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Antifungal effects of basil and camphor essential oils against *Aspergillus flavus* and *A. parasiticus*.

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Abstract

Aflatoxins, a worldwide health hazard to humans and animals, are among the most potent mutagenic and carcinogenic compounds known to be produced in nature. The present study aimed to determine the frequency of occurrence of mycotoxigenic fungi associated with peanut seeds. Under UV light of 365 nm wavelength, the presence or absence of fluorescence which surrounding the colonies of *A. flavus* or *A. parasiticus* isolates was determined and expressed as positive (+) or negative (-) signs. The efficacy of five concentrations of basil and camphor essential oils on growth and aflatoxin production for isolates of *A. flavus* and *A. parasiticus* were evaluated under laboratory conditions using UV light. Results indicated that the most prevalent fungi, associated with peanut cvs. were *A. flavus* and *A. parasitica* with frequency of occurrence (FO) 22.5- 40.0 %. *A. flavus* and *A. parasiticus* showed high bright fluorescence, which expressed as a high potential of aflatoxin production. Treatments with the fungicide Benlate® and the higher tested concentration of 3.0 µl/ml of basil and camphor essential oils resulted in great inhibitions (73.3-93.3%) in linear growth of both *A. flavus* and *A. parasiticus*. Treatments with the three concentrations (1.5, 2.0 and 2.5 µl/ml) of either essential oil caused 37.5-71.1% inhibition, followed by the lowest concentration (1.0 µl/ml), which showed 16.7-33.3% inhibition compared to the check treatment. However, the higher concentrations of 2.5 and 3 µl/ml of the tested essential oils completely inhibited aflatoxin production. This study proved that essential oils of basil and camphor can be used as a protective agent against aflatoxin production by *A. flavus* and *A. parasiticus*.

Keywords: Aflatoxin; control; florescence UV light; peanut cultivars; mycotoxins; mycotoxigenic fungi.
Abbreviations: cv._cultivar; cvs._cultivars; WA_water agar; CZ_Czapek-Dox; FO_Frequency of occurrence; PDA_Potato dextrose agar; CAM_Coconut agar medium.

Introduction

Peanut *Arachis hypogaea* L. is considered one of the important food crops produced in many subtropical and tropical countries. In addition, it is a high value cash crop for small and large growers alike. It was listed as one of the twenty crop plants that stand between man and starvation (Wittwer, 1981). Peanut seeds are highly susceptible to several fungi species, such as: *Alternaria* spp., *Aspergillus* spp., *Botrytis cinerea*, *Chaetomium* sp., *Cladosporium* sp., *Curvularia* sp., *Fusarium* spp., *Helminthosporium* spp., *Macrophomina phaseolina*, *Mucor* spp., *Pythium* spp., *Penicillium* spp., *Rhizoctonia solani*, *Rhizopus* spp., *Sclerotium rolfsii*, *S. bataticola* and *Verticillium* sp. (El-Wakil and El-Metwaly, 2001; Horn, 2005; Sultan and Magan, 2010; El-Mohamady et al., 2014). Aflatoxin is the term used to refer to a group of chemicals that show severe toxicity to humans and animals. Aflatoxin producing fungi are widely distributed in nature and can grow over a wide range of environmental conditions (Holmquist et al., 1983; Bennet and Klich, 2003). The Food and Agriculture Organization (FAO) stated that 25% of world food products are affected by mycotoxins, most of them are aflatoxins (Khorasgani et al., 2013). Aflatoxins are the major concern for peanut industry because they are potent carcinogens. Therefore, exposure through food should be kept as low as possible (Abdel-Wahhab et al., 1999; Khorasgani et al., 2013). More than twenty five fungal genera are involved in pathway synthesized aflatoxins, and *Aspergillus* is one of the most important genera (Pearson et al., 1999; Bennet and Klich, 2003). *A. flavus* and *A. parasiticus* are the primary causal agents of food and feed contamination with the severely toxic fungal metabolites, aflatoxins (Reddy and Raghavender, 2007; Probst et al., 2010 and 2011). Peanut seeds are susceptible to colonization by *A. flavus* and *A. parasiticus* prior to harvest. After colonization of peanut seeds, these fungi can produce aflatoxins under certain conditions (Sanders et al., 1985). The main four aflatoxins produced by *Aspergillus* spp. are: B1, B2, G1 and G2. They are defined as toxigenic, mutagenic and carcinogenic toxins. Aflatoxin B1 is the most prevalent and carcinogenic of aflatoxins, because of its hepatotoxic, teratogenic, immunosuppressive and mutagenic nature (Abdel-Wahhab et al., 1999; Othman and Al-Delamiy, 2012; Williams et al., 2004). The International Agency for Research on Cancer (IARC) classifies aflatoxin B1 as a group I carcinogen (IARC, 1993). The control of aflatoxins producing fungi is urgently needed, since their occurrence in foods and feeds is continuously posing threats to both health and economics all over the world (Basappa and Shantha, 1996). Numerous diverse compounds and extracts

containing inhibitory activity to aflatoxin biosynthesis have been reported. Most of these inhibitors are plant-derived such as phenylpropanoids, terpenoids and alkaloids (Holmes et al., 2008). A group of plant-derived inhibitors is plant essential oils that possess antimicrobial activities against *A. flavus* and *A. parasiticus* (Rasoli and Owlia, 2005; Kumar et al., 2007; Rasoli et al., 2008; Bluma et al., 2008). Essential oils are known to contain a natural cocktail of antifungal (Daferera et al., 2000; Adjou et al., 2012) and antimicrobial (Cowan, 1999; Hammer et al., 1999) activities. Essential oils have shown promising results in controlling fungi producing aflatoxins (Kumar et al., 2007; Ceylan and Fung, 2004; Burt, 2004; Alpsoy, 2010). The present study was undertaken to (i) determine frequency of occurrence (FO) of fungi associated with seeds of five peanut cultivars (ii) select specific *A. flavus* and *A. parasiticus* isolates from five peanut cvs. capable of producing aflatoxins under UV light, (iii) evaluate the effects of basil and camphor essential oils and the fungicide Benlate® on growth of isolates of *A. flavus* and *A. parasiticus*, (iv) evaluate the effects of basil and camphor essential oils on aflatoxin production by the selected isolates of *A. flavus* and *A. parasiticus* using UV light.

Results

Data presented in Table (1) indicated the presence of 14 genera of fungi isolated from seeds of five peanut cultivars. The most prevalent fungi were *A. flavus* and *A. parasitica* with 22.5 - 40.0 FO%, followed by *Cephalosporium* sp., *Chaetomium* sp., *F. oxysporum*, *Penicillium* sp. and *Rhizoctonia solani*, which showed 11.7- 15.8 FO %. However, *Alternaria alternate, Asergillus nidulans, A. niger, A. terreus, Botrytis* spp., *Cladosporium* spp., *Fusarium moniliforme, F. semitectum, F. solani, Macrophomina* spp., *Mucor* sp., *Rhizopus stolonifer, Sclerotium bataticola* and *Verticillium* sp. were less common with 1.7- 9.2 FO % (Table 1). The presence or absence of fluorescence in the agar media surrounding the assayed fungal colonies was determined using UV light at 365 nm and expressed as positive (+) or negative (-) signs. Under UV light of 365 nm, *A. flavus* isolated from Balady cv. showed high bright fluorescence (+++), which represents a high potential of producing aflatoxin, and *A. parasiticus* from Giza 6 cv. showed moderate fluorescence (++). Meanwhile, weak fluorescence (+) was observed with *A. flavus* from Giza 5 cv. and *A. parasiticus* from Giza 4 cv. (Table 2). The effects of basil and camphor essential oils and the fungicide Benlate® on linear growth of *A. flavus* and *A. parasiticus* were presented in Table (3 and 4). Treatment with Benlate® resulted in great inhibitions of 80.0-93.3% on linear growth of *A. flavus* and *A. parasiticus* followed by treatment with the highest concentration (3.0 µl/ml) of basil or camphor oils, which showed 73.3-78.9 % inhibition. In addition, treatments with the three concentrations (1.5, 2.0 and 2.5 µl/ml) of basil or camphor oils caused 37.5-71.1 % inhibition on linear growth of *A. flavus* and *A. parasiticus*. Meanwhile, treatment with the lowest concentration of 1.0 µl/ml of basil or camphor essential oils showed 19.4-33.3 % inhibition on linear growth of *A. flavus* and *A. parasiticus*, except after 2days with *A. flavus*, as compared with the check treatment (Tables 3 and 4). Data presented in Table (5) showed that treatments with the higher concentrations of 2.5 and 3 µl/ml of basil or camphor essential oils completely inhibited *A. flavus* and *A. parasiticus* aflatoxin production. Treatments with 1.0 and 1.5 µl/ml of the tested essential oils showed medium and high fluorescence for both tested *Aspergillus* species started from the 2nd day of incubation until the end of experiment (5 days). Treatment of 2.0 µl/ml of the tested essential oils showed weak fluorescence intensity for both tested *Aspergillus* species started from the 4th and 3rd days of incubation with *A. flavus* and *A. parasiticus*, respectively until the end of experiment (5 days) (Table 5).

Discussion

The present research showed the presence of 14 genera of fungi associated with seeds of the five tested peanut cultivars and the most prevalent fungi were *A. flavus* and *A. parasitica*. These results are in agreement with those of other workers (El-Wakil and El-Metwaly, 2001; El-Mohamady et al., 2014). Several investigations have listed a large number of fungi which could be isolated from peanut seeds. *Aspergillus* spp. were the dominant storage fungi colonizing peanuts, capable of causing seed rots, molding of seeds, pre and post emergence damping off and reducing seed viability and seedlings growth (Horn and Greene, 1995; Horn, 2005).

Our data indicated that different concentrations of both basil and camphor essential oils have a great ability to inhibit linear growth and aflatoxin production of *A. flavus* and *A. parasiticus*, under laboratory conditions. These results are in agreement with those of other workers (Rasoli and Owlia, 2005; Kumar et al., 2007; Rasoli et al., 2008; Adjou et al., 2012; Bluma et al., 2008; Moghtader et al., 2011). Essential oils are liquid products of aromatic plants, particularly rich in essential oils, belonged to families Pinaceae, Zingiberaceae, Umbellifereae, Lamiaceae, Apiaceae, Myrtaceae, Asteraceae, Rutaceae, and Laureace (Kohlert et al., 2000). These compounds build glycosides, saponins, tannins, alkaloids, organic acids, and other compounds that constitute the plant's defense system against microbial infections (Ceylan and Fung, 2004). The main component make up to 85% of the basil essential oil are D-linalool and methyl chavicol (Burt, 2004). Camphor is a waxy, white or transparent solid with a strong aromatic odor (Mann et al., 1994) which sublimates at room temperature and melts at 180 $^{\circ}$C. It is practically insoluble in water, but soluble in alcohol, ether, chloroform and other organic solvents. It is a terpenoid (1,7,7-trimethylbicyclo[2.2.1]-2heptanone) with a chemical formula of $C_{10}H_{16}O$ (Kumar and Ando, 2003). Antimicrobial activity of essential oils depends not only on their components but also on the chemical structure of these components (Farag et al., 1989). Plant essential oils could be safely used as a protective materials on some foods because they stopped fungal growth and aflatoxin (B1) accumulation. They may also prove valuable as 'lead structure' for the development of synthetic compounds as they are natural and nontoxic to humans and animals alike (Soliman and Badeaa, 2002; Bluma et al., 2008). The possible mechanism of action of essential oils components on the growth of fungi was reported in several studies (Bluma et al., 2008; Alpsoy, 2010; Hassan et al., 2011). The antiaflatoxin actions of plant essential oils may be related to inhibition of the ternary steps of aflatoxin biosynthesis involving lipid peroxidation and oxygenation (Bluma et al., 2008). It is generally accepted that the essential oils components act on the functionality and the structure of the cell membrane (Viuda-Martos et al., 2008). Daferera et al. (2000) indicated that fungitoxic effect of essential oils is a consequence of hydrogen bonds formation between hydroxyl groups of phenolic compounds and active sites of cellular enzymes. Also, Hashim et al. (1994) reported that the enzymatic modulation is perhaps due to the chemical constituents of the oils and this could form a basis for their potential anti-carcinogenic roles.

Table 1. Frequency of occurrence (FO) % of fungal species isolated from seeds of five peanut cultivars.

Fungal isolate	Peanut cultivar				
	Balady	Ismailia 1	Giza 4	Giza 5	Giza 6
Alternaria alternata	2[a], 1.7[b]			4, 3.3	
Aspergillus flavus	48, 40.0	29, 24.2	32, 26.7	31, 25.8	35, 29.2
A. nidulans	3, 2.5		2, 1.7		
A. niger			9, 7.5		10, 8.3
A. parasitica	33, 27.5	35, 29.2	30, 25.0	27, 22.5	42, 35.0
A. terreus	4, 3.3		5, 4.2		
Botrytis spp.		3, 2.5			
Cephalosporium sp.		15, 12.5		18, 15.0	
Chaetomium sp.			14, 11.7		17, 14.2
Cladosporium spp.		2, 1.7		5, 4.2	3, 2.5
Fusarium moniliforme	8, 6.7			11, 9.2	
F. oxysporum		16, 13.3		19, 15.8	
F. semitectum	10, 8.3		11, 9.2		
F. solani				7,5.8	
Macrophomina spp.	9, 7.5				11, 9.2
Mucor sp.			11, 9.2	9, 7.5	
Penecillium sp.		17, 14.2			18, 15.0
Rhizoctonia solani	19, 15.8			16, 13.3	
Rhizopus stolonifer	5, 4.2	7, 5.8			6, 5.0
Sclerotium bataticola		10, 8.3		11,9.2	
Verticillium sp.			7, 5.8		

[a] = Number of infected samples. [b] = FO % = Number of infected samples/number of total samples ×100.

Table 2. Detection of aflatoxin production from *Aspergillus flavus* and *A. parasiticus* under UV light.

Treatment	Aflatoxin production (Fluorescence intensity)									
	A. flavus					*A. parasiticus*				
	Peanut cultivar									
	Balady	Ismailia1	Giza4	Giza5	Giza6	Balady	Ismailia1	Giza4	Giza5	Giza6
UV light	+++	-	-	+	-	-	-	+	-	++

(+++) = bright fluorescence; (++) = moderate fluorescence; (+) = weak fluorescence; (-) = no fluorescence.

Table 3. Effect of basil essential oils on growth and inhibition % of *Aspergillus flavus* and *A. parasiticus.*

Treatment	Linear growth (cm)/days and Inhibition %							
	Aspergillus flavus				*A. parasiticus*			
	2 days	I %**	4 days	I %**	2 days	I %**	4 days	I %**
Check*	3.0 a	0.0	9.0 a	0.0	3.6 a	0.0	9.0 a	0.0
Basil essential oil concentration:								
1.0 µl/ml	2.5 ab	16.7	7.2 b	20.0	2.9 b	19.4	6.4 b	28.9
1.5 µl/ml	1.4 c	53.3	4.0 c	55.6	1.6 c	55.6	4.6 c	48.9
2.0 µl/ml	1.3 c	56.7	3.3 cd	63.3	1.5 c	58.3	3.2 d	64.4
2.5 µl/ml	1.1 cd	63.3	2.8 d	68.9	1.2 cd	66.7	2.9 d	67.8
3.0 µl/ml	0.8 d	73.3	1.9 e	78.9	0.9 d	75.0	2.0 e	77.8
Benlate® 25 µg/ml	0.6 d	80.0	0.7 f	92.2	0.6 e	83.3	0.6 f	93.3

*Check = Untreated plates. **I %= Inhibition % = growth zone in check plate-growth zone in test plate/growth zone in check plate. Data are averages of 10 replicates. Values, within each column, followed by the same letter(s) are not significantly different at ($P \leq 0.05$).

Materials and Methods

Sources of peanut seeds

Seeds of five peanut cvs., commonly grown in Egypt, were obtained from Crop Institute Agriculture Research Center, Giza, Egypt. The five cultivars were Balady, Ismailia 1, Giza 4, Giza 5 and Giza 6. The seeds (13% relative humidity) were stored in paper bags. The peanut seed were kept at room temperature (28±2 °C) for 3 months before fungal isolation and aflatoxin detection.

Essential oils collection

Essential oils of basil (*Ocimum basilicum* L.) belonged to family Lamiaceae and camphor (*Cinnamomum camphora* L.) belonged to family Lauraceae, used in this study, were obtained from El-Gomhoria Chemicals Company, Egypt.

Fungal isolation from seeds of peanut cultivars

The infected and associated fungi presented with seeds of the five peanut cvs. Balady, Ismailia 1, Giza 4, Giza 5 and Giza 6 were isolated under laboratory condition. One hundred and twenty peanut seeds were used for each cultivar (twelve seeds/each Petri plate). Peanut seeds were surface sterilized in 0.5% sodium hypochlorite solution for 30 seconds. Seeds were then rinsed in three changes of sterile distilled water to remove excess sodium hypochlorite and dried using sterile filter papers. Fifty Petri plates filled with water agar (WA) were used in this experiment. Seeds were placed on the surface of WA and incubated at 28°C. After 3-5 days of incubation, hyphal tips were transferred onto Czapek-Dox

Table 4. Effect of camphor essential oil on linear growth and inhibition % of *Aspergillus flavus* and *A. parasiticus.*

Treatment	Linear growth (cm)/days and Inhibition % (I)**							
	A. flavus				*A. parasiticus*			
	2 days	I %**	4 days	I %**	2 days	I %**	4 days	I %**
Check*	4.0 a	0.0	9.0 a	0.0	4.3 a	0.0	9.0 a	0.0
Camphor essential oil concentration:								
1.0 µl/ml	2.9 b	27.5	6.8 b	24.4	3.1 b	27.9	6.0 b	33.3
1.5 µl/ml	2.5 b	37.5	4.2 c	53.3	2.4 bc	44.2	4.5 c	50.0
2.0 µl/ml	1.5 c	62.5	3.0 d	66.7	1.6 bc	62.8	2.9 d	67.8
2.5 µl/ml	1.3 c	67.5	2.6 de	71.1	1.2 bc	72.1	2.5 de	72.2
3.0 µl/ml	1.0 c	75.0	2.0 e	77.8	1.1 bc	74.4	2.2 e	75.5
Benlate® 25 µg/ml	0.6 c	85.0	0.7 e	92.2	0.6 c	90	0.8 e	91.1

Legend as in Table (4).

Table 5. Presence (+) or absence (-) of aflatoxin production of *Aspergillus flavus* and *A. parasiticus* treated with basil or camphor essential oils under UV light.

Treatment	Fluorescence intensity** and incubation period (days)									
	Aspergillus flavus					*A. parasiticus*				
	1	2	3	4	5	1	2	3	4	5
Check*	-	++	+++	+++	+++	-	++	++	+++	+++
Basil oil concentration										
1.0 µl/ml	-	++	++	+++	+++	-	++	++	+++	+++
1.5 µl/ml	-	++	++	++	++	-	++	++	++	++
2.0 µl/ml	-	-	-	+	+	-	-	+	+	+
2.5 µl/ml	-	-	-	-	-	-	-	-	-	-
3.0 µl/ml	-	-	-	-	-	-	-	-	-	-
Camphor oil concentration										
1.0 µl/ml	-	++	++	++	+++	-	++	++	++	+++
1.5 µl/ml	-	++	++	++	++	-	-	++	++	+++
2.0 µl/ml	-	-	-	+	+	-	-	+	+	+
2.5 µl/ml	-	-	-	-	-	-	-	-	-	-
3.0 µl/ml	-	-	-	-	-	-	-	-	-	-

Legend as in Table (3).

(CZ) agar plates. Seven days later, plates were examined, and the isolated fungi were identified according to Barnett (1960) and Melone and Maskett (1964). The frequency of occurrence % of the isolated fungi was calculated and recorded. *Aspergillus* isolates were examined under the compound microscope, and the identification was performed in Plant Pathology Department, Faculty of Agriculture, Alexandria University, Egypt according to the taxonomic keys and guides for the *Aspergillus* genus (Pitt and Hocking, 1997; Klich, 2007). Pure cultures of *A. flavus* and *A. parasiticus* were maintained on WA slants and kept at 4°C, until examinations for their aflatoxins production.

Preparation of spore suspension of Aspergillus flavus and A. parasiticus

Spore suspension of *A. flavus* and *A. parasiticus* isolates, which isolated from peanut cvs. were prepared at Biology Department, Jazan University, Saudi Arabia by culturing *A. flavus* and *A. parasiticus* on PDA medium for seven days at 28±2°C. The spores of 7-day-old cultures were removed by sterile distilled water supplemented with 0.1 ml/l of Tween 80. The spore suspensions were then collected and filtered through sterile cheesecloth to remove mycelia and agar fragments, and the aliquot was diluted with sterile distilled water to a concentration of (1×10^5 spores/ml). The conidial concentrations were counted using a haemocytometer slide.

Coconut agar medium (CAM) preparation

One hundred grams of shredded coconut was homogenized for 5 minutes with 300 ml of hot distilled water. The homogenate was filtered through four layers of cheesecloth, and the clear filtrate was adjusted to pH 7.0 using 2 N NaOH. Agar was added (20 g/l), and the mixture was autoclaved (Attanda et al., 2011).

Detection for aflatoxin production in Aspergillus flavus or A. parasiticus isolates under UV light

Fifty Petri plates, containing 10 ml of CAM, were used as a culture media for growing isolates of *Aspergillus* species. Plates were inoculated with 1×10^5 spores/ml of each isolate of *Aspergillus flavus* or *A. parasiticus*, which previously isolated from peanut cultivars. Plates were incubated at 28±2°C for seven days under UV light of 365 nm wavelength, in a dark cabinet. After incubation period, the presence or absence of fluorescence in the CAM media surrounding the assayed colonies was determined and expressed as positive (+) or negative (-) signs, according to Davis et al. (1987) and Franco et al. (1998). Isolates of *A. flavus* or *A. parasiticus*, which showed high or moderate fluorescence were used in the further studies.

Evaluate of basil and camphor essential oils and the fungicide Benlate® on the growth of A. flavus and A. parasiticus

Two laboratory experiments were conducted to study the effect of five concentrations of 1.0, 1.5, 2.0, 2.5 and 3.0 µl/ml of basil and camphor essential oils and one concentration, the recommended dose, of the fungicide Benlate® (25 µg/ml) on linear growth of *A. flavus* isolated from peanut cv. Balady and *A. parasiticus*, isolated from peanut cv. Giza 6. One

hundred and forty Petri plates (9 cm) were used (70 plates/each experiment). Plates containing 20 ml of CZ medium/each were inoculated at the centre with 1×10^5 spores/ml distilled water of each *Aspergillus* species. Plates were incubated at 28±2°C for 4 days. Data were collected at 2 and 4 days from incubation. Plates without basil and camphor essential oils were inoculated following the same procedure to serve as a check treatment. Treatments were replicated ten times for each essential oil and the fungal inhibition growth percentage, for each treatment, was determined and recorded.

Evaluate of basil and camphor essential oils for inhibiting aflatoxin production secreted by A. flavus and A. parasiticus under UV light

The ability of basil and camphor essential oils to inhibit production of aflatoxin by *A. flavus* and *A. parasiticus* was determined using CAM medium (Attanda et al., 2011). CAM media with concentrations of 1.0, 1.5, 2.0, 2.5 and 3.0 µl/ml of basil or camphor essential oils were prepared by adding appropriate quantity of each essential oil and Tween 80 to melted CAM medium then vortex to disperse the oil in the medium. A total of 120 Petri plates were used in this experiment. Each plate containing 20 ml of CAM medium, was inoculated at the center with 1×10^5 spores/ml of *A. flavus* or *A. parasiticus*. Treatments were replicated ten times. Plates were incubated at 28±2°C for 5 days. Plates without essential oils were saved as a check treatment. After the incubation period, single large colonies, showed on the bottom side plate, were observed daily under 365 nm long wavelength of UV light for blue/blue green fluorescence in a dark cabinet according to Attanda et al. (2011).The data of presence or absence of aflatoxin were expressed as positive (+) or negative (-) signs (Davis et al., 1987 and Franco et al., 1998).

Statistical analysis

Data obtained were statistically analyzed according to SAS software program (SAS, 1997). Comparison among means was made via the least significant difference test (LSD) at ≤ 5% level of probability.

Conclusion

The use of plant essential oils is a promising method, which provides an opportunity to avoid synthetic chemical fungicides preservatives and offers novel approach to the management of mycotoxigenic fungi. However, the appropriate application of essential oils should further be investigated.

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