fill within the ranges found in a range of species by other workers (Lebai *et al*., 1985; Bhat *et al*., 1987; Geeta and Kulkarni, 1987; El-Kady *et al*., 1992; Abdulkadir *et al*., 2003).

Fifteen genera and 31 species were isolated from spices samples during this investigation. *Aspergillus* was the most common genus in the different spices tested. Nine species in addition to one species variety were collected from *Aspergillus*. The genus was represented in 100% of the samples constituting 76.5% of the gross total count of fungi. *Aspergillus flavus* and *A. niger* were the most prevalent. They emerged from 100% of the samples contributing 27.4 and 39.5 of total count of fungi. *Aspergillus flavus* and *A. niger* were the most frequently encountered and widely distributed in spices and herbal drugs (Pal and Kundu, 1972; Udagawa *et al*., 1976; Hitokoto *et al*., 1978; Sharma and Sharma, 1984; Lee and Lim, 1985; Garrido *et al*., 1988; Moharram *et al*., 1989; Abdel-Hafez and El-Said, 1997; El-Kady *et al*., 1992; Abdulkadir *et al*., 2003). From *Aspergillus* 7 species and one variety (*A. ochar-ceus*, *A. fumigatus*, *A. flavus* var. *columnaris*, *A. nidulans*, *A. terreus*, *A. versicolor*, *A. sydowii* and *A. tamarii*) were isolated in moderate or low frequency of occurrence. These species were previously isolated from different kind of spices by several researchers (Flannigan and Hui, 1976; Ayres *et al*., 1980; Misra, 1981a, b; Hassan, 1984; Moharram *et al*., 1989; El-Kady *et al*., 1992; Abdulkadir *et al*., 2003). *Penicillium* was isolated in high frequency of occurrence and accounting 80% of the samples and 9.8% of total fungi. It was represented by 5 species of which *P. chrysogenum* and *P. corylophilum* were the most prevalent. These species were previously recovered but with variable densities and frequencies (Hassan, 1984; Garrido *et al*., 1988; Moharram *et al*., 1989; El-Kady *et al*., 1992; Abdulkadir *et al*., 2003). *Fusarium* were recovered in moderate frequency constituting 2.9% of total fungi. It was represented by 5 species. All *Fusarium* species recovered in the present work were isolated in rare frequency of occurrence. These results came in agreement with El-Kady *et al.* (1992) findings. They reported that *Fusarium* was isolated in moderate frequencies from 24 kind of spices in Egypt, and it was represent by 8 species and 2 species varieties all of them were isolated in rare frequency of occurrence. Srivastava and Chandra (1985) recorded that *Aspergillus* followed by *Fusarium* were the most frequent members of the mycobiota of coriander, cumin, fennel and fenugreek. Contrary to this finding, Hassan (1984) reported that members of *Fusarium* was completely absent in 12

kind of spices tested. The remaining genera and species were isolated from one or two substrates with low frequency and total counts as represented in Table 1. Most of these fungi were isolated previously from various kind of spices (Moharam *et al*., 1989; El-Kady *et al*., 1995; Bokhari, 1992, 2001; Abdulkadir *et al*., 2003).

Spices mycotoxins. The natural occurrence of mycotoxins in herbs and spices has become of increasing interest because of wide spread use of these substances in the world today (Arcos, 1978). Analysis of 50 different samples belonging to 10 kinds of spices for the natural occurring mycotoxins, showed that 10 samples belonging to 5 kinds of spices were contaminated by aflatoxins. Aflatoxins were detected in the 3 samples of each of anise samples, and of black pepper seeds; 2 samples black cumin and one sample only of each of peppermint and marjoram leaves (Table 2). Finding similar to the results obtained in the present study, has been reported by El-Kady *et al.* (1995). They analyzed 24 kinds of spices and reported the presence of low concentrations of aflatoxin in some samples of anise, black pepper, caraway, black cumin, fennel seeds, peppermint, coriander and marjoram. Suzuki *et al.* (1973) reported the same results in the samples of black pepper.

Aflatoxins in concentrations of 12~40 μg/kg, were detected in all the positive samples during this work. Minor concentrations of aflatoxins contaminated various kind of spices were previously reported by Majerus *et al.* (1985), 5.2~24 μg/kg; Misra (1987), 3~37 μg/kg; Misra and Batra (1987), 8~19 and El-Kadey *et al.* (1995), 8~35 μg/kg. Martins *et al*. (2001) detected Aflatoxin B (AFB) in 34 samples (out of 79 samples) of prepackaged spices (43.0%) from supermarkets and ethnic shops in Lisbon (Portugal). Paprika contained levels of aflatoxin B_1 ranging from 1 to 20 μg/kg. Chilli, cumin, curry powder, saffron and white pepper samples had levels ranging from 1 to 5 μg/kg. Aflatoxins were not detected in cardamom, cloves, and ginger. None of the samples analyzed contained aflatoxins, G_1 and G_2.

Sterigmatocystin in concentrations of 15~20 μg/kg was detected in some samples of red pepper, cumin and marjoram. This results came in agreement with El-Kady *et al.* (1995) results. They reported that presence of sterigmatocystin (10~23 μg/kg) in some samples of red pepper, caraway, cumin, marjoram and citrinin (8~12 μg/kg) in samples of black cumin. In contrary to these findings, several previous analysis failed to detect any mycotoxin other than aflatoxin in different kinds of spices (Hitokoto *et al.*, 1978; Majerus *et al.*, 1985).

Inhibitory effects of the tested spices against growth and toxin production by toxigenic fungi. The inhibitory effects of 10 kinds of spices were tested against three isolates of toxigenic fungi namely *A. flavus*, *A. versicolor* and *P. citrinum* which produced 3 different mycotoxins (aflatoxins B_1, B_2, G_1 & G_2, sterigmatocystin and citrinin). Clove proved to be the strongest antifungal spices. It inhibited growth of all three mould isolates tested (Table 3). Azzouz (1981) studied the effect of some spices on several toxigenic species of *Aspergillus* and *Penicillium* and found that at 2% level in (YES) agar, cloves and cinnamon completely inhibited growth of all fungi tested. At level of 8 mg/*ml* (0.8%) clove and cinnamon completely inhibited growth and mycotoxin production. Mostafa (1990) examined the inhibitory effects of 24 commercial spices and toxin production of four toxigenic *Aspergillus* (*A. flavus*, *A. versicolor*) and *Penicillium* (*P. citrinum* and *P. corylophilum*) and found that Chinese cassia, cinnamon, clove and thyme completely inhibited the fungal growth of the tested isolates. Mabrouk and El-Shayeb (1980) reported that cloves as a concentration of 0.1% in a medium of rice powder and corn steep liquor, partially inhibited both mould growth and aflatoxin production by *A. flavus* and completely inhibited mould growth at concentrations of 0.5% or higher. Atanda *et al.* (2007) shows that aflatoxins can be completely inhibited in culture medium with sweet basil leaves and there is the possibility of its being used to protect sorghum against *Aspergillus* contamination.

Table 3 showed that five kinds of spices could be classified as antiaflatoxigenic compounds, they did not affect

Table 2. Natural occurrence of mycotoxins in the different spices samples tested

Kind of spices	Number of positive samples	Toxins detected	Concentration of toxins (μg/kg)
Anise	3	Aflatoxins B_1 and B_2	28~38
Black cumin	2	Aflatoxins B_1 and B_2	24~35
Black pepper	3	Aflatoxins B_1 and B_2	25~40
Red pepper	3	Sterigmatocystin	11~25
Peppermint	1	Aflatoxins B_1 and B_2	17
Cardamom	–	–	
Clove	–	–	
Cumin	3	Sterigmatocystin	15~20
Ginger	–	–	
Marjoram	1	Aflatoxins B_1 and B_2	12

Table 3. Inhibition effect of different kinds of spices on growth and mycotoxins production by 3 strains of toxigenic fungi

Kind of spices	*Aspergillus flavus*			*A. versicolor*			*Penicillium citrinum*		
	Mycelial growth mg/50 *ml*	Aflatoxins B_1, B_2, G_1 and G_2		Mycelial growth mg/50 *ml*	Sterigmatocystin		Mycelial growth mg/50 *ml*	Citrinin	
		*μ*g/50 *ml*	Inhibition (%)		*μ*g/50 *ml*	Inhibition (%)		*μ*g/50 *ml*	Inhibition (%)
Control	1560 (+++)	2540.0	–	2850 (+++)	758.5	–	1158 (+++)	353	–
Anise	+++	255.0	0	+++	0.0	100	+++	235.0	45
Black cumin	+++	956.0	65	+++	225.0	70	+++	125.0	70
Black pepper	+++	0.0	100	+++	0.0	100	+++	253.0	36
Red pepper	+++++	3125.0	30	+++++	955.0	30	+++	354.0	0
Peppermint	+++	0.0	100	+++	735.0	0	+++	0.0	100
Cardamom	+++	0.0	100	+++	0.0	100	+++	135.0	62
Clove	0	0.0	100	0	0.0	100	0	0.0	100
Cumin	+++	0.0	100	+++	735.0	0	+++	199.0	60
Ginger	+++++	256.0	0	+++++	0.0	100	+++	201.0	42
Marjoram	+++	0.0	100	+++	738.0	0	+++	122.0	67

growth of *A. flavus*, however they completely prevented aflatoxin production. The five kinds of spices are black pepper, peppermint, cardamom, cumin and marjoram. Black pepper and cardamom, also completely inhibited sterigmatocystin production, however the growth of *A. versicolor* did not affect. Inhibition of aflatoxin production of different strains of *A. flavus* and or *A. parasiticus* by powdered black and white pepper; Cardamom was also recorded by several authors (Hitokoto *et al*., 1980; Mabrouk and El-Shayeb, 1980; Madhyastha and Bhat, 1985; Mostafa, 1990). Peppermint, cumin and margoram was not affected sterigmatocystin production. Peppermint leaves also completely inhibited production of citrinin by *P. citrinum*. Cumin and marjoram retarded citrinin production (Table 3). These results are in harmony to that obtained by many investigators (Hitokoto *et al.*, 1980; Mabrouk and El-Shayeb, 1980; Mostafa, 1990; Krishnamurthy and Shashikala, 2006).

In conclusion, the results indicated that many samples of spices, although pure in the sense that they are not grossly adulterated with foreign matter, are far from pure microbiology. Mycotoxin contamination of the spices is considerably of low incidence and of minor concentrations if present. Although aflatoxin present as a natural contaminant of spices is of minor concentrations, the health risk is increased because anise and cumin are used as carminative, as expectorant, treatment colic and flatulence for children. Certain spices contain natural substances with substantial antimycotic or antitoxigenic activities. Further work for identification of these substances and confirmation of their actual activities will be needed; which may lead to control of mould growth and toxin production in foods, feeds or other materials.

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