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Frustration of Mycotoxins with Spices used for Coffee Spicing

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ABSTRACT

This study was conducted to examine the ability of seven fungal isolates from the coffee beans which are: *A. niger*; *A. alliaceus* (1); *A. flavus* (1); *A. melleus* (1); *A. melleus* (2) To produce active biological materials as well as effect of spices coffee (ginger, cinnamon, lichen, cardamom, cloves, ajwain, saffron, ready-mix spices) on inhibition biological inhibitory effect of fungal filtrations against *Bacillus subtilis* and destroyed Aflatoxin and Ochratoxin A in filtrates. All filtrations had an effect on inhibiting the bacterial growth of *Bacillus subtilis* to varying degrees which confirms the presence of biological active secreted by tested fungi. The experiments were carried out to determine and focus the types which results proved that the fungal isolates were able to produce. All fungal isolates studied were able to grow on the three tested media (sucrose magnesium potassium yeast extract medium, sucrose caffeinated coffee and decaffeinated coffee medium) and the caffeinated coffee medium was the first candidate to increase the ability of these isolates on the production. Spices Coffee (ginger, cinnamon, Lichen, cardamom, cloves, Ajwain, saffron, Ready-mix spices) had a positive effect to block the biological Inhibitory effect of fungal filtrations against bacteria *Bacillus subtilis*. This effect differed depending on the spice and concentration of spice.

Key words: Ginger, cinnamon, lichen, cardamom, cloves, ajwain, saffron, aflatoxin, ochratoxin A, *A. niger*, *A. alliaceus*, *A. flavus*, *A. melleus*

INTRODUCTION

Mycotoxins are harmful substances produced by fungi in many foods, Approximately 25% of the world's crops annually are estimated as losses due to these toxins. Most of mycotoxins are produced by three genera of fungi: *Aspergillus, Penicillium* and *Fusarium*. The existence of fungi producing mycotoxins in materials, food and feed is considered as international health problem all over the world (Smith and Hacking, 1983). These toxins may be carcinogenic, mutagenic, teratogenic, oesterogenic, In addition it has an impact on the growth rates of small animals and weaker immune responses and become more susceptible to diseases (Pier *et al*., 1980).

Ochratoxin was formed in seeds during the drying process and many researchers have studied that including (Bucheli and Taniwaki, 2002; Urbano *et al*., 2001). They noticed the presence of these toxins in the seeds prior to storage and pointed out the possibility that the harvest and post-harvest and what happens of the mistakes are the cause of such pollution (Urbano *et al*., 2001; Bucheli and Taniwaki, 2002). Le-Bars and Le-Bars (2000) stated that as the main polluter of coffee seeds. While Pitt (2000) declared that *Aspergillus ochraceus* is the most important producer for Ochratoxin A in coffee seeds.

Belli *et al.* (2004) found that the same fungus strains have produced of Ochratoxin (OTA) after 5 days of incubation and produced with a concentration of 4-5 micrograms/gram. And in a study conducted by Burmeister and Hesseltine (1966); 20 microgram mL of Aflatoxin was frustrate the growth of *Bacillus subtilis*. Moslem *et al.* (2010) confirmed that 80% of the isolates of *A. flavus* isolated from coffee beans were able to produce Ochratoxin A.

Several researchers have studied the effect of natural antimicrobial from plants and has been used to thwart microbial activity as a safe and alternative option for industrial preservatives (Nilsen and Rios, 2000; Smith-Palmer *et al.,* 2001; Lopez-Malo *et al.,* 2002; Moreira *et al.,* 2005; Al Talib, 2012). Guynot *et al.* (2003) tested that 16 types of spices and their effects on the growth of *E. herbariorum*; *E. repens*; *E. rubrum*; *E. amstelodami*; *Eurotium* sp. the results showed that the full frustrating effect is due to the lemon, cloves, cumin, bay leaf and thyme.

Thanaboripat *et al.* (2004) found that citronella oil in concentration of 0.2% has inhibited the growth of three isolates of *A. flavus* and *A. parasiticus* in Potato decstrose agar medium.

Thanaboripat *et al.* (2004) found that Starunella oil in concentration of 0.2% has inhibited the growth of three isolates of A. flavus and isolation of A. parasiticus growing with the medium of PDA.

Dimia *et al.* (2007) tested the impact of caraway seeds, garlic and marjoram on *Eurotium herbariorum; E. amstelodami A. flavus; A. sydowii* and has proved that caraway seed extract is highly effective in inhibiting the growth of all tested types. Also it had an impact on all four toxins produced by fungal *Eurotium, Aspergillus*; during 7 days incubation at temperature of 25 °C. Also Thanaboripat *et al.* (2007) tested inhibitory effect of 16 essential oil of aromatic plants on growth of *A. flavus*, where the results proved that the oil extracted from the white wood has high inhibition followed by cinnamon oil and lavender oil, respectively.

Mitchell *et al.* (2010) confirmed that marjoram extract may inhibited growth of *A. flavus; A. parasiticus; A. fumigatus; A. terreus; A. ochraceus* in a significant effect, also it gave a significant effect in inhibiting the growth of all tested fungi and the minimum inhibitory concentration was 0.6 Ug mL^{-1}.

Al Talib (2012) tested the effect of ginger, cinnamon, cardamom, cloves, saffron on fungal culture extracts if frustrating activity of bacteria and noted that the greater the concentration of spices increased, the more the frustrating ability increased. In this study aimed to identify the biological material with the inhibitory activity of bacteria and estimating them.

MATERIALS AND METHODS

Biological tests: The isolates were evaluated for their ability to produce toxigenic materials, namely (Table 1): *A. niger*; *A. alliaceus* (1); *A. flavus* (1); *A. melleus* (1); *A. melleus* (2) The effect of culture filtrate on the growth of *Bacillus subtilis* was studied (Olivigni and Bullerman, 1978) liquid SMKY medium (Diener and Davis, 1966) and consists of: (200 g sucrose 0.5, 0 grams magnesium sulfate, 3 g potassium nitrate, 7 g yeast extract per liter of water), caffeinated coffee medium (200 g sucrose, 25 g caffeinated coffee and complement components of the medium to one liter with the distilled water) and the medium of decaffeinated coffee (200 g sucrose, 25 g decaffeinated coffee and complement components to one liter with distilled water). then incubated at 25+2°C for 14 days. Then, the filtrate of each isolate was extracted three times with equal volumes of ethyl acetate. The ethyl acetate was removed by evaporation and residue was brought up in sterilized distilled water. The method described by Lenz *et al.* (1986) was used as follows: one species of specific bacteria, that is, *B. subtilis,* was obtained from Bacterial Disease Department,

Table 1: Fungal isolates and varieties of coffee and frequency (%) (Al Talib, 2012)

Fungi isolates	Variety	City	Frequency (%)
A. niger	Harary coffee beans	Dammam	74.89
(1) *A .alliaceus*	Harary coffee beans	Dammam	7.34
A. flavus (1)	Habbashy coffee beans	Dammam	2.01
(2) *A. alliaceus*	Moca coffee (Rubesta)	Dammam	7.33
(2) *A. flavus*	Nescafe coffee (Rubesta)	Qatif	2.01
(1) *A. melleus*	Harary coffee beans	Al-hsa	3.97 (2)
A. melleus	Habbashy coffee beans	Al-hsa	5.10

Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt. Equal disks (cm) of the tested bacterium were prepared from 10-days old bacterial cultures grown on TYG solid medium (Scott and Kennedy, 1975) which consists of 50 g tryptone, 2.5 g yeast extract, one g glucose and 20 g agar dissolved in one liter of distilled water. A liquid medium of TYG was prepared, distributed into 500 mL Erlenmeyer flasks (200 mL/each) and autoclaved. After cooling, one disk (0.5 cm) of bacterial culture was added to each flask. All the flasks were incubated for 48 h at 30°C. Other flasks (250 mL) containing TYG solid medium (100 mL/each) were prepared and autoclaved. After cooling and before solidification, one mL of the previous bacterial suspension was added to each flask and shaken well. The inoculated medium was distributed into Petri dishes (20 cm) at the rate of 10 mL medium/plate. Diffusion through agar pore technique (Rojas *et al*., 2003) was used to study the effect of culture filtrate against the efficiency of aflatoxins by using a cork borer (0.3 cm in diameter). A pore was made in the middle of each plate. One mL of the aforementioned filtrate of each tested fungal isolate was added to the pore and all the dishes were incubated for 48 h at 30°C. The diameters of the inhibition zones were measured (in cm^2) as an indicator for aflatoxin production.

Qualitative and quantitative assessment of mycotoxins in fungal culture filtrate: The High Performance Liquid Chromatography (HPLC) system consisted of Waters Binary pump Model 1525, a Model Waters 1500 Rheodyne manual injector, a Waters 2475 Multi-Wavelength Fluorescence Detector and a data workstation with software Breeze 2. A phenomenex C_{18} (250×4.6 mm i.d), 5 um from Waters corporation (USA) for aflatoxins. A HyperClone 5 µ ODS column (C_{18}) 120A°, DIM: 250×4.60 mm. (Phenomenex)

Extraction of Aflatoxins by VICAM (2000)

Sample extraction: Weigh 25 g sample with 5 g salt sodium chloride and place in blender jar. Add to jar 125 mL methanol: water (70:30). Cover blender jar and blend at high speed for 1 min. Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

Extract dilution: Pippet or pour 15 mL filtered extract into a clean vessel. Dilute extract with 30 mL of purified water, mix well. Filter diluted extract through glass microfiber filter into a glass syringe barrel using markings on barrel to measure 4 mL.

Immunoaffinity Chromatography: Pass 15 mL filtered diluted extract (15 mL = 1 g sample equivalent) completely through AflaTest®-P affinity column at a rate of about 1-2 drops/second until air comes through column. Pass 5 mL of purified water through the column at a rate of about 2 drops/second. Elute affinity column by passing 1.0 mL HPLC grade methanol through column

at a rate of 1-2 drops/second and collecting all of the sample elute (1 mL) in a glass vial. Evaporated to dryness under stream of nitrogen and was determination of HPLC.

Detection and determination of Aflatoxins by HPLC

Derivatization: The derivatives of samples and standard were done as follow: 100 µL of Trifluoracetic Acid (TFA) were added to samples and mixed well for 30 sec and the mixture stand for 15 min. 900 µL of water: acetonitrile (9:1 v/v) were added and mixed well by vortex for 30 sec .and the mixture was used for HPLC analysis.

HPLC conditions: The mobile phase consists of Acetonitile/Water/ methanol (1:6:3). The separation was performed at ambient temperature at a flow rate of 1.0 mL min^{-1}. The injection volume was 20 µL for both standard solutions and sample extracts. The fluorescence detector was operated at an excitation wavelength of 365 nm and an emission wavelength of 450 nm. AFB_1 concentration in samples was determined from the standard curve, using peak area for quantification.

Ochratoxin analysis

HPLC equipment: The HPLC system consisted of Waters Binary pump Model 1525, a Model Waters 1500 Rheodyne manual injector, a Watres 2475 Multi-Wavelength Fluorescence Detector and a data workstation with softwear Breeze 2.

Chemicals and reagents: OTA standard, Chartist, microfiber filter 1.5 µm and filter papers were purchased from VICAM. Milford, MA USA. Acetonitrile, glacial acetic acid HPLC grade were obtained from BDH, England. Sodium chlorid, sodium biocarbonate, sodium hydrogen phosphate, potassium dihydrogen phosphate and potassium chloride were purchased from (BDH, Merck chemicals). And tween -20 obtained from Sigma (St. Louis, MO, USA).

HPLC condition: A Symmetry C_{18} (5 µm particle size, 150×4.6 mm i.d.) from Waters corporation (USA), were used along with a mobile phase of Acetonitile/Water/ acetic acid (55:43:2). The separation was performed at ambient temperature at a flow rate of 1.0 mL min^{-1}. The injection volume was 50 µL for both standard solutions and sample extracts. The fluorescence detector was operated at an excitation wavelength of 330 nm and an emission wavelength of 470 nm. OTA concentrations in coffee extracts were determined from the standard curve, using peak area for quantitation.

Study of the effect of some commonly used coffee spices on studied fungal culture filtrates: The purpose of the experiment is to find out the effect of adding some of the spices commonly used in the Arabic coffee on the effectiveness of the active substances in the fungal culture filtrates under study by testing the effect of adding them to these filtrates and with different concentrations.

Prepare water extract of tested spices which include (cardamom *Elletaria cardamomum*; ginger *Zingiber officinaie*; Roscos, cinnamon *Cinnamomum zeylanicum*; ajwain; lichen; cloves *Syzygium aromaticum* L.; saffron *Crocus sativus* and a mixture of spices) (Table 2) to be cold depending on the method of El-Astal *et al.* (2005) by adding 5 g of ground condiment in 100 mL of distilled sterilized water so as to obtain a concentration of (5%) and then put it in the refrigerator

Table 2: Most important spices commonly used in the preparation of Arabic coffee

Spices	Effective compounds	Scientific name	Family	Parts used
Cardamon	Volatile oil is composed of several turbines and Althenol eucalyptus Albornjul	*Elletaria cardamomum*	Zingiberaceae	Fruits
Cinnamom	Cinnamon oil containing aldehyde Alqrfa and acidic Aleugenik and Alsnobrin	*Cinnamomum zeylanicum*	Lauraceae	Inner bark
Clove	Clove oil is extracted from the buds and the most important compounds that it contains eugenol and a little turbine	*Syzygium aromaticum* (L.)	Myrtaceae	Floral buds
Ajwain	Volatile oils and most important of them is thymol	*Trachyspermum ammi* (L.)	Apiaceae	Fruits
Ginger	Volatile oil composed of Alcamvin and Allinalol including resinous material and a large amount of starch and hot taste of Gangrol and Alzenjron	*Zingiber officinaie* Roscoe	Zingiberaceae	Rhizoms
Saffron	Jlecoqsid amorphous in saffron oil is Bakarockerocn	*Crocus sativus*	*Iridaceae*	Stigma and stylus
Shibah (Lichen)	Different kind of lichen containing many essential oils			
Ready-mix spices	A combination of the above ingredients in different proportions commercialized with Arabic coffee			

at a temperature of (4°C) for 24 h. Then put extract in the centrifuge for 10 min and then the pure part was filtered through the membrane filter. Then the two concentrations of (5.0, 3%) were equipped, then a working control group has been made which is about sterile distilled water+ extracts of mycotoxins with the same calculated proportions.

A biological test has been made to study the impact of biologically active substances in different concentrations of spices, through the study of the effectiveness of these active substances containing aqueous extracts concentrations of spices on the growth of *B. subtilis* with the same way used in biological tests.

Statistical analysis: Data obtained were statistically using the 16 version of program SPSS where transactions averages were compared at the abstract level of (0.05) using the test Least Significant Difference (LSD) designed by Norusis (1999).

RESULTS

The biological tests to estimate the toxicity of fungal culture extracts: This test was conducted to seven different isolates of Aspergilli were isolated from different samples of arabic coffee and rubesta. These isolates are *A. niger*, *A. alliaceus* (1); *A. flavus* (1); *A. melleus* (1); *A. melleus* (2) all of which are isolated from the seeds of coffee of harari, adding to the isolation of *A. alliaceus* (2) were isolated from samples of milled mocha coffee of rubesta and isolation of *A. flavus* (2) isolated from nescafé coffee without caffeine which is also rubesta coffee.

Notes from the results recorded in Table 3 of the study of the impact of fungal culture filtrate of above isolates on the growth of *B. subtilis* as an indicator of the presence of biological material inhibitor to the growth of these bacteria in case get the inhibition zone and the results showed the following All isolates of were able to grow on the three media but differed ability to produce materials active biologically and inhibitory to the growth of bacteria but caffeinated coffee medium was the first candidate to increase the ability of these isolates on production as the average size of the inhibitory areas generally was 34.83 in the medium of caffeine coffee followed by SMKY medium area of 19.45 cm^2, then decaffeinated coffee medium area of 12.62 cm^2.

Table 3: Effect of culture filtrates of tested fungi on growth of *Bacillus subtilis*

Fungi isolates	Variety which each fungus was isolated	Inhibition zone (cm^2) Coffee without caffeine medium	Caffeinated coffee medium	SMKY medium
A. niger	Harary coffee beans	32.71	41.13	37.88
(1) *A. alliaceus*	Harary coffee beans	-	49.01	34.93
A. flavus (1)	Habbashy coffee beans	10.85	49.01	35.69
(2) *A. alliaceus*	Moca coffee (Rubesta)	17.33	19.29	14.37
(2) *A. flavus*	Nescafe coffee (Rubesta)	15.92	29.54	13.25
(1) *A. melleus*	Harary coffee beans	11.76	34.9-	
(2) *A. melleus*	Habbashy coffee beans	-	20.96	-
	Average	12.65	34.83	19.45
	L.S.D. at 0.05%	2.98	7.51	3.1

Estimated quantity tests for aflatoxins and ochratoxin in extracts of the tested fungal culture filtrates: The isolation and purification of Aflatoxins and Ochratoxin was made from fungal culture filtrates where results in Table 4 shows the ability of isolates to produce mycotoxins and affected by differences in installation medium on which they grow but all prefer caffeine coffee medium which fungal isolates expressed more ability on produce more than one type of these toxins as noted, for example, *A. niger* was produced aflatoxin B1 in SMKY medium with the concentration of 0.521 Mg 1 g, while on caffeinated coffee medium produced 0.497 Mg grm of aflatoxin B1 and 0.05 Mg 1 g of aflatoxin G1 and gave in a coffee without caffeine 0.043 Mg 1 g of aflatoxin G1.

Also it is noted from results the ability of *A. alliaceus* (1) on the production of all four types of Aflatoxins (B1; B2; G1; G2) in caffeinated coffee medium while it did not produce any type in decaffeinated coffee medium and gave 0.728 Mg 1 g concentration of B1 type in SMKY medium. The results also shows that the isolation of *A. alliaceus* (2) only was able to produce B1 in medium of coffee without caffeine and SMKY medium, as well as *A. melleus* (2) and which was not unable to produce even in the mediums of coffee without caffeine and SMKY medium and this was confirmed also by the results of biological previous tests.

Also the fungi of *A. melleus* (1) was not able to produce any kind on the SMKY medium. It was also noted that *A. flavus* (1,2) were not able to produce any kind of Aflatoxins in decaffeinated coffee medium.

Regarding toxins of Ochratoxin, results recorded in the Table 4 show the inability of all fungal isolates tested on the production of Ochratoxin in liquid the three tested media.

Study of the effect of some spices of coffee drink on the activity of biological activity material in studied fungi culture filtrate: Results in the Tables 5, 6 and 7 of the effect of different concentrations of spices of coffee drink in controlling the activity of biologically active substances in fungal culture filtrate and bacterial growth *B. subtilis* confirm the existence of a positive impact varies according to the used condiment and that the more focus condiment increased accordingly the more rate of decline in inhibitory activity. For example when using focus 0.5% of these spices as in Table 5 the overall average for the decline was dissentingly as follows: 59.42, 58.86, 57.07, 53.06, 46.89, 45.20, 40.67, 40.59% for each of ginger, cinnamon, lichen, cardamom, cloves, ajwain, saffron, ready-mix pepper, respectively.

The increase in the average rate of decline was observed with the increase to 3% concentration of spices as in Table 6, reaching 77.67, 74.61, 71.29, 70.46, 62.54, 60.82, 51.02, 50.45% of the ajwain, ginger, lichen, a mixture of ready pepper, cloves, cardamom, saffron, cinnamon,

Table 4: Toxins concentration (Aflatoxins and Ochratoxins) in the filtrate of tested fungi

		Caffeinated coffee medium						Coffee without caffeine medium						SMKY medium					
Isolates	Isolates source	OTA	Total	G2	G1	B2	B1	OTA	Total	G2	G1	B2	B1	OTA	Total	G2	G1	B2	B1
A. niger	Harri beans	ND	0.551	ND	0.050	ND	0.497	ND	0.043	ND	0.043	ND	ND	ND	0.521	ND	ND	ND	0.521
(1) A. alliaceus	Harri beans	ND	0.426	0.176	0.167	0.187	0.592	ND	ND	ND	ND	ND	ND	ND	0.728	ND	ND	ND	0.728
A. flavus(1)	Harri beans	ND	0.426	ND	ND	ND	0.426	ND	ND	ND	ND	ND	ND	ND	0.711	ND	ND	ND	0.711
A. alliaceus(2)	Harri beans	ND	0.434	0.010	0.021	ND	0.403	ND	0.332	ND	ND	ND	0.332	ND	0.806	ND	ND	ND	0.806
A. flavus(2)	Moca rubesta	ND	0.402	ND	ND	ND	0.402	ND	ND	ND	ND	ND	ND	ND	0.711	ND	ND	ND	0.711
A. melleus(1)	Nescafa with out cafaine	ND	0.403	ND	ND	ND	0.403	ND	0.901	ND	ND	ND	0.901	ND	ND	ND	ND	ND	ND
A. melleus(2)	Harri powder	ND	0.246	ND	0.46	0.200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Average		-	0.413	0.027	0.041	0.055	0.389	-	0.182	-	0.006	-	0.176	-	0.497	-	-	-	0.497
LSD (0.05)		-	12.21	1.07	1.790	1.550	5.550	-	1.420	-	1.000	-	1.360	-	3.760	-	-	-	3.760

OTA: Ochratoxin A, B1: Aflatoxin B1, B2: Aflatoxin B2, G1: Aflatoxin G1, G2: Aflatoxin G2, ND: Non Detected

Table 5: Effect of some spices of coffee drink (0.5% concentration) on the activity of biological activity material in studied fungi culture filtrate

	Coffee spices										
Filtrate	Control	Cardamom	Ginger	Cinnamon	Ajwain	Lichen	Cloves	Ready-mix spices	Saffron	Average of reduce\fungi	LSD
A. niger	28.29	19.24	20.96	19.630	20.960	21.98	20.840	21.65	20.56	21.57	24.28
	-%	-31.99	-25.91	-99.310	-25.910	-22.31	-26.340	-23.47	-27.33	-35.32	
A. alliaceus (1)	50.70	45.76	43.69	36.500	49.840	48.40	44.995	42.62	42.86	45.04	30.97
	-%	-9.74	-13.83	-28.010	-1.700	-4.54	-11.250	-15.94	-15.46	-12.56	
A. flavus (1)	47.39	13.10	7.47	8.900	4..20	11.34	21.930	9.81	11.65	15.15	3.5
	-%	-72.36	-84.24	-81.220	-9.000	-76.07	-53.730	-79.30	-75.42	-66.42	
A. alliaceus (2)	26.72	22.13	8.34	19.240	11.560	5.38	6.840	23.75	11.85	15.09	5.68
	-%	-17.18	-68.79	-27.995	-56.740	-79.87	-74.400	-11.12	-55.65	-48.97	
A. flavus (2)	26.65	5.94	12.76	20.690	6.910	13.65	19.650	22.36	24.48	17.01	6.79
	-%	-77.71	-52.12	-22.360	-74.070	-48.78	-26.270	-16.10	-8.14	-40.69	
A. melleus (1)	33.53	12.59	9.71	15.710	7.720	10.75	21.380	3.14	16.98	14.61	4.93
	-%	-62.45	-71.04	-53.150	-76.980	-67.94	-36.240	-90.64	-49.36	-63.48	
A. melleus (2)	20.96	0.00	0.00	0.000	5.870	0.00	0.000	10.99	8.13	5.13	2.10
	-%	-100.00	-100.00	-100.000	-71.995	-100.00	-100.000	-47.57	-60.35	-84.99	
Average of reduce\spice	-%	-53.06	-59.42	-58.860	-45.200	-57.07	-46.89	-40.59	-40.67		
Average	33.46	16.97	14..70	17.24	15.370	15.93	19.370	19.19	19.53		
LSD (0.05)	7.84	3.04	2.73	4.05	2.520	2.66	3.620	3.94	4.41		

Table 6: Effect of some spices of coffee drink (3% concentration) on the activity of biological activity material in studied fungi culture filtrate

	Coffee spices										
Filtrate	Control	Cardamom	Ginger	Cinnamon	Ajwain	Lichen	Cloves	Ready-mix spices	Saffron	Average of reduce\fungi	LSD
A. niger	28.29	11.06	22.34	7.87	17.12	19.63	15.94	14.74	14.19	16.8	8.29
	- %	-60.91	-21.03	-72.18	-39.48	-30.61	-43.66	-47.9	-49.84	-45.7	
A. alliaceus (1)	50.7	42.23	35.25	35.82	48.55	43.23	38.86	42.66	42.86	42.24	24.48
	-%	-16.71	-30.47	-29.35	-4.24	-14.73	-23.35	-15.86	-15.46	-18.77	
A. flavus (1)	47.39	3.74	4.03	4.92	0	4.03	13.995	5.13	11.65	10.54	2.19
	-%	-92.11	-91.5	-89.62	-100	-91.5	-70.47	-89.18	-75.42	-87.48	
A. alliaceus (2)	26.72	18.44	0.87	18.88	0	0	6.75	0	0.39	8.01	2.29
	-%	-30.99	-96.74	-29.34	-100	-100	-74.74	-100	-98.54	-78.79	
A. flavus (2)	26.65	3.77	4.65	16.77	0	4.34	14.19	15.91	24.48	12.31	3.85
	-%	-85.85	-82.55	-37.08	-100	-83.72	-57.68	-40.3	-8.14	-61.92	
A. melleus (1)	33.53	0	0	11.54	0	7.22	10.77	0	16.98	8.89	2.38
	-%	-100	-100	-65.58	-100	-78.47	-67.88	-100	-49.36	-82.66	
A. melleus (2)	20.96	0	0	0	0	0	0	0.01	8.31	3.25	1.36
	-%	-100	-100	-100	-100	-100	-100	-99.95	-60.35	-95.04	
Average of reduce\spice		-60.82	-74.61	-50.45	-77.67	-71.29	-62.54	-70.46	-51.02		
Average	33.46	11.32	9.59	13.69	9.38	11.21	14.36	11.21	16.98		
LSD (0.05)	7.84	1.98	1.84	3.08	1.35	1.9	3.14	1.92	3.3		

respectively and also the average rate of decline in activity after using inhibitory concentration of 5% increased as in Table 7 where it reached 91.24, 88.38, 80.20, 77.66, 75.59, 74.39, 72.45, 70.61% for ginger, ajwain, cardamom, Lichen, a mixture of spices, cloves, saffron, cinnamon, respectively. Regarding steadily of these active substances of different fungi it were observed that they differed

Table 7: Effect of some spices of coffee drink (5% concentration) on the activity of biological activity material in studied fungi culture filtrate

	Coffee spices										
Filtrate	Control	Cardamom	Ginger	Cinnamon	Ajwain	Lichen	Cloves	Ready-mix spices	Saffron	Average of reduce\fungi	LSD
A. niger	28.29	5.24	10.88	6.73	0.04	19.11	0.00	14.74	8.51	10.39	3.40
	-%	-81.48	-61.54	-76.21	-99.86	-32.45	-100.00	-47.90	-69.92	-71.17	
A. alliaceus (1)	50.7	33.86	0.00	33.18	41.16	39.79	32.84	38.66	31.76	33.55	7.24
	-%	-33.22	-100.00	-34.56	-18.82	-21.52	-35.23	-23.75	-37.36	-38.06	
A. flavus (1)	47.39	0.00	3.46	0.00	0.00	0.00	13.34	0.00	7.06	7.92	1.53
	-%	-100.00	-92.70	-100.00	-100.00	-100.00	-71.85	-100.00	-85.10	-93.71	
A. alliaceus (2)	26.72	11.10	0.00	17.49	0.00	0.00	6.60	0.00	0.00	6.88	2.12
	-%	-58.46	-100.00	-34.54	-100.00	-100.00	-75.30	-100.00	-100.00	-83.54	
A. flavus (2)	26.65	3.14	4.15	5.58	0.00	2.75	11.75	11.34	9.26	8.29	3.12
	-%	-88.22	-84.43	-79.06	-100.00	-89.68	-55.91	-57.45	-65.25	-77.50	
A. melleus (1)	33.53	0.00	0.00	10.09	0.00	0.00	5.89	0.00	13.21	6.97	1.87
	-%	-100.00	-100.00	-69.91	-100.00	-100.00	-82.43	-100.00	-60.60	-89.12	
A. melleus (2)	20.96	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.32	2.59	1.12
	-%	-100.00	-100.00	-100.00	-100.00	-100.00	-100.00	-100.00	-88.93	-98.62	
Average of reduce\spice		-80.20	-91.24	-70.61	-88.38	-77.66	-74.39	-75.59	-72.45		
Average	33.46	7.62	2.64	10.44	5.89	8.81	10.06	9.25	10.30		
LSD (0.05)	7.84	1.65	1.72	2.36	1	1.52	2.36	1.7	2.61		

in the degree of vulnerability by adding different spices and results recorded in the above the vulnerability of these materials noted in ascending order *A. melleus* (2), *A. flavus* (1), *A. melleus* (1), *A. alliaceus* (2), *A. flavus* (2), *A. niger*. Finally *A. alliaceus* (1) and this sequence were observed in all concentrations of used spices.

DISCUSSION

Ochratoxin A (OA) is an emerging problem in the production of coffee all over the world. In the past decade the importing companies for coffee as well as researchers have made a considerable interest of the fungal properties of coffee seed to estimate the risk to human exposure by toxins produced by fungi and the low levels of Ochratoxin are not dangerous while high concentration of these toxins in food is considered one of the reasons of potential cancer (Moss, 1996; Castegnaro and McGregor, 1998; Fink-Gremmels, 1999).

The minimum allowable levels of Aflatoxins in food are identified globally of no more than 20 micrograms/liter, according to SASO (1998).

The results showed that for all isolates filtrate led to the inhibition of the growth of *B. subtilis* with various degrees and such results corresponds with the results of many researchers such as Burmeister and Hesseltine (1966) and Al-Abdalall (2009).

These results are the same of many researchers regarding *A. niger*; A. *flavus* including Smedsgaard (1997), Payne and Brown (1998), Silva *et al.* (2000) and Premila and Sanchez (2006). Cabanes *et al.* (2002) confirmed the influence played by the food medium of in toxin production of Ochratoxin as the ability of isolates of *A. carbonarius* was tested to produce OTA on medium of Agar sucrose yeast extract and agar entanglement yeast extract and incubator for 14 days, where the toxins was found in farm after 7 days of incubation and this is the focus of both Calvo *et al.* (2002) and Bhatnagar *et al.* (2003), where they mentioned that the most important mediumal factors affecting the production of mycotoxins the food medium components especially used sources of carbon and nitrogen as well as pH, temperature, water activity and plant metabolism.

Aflatoxins and ochratoxin were isolated from fungal culture filtrates where results proving the ability of these fungal isolates to produce mycotoxins and affected by differences in components of medium on which they grow but all prefer medium of caffeine coffee which expressed fungal isolates ability more on the production of more than one type of these toxins it is noted from the obtained results by many researchers that the addition of caffeine to the medium contributes to disrupt or prevent the production of toxins by fungi. Regarding Ochratoxin, results show no ability of all tested isolates to produce Ochratoxin in the three liquid tested media and this result contrary to that obtained by a lot of researchers and including Pardo *et al.* (2004) where stated that all isolates of *A. carbonarius* isolated from coffee seeds were able to produce this toxin.

Regarding the results of the study of the effect of different concentrations of coffee drink spices in controlling the activity of biologically active substances in fungal culture filtrate and bacterial growth retardant *B. subtilis*, the results confirm the existence of a positive impact varies according to used spices and that the more focus of spices the more rate of decline in the frustration activity. The metabolism of plant plays a key role in the evolution of fungal growth and production of toxins.

The previous research in this area show that the plant ingredients or extracts can develop fungi of *Aspergillus* and production of Aflatoxin such as Alldhidat and vitamin C (Clevstrom *et al.*, 2004) and caffeine as mentioned by Buchanan *et al.* (1983).

In a study conducted by Dimia *et al.* (2007) on the types belonging to *Aspergillus* it was clear that exposing fungal cells of extracts of caraway seeds and also the garlic and marjoram has led to a cosmetic changes in the fungi and also led to growth inhibition of fungal colonies which may be due to changes at the cellular level. While Rasooli *et al.* (2006) stated the occurrence of damage to the cell walls and the cell membranes of *A. niger* after exposure to thyme oil while spores lost effectiveness with concentration of 250 and 500 ppm of essential oils for two varieties of thyme.

Thanaboripat *et al.* (2004) confirmed that the benefit of natural plant extracts lies in mind as alternative sources more effective and safer than of industrial antimicrobial and can be an alternative way to prevent food and feed of fungal contamination. And Velluti *et al.* (2003) attributed inhibitory activity to marjoram oil against microbes to contain aromatic aggregates and known to its interactive activity forming hydrogen bonds with important positions in enzymes and this was confirmed by Dimitrijevic *et al.* (2007) and Souza *et al.* (2007).

CONCLUSION

Seven isolates of fungi, namely: -*A. niger; A. alliaceus* (1); *A. flavus* (1); *A. alliaceus* (2); *A. flavus* (2); *A. melleus* (1); *A. melleus* (2) were tested for their ability to give biological active materials in-vitro. Data showed that all the tested fungi had the ability to give biological active materials in the culture filtrations. All filtrations had an effect on inhibiting the bacterial growth of *Bacillus subtilis* to varying degrees which confirms the presence of biological active secreted by tested fungi. The experiments were carried out to determine and focus the types which results proved that the fungal isolates were able to produce. All fungal isolates studied were able to grow on the three tested media (sucrose magnesium potassium yeast extract medium, sucrose caffeinated coffee and decaffeinated coffee medium) and the caffeinated coffee medium was the first candidate to increase the ability of these isolates on the production. Spices Coffee (ginger, cinnamon, Lichen, cardamom, cloves, Ajwain, saffron, Ready-mix spices) had a positive effect to block the biological Inhibitory effect of fungal filtrations against bacteria *Bacillus subtilis*, This effect differed depending on the spice and concentration of spice.

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