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ORIGINAL PAPER

Effect of essential oil from fresh leaves of *Ocimum gratissimum* L. on mycoflora during storage of peanuts in Benin

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Abstract The aim of this study was to evaluate the effect of essential oil from fresh leaves of Sweet Fennel (*Ocimum gratissimum*) on mycoflora and *Aspergillus* section *Flavi* populations in stored peanuts. *Aspergillus*, *Fusarium* and *Mucor* spp. were the most common genera identified from peanuts at post-harvest in Benin by using a taxonomic schemes primarily based on morphological characters of mycelium and conidia. The isolated fungi include *Aspergillus niger*, *A. parasiticus*, *A. flavus*, *A. ochraceus*, *Fusarium graminearum*, *F. solani*, *F. oxysporum* and *Mucor* spp. The most prevalent fungi recorded were *A. niger* (94.18 %), *A. flavus* (83.72 %), *A. parasiticus* (77.90 %), *A. ochraceus* (72.09 %), *F. graminearum* (59.30 %) and *F. oxysporum* (51.16 %). Antifungal assay, performed by the agar medium assay, indicated that essential oil exhibited high antifungal activity against the growth of *A. flavus*, *A. parasiticus*, *A. ochraceus* and *F. oxysporium*. The minimal inhibitory concentration (MIC) of the essential oil was found to be 7.5 μl/ml for *A. flavus* and *A. parasiticus* and 5.5 μl/ml for *A. ochraceus* and *F. oxysporium*. The minimal fungicidal concentration (MFC) was recorded to be 8.0 μl/ml for *A. flavus* and *A. parasiticus*, 6,5 μl/ml for *A. ochraceus* and 6.0 μl/ml for *F. oxysporium*. The essential oil was found to be strongly fungicidal and inhibitory to aflatoxin production. Chemical analysis by GC/MS of the components of the oil led to the identification of 31 components characterized by myrcene (6.4 %), α-thujene (8.2 %), p-cymene (17.6 %), γ-terpinene (20.0 %), and thymol (26.9 %) as major components. The essential oil of Sweet Fennel, with fungal growth and mycotoxin inhibitory properties, offers a novel approach to the management of storage, thus opening up the possibility to prevent mold contamination in stored peanuts.

Keywords Essential oil · *Ocimum gratissimum* · Aflatoxin · Fungi and peanuts · Benin

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Introduction

Fungal deterioration of stored seeds and grains is a main problem in the storage system in Benin. Harvested seeds are colonized by various species of *Aspergillus*, under conditions leading to deterioration and mycotoxin production (Reddy and Raghavender 2007). Among the mycotoxins, aflatoxin B1 (AFB_1) is the most toxic form for mammals and presents hepatotoxic, teratogenic and mutagenic properties, causing damage such as toxic hepatitis, hemorrhage, edema, immunosuppression, and hepatic carcinoma (Magan et al. 2004).

Peanuts (*Arachis hypogaea*) are one of the most important food and oilseed crops cultivated and utilized in most parts of the world. They are widely accepted as an excellent source of nutrition due to their high protein content. The seed has several applications such as peanut butter, oil, and other products (Yaw et al. 2008).

In Benin, peanuts are an important legume and are eaten with boiled maize to reduce the impact of hunger during the

lean season when most foodstuffs are in short supply (Honfo et al. 2010). Extracting oil from peanuts provides an income for women and their dependants in a small-scale cottage industry (Bankole et al. 2005). However, the structure and chemical composition of peanuts allows the growth of several fungal species. This contamination occurrs before and during harvesting, as well as during storage (Mutegi et al. 2009).

To overcome these problems, the usual practice is to fumigate or treat the stored commodities using different synthetic preservatives. However, most of the synthetic chemicals used during post-harvest treatment of food commodities are novel man-made xenobiotics. Hence, most of the synthetic antimicrobials are not easily biotransformed into simpler forms and, as a result, remain in the food chain for longer periods, causing adverse effects to different mammalian systems through residual toxicities (Moosavy et al. 2008).

There is considerable on-going research on methods to prevent pre-harvest contamination of crops. These approaches include developing host resistance through plant breeding and through enhancement of antifungal genes by genetic engineering and targeting regulatory genes in mycotoxin development (Brown et al. 2001). However, none of these methods has solved the problem (Atanda et al. 2012). Methods for managing mycotoxins are largely preventive. They include good agricultural practices, sufficient drying of crops after harvest, and also knowledge about fungal sources and their control is needed (Lisker and Lillehoj 1991).Several strategies have been investigated for lowering mycotoxin contamination of foods at post-harvest, which can be divided into natural, biological, chemical, and physical methods. However, restrictions imposed by the food industry and regulatory agencies on the use of some synthetic food additives have led to renewed interest in searching for alternatives, such as natural antimicrobial compounds, particularly those from plants (Hammer et al. 1999).

Essential oils (EOs) as well as derived compounds possess a wide range of activities of which the antimicrobial activity is most studied (Hammer et al. 2003; Adjou et al. 2012a, b). Their applications as preservatives in food or antiseptics and disinfectants have been widely investigated (Yehouenou et al. 2010). The biological activities of EOs depend on the qualitative and quantitative characteristics of their components, which are affected by the plant genotype, plant chemotype, organ of plant, geographical origin, season, environmental, agronomic conditions, extraction method, and storage conditions of plants and the EOs (Suhr and Nielsen 2003). The importance of alternative indigenous products to control phytopathogenic fungi is urgently needed (Bankole 1997).

Plants belonging to Lamiaceae family like Sweet Fennel (*Ocimum gratissimum* Linn.) have retained the attention of researchers, not only because of their high diversity and their distribution around the world but also for their variable use as popular medicines to treat diseases. Several studies have revealed the antimicrobial and fungicidal activities of *O. gratissimum* (Nguefack et al. 2009; Kpadonou-Kpoviessi et al. 2012).

Ethnobotanic studies and preliminary surveys revealed that plant leaves are also used to preserve food. For this, fresh leaves are introduced into grain barns to preserve stored cowpea and maize from insect and fungal damage (Illiassa 2004). The present work aims to evaluate the effects of EO extracted from fresh leaves of *O. gratissimum* on the mycelial growth and aflatoxin production by strains of *Aspergillus parasiticus* and *A. flavus* isolated from peanuts at post-harvest in Benin.

Materials and methods

Collection of plant leaves

Plant materials used for EO extraction were fresh leaves from *Ocimum gratissimum* L. Plants were collected at Abomey-calavi (south Benin) and identified at the Benin national herbarium, where voucher specimens are deposited (AA 6430/HNB).

Essential oil extraction

The EO tested was extracted by the hydro-distillation method using Clevenger-type apparatus. The oil recovered was dried over anhydrous sodium sulfate and stored at 4 °C until it was used (de Billerbeck et al. 2001).

Gas chromatography–mass spectrometry analysis

The EO were analyzed by gas chromatography (PerkinElmer Auto XL GC; Waltham, MA, USA) equipped with a flame ionisation detector, and the GC conditions were EQUITY-5 column (60 m x 0.32 mm x 0.25 μm); H_2 was the carrier gas; column head pressure 10 psi; oven temperature programme isotherm 2 min at 70 °C, 3 °C/min gradient 250 °C, isotherm 10 min; injection temperature, 250 °C; detector temperature 280 °C. Gas chromatography–mass spectrometry (GC-MS) analysis was performed using a Perkin Elmer Turbomass GC-MS. The GC column was EQUITY-5 (60 m x 0.32 mm x 0.25 μm); fused silica capillary column. The GC conditions were injection temperature, 250 °C; column temperature, isothermal at 70 °C for 2 min, then programmed to 250 °C at 37 °C/min and held at this temperature for 10 min; ion source temperature, 250 °C. Helium was the carrier gas. The effluent of the GC column was introduced directly into the source of MS and spectra obtained in the EI mode with 70 eV ionisation energy. The sector mass analyzer was set to scan from 40 to 500 amu for

2 s. The identification of individual compounds is based on their retention times, retention indices relative to C_5–C_{18} n-alkanes, and matching spectral peaks available in the published data (Adams 2007).

Collection of peanut samples

A total of 86 samples (each 500 g) of peanut in shell samples were purchased from street hawkers, markets and retail shops in the different locality of peanut production in Benin (*Avrankou*, *Adjarra*, *Pahou*, *Ouidah*, *Bohicon*, *Glazoué*, *Savalou*, *Wèssè*, *Kèrè*, *Dassa*, *Banigbé*, *Bassila*, *Pira*, *Natitingou*, *Takissari*, *Tchoudigou*). The samples were purchased from five different points in each locality, except in *Bohicon* and *Savalou* where eight collection points were investigated, because these two localities were the major peanut production localities in Benin. Each sample was shelled in a sterile flow bench to obtain the peanut seeds which were kept at 4 °C until fungal enumeration.

Preparation of media

Three different media were used in this study: Potato Dextrose Agar (PDA) for isolation of toxigenic fungi, Yeast Extract Sucrose Agar (YES) for testing antifungal potential of essential oil, and the conventional Dessicated Coconut Agar medium (DCA) for the detection and visualization of aflatoxin production. PDA and YES was prepared as described by Nguyen (2007). DCA was prepared by modification of the method of Davis et al. (1987) as reported by Atanda (2005), as follows: 200 g of desiccated coconut were soaked in 1 L of hot distillated water for 30 min and filtered through four layers of cheese cloth. Two percent of bacteriological agar was added to the filtrate and heated to boiling. The media was then sterilized at 121 °C for 15 min.

Fungal isolation and identification

The samples were examined by the direct plating technique described by Pitt et al. (1994). One hundred peanut seeds per sample were surface disinfected in 0.4 % active chlorine solution for 1 min at room temperature. Then, they were placed directly on Yeast Extract Sucrose Agar medium (YES). Plates were incubated at 25 °C for 5 to 7 days. This method permits recovery of the fungi actually growing in the particles. The dilution plating method was also used in other to recovery of the fungi growing on the particles as described by Nguyen (2007). Fungi that developed were purified by repeated subcultures. Pure cultures of fungi were examined macroscopically and microscopically and their identification was carried out by using a taxonomic schemes primarily based on morphological characters using the methods given by Singh et al. (1991), Filtenborg et al. (1995), and Tabuc (2007). After identification, the frequency of fungi was recorded. The isolates of Aspergilla were collected and maintained on YES at 4 °C, until examination for their aflatoxigenic potential.

Toxinogenic potential assay

The aflatoxinogenic potential of Aspergilla strains isolated from the peanuts was investigated using DCA medium according to the method described by Atanda et al. (2011) as follows: about 20 ml of DCA medium were poured into glass Petri dishes. Care was taken to avoid trapping air bubbles in the media. Each Petri dish was inoculated with 40 μl of spore suspension of *A. flavus*, *A. parasiticus*, *A. ochraceus*, or *Aspergillus* spp. Each spore suspension was harvested by adding 10 ml of sterile distilled water to cultures of fungi maintained on YES at 4 °C. The inoculated plates were incubated at 30 °C for 48 h. Thereafter, the plates were examined with some media characteristics. The reverse side of each plate, which consisted of a single large colony, was observed daily for 8 days at 25 °C under long wave (365 nm) UV light in a dark cabinet to verify the presence of a blue/blue green fluorescence ring which indicates the presence of aflatoxins (Sultan and Magan 2010; Atanda et al. 2011).

Antifungal assay

Antifungal assay was performed by the agar medium assay (de Billerbeck et al. 2001). Yeast Extract Sucrose (YES) medium with different concentrations of EO (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, or 8.0 μL/mL) were prepared by adding appropriate quantity of EO and Tween 20 to melted medium, followed by manual rotation of Erlenmeyer to disperse the oil in the medium. About 20 ml of the medium were poured into glass Petri dishes (9 cm). The molds (*A. flavus*, *A. parasiticus*, *A. ochraceus*, or *F. oxysporium*) grown on YES for 48 h are transplanted (subcultured), using a disc of 6 mm in diameter which carries spores from the anamorph

Table 1 Fungi isolated from peanuts in Benin

Fungi	NCI	Occurrence (%)
Aspergillus niger	81	94.18
Aspergillus flavus	72	83.72
Aspergillus parasiticus	67	77.90
Aspergillus ochraceus	62	72.09
Fusarium graminearum	51	59.30
Fusarium oxysporum	44	51.16
Fusarium solani	31	36.04
Aspergillus oryzae	27	31.39
Mucor spp.	16	18.60
Aspergillus spp.	3	3.48
Fusarium spp.	1	1.16

NCI Number of cases of isolation out of 86 samples

Table 2 Aflatoxinogenic assay of *Aspergillus* strains isolated from peanuts during storage

Items	Aspergillus strains isolated from peanut			
	A.flavus	*A.parasiticus*	*A.ochraceus*	*Aspergillus* spp.
Number of cases of isolation from 86 samples	72	67	62	3
Number of cases exhibited fluorescence under UV light	65	62	00	00
Percentage of toxinogenic fungi (%)	90.27	92.53	00	00
Percentage of non-toxinogenic fungi (%)	9.73	7.47	100	100
Ratio of producing/non producting fungi	9.27	12.38	00	00

mold, on the surface of a Petri dish containing the former medium YES and EO at different concentrations. Control plates (without essential oil) were inoculated following the same procedure. Plates were incubated at 25 °C for 8 days and the mycelial growth was appreciated every day by measuring the average of two perpendicular diameters passing by the middle of the disc (Khallil 2001; Yehouenou et al. 2012). The percentage inhibition (PI) of fungal growth was evaluated by the following equation: $PI = [1- (d/dc)] \times 100$ (Kumar et al. 2007), where d is the diameter of growth zone in the test plate, and dc the diameter of growth zone in the control plate (Petri dish without essential oil).

Determination of the fongiostatic or fungicidal activity

With the experimental concentrations where neither growth nor germination was observed, the fungiostatic or fungicidal activity was tested. This assay consisted by taking the mycelial disc not germinated at the end of the incubation of the Petri dish and reintroducing it in a new culture medium (former one) without essential oil. If the mycelial growth is always inhibited, the plant extract is fungicidal at this concentration and allows the determination of the Minimal Fungicide Concentration (MFC). In the contrary case, it becames fungiostatic activity which is related to the Minimal Inhibitory Concentration (MIC) (Yehouenou et al. 2012)

Antifungal activity of the oil compared to the *Nystatine* fungicide assay

The disk diffusion method was used as described by Yin and Tsao (1999). Filter paper disks (6 mm diameter) containing 5.0 µL of the crude EO of *O. gratissimum* were applied on the surface of the Yeast Extract Sucrose (YES) medium plates previously inoculated at surface with 0.1 mL of spore suspension of fungi strains *A. flavus*, *A. parasiticus*, *A. ochraceus* or *F. oxysporium*. The inoculated plates were incubated at 25 °C for 5 days. At the end of the period, antifungal activity was evaluated by measuring the zone of inhibition (mm) against the tested fungi. The fungicide Nystatine disc (Bio Merieux) was used as a positive control. All treatments consisted of three replicates, and the averages of the experimental results were determined.

Table 3 Major components identified as constituents of essential oil of *Ocimum gratissimum* from Benin

Constituents	IR	Percentage
α-thujene	992	8.2
α-pinene	934	1.2
Camphene	948	0.3
β-pinene	974	0.7
Myrcene	985	6.4
α-phellendrene	997	0.5
α-terpinene	1,011	4.2
p-cymene	1,018	17.6
Limonene	1,024	2.5
1,8-cineole	1,025	2.1
(E)-β-ocimene	1,041	0.3
γ-terpinene	1,054	20.0
p-cyménene	1,077	2.2
Terpinolene	1,085	0.1
Linalol	1,091	0.2
Borneol	1,160	0.2
terpinen-4-ol	1,180	1.2
p-cymen-8-ol	1,185	0.2
α-terpineol	1,188	0.1
thymol méthylether	1,235	0.3
Thymol	1,281	26.9
Carvacrol	1,288	0.7
α-copaene	1,370	0.1
β-caryophyllene	1,417	1.2
trans-α-bergamotene	1,429	0.1
α-humulene	1,449	0.2
germacrene D	1,478	0.1
β-selinene	1,485	0.4
α-selinene	1,592	0.2
δ-cadinene	1,515	0.1
oxyde of caryophyllene	1,611	0.2
Total		99.0

RI Retention index (KI)

Table 4 *Aspergillus flavus* colony diameters recorded (mm) with essential oil of *Ocimum gratissimum*

Days	Essential oil of *Ocimum gratissimum*						
	5.0 µl/ml	5.5 µl/ml	6.0 µl/ml	6.5 µl/ml	7.0 µl/ml	7.5 µl/ml	8.0 µl/ml
1	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
2	9.1±0.04 b	7.5±0.06 b	7.2±0.01 b	6.6±0.01 b	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
3	18.6±0.04 c	12.4±0.01 c	10.5±0.09 c	7.3±0.08 c	6.9±0.02 a	6.0±0.00 a	6.0±0.00 a
4	32.8±0.02 d	27.3±0.08 d	21.6±0.02 d	11.3±0.07 d	10.2±0.07 a	6.0±0.00 a	6.0±0.00 a
5	50.7±0.06 e	41.3±0.09 e	33.7±0.06 e	19.1±0.04 e	11.6±0.09 a	6.0±0.00 a	6.0±0.00 a
6	68.6±0.08 f	52.1±0.04 f	46.3±0.01 f	28.2±0.05 f	14.4±0.06 a	6.0±0.00 a	6.0±0.00 a
7	74.2±0.05 g	61.5±0.08 g	54.8±0.06 g	35.8±0.02 g	16.0±0.04 a	6.0±0.00 a	6.0±0.00 a
8	84.8±0.04 h	79.3±0.02 h	64.9±0.07 h	43.7±0.06 h	21.6±0.03 a	6.0±0.00 a	6.0±0.00 a

Diameter of 6 mm is the inoculation point. Values (mm) are mean (n=3) ± SE. The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests

In vitro antiaflatoxin assay

Antiaflatoxin assay was performed using DCA medium and the method described by Tatsadjieu et al. (2009) and Adjou et al. (2012a, b). DCA medium with different concentrations of EO (5.0, 5.5, 6.0, 6.5, or 7 µl/ml) were prepared by adding an appropriate quantity of essential oil and Tween 20 to the melted medium, followed by manual rotation to disperse the oil in the medium. About 20 ml of the medium were poured into glass Petri dishes. Care was taken to avoid trapping air bubbles in the media. Each Petri dish was inoculated with spore suspension of aflatoxinogenic strains, *A. flavus* and *A. parasiticus*, isolated from peanuts and incubated at 30 °C for 8 days. Control plates (without EO) were inoculated following the same procedure. Thereafter, the plates were examined with some media characteristics. The reverse side of each plate, which consisted of a single large colony, was observed under long wave (365mn) UV light for blue/blue green fluorescence each day (Nguyen 2007; Atanda et al. 2011).

Statistical analysis

Experiments were performed in triplicate, and data analyzed are means ± SE subjected to one-way Anova. Means are separated by the Tukey's multiple range test when Anova was significant (P<0.05) (SPSS 10.0; Chicago, IL, USA).

Results

The result of microbial analysis and isolation of fungi in pure culture revealed that peanut samples collected from street hawkers, markets and retail shops were highly contaminated by fungi (Table 1). Fungal isolates include *A. niger*, *A. parasiticus*, *A. flavus*, *A. ochraceus*, *F. solani*, *F.oxysporum* and *Mucor* spp. The most prevalently fungi recorded are *A. niger* (94.18), *A. flavus* (83.72 %), *A. parasiticus* (77.90 %), *A. ochraceus* (72.09 %), *F. graminearum* (59.30 %). and *F. oxysporum* (51.16 %).

Table 5 *Aspergillus parasiticus* colony diameters recorded (mm) with essential oil of *Ocimum gratissimum*

Days	Essential oil of *Ocimum gratissimum*						
	5.0 µl/ml	5.5 µl/ml	6.0 µl/ml	6.5 µl/ml	7.0 µl/ml	7.5 µl/ml	8.0 µl/ml
1	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
2	11.4±0.06 b	10.7±0.08 b	9.1±0.04 b	9.4±0.02 b	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
3	33.7±0.09 c	21.6±0.02 c	22.5±0.04 c	13.9±0.06 c	9.5±0.04 a	6.0±0.00 a	6.0±0.00 a
4	41.7±0.06 d	36.5±0.06 d	34.2±0.06 d	21.4±0.08 d	15.6±0.01 a	6.0±0.00 a	6.0±0.00 a
5	57.5±0.09 e	54.2±0.09 e	48.6±0.07 e	29.7±0.04 e	19.3±0.04 a	6.0±0.00 a	6.0±0.00 a
6	69.8±0.03 f	66.1±0.01 f	54.3±0.07 f	33.8±0.02 f	21.6±0.02 a	6.0±0.00 a	6.0±0.00 a
7	79.3±0.01 g	73.8±0.05 g	68.4±0.04 g	46.3±0.08 g	23.7±0.04 a	6.0±0.00 a	6.0±0.00 a
8	89.3±0.07 h	81.7±0.05 h	72.9±0.07 h	51.2±0.04 h	38.8±0.07 a	6.0±0.00 a	6.0±0.00 a

Diameter of 6 mm is the inoculation point. Values (mm) are mean (n=3)±SE. The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests

Table 6 Colony diameters recorded (mm) of *Aspergillus ochraceus* isolated from peanut exposed to the essential oil of *Ocimum gratissimum*

Days	Essential oil of *Ocimum gratissimum*						
	5.0 µl/ml	5.5 µl/ml	6.0 µl/ml	6.5 µl/ml	7.0 µl/ml	7.5 µl/ml	8.0 µl/ml
1	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
2	8.2±0.05 b	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
3	14.5±0.01 c	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
4	17.3±0.04 d	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
5	25.4±0.06 e	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
6	30.6±0.03 f	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
7	41.6±0.04 g	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
8	45.3±0.07 h	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a

Diameter of 6 mm is the inoculation point. Values (mm) are mean (n=3) ± SE. The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests

Results of aflatoxinogenic assay based on the rapid detection of aflatoxinogenic fungi and the visual determination of aflatoxins method showed that the major part of strains of *A. flavus* (90.27 %) and *A. parasiticus* (92.53 %) exhibited very bright and moderate blue fluorescence under UV light after 4 days of incubation (Table 2). However, no fluorescence was detected with strains of *A. ochraceus* and *Aspergillus* spp. after 8 days of incubation.

By hydrodistillation, fresh leaves of *O. gratissimum* yielded 1.24 % of EO. Chemical analysis by GC and GC-MS analysis of EO enabled the identification of 31 components, (Table 3) representing 99.0 % of the EO. In the volatile extract, different groups of terpene and terpenoid were detected. The EO has chemical composition characterized by Myrcene (6.4 %), α-thujene (8.2 %), p-cymene (17.6 %), γ-terpinene (20.0 %), and thymol (26.9 %) as major components.

EO exhibited pronounced antifungal activity against the growth of *A. flavus* and *A. parasiticus*. The MIC of the EO was found to be 7.5 µl/ml for *A. flavus* and *A. parasiticus* (Tables 4, 5) and 5.5 µl/ml for *A. ochraceus* and *F. oxysporium* (Tables 6, 7). The MFC was recorded to be 8.0 µl/ml for *A. flavus* and *A. parasiticus*, 6,5 µl/ml for *A. ochraceus*, and 6.0 µl/ml for *F. oxysporium*. The radial growth of strains was totally inhibited by the essential oil. Percentage of growth inhibition (PI) evaluated by direct method was significantly (P<0.05) affected by incubation time and EO concentration. Mycelia growth was considerably reduced with increasing concentration of EO while their growth increased with incubation time. The EO was more active on the mycelia growth of *A. ochraceus* and *F. oxysporium* than *A. parasiticus* and *A. flavus*. The percentages of inhibition (PI) of the EO were 76 and 56.9 %, respectively, on *A. flavus and A. parasiticus* at 7.0 µl/ml while the radial growth of *A. ochraceus* and *F. oxysporium* was totally inhibited (PI: 100 %) by the oil at lower concentration (5.5 µl/ml) after 8 days of incubation (Table 8).

The influence of standard fungicide (Nystatine) and the EOs on the inhibitory zone against the tested fungi strains ranged from 2.6 to 4.0 mm (average n=3) for the fungicide

Table 7 Colony diameters recorded (mm) of *Fusarium oxysporium* isolated from peanut exposed to the essential oil of *Ocimum gratissimum*

Days	Concentration of the essential oil						
	5.0 µl/ml	5.5 µl/ml	6.0 µl/ml	6.5 µl/ml	7.0 µl/ml	7.5 µl/ml	8.0 µl/ml
1	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
2	8.2±0.05 b	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
3	9.7±0.01 c	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
4	10.3±0.04 d	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
5	15.7±0.06 e	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
6	23.6±0.03 f	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
7	25.4±0.02 g	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a
8	30.2±0.03 h	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a	6.0±0.00 a

Diameter of 6 mm is the inoculation point. Values (mm) are mean (n=3) ± SE. The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests

Table 8 Percentage of mycelial growth inhibition (PI)

Concentration of essential oil	Percentage of inhibition (%)			
	Strains isolated from peanut			
	A. flavus	*A. parasiticus*	*A. ochraceus*	*F. oxysporium*
5.0 µl/ml	5.77±0.7 a	0.77±0.5 a	49.66±0.2 a	66.44±0.5 a
5.5 µl/ml	11.88±0.9 b	9.22±1.1 b	100±0.00 b	100±0.00 b
6.0 µl/ml	27.88±0.3 c	19.0±0.3 c	100±0.00 b	100±0.00 b
6.5 µl/ml	51.44±0.5 d	43.11±0.3 d	100±0.00 b	100±0.00 b
7.0 µl/ml	76.0±0.2 e	56.88±0.7 e	100±0.00 b	100±0.00 b
7.5 µl/ml	100±0.00 f	100±0.00 f	100±0.00 b	100±0.00 b
8.0 µl/ml	100±0.00 f	100±0.00 f	100±0.00 b	100±0.00 b

Values are mean (n=3) ± SE. The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests

and 1.4 to 3.3 mm for the EO. The results obtained by the disk diffusion method showed, respectively, 53.84, 60.71, 82.5, and 78.1 % of inhibition on *A. parasiticus*, *A. flavus*, *A. ochraceus*, and *F. oxysporium* growth for the EO, when compared with control (Table 9).

The results of antiaflatoxinogenic assay listed in Table 10 show that EO of *O.gratissimum* has important aflatoxin inhibition potential on toxigenic strains *A. flavus* and *A. parasiticus*. At the concentration of 7.0 µl/ml, aflatoxin production by *A. flavus* and *A. parasiticus* was totally inhibited.

Discussion

The results of microbial analysis clearly showed that the peanut samples we collected were highly contaminated with fungi. These results were similar to those obtained by Nesci et al. (2011) in stored peanut samples from Argentina. As shown in Table 2, *A. flavus* and *A. parasiticus* isolated from peanuts were aflatoxin-producing. This could explain the high level of aflatoxin contamination as reported by Ediage et al. (2011) and Adjou et al. (2012c) in peanut samples from Benin, Bankolé et al. (2005) in dry-roasted peanuts from Nigeria, Mutegi et al. (2009) in stored peanuts from western Kenya, and Ding et al. (2012) in post-harvest peanuts from China. The presence of *Aspergillus ochraceus* strains in peanut samples could also constitute a serious threat to food safety due to their toxigenic potential for producing ochratoxins A (Awad et al. 2012). These findings underline the possible coexistence of aflatoxins and ochratoxin A in peanuts and should be taken into consideration as stated by the European community (CEC 1998). This is also particularly important with regard to possible synergism and additive effects of these mycotoxins. Such co-contamination has been previously observed with other food samples (Ediage et al. 2011) and, accord to Atanda et al. (2012), some molds are capable of producing more than one mycotoxin and some mycotoxins are produced by more than one fungal species. The present study also explores the bioefficacy of EOs of *O. gratissimum* as the promising plant-based antimicrobial against peanut-infecting fungal growth and aflatoxin production. This EO was found to be effective against all *Aspergillus* and *Fusarium* strains tested. The antifungal activity was very pronounced on *F. oxysporium* and *A. ochraceus* compared with *A. flavus* and *A. parasiticus*. This bioefficacy may be due to the presence of some highly fungitoxic components in the oil such as terpenoids. Indeed, *Ocimum gratissimum* oil has a chemical composition characterized by terpenes (*p*-cymene and γ-terpinene) and terpenoids (thymol) as the main chemical groups. Several studies have indicated that terpenes do not represent a group of constituents with a high inherent antimicrobial activity. For example, Koutsoudaki et al. (2005) compared the effect of α-pinene, β-pinene, *p*-cymene, β-myrcene, β-caryophyllene, limonene, and γ-terpinene against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* and reported that their antimicrobial activity was low or absent. Rao et al. (2010) also reported that *p*-cymene and γ-terpinene were ineffective as fungicides against *Saccharomyces cerevisiae*. In contrast, terpenoids are a large group of antimicrobial compounds that are active against a broad spectrum of microorganisms (Dorman and Deans 2000). Their antimicrobial activities are linked to their functional groups and it has also

Table 9 Antifungal assay (disk diffusion method)

Inhibition zone (cm)		
Fungi species	EO of *Ocimum gratissimum*	*Nystatine* fungicide
A. flavus	1.7±0.02 a	2.8±0.05 a
A. parasiticus	1.4±0.09 a	2,6±0.07 a
A. ochraceus	3.3±0.07 b	4.0±0.09 b
F. oxysporium	2.5±0.03 c	3.2±0.05 c

Values are mean (n=3) ± SE. The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests

Table 10 Antiaflatoxinogenic assay of toxinogenic *A. flavus* and *A. parasiticus* strains isolated from peanuts

Days	Fluorescence intensity									
	A. flavus					*A. parasiticus*				
	5.0 µl/ml	5.5 µl/ml	6.0 µl/ml	6.5 µl/ml	7.0 µl/ml	5.0 µl/ml	5.5 µl/ml	6.0 µl/ml	6.5 µl/ml	7.0 µl/ml
1	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−
3	+	+	−	−	−	+	+	−	−	−
4	+++	+	−	−	−	+++	+++	−	−	−
5	+++	+	−	−	−	+++	+++	++	−	−
6	+++	+++	+	−	−	+++	+++	+++		−
7	+++	+++	+++	−	−	+++	+++	++	++	−
8	+++	+++	+++	−	−	+++	+++	+++	+++	−

Bright fluorescence (+++); moderate fluorescence (++); weak fluorescence (+); no fluorescence (−)

been reported that the hydroxyl group of phenolic terpenoids and the presence of delocalized electrons are important for the antimicrobial activity (Dorman and Deans 2000). The most active monoterpenoids identified so far are carvacrol and thymol (Hyldgaard et al. 2012). Moreover, the antimicrobial activity of carvacrol, thymol, linalool, and menthol were evaluated against *Listeria monocytogenes*, *Enterobacter aerogenes*, *Escherica coli*, and *Pseudomonas aeruginosa*. The most active compound was carvacrol followed by thymol with their highest MIC being 300 and 800 µg/mL, respectively (Bassole et al. 2010). These results confirm the high antimicrobial activity of a broad collection of terpenoids especially of thymol which is the major component of the EO of *O. gratissimum*. A range of EO components (linalool, thymol, eugenol, carvone, cinnamaldehyde, vanillin, carvacrol, citral, and limonene) have been accepted by the European Commission for their intended use as flavorings in food products (Hyldgaard et al. 2012). The United States Food and Drug Administration (FDA) also classify these substances as generally recognized as safe (GRAS). In our study, GC–MS data depicted remarkable variation in the earlier reports on the oils (Kpadonou-Kpoviessi et al. 2012). The chemical profile of EO is reported to be influenced by the harvest period, and by climatic, seasonal, and geographical conditions, which can significantly affect the amount and composition of the active constituents(Bakkali et al. 2008; Kpadonou-Kpoviessi et al. 2012). Thus, the biologically active EOs should be qualitatively standardized before their recommendation for practical exploitation as has been done in the present investigation. The findings of the present study clearly showed that aflatoxin production was significantly inhibited at concentrations lower than MIC of the oil. Hence, the EO would be acting by two different modes of action as an inhibitor of fungal growth and aflatoxin production (Rasooli and Abyaneh 2004). Based on such observations, it may be also concluded that the EOs are more active as aflatoxin inhibitors than as fungal growth suppressors as emphasized in the literature (Kumar et al. 2009; Prakash et al. 2010). Several strategies are used in controlling fungal growth and mycotoxin biosynthesis in stored grains using chemical, physical, and biological methods. These methods require sophisticated equipment and expensive chemicals or reagents. Attempts have been devoted to researching for new antifungicides from natural sources for food preservation (Reddy et al. 2010). Awuah (1996) reported that the following plants: *Cymbopogon citratus*, *Xyloppia aethiopica*, and *Cinnamomum verum* are effective in inhibiting the formation of norsolorinic acid, a precursor in the aflatoxin synthethis pathway. Fandohan et al. (2004) showed that *Ocimum basilicum* EO from Benin possesses significant inhibitory effects on the growth of *Fusarium verticilloides* and fumonisin B_1 production. According to Sánchez-González et al. (2011), one option is to use EOs in active packaging rather than as an ingredient in the product itself. EOs can also be encapsulated in polymers of edible and biodegradable coatings or sachets that provide a slow release to the food surface or to the headspace of the packages (Pelissari et al. 2009). The EO from fresh leaves of *O. gratissimum*, having fungal growth and mycotoxin inhibitory properties, offers a novel approach to the management of storage fungi. It is a promising method for preserving stored products in rural areas, which do not have access to modern storage systems.

Conclusion

This work underlined the bioactivity of EO of fresh leaves of *O. gratissimum* from Benin as an aflatoxin inhibitor and fungal growth suppressor. Different major components such as myrcene (6.4 %), α-thujene (8.2 %), p-cymene (17.6 %), γ-terpinene (20.0 %), and thymol (26.9 %) were present in

the volatile extract. Based on its antifungal and antiaflatoxin potentials, this natural plant product may successfully replace synthetic chemicals and provide an alternative method to protect peanuts as well as other agricultural commodities of nutritional significance from toxigenic fungi and aflatoxin production.

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Conflict of interest The authors declare that there are no conflicts of interest.

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