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Effect of Essential Oils on Growth and Aflatoxin Production by *A. parasiticus* Isolated from Walnuts

Monira R. Al-Othman[1], Abeer R.M. Abd El-Aziz[1]*, Mohamed A. Mahmoud[2] and Munirah F. Al-Gahtani[1]

[1]Botany and Microbiology Department, College of Science, King Saud University, Riyadh 1145, Kingdom of Saudi Arabia.
[2]Plant Pathology Research Institute, ARC, Giza, Egypt.

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Effects of five essential oils (Cinnamon, Garlic, Mint, Rosemary and thyme) that seemed to have powerful antifungal and antiaflatoxin characteristics on *Aspergillus parasiticus* were studied in this research. *A. parasiticus* is one of the most important species that produces aflatoxin in walnuts. We used three concentrations from each tested essential oils (1,2 ,4). The all tested essential oils appeared more effectiveness on the growth and aflatoxin B and G at three tested concentrations compare to control. Cinnamon oil at 4 % was the highest effective essential oils on inhibition aflatoxin B and G were ranged from 62.7 to 71.0 and 46.4 to 74.4 % and 58.2 to 69.1%, respectively followe by and thyme oil . Mint and Rosemary oil gave moderate effect while garlic oil was the least. As well as aflatoxin was extracted from samples of walnuts then determined by high performance liquid chromatography (HPLC). Their concentrations ranged from 27.8 to 105.1 μg/kg. The highest levels were found in sample no.4.

Key words: Essential oils, Aflatoxin, growth, *A. parasiticus* , walnuts , HPLC.

Walnuts (*Juglans regia*) belong to the family Juglandaceae. Walnut contained poly unsaturated fatty acids like linoleic acid ,oleic acid and linolenic acid. Among the saturated fatty acids are palmitic acid and stearic acid (Martinez *et al*., 2005). Content of omega-3 fatty acid in walnuts are 40 to 500 times greater than most other nuts (McKay and Sibley, 2007).

Mycotoxins are secondary metabolites produced by fungi which mostly belong to the *Aspergillus, Penicillium* and *Fusarium* species. Aspergillus mycotoxins of greatest significance in foods and feeds are aflatoxins (produced by *A. flavus, A. parasiticus, A. nomius*) (Sekar *et al*.,2008).

Various strains of *Aspergillus* species may result in production of aflatoxins, which are decrease food safety and quality of walnuts (Mahoney *et al.*, 2003; Zubair *et al*.,2011). Aflatoxins are a group of chemically related mycotoxins, which are carcinogenic in nature. The aflatoxin-producing moulds occur widely, in temperate, sub-tropical and tropical climates (Coker, 97). *A. parasiticus* produce both B and G aflatoxins (Klich and Pitt 1988; Pitt, 1993). *Aspergillus flavus* and *A. parasiticus* are the most important aflatoxigenic species naturally occurring in agricultural commodities (Pildain *et al*., 2008; Pitt and Hocking, 2009). Condition of storage like as temperature and humidity contribute to mycotoxin contamination of foods and their spoilage (Riba *et al.*, 2013).

* To whom all correspondence should be addressed.
E-mail: aabdelaziz@ksu.edu.sa

MATERIALS AND METHODS

Collection of samples

Fifteen samples from Walnuts were collected randomly from different markets in Riyadh Saudi Arabia during 2012 for this experimental work. The samples were stored at 2 °C until used (Czerwiecki *et al*., 2002).

Isolation, purification and identification of *A. parasiticus* associated with Walnuts

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 9 cm diameter containing potato dextrose agar (PDA), and each entry replicated three times. Petri dishes were incubated at 25±2ºC and observed daily for emergence of colonies, After which the colonies were counted. Isolates were purified either by single spore method. The isolates were identificated by Regional Center of the Fungi and their Applications, Al-Azhar university, Cairo, Egypt.

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) (Diener and Davis 1966) . The flasks were inoculated with discs of 6 mm diameter of *A. parasiticus* at 25 ± 2°C for 7 days (Paranagama *et al*., 2003) three replicates were performed. After incubation, content of each flask was filtered (Whatman, No. 1). For aflatoxins extractions, the filtrates of each flask were treated three times with 50 ml of chloroform in a separating funnel. The chloroform extract was separated and dehydrated with anhydrous sodium sulfate and evaporated till dryness on water bath at 50°C under vacuum. The residues were dissolved in 10 ml methanol and stored in dark vials.

Extraction of aflatoxin from Walnuts

For extraction of aflatoxin, 100 ml of 4% acetonitrile aqueous solution of potassium chloride (9:1) was mixed with 20 g of each sample. Extraction was followed by shaking for 20 min and filtered through Whatman No.4 filter paper. For purification, 100 ml of n-hexane were added to the filtrate then shaken for 10 min., 50 ml deionized water and 50 ml chloroform were added to the lower phase and this solution was shaken for 10 min. After separation, the upper phase was re-extracted twice with 25 ml of chloroform by using the above conditions. Then the chloroform was evaporator in a 40°C water bath at low speed. Methanol at the rate of 2 ml was added and the solution filtered through a 0.45 µl filter . (Zaboli *et al.,* 2011).

Effect of essential oils on growth and aflatoxin produced by *A. parasiticus*

Antiaflatoxigenic efficacy values of each tested oil were determined using SMKY liquid medium (sucrose, 20 g; magnesium sulfate, 0.5 g; potassium nitrate, 3 g; yeast extract, 7 g and distilled water, 1000 ml) .The surfactant (25% Tween in sterile water) were add to oils then different concentrations of each oil (1,2 and 4% %) were prepared and added to media, the flasks were inoculated with discs of 6 mm diameter of *A. parasiticus* isolates at 25 ± 2°C for 7 days (Paranagama *et al.,* 2003). Three replicates were performed for each concentration and the control set was kept parallel to the treatment without oils. After incubation, content of each flask was filtered (Whatman, No. 1) and biomass of filtered mycelium was dried at 70°C for 4 days till their weights remains constant. Mycelial dry weights of treatments and control was determined . For aflatoxins extractions, the filtrates of each flask were treated three times with 50 ml of chloroform in a separating funnel. The chloroform extract was separated and dehydrated with anhydrous sodium sulfate and evaporated till dryness on water bath at 50°C under vacuum. The residues were dissolved in 10 ml methanol. (Mostafa *et al.* 2011).

Determination of aflatoxins by High -performance liquid chromatography (HPLC)

Analysis of compounds was performed on HPLC model (model PerkinElmer series 200 UV/ VIS) with a C18 column with an internal diameter of 300 mm x 3.9 mm, 4 micron. The HPLC was equipped with an UV detector and fluorescence with 365 nm excitation and 430 emission wavelengths.). The total run time for the separation was approximately 25 min at a flow rate of 1 ml/min. The mobile phase consisting of methanol: acetic acid: water (20:20:60 v/v/v (Christian, 1990). The aflatoxin inhibition was calculated as follows: percentage of inhibition toxin = [A – a / A] x 100, where" A" is the concentration of aflatoxin in the treated sample and "a" is the concentration of aflatoxin in the control.

Statistical analysis

All of the data from three independent replicate trials were subjected to analysis using Statistical Package for the Social Sciences (SPSS) 10.0 statistical software (Chicago, USA). The data are reported as the mean ± standard deviations, and significant differences between mean values were determined with Duncan's Multiple Range test ($p<0.05$), followed by one-way ANOVA.

RESULTS

Data in table (1) obtained that concentrations of aflatoxins (AFB1, AFB2, AFG1 and AF G2) ranged from 13.5-42.4, 11.7-27, 12.5-27.1 and 15.8-27.1 µg/kg, respectively. The highest total contamination of aflatoxins B1, B2, G1 and G2 were found in samples No. 4 (105.1 µg/kg). Sample no.2 failed to produced aflatoxin B1, sample no 5 was produced B1 only. whereas, three samples produced all four aflatoxins (B1, B2, G1 and G2) in varying amounts.

Effect of essential oils

A-Effect of five essential oils at three different concentrations on dry weight of *A. parasiticus* isolated from Walnuts

Effect of different concentrations of essential oils on dry weight of mycelia after incubation at 25±2°C for 7 days. The all tested essential oils appeared more effectiveness on the growth at three tested concentrations compare to control . % inhibition of dry weight decreased with increasing concentrations of all treatments by essential oils. Table (2 &3) show that the highest growth inhibition rate of the tested fungi were isolated from walnuts observed with the Cinnamon oil and thyme oil at 4 % were ranged from 60.0 to 80.7% and 60.0 to 73.7% respectively. Mint and Rosemary oil gave moderate effect (ranged from 45.1 to 66.7 and 51.2 to 66.3% respectively) while garlic oil was the least (ranged from 34.5 to 56.9%). Statistical results showed that kind and amount of essential oils have a significant influence on dry weight $p<0.05$.

D-Effect of five essential oils at 4% on aflatoxin B (µg/ml) produced by *A. parasiticus* were isolated from Walnuts

Data in table (4) obtained that five tested essential oils lead to decrease aflatoxin (B) were produced by *A. parasiticus* when compared with control. Cinnamon oil and thyme oil were the highest effective essential oils on inhibition aflatoxin B were ranged from 62.7 to 71.0 and 58.2 to 69.1%, respectively. Mint oil and rosemary oil gave the third rank were inhibition ranged from 52.6 to 68.7 and 47.8 to 61.8 % respectively whereas garlic essential oil was ranged from (36.9 to 49.7%). The highest level of inhibition was observed in isolate no.4 when treatment with cinnamon, garlic, mint, rosemary and thyme and oil (71.0, 49.7,68.7,67.8 and 69.0%, respectively). The least of inhibition was observed in isolate no.3.

E-Effect of five essential oils at 4% on aflatoxin G (µg/ml) were produced by *A. parasiticus* isolated from Walnuts

Data in table (5) obtained that cinnamon oil was the highest effective essential oil on inhibition aflatoxin G followed by thyme oil at 4 % were lead to the highest level of inhibition were ranged from 46.4 to 74.4 and 41.4 to 63.0% , Mint oil gave the third rank followed by Rosemary oil, inhibition ranged from 42.8 to 59.4 and 36.6 to 51.6%, respectively. Whereas garlic oil gave the least (28.9 to 41.7%).

DISCUSSION

Climatic conditions and to agricultural practices that increase aflatoxin contamination by *Aspergillus* spp. (Nawar, 2008) and improper postharvest handling and storage (Nakai *et al.*, 2008). inhibition of fungal growth and mycotoxin production was dependent on the concentration of essential oils (Soliman & Badeaa, 2002; Al-Gahtani *et al.*,2013). A direct correlation was found between fungal growth and aflatoxin production. (El-Habib, 2012).

The results indicate that the test toxigenic fungi more sensitive to thyme and cinnamon. This result is confirmed by (Omidbeygi *et al.* 2006; Rad *et al.*,2011; Eweis *et al.*, 2012; Abd El- Aziz *et al.*, 2012; Al-Gahtani *et al.*,2013).

The effect of essential oils as antimicrobial may be due to the presence of active component as phenols, alkaloids and tannins (Ebana *et al.*,1991) might be penetrating into the interior of the cell and interacting with critical sites (Cristani *et al.*, 2007).the loss of aflatoxigenic capabilities of *A. parasiticus* correlated with

Table 1. Determination of aflatoxins from Walnuts (µg/kg)

Sample	aflatoxins from Walnuts (µg/kg)				
	G1	G2	B1	B2	Total
1	23.1	26.7	13.5	27	90.3
2	16.2	26.6	0	15.3	58.1
3	12.5	15.8	32.3	11.7	72.3
4	27.1	19.5	42.4	16.1	105.1
5	0	0	27.8	0	27.8

Table 2. Effect of five essential oils at three different concentrations on dry weight of mycelia (g) of *A. flavus* and *A. parasiticus* isolated from walnuts

Isolates	Control	Cinnamon			Garlic			Mint			Rosemary			Thyme		
		1%	2%	4%	1%	2%	4%	1%	2%	4%	1%	2%	4%	1%	2%	4%
1	3.61^{ab}	2.56^{ac}	1.59^{d}	1.14^{e}	3.29^{ab}	2.65^{ed}	2.10^{def}	2.50^{cb}	1.57^{ad}	1.54^{d}	2.60^{bc}	1.61^{d}	1.50^{d}	2.63^{abc}	1.24^{cd}	1.18^{e}
	±0.11	±0.02	±0.31	±0.03	±0.15	±0.05	±0.04	±0.13	±0.03	±0.05	±0.11	±0.07	±0.04	±0.08	±0.08	±0.05
2	4.36^{e}	2.23^{ab}	1.90^{abcd}	1.13^{ef}	3.60^{abc}	2.96^{ed}	2.53^{edf}	3.07^{e}	2.17^{ab}	1.52^{b}	3.21^{ef}	2.21^{bc}	1.81^{d}	2.93^{ef}	1.67b^{d}	1.22^{a}
	±0.20	±0.08	±0.14	±0.06	±0.07	±0.26	±0.22	±0.18	±0.15	±0.30	±0.31	±0.22	±0.10	±0.18	±0.12	±0.10
3	3.47^{cd}	2.97^{bd}	2.65^{e}	1.11^{f}	3.08^{cd}	2.91^{cde}	2.01^{ab}	3.10^{cd}	2.78^{ab}	1.48^{b}	2.45^{d}	1.93^{b}	1.52^{b}	2.98^{cde}	2.42^{a}	1.34^{b}
	±0.16	±0.15	±0.06	±0.08	±0.11	±0.09	±0.06	±0.14	±0.06	±0.07	±0.05	±0.10	±0.03	±0.11	±0.02	±0.07
4	5.71^{cd}	3.06^{b}	2.53^{ef}	1.19^{f}	4.19^{bc}	3.36^{b}	2.46^{d}	3.22^{e}	2.65^{f}	1.92^{ef}	3.22^{ef}	2.65^{c}	1.92^{e}	2.77^{ab}	1.98^{abc}	1.57^{ab}
	±0.10	±0.27	±0.10	±0.03	±0.15	±0.12	±0.09	±0.05	±0.15	±0.02	±0.21	±0.27	±0.07	±0.08	±0.10	±0.10
5	4.25^{cd}	3.01^{abc}	2.47^{b}	1.79^{a}	3.62^{b}	3.0^{ab}	2.78^{e}	3.19^{e}	2.39^{ef}	1.87^{ef}	3.45^{a}	2.68^{b}	2.07^{ab}	1.98^{abc}	1.91^{abc}	1.71^{bc}
	±0.29	±0.30	±0.15	±0.34	±0.10	±0.16	±0.13	±0.15	±0.12	±0.04	±0.21	±0.13	±0.13	±0.11	±0.09	±0.06
6	3.28^{b}	2.12^{bc}	1.88^{f}	1.23^{ad}	3.05^{b}	2.51^{ab}	2.07^{a}	2.88^{ab}	1.97^{abc}	1.81^{ef}	2.41^{c}	1.71^{e}±	1.60^{f}	2.19^{d}	1.54^{ab}	1.32^{b}
	±0.14	±0.14	±0.04	±0.03	±0.14	±0.05	±0.05	±0.05	±0.09	±0.03	±0.11	0.06	±0.04	±0.12	±0.06	±0.04
7	4.09^{a}	2.78^{bd}	1.77^{bd}	1.22^{ad}	3.36^{bc}	3.0^{e}±	2.08^{bc}	2.86^{f}	2.76^{f}	1.56^{b}	3.11^{b}	2.16^{ab}±	1.73^{a}	2.99^{d}	1.68^{ab}	1.42^{b}
	±0.12	±0.11	±0.08	±0.06	±0.33	0.28	±0.26	±0.14	±0.23	±0.21	±0.20	0.16	±0.17	±0.30	±0.29	±0.08
8	4.18^{a}	2.74^{bc}	2.17^{b}	1.13^{f}	3.16^{bc}	2.55^{d}	2.0^{bc}	2.77^{f}	1.91^{abc}	1.50^{b}	2.92^{f}	2.33^{bc}	1.90^{b}	2.25^{bc}	1.62^{ab}	1.28^{b}
	±0.12	±0.17	±0.15	±0.22	±0.15	±0.32	±0.21	±0.16	±0.04	±0.10	±0.07	±0.10	±0.34	±0.35	±0.05	±0.07
LSD0.05	0.460	0.351	0.322	0.405	0.319	0.511	0.372	0.321	0.195	0.362	0.499	0.411	0.243	0.247	0.345	0.189

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

Table 3. Effect of five essential oils at three different concentrations on % inhibition of dry weight of *A. parasiticus* isolated from walnuts

Isolates	Cinnamon			Garlic			Mint			Rosemary			Thyme		
	1%	2%	4%	1%	2%	4%	1%	2%	4%	1%	2%	4%	1%	2%	4%
1	30.7	55.9	69.5	11.3	27.9	41.8	30.7	58.4	58.4	27.9	55.6	58.4	27.9	66.7	69.5
2	49.5	56.4	74.7	17.4	33.4	41.9	31.1	51.8	65.5	26.6	49.5	58.4	33.4	63.3	72.4
3	16.4	23.6	68.2	13.5	16.4	42.1	10.6	22.1	59.6	30.8	45.2	56.1	16.4	30.8	62.5
4	47.4	55.6	80.7	28.1	42.2	56.9	43.9	54.4	66.7	43.9	54.4	66.3	52.7	66.7	73.7
5	29.4	41.8	60.0	15.2	29.4	34.5	27.0	45.8	57.6	20.0	38.8	51.2	55.2	55.2	60.0
6	35.9	42.6	63.4	8.53	23.7	36.8	14.6	42.0	45.1	26.8	48.1	51.2	35.9	54.2	60.3
7	33.9	56.7	70.6	19.3	26.6	49.1	31.5	33.9	63.3	24.2	48.6	57.7	29.0	60.8	65.7
8	35.4	48.1	73.6	25.8	40.1	52.1	35.4	54.5	64.1	30.6	44.9	54.5	47.3	61.7	71.2

Table 4. Effect of five essential oils at 4% on aflatoxin B (µg/ml) produced by *A. parasiticus* isolated from walnuts

Isolate	Control		Cinnamon		%	Garlic		%	Mint		%	Rosemary		%	Thyme		%
	B1	B2	B1	B2	Inhibation	B1	B2	Inhibation	B1	B2	Inhibation	B1	B2	Inhibation	B1	B2	Inhibation
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	37.5	23.8	15.1	7.9	62.7	25.5	11.6	39.5	17.2	9.3	56.8	17.2	9.2	56.9	15.7	8.1	61.2
3	47.3	10.4	17.5	4.8	61.4	29.3	7.1	36.9	21.7	5.6	52.6	24.0	6.1	47.8	19.1	5.0	58.2
4	56.8	30.2	16.5	8.7	71.0	30.1	13.6	49.7	18.0	9.2	68.7	19.2	8.8	67.8	17.0	10.0	69.0
5	41.2	15.5	13.9	6.4	64.2	23.6	9.3	41.9	16.6	7.0	57.4	20.0	7.3	51.8	12.6	2.4	63.6
6	11.0	0	4.9	0	64.5	6.5	0	40.1	5.1	0	53.6	4.2	0	61.8	2.3	1.1	69.1
7	7.5	0	2.4	0	68.0	4.2	0	44.0	4.0	0	46.7	3.7	0	50.7	2.7	0	64.0
8	14.1	7.2	5.1	2.8	62.9	8.5	4.6	38.5	7.1	3.8	48.8	6.2	4.1	51.6	6.5	2.0	60.1

Table 5. Effect of five essential oils at 4% on aflatoxin G (µg/ml) produced by *A. parasiticus*. isolated from walnuts

Isolate	Control		Cinnamon		%	Garlic		%	Mint		%	Rosemary		%	Thyme		%
	G1	G2	G1	G2	Inhibation	G1	G2	Inhibation	G1	G2	Inhibation	G1	G2	Inhibation	G1	G2	Inhibation
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	24.6	17.3	6.9	4.3	73.3	14.0	12.3	38.0	9.0	8.0	59.4	11.3	9.0	51.6	8.3	7.2	63.0
3	31.8	19.3	14.2	13.1	46.6	21.3	15.0	28.9	14.9	14.3	42.8	19.0	17.1	38.1	16.1	14.0	41.1
4	29.3	14.0	15.9	2.9	56.5	19.0	7.2	41.6	17.3	6.0	44.2	17.5	4.1	48.3	18.1	3.5	48.3
5	29.4	12.5	13.4	0	68.0	18.4	6.0	41.7	15.2	5.4	50.7	17.0	6.0	42.6	15.0	4.1	54.3
6	9.1	0	2.3	0	74.7	6.4	0	29.6	4.4	0	51.6	5.0	0	45.0	3.7	0	59.3
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	12.3	0	5.1	0	58.8	8.2	0	33.3	7.1	0	42.2	7.8	0	36.6	4.6	0	62.6

conidial morphology (Kale *et al.*, 1996) . Essential oils were act on the mycellium lead to lysis of the hyphae and spores and loss of rigidity of hypha cell wall which cause aflatoxin deactivation process. (Namazi *et al*., 2002 Nicola *et al*., 2005; Sharma &Tripathi, 2008).

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