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Efficacy of *Rosmarinus officinalis* Essential Oil on *Aspergillus flavus* and *parasiticus*

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***Aspergillus flavus* and *parasiticus* associated with almonds were isolated and three different culture media were used to qualitatively measure aflatoxin production by *Aspergillus* via UV light (365 nm). Commercially rosemary essential oil was tested to determine their influence on growth and aflatoxin production in *A. flavus* and *A. parasiticus* by performed through high-performance liquid chromatography (HPLC). The results showed that the tested essential oils caused highly significant inhibition of fungal growth and aflatoxin production in *A. flavus* and *A. parasiticus*.**

Key words: Anti-aflatoxigenic, High-performance liquid chromatography, Rosemary, Almonds.

Almond tree, *Prunus dulcis* (Miller) D.A. Webb, is a cultivated tree originating from wild trees from Central Asia, which is currently dispersed throughout the world. The almond tree is adapted to dry and hot climates, and for that reason it is mainly established in Mediterranean countries (Portugal, Spain, Italy, and France) and others with similar climatic characteristics, like USA (specifically California) Rodrigues *et al*., 2012).The world produced 2.00 million tonnes of almonds in 2011 according to Food and Agriculture Organization, with United States the largest producer at 0.73 million tonnes (FAOSAT, 2013). The almond is a nutritionally dense food and is a rich source of vitamin E, containing 26 mg per 100 g. They are also rich in dietary fiber, B vitamins, essential minerals such as magnesium, copper, manganese, calcium, and potassium as well as monounsaturated fats and polyunsaturated fats (see nutrient table), fats which potentially may lower LDL cholesterol. Typical of nuts and seeds, almonds also contain phytosterols such as Beta-sitosterol, stigmasterol, campesterol, sitostanol, and campestanol, which have been associated with cholesterol-lowering properties (Berryman *et al*., 2011). Aflatoxins (AFs) are toxic secondary metabolites produced by species of *Aspergilli*, especially *Aspergillus flavus* and *Aspergillus parasiticus*. These fungi can grow on certain foods and feeds under favorable conditions of temperature and humidity and generate AFs before and=or during harvest, handling, shipment, and storage (Frisvad and Thrane, 2004). The four major naturally occurring AFs are known as aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2). AFs have been shown to be potent carcinogens, mutagens, and teratogens (Kotsonis *et al*., 2001). AFs can enter the food chain in the field, during storage, or later, under favorable conditions of temperature and humidity. The most potent of the four naturally occurring AFs (B1, B2, G1 and G2) is B1, which is listed as a group I carcinogen by the International Agency for Research on Cancer (IARC, 1982). Rosmarinus officinalis essential oil has been reported to be among the most active compounds against a number of food spoilage and pathogen microorganisms (Mangena & Muyima, 1999). The major components of R. officinalis and T. copticum L. oils were Piperitone (23.65%), alpha-pinene

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(14.94%), Limonene (14.89%), 1,8-Cineole (7.43%) and Thymol (37.2%), P-Cymene (32.3%), gamma-Terpinene (27.3%) respectively. It is concluded that the essential oils could be safely used as preservative materials on some kinds of foods to protect them from toxigenic fungal infections (Rasooli *et al*., 2008).

The antimicrobial activity of *R. officinalis* was also demonstrated (Abutbul, Golan-Goldhirsh, Barazani, & Zilberg, 2004, Yesil, Hames, Bedir, Vardar, Ozek & Baser, 2007).

In this study, the effects of essential oils of *Rosmarinus officinalis* (Rosemary) on growth and AFB1 production in *Aspergillus flavus* and *A. parasiticus* are evaluated

MATERIALS AND METHODS

Isolation of fungi from almonds

Fifteen samples of almond were collected from three locations from Saudi Arabia (Riyadh, Al- Damam and Abha) during 2012. Sodium hypochlorite 5% were used for sterilized samples superficially for one minute, then wash these samples three times with sterilized distilled water. Four pieces were placed on the surface of potato dextrose agar petri dishes and incubated at 25º± C for 7 days. Isolates were purified either by single spore methods. *Aspergillus flavus* and *A. parasiticus* isolates have been identified by Regional Center of the Fungi and their Applications, Al-Azhar University, Cairo, Egypt.

Extraction of aflatoxin from Almonds

For extraction of aflatoxin, 20 g of Almonds were mixed with 100 ml of 4% acetonitrile aqueous solution of potassium chloride (9:1). Extraction was followed by shaking for 20 min and filtered through Whatman No.4 filter paper under vacuum condition. 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. To the lower phase, 50 ml deionized water and 50 ml chloroform were added and this solution was shaken for 10 min. After separation, the lower phase was collected and 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 ¼l filter (Zaboli *et al*, 2011).

Aflatoxin production in different culture media

For determination of aflatoxin production based on fluorescence media, all Aspergillus strains were cultivated in PDA at 25 c for 7 days. A mycelium Plug of each strain was placed at the centre of a petri dish containing tested media (potato dextrose agar (PDA), Czapek agar (CZ) and Malt extract agar (MEA) (Davis *et al*., 1987).The plates were incubated at 25 c for 4 days in the dark, the presence or absence of fluorescence in the agar surrounding the growing Aspergillus colonies was determined by exposing the petri dishes to ultraviolet light (365nm)and expressed as positive or negative.

Detection of aflatoxins with fluorescence (UV)

The culture media used were Cazpek's agar, YES agar and potato dextrose agar sodium chloride Medium detection were, at 25°C for 4 days in darkness. The presence or absence of fluorescence in the agar surrounding the colonies assayed was determined under UV radiation (365 nm) and expressed as positive or negative according to Franco *et al*., (1998).

Efficacy of Rosemary oil on growth and aflatoxin production

Antiaflatoxigenic efficacy values 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) (Diener and Davis 1966). The surfactant (25% Tween in sterile water) were add to oil 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 Aspergillus spp. at 25 ± 2°C for 7 days (Paranagama *et al.*, 2003). Control was carried out with Tween 80 (Lima *et al*., 1993). After incubation, the 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 aflatoxin extraction : content of each flask was filtered (Whatman No. 1) then treated with 50 ml of chloroform three times in a separating funnel afterwards dehydrated with anhydrous sodium sulfate and evaporated on water bath at 50°C under vacuum. Later, the residues were dissolved in 10 ml methanol.

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

The sample was passed through a 0.45

¼m micro-filter. 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 (model PerkinElmer series 200 UV/VIS) was equipped with an UV detector and fluorescence with 365 nm, methanol: acetic acid: water (20:20:60 v/v/v) as mobile phase, the total run time approximately 25 min at a flow rate of 1 ml/ min for separation (Christian, 1990).

RESULTS AND DISCUSSION

Determination of aflatoxins from Almonds

Data in Table (1) obtained that concentrations of aflatoxins (AFB1, AFB2, AFG1 and AFG2) ranged from 4-47.5, 2.1-14.2, 2.3-30 and 2.4-20 µg/kg, respectively. The highest contamination levels of aflatoxins were found in samples No. 3 was collected from Dammam and contaminated with aflatoxins B1, B2, G1 and G2 (47.5, 10.4, 18.1 and 7.5 µg/kg) respectively. Five samples failed to produced aflatoxins G1 or G2, sample no 4 was collected from Riyadh and sample no. 2 was collected from Dammam whereas sample no. 2, 3, 5 were collected from Abha region. Eight samples failed to produced aflatoxins B1 or B2, samples no 2, 3, 5 were collected from Riyadh, samples no 1, 5 were collected from Dammam and Abha regions. Four samples produced all four aflatoxins (B1, B2, G1 and G2) in varying amounts.

Table 1. Determination of aflatoxin from Almonds (µg/kg) in Riyadh, Dammam and Abha

Sample	Riyadh				Dammam				Abha			
	G1	G2	B1	B2	G1	G2	B1	B2	G1	G2	B1	B2
1	16.8	20	15	8.1	12.1	2.5	36	0	6.4	2.4	4	0
2	3.9	9.5	42	0	30	0	37.3	1.2	0	0	27.4	2.1
3	10.2	12.3	28.3	0	18.1	7.5	47.5	10.4	10.8	0	18.9	11.1
4	8.3	0	38.2	14.2	27.5	14.5	24.2	3.1	2.6	10.2	32.1	7.3
5	4.9	6.8	0	0	23.4	11.2	0	0	2.3	0	0	0

Table 2. Detection of Aflatoxins produced by *Aspergillus* spp isolates from Almonds under UV light

Region	No. of isolates	*Aspergillus* spp.	Culture Media		
			Yeast Extract	Czapek – Dox- Agar	PDA+ NaCl
Riyadh	RA1	*A. parasiticus*	+	-	+
Riyadh	RA2	*A. parasiticus*	-	-	-
Riyadh	RA3	*A. parasiticus*	-	-	-
Riyadh	RA4	*A.flavus*	+	-	-
Riyadh	RA5	*A.flavus*	-	-	-
Riyadh	RA6	*A.flavus*	+	-	-
Riyadh	RA7	*A.flavus*	-	-	-
Riyadh	RA8	*A.flavus*	-	-	-
Dammam	DA6	*A. parasiticus*	+	-	-
Dammam	DA7	*A. parasiticus*	+	-	-
Dammam	DA8	*A.flavus*	+	+	-
Dammam	DA9	*A.flavus*	-	-	-
Dammam	DA10	*A.flavus*	-	-	-
Dammam	DA11	*A.flavus*	-	-	-
Abha	AA1	*A.flavus*	+	-	-
Abha	AA2	*A.flavus*	-	-	-
Abha	AA3	*A.flavus*	-	-	-
Abha	AA4	*A.flavus*	+	+	-
Abha	AA8	*A. parasiticus*	-	-	-
Abha	AA9	*A. parasiticus*	-	-	-

Detection Aflatoxins produced by *Aspergillus* spp isolates from Almonds from Riyadh, Dammam and Abha under UV light

Data in Table (2) show that three isolates of *A. parasiticus* and five isolates of *A.*flavus were isolated from **Riyadh** and detected under UV light whereas, three isolates (RA1 RA4, and RA6) of *Aspergillus* spp expressed as positive for aflatoxin production. Two isolates of *A. parasiticus* and four isolates of *A. flavus* were isolated and detected under UV light whereas, two isolates (DA6 and DA8) of *Aspergillus* spp expressed as positive for aflatoxin production. In Abha: two isolates of *A. parasiticus* and four isolates of *A. flavus* were isolated and detected under UV light whereas, two isolates (AA1 and AA4) of *Aspergillus* spp expressed as positive for aflatoxin production.

Effect of Rosemary oil at three different concentrations on dry weight of *A. flavus* and *A. parasiticus*

Data in Table (3 &4) show that effect of different concentrations of Rosemary oil on dry weight of mycelia after incubation at 25°C for 7 days of *A. flavus* and *A. parasitica*. The tested essential oil 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

Table 3. Effect of Rosemary oil at three different concentrations on dry weight of mycelia (g) of *A. flavus* and *A. parasiticus* isolated from almonds

Isolates	Control	1%	2%	4%
RA3	3.30^{b} ±0.10	2.71^{ab} ±0.13	2.36^{e} ±0.06	1.90^{bc} ±0.08
RA5	2.83^{b} ±0.14	2.31^{b} ±0.11	1.95^{ab} ±0.23	1.74^{abc} ±0.13
DA6	3.96^{a} ±0.06	3.14^{f} ±0.08	2.0^{e}±0.05	1.77abc ±0.08
DA8	5.19^{e} ±0.06	3.65^{e} ±0.04	2.45^{e} ±0.08	2.11^{b} ±0.14
AA1	4.11^{a} ±0.02	3.0^{f} ±0.05	2.30^{e} ±0.04	1.88^{cd} ±0.06
AA4	4.58^{a} ±0.11	3.08^{f} ±0.06	2.35^{e} ±0.05	2.18^{c} ±0.05
LSD0.05	0.563	0.119	0.506	0.258

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 4. Effect of Rosemary oil at three different concentrations on dry weight of mycelia (g) of *A. parasiticus* isolated from almonds

Isolates	Control	1%	2%	4%
RA1	4.02^{a} ±0.08	3.11^{f}±0.06	2.36^{e} ±0.08	2.11^{b}±0. 11
DA6	4.74^{b} ±0.11	3.92^{e}±0.32	3.11^{b} ±0.18	2.85^{f}±0.06
DA7	5.19^{a}±0.09	4.12^{b}±0.16	3.52^{a} ±0.08	3.14^{f}±0.07
LSD0.05	0.425	0.108	0.422	0.108

Table 5. Effect of Rosemary oil at 4% on aflatoxin B (µg/ml) produced by *A. flavus* isolated from Almonds

Isolates	Control		Rosemary		% Inhibition
	B1	B2	B1	B2	
RA4	35.2	7.8	15.9	6.3	48.3
RA6	27.1	13.7	11.0	5.5	59.5
DA8	46.6	0	29.1	0	37.6
AA1	24.9	9.1	18.4	6.0	28.2
AA4	30.1	0	17.7	0	41.1

essential oil. The highest growth inhibition rate was observed at 4% in both of *A. flavus* and *A. parasiticus*. However, the inhibitory effect of the oils increased in proportion to their concentrations. Statistical results showed that kind and amount of essential oils have a significant influence on dry weight $p<0.05$.

Effect of Rosemary oil on aflatoxin B (µg/ml) produced by *A. flavus* and *A. parasiticus* were isolated from Almonds

Data in Table (5 &6) obtained that rosemary oil lead to decrease aflatoxin (B) were produced by *A. flavus* and *A. parasiticus* when compared with control. Rosemary oil was inhibition ranged from 28.2 to 59.5 % and 41.5 to 52.4 respectively. The results indicate that the tested toxigenic fungi are sensitive to the tested essential oil, particularly isolates no. (AA1) and (RA1) were more sensitive.

Effect of Rosemary oil on aflatoxin G (µg/ml) were produced by *A. flavus* and *A. parasiticus* isolated from Almonds

Data in Table (7 &8) obtained that , treatment with Rosemary oil at 4 % were lead to the highest level of inhibition on aflatoxin G (µg/ml) were produced by *A.flavus* and *A. parasiticus* were ranged from 57.9 to 42.1 and 43.9 to 60.1 respectively. In addition that, the results indicate that the highest level of inhibition % were observed in isolate no. AA1 and RA1.

Climatic conditions and to agricultural practices that increase effect on aflatoxin contamination by *Aspergillus* spp. (Nawar, 2008) and improper postharvest handling and storage (Nakai *et al.*, 2008). Efficacy of essential oils as antimicrobial 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) lead to lysis of the hyphae and spores which cause aflatoxin deactivation process (Sharma and Tripathi, 2008).

Table 6. Effect of Rosemary oil at 4% on aflatoxin B (µg/ml) produced by A. parasiticus isolated from Almonds

Isolates	Control		Rosemary		% Inhibition
	B1	B2	B1	B2	
RA1	12.6	10.3	6.2	4.7	52.4
DA6	23.4	7.6	13.9	3.6	43.5
DA7	13.5	0	7.9	0	41.5

Table 7. Effect of Rosemary oil at 4% on aflatoxin G (µg/ml) produced by A.flavus isolated from Almonds

Isolates	Control		Rosemary		% Inhibition
	G1	G2	G1	G2	
RA4	27.1	0	15.7	0	42.1
RA6	12.3	19.1	8.2	9.0	45.2
DA8	29.2	8.6	17.1	4.1	43.9
AA1	9.7	6.7	5.0	1.9	57.9
AA4	0	0	0	0	0

Table 8. Effect of Rosemary oil at 4% on aflatoxin G (µg/ml) produced by A. parasiticus. isolated from Almonds

Isolates	Control		Rosemary		% Inhibition
	G1	G2	G1	G2	
RA1	0	17.8	0	7.1	60.1
DA6	12.3	19.1	8.2	9.0	45.2
DA7	29.2	8.6	17.1	4.1	43.9

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