Antifungal activity of *Ocimum canum* Essential oil against Toxinogenic Fungi isolated from Peanut Seeds in post-harvest in Benin

Adjou Euloge S., Kouton Sandrine, Dahouenon-Ahoussi Edwige, Sohounhloue Dominique C.K., Soumanou Mohamed M.*
Laboratory of Research and Study in Applied Chemistry, Polytechnic School of Abomey-calavi, University of Abomey-Calavi, Cotonou, BÉNIN

Abstract

The aim of this study is to evaluate the inhibition of Aspergillus flavus and Aspergillus parasiticus isolated from peanut and their aflatoxin production exposed to the essential oils extracted from fresh leaves of Ocimum canum. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of the oil were determined. The essential oil was found to be strongly fungicidal and inhibitory to aflatoxin production. Through GC/MS analysis, an amount of 30 components were identified, representing almost 95.2% of the oil. Essential oil of O. canum was characterized by major components such as terpinene-4-ol (41.18%), linalol (14.7%) and γ-terpinène (6.9%). This plant offers novel approach to the management of storage fungi

Key words: Bioactivity, essential oils, aflatoxin, antifungal, peanut, Benin.

Introduction

Investigations into the chemical and biological activities of plants during the past two centuries have yielded compounds for the development of modern synthetic organic chemistry and the emergence of medicinal chemistry as a major route for the discovery of novel and more effective therapeutic agents[1]. Thus, plants are considered as one of the most important and interesting subjects that should be explored for the discovery and development of newer and safer drug products.

In tropical areas, such as Benin, fungal deterioration of stored seeds and grains is a chronic problem. Harvested grains are colonized by various species of fungi, such as *Aspergillus flavus* and *Aspergillus parasiticus*, under such conditions leading to deterioration and mycotoxin production[2]. Among all the mycotoxins, particularly aflatoxin B1 (AFB1) 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[3]. It has been classified as a class 1 human carcinogen by the International Agency for Research on Cancer[4]. The presence and growth of fungi may cause spoilage of food and mycotoxin production[5]. Therefore, the control of fungi and of aflatoxin biosynthesis is extremely important for agriculture and public health. To overcome these problems, the usual practice is to fumigate or treat the stored commodities using different synthetic preservatives. However, none of these methods has solved the problem[6]. The increase of demand for safe and organic food, without chemical preservatives, incites many researchers to investigate the antimicrobial effects of natural compounds. Numerous investigations have confirmed the antimicrobial action of essential oils in model food systems and in real food[7]. Essential oils are a rich source of biologically active compounds and they are potential sources of novel antimicrobial compounds. It was demonstrated that essential oils have been shown to possess antibacterial, antifungal, antiviral insecticidal and antioxidant properties[8]. *Ocimum canum* is grown for its medicinal and culinary value and it is highly useful in treating various types of diseases and in lowering blood glucose, especially in type 2 diabetes levels. The traditional medicine recognized its value in the treatment of fevers, dysentary and tooth problems. It was used as an insect repellent to counter the insect damages post harvest. The herb has known antibacterial, and acts like an analgesic and rubefacient[9]. The present study was undertaken to investigate the bioactivity potential of essential oil extracted from leaves of *O. canum* as antifungal agent using toxinogenic strains of *Aspergillus parasiticus* and *Aspergillus flavus* strains infecting peanut at post harvest in Benin.

Material and Methods

Collection of plant leaves: Plant materials used for essential oils extraction were fresh leaves of *Ocimum canum*. Plants were collected at Dassa (center of Benin) and identified at the Benin national herbarium, where voucher specimens are deposited.

Essential oil extraction: Essential oils tested were extracted by the hydro-distillation method using Clevenger-type apparatus. Oils recovered was dried over anhydrous sodium sulphate and stored at 4°C until it was used[9].

Gas chromatography-mass spectrometry analysis: The EOs were analysed 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 program 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 PerkinElmer 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 relative to those of authentic samples and matching spectral peaks available with the published data[10].

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 N'Guyen[11]. DCA was prepared by modification of the method of Davis et *al*.[12], as reported by Atanda[13] as follows: two hundred grams of desiccated coconut were soaked in 1L of hot distillated water for 30 min and filtered through four layers of cheese clothes. Two percent of bacteriological agar was added to the filtrate and heated for boiling. The media was then sterilized at 121°C for 15 min.

Fungal isolation: All target toxinogenic fungi strains were isolated originally from infected peanuts collected in different agro ecological zones of Benin[14]. Strains were preserved on the Potato Dextrose Agar (Oxoid Basingstoke) at 4°C. Subcultivations on Petri dishes and other manipulations with these strains were carried out in the Bio Security Level two (BSL 2) Laboratories with respect to the BSL of *Aspergillus* species used in our experiment.

Antifungal assay (Direct method): Antifungal assay was performed by the agar medium assay[15]. Yeast Extract Sucrose (YES) medium with different concentrations of essential oil (1.5, 2.0 or 2.5 µL/ml) were prepared by adding appropriate quantity of essential oil and *Tween 80*, 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). Each Petri-dish was inoculated at the centre with a mycelial disc (6 mm diameter) taken at the periphery of *A. parasiticus* and *A. flavus* colonies grown on PDA for 48 h. Control plates (without essential oil) were inoculated following the same procedure. Plates were incubated at 25°C for 8 days and the colony diameter was recorded each day. Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration of essential oil in which no growth occurred. The inhibited fungal discs of the oil treated sets were re-inoculated

into the fresh medium, and revival of their growth was observed. Minimal Fungicide Concentration (MFC) is the lowest concentration at which no growth occurred on the plates. Diameter of fungal colonies of treatment and control sets was measured, and percentage inhibition (PI) of fungal growth was calculated according to following formula[16].

$$PI = 1 - \frac{Dt}{Dc} \times 100$$

Dt: the diameter of growth zone in the test plate; Dc: the diameter of growth zone in the control plate.

Antifungal assay (Disk diffusion assay): Filter paper disks (6 mm diameter) containing 5.0 µL of the crude essential oil of *O.canum* was applied on the surface of Yeast Extract Sucrose (YES) medium plates previously inoculated with *A. parasiticus* or *Aspergillus flavus*. 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 tested fungi[17]. 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.

Antiaflatoxin assay: Antiaflatoxin assay was performed using DCA medium according to the method described by Atanda et *al*.[18] as followed: DCA medium with different concentrations of essential oil (1.0, 1.5, 2.0, 2.5, 3.0 or 3.5 µl/ml) were prepared by adding appropriate quantity of essential oil and *Tween 80* to 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 single spores of *Aspergillus parasiticus* or *Aspergillus flavus* and incubated at 30°C for 48 hours. Control plates (without essential oil) were inoculated following the same procedure. Thereafter, the plates were examined with some media characteristics. The reverse side of each plate, which consists of a single large colony, was observed under the long wave (365mn) UV light for blue / blue green fluorescence[11,18,19].

Statistical analysis: Experiments were performed in triplicate, and data analyzed are mean ± 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 and Discussion

By hydrodistillation, leaves of *Ocimum canum* yielded 1.2% (v/w) of essential oils. Chemical analysis by GC/MS of the components of the oils led to identification of 30 components, representing 95.2% of the essential oils of *Ocimum canum*. The results are given in table-1. *Ocimum. canum* oil has chemical compositions characterized by terpinene-4-ol (41.18%), linalol (14.7%), γ-terpinène (6.9%), as the major components. Essential

oils exhibited pronounced antifungal activity against the growth of *Aspergillus flavus* and *A. parasiticus*. The results are given in table-2 and 3. MIC of essential oil of *O.canum,* was found to be 1.5 µl/ml and 2.0 µl/ml respectively against toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus*. The MFC was recorded to be 2.0µl/ml and 2.5 µl/ml respectively against *Aspergillus flavus* and *Aspergillus parasiticus*. The results of mycelial percentage growth inhibition (PI) are given in table-4 and indicated that the radial growth of strains was totally inhibited by the essential oil. Percentage of growth inhibition (PI) was significantly ($P < 0.05$) influenced by incubation time and essential oil concentration. Mycelia growth was considerably reduced with increasing concentration of essential oil while their growth increased with incubation time. The oil

was more active on the mycelia growth of *A. flavus* than *A. parasiticus*. 21.33%, 72.33%, 83.44% and 100% were the PI of the oil respectively at 1, 1.5, 2.0, 2.5µl/ml on *A. parasiticus* after 8 days of incubation. The influence of standard fungicide (Nystatine) and the essential oil on the inhibitory zone against *A. parasiticus*, given in table-5, was measured at 3.2 mm and 2.4 mm (average n=3) for the fungicide and the essential oil respectively. The results obtained by the disk diffusion method showed 75% of inhibition of *A. parasiticus* growth for the essential oil when compared with control (Nystatine). The results of antiaflatoxinogenic assay, given in table-6, showed that EO of *O. canum* has important aflatoxin inhibition potential on toxigenic strain *Aspergillus parasiticus*. At 1.5µl/ml, aflatoxin production by *A. parasiticus* was inhibited.

Table- 1
Major components identified as constituents of essential oil of *Ocimum canum*

Compounds	RT	[%]
α-thujène	928	1,3
α-pinène	937	2,0
camphène	952	0,3
sabinène	968	0,2
β-pinène	972	0,1
acetate de (Z)-3-hexényle	979	0,2
myrcène	985	2,1
α-phellandrène	1016	1,4
α-terpinène	1020	1,7
limonène	1030	3,4
γ-terpinène	1058	6,9
hydrate de sabinène	1065	1,0
terpinolène	1087	1,3
linalol	1097	14,7
acétate d'octen-3-yle	1101	0,6
camphre	1139	1,0
bornèol	1150	0,3
terpinèn-4-ol	1189	41,1
p-cymèn-8-ol	1192	0,6
α-terpinéol	1205	0,4
acétate de fenchyle	1219	0,9
acétate de phenyl éthyle	1238	0,2
acétate de bornyle	1282	0,4
acétate de myrtényle	1318	0,4
butyrate de (Z)-3-hexényle	1368	0,2
β-caryophylléne	1439	4,1
trans- α –bergamotène	1446	4,8
α-humulène	1470	0,5
germacrène D	1486	2,4
β-bisabolène	1510	0,2
nérolidol	1598	0,4
oxyde de caryophyllène	1611	0,1
Total		**95.2**

Table-2
Aspergillus flavus* colony diameters recorded (mm) with essential oil of *Ocimum canum

Days	Essential oil of *Ocimum canum*					
	1.µl/ml	1.5µl/ml	2.0µl/ml	2.5µl/ml	3.0µl/ml	3.5µl/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]
2	8.9±0.04[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]
3	15.6±0.04[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]
4	27.8±0.02[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]
5	30.7±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	38.6±0.08[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]
7	44.2±0.05[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]
8	49.8±0.04[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]

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- 3
Aspergillus parasiticus* colony diameters recorded (mm) with essential oil of *Ocimum canum

Days	Essential oil of *Ocimum canum*					
	1.0µl/ml	1.5µl/ml	2.0µl/ml	2.5µl/ml	3.0µl/ml	3.5µl/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]
2	8.5±0.07[b]	6.2±0.07[b]	6.0±0.00[a]	6.0±0.00[a]	6.0±0.00[a]	6.0±0.00[a]
3	27.4±0.08[c]	8.2±0.04[c]	6.0±0.00[a]	6.0±0.00[a]	6.0±0.00[a]	6.0±0.00[a]
4	34.8±0.02[d]	17.5±0.06[d]	6.0±0.00[a]	6.0±0.00[a]	6.0±0.00[a]	6.0±0.00[a]
5	39.7±0.08[e]	17.9±0.02[d]	6.0±0.00[a]	6.0±0.00[a]	6.0±0.00[a]	6.0±0.00[a]
6	50.4±0.06[f]	24.4±0.05[f]	6.0±0.00[a]	6.0±0.00[a]	6.0±0.00[a]	6.0±0.00[a]
7	62.4±0.08[g]	24.5±0.02[f]	6.0±0.00[a]	6.0±0.00[a]	6.0±0.00[a]	6.0±0.00[a]
8	70.8±0.06[h]	24.9±0.05[f]	6.0±0.00[a]	6.0±0.00[a]	6.0±0.00[a]	6.0±0.00[a]

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-4
Percentage of mycelial growth inhibition (PI)

Concentrations of EO	*A. flavus*	*A. parasiticus*
1.0µl/ml	44.66± 0.2	21.33± 0.5
1.5µl/ml	100 ± 0.00	72.33± 0.1
2.0 µl/ml	100 ± 0.00	83.44 ± 0.3
2.5 µl/ml	100 ± 0.00	100 ± 0.00
3.0 µl/ml	100 ± 0.00	100 ± 0.00
3.5µl/ml	100 ± 0.00	100 ± 0.00

Table-5
Antifungal assay (disk diffusion method)

	Nystatine fungicide		EO of *Ocimum canum*	
	A. flavus	*A. parasiticus*	*A. flavus*	*A. parasiticus*
Inhibition zone (mm)	4.0	3.2	2.8	2.4

Table-6
Antiaflatoxinogenic assay with essential oil of *Ocimum canum*

Days	Fluorescence intensity (Essential oil of *Ocimum canum*)			Control
	A. flavus	*Aspergillus parasiticus*		
	1.0µl/ml	1.0µl/ml	1.5µl/ml	
1	-	-	-	-
2	-	-	-	-
3	-	+	-	-
4	-	++	-	-
5	-	+++	-	+
6	-	+++	-	+++
7	-	+++	-	+++
8	-	+++	-	+++

Bright fluorescence (+++); moderate fluorescence (++); weak fluorescence (+); No fluorescence (-)

Essential oils are natural mixtures of hydrocarbons and oxygen (alcohols, aldehydes, ketones, carboxylic acids, esters, and lactones) containing organic substances of plants. Their constituents and derivatives have a long history of application as antimicrobial agents in the areas of food preservation and medicinal antimicrobial production[20]. Biological activities of essential oils depends on the qualitative and quantitative characteristics of their components, which is affected by the plant genotype, plant chemotype, organ of plant, geographical origin, season, environmental, agronomic conditions, extraction method and storage condition of plant and essential oils[21,22]. The present study explores the bioefficacy of essential oils of *O.canum* as the promising plant-based antimicrobials against toxinogenic fungi and their aflatoxin production. The essential oil was found to be effective against *A. flavus* and *A. parasiticus.* The antifungal activity was very pronounced on *A. flavus* than *A. parasiticus.* The bioactivity of the essential oil may be due to the presence of some highly fungitoxic components in the oil. Indeed *O. canum* essential oil has monoterpenes alcohol as the major components. Terpenes are hydrocarbons produced from combination of several isoprene units (C_5H_8) and have a hydrocarbon back bone which can be rearranged into cyclic structures by cyclases, thus forming monocyclic or bicyclic structures[23]. The main terpenes are monoterpenes ($C_{10}H_{16}$) and sesquiterpenes ($C_{15}H_{24}$), but longer chains such as diterpenes ($C_{20}H_{32}$), triterpenes ($C_{30}H_{40}$), etc., also exist. Terpenes do not represent a group of constituents with high inherent antimicrobial activity. For example, *p*-cymene, one of the major constituents in thyme, had no antimicrobial activity against several Gram-negative pathogens even at 85700μg/mL concentration[24]. In a large scale experiment, limonene, α-pinene, β-pinene, δ-3-carene, (+)-sabinene, and α- terpinene showed no or low antimicrobial activity against 25 different genera of bacteria that pose problems in animals, plants, and food products[25]. These *in vitro* tests indicate that terpenes are inefficient as antimicrobials when applied as single compounds. Terpenoids are terpenes that undergo biochemical modifications via enzymes that add oxygen molecules and move or remove methyl groups[23]. Terpenoids can be subdivided into alcohols, esters, aldehydes, ketones, phenols, and epoxides. The antimicrobial activity of most terpenoids is linked to their functional groups, and it has been shown that the hydroxyl group of phenolic terpenoids and the presence of delocalized electrons are important for antimicrobial activity. For example, the antimicrobial activity of the carvacrol derivatives carvacrol methyl ether and *p*-cymene were much lower than carvacrol[25,26,27]. Exchanging the hydroxyl group of carvacrol with methyl ether affects its hydrophobicity, antimicrobial activity, and changes how the molecule interacts with the membrane[28]. Carvacrol's antimicrobial activity is comparable to that of 2-amino-*p*-cymene, which indicates that the hydroxyl group is important, but not essential for carvacrol's activity[28]. The antimicrobial activity of essential oils can often be correlated to its content of phenolic constituents[29]. Dorman and Deans[25] investigated the effect of many terpenoids against 25 different bacterial strains, and showed that all terpenoid compounds, except borneol and carvacrol methyl ester, exhibited abroad antimicrobial activity. The antimicrobial activity of carvacrol, thymol, linalool, and menthol were evaluated against *Listeria monocytogenes*, *Enterobacter aerogenes*, *E. coli*, and *Pseudomonas aeruginosa*. Themostactive compound was carvacrol followed by thymol with their highest MIC being 300 and 800μg/mL, respectively[30]. These results confirm the high antimicrobial activity of a broad collection of terpenoids, and because their chemical structures are closely related to that of terpenes. The increased activity compared to terpenes can be attributed to the functional moieties. In our study, GC–MS data, depicted remarkable variation with the earlier reports on the oils[31]. The chemical profile of EOs is reported to be influenced by the harvest period. Climatic, seasonal and geographical conditions and the amount and composition of active constituent can be significantly affected[32-35]. Thus, the biologically active EO should be qualitatively standardized before their recommendation for practical exploitation as has been done in the present investigation. The findings of the present investigation clearly showed that aflatoxin production was significantly inhibited at concentrations lower than MIC of oil (*O. canum*). Hence, essential oil would be acting by two different modes of action as inhibitor of fungal growth and aflatoxin production[5]. Based on such observation, it may be also concluded that the EO is more active as aflatoxin inhibitors than as fungal growth suppressors as emphasized by the earlier workers[32]. The use of natural plant extract provides an opportunity to avoid synthetic chemical preservatives and offers novel approach to the management of storage fungi. It was a promising method for preserving stored products in rural areas, which do not have access to modern storage system.

Conclusion

This survey underlined the bioactivity of essential oil of fresh leaves of *O. canum* from Benin as aflatoxin inhibitor and fungal growth suppressor. Monoterpene hydrocarbons were the main components present in the volatile extract. Based on their antifungal and antiaflatoxin potentials, essential oil of *O. canum* from Benin may be recommended as preservative of stored food commodities from fungal and aflatoxin contamination in storage system. This research gives also justification to the use of the leaves of *O. canum* in traditional medicine practices for the cure of different ailments. The leaves of this plant therefore can be used as a potential source of useful drugs.

Acknowledgements

References

1. Siddiqui H.H., Safety of herbal drugs-an overview, *Drugs News and Views*, **1(2),** 7–10 **(1993)**

2. Reddy B.N., Raghavender C.R., Outbreaks of aflatoxicoses in India, *Afr. J. Food Agric., Nutr. Dev.*, **7(5),** 1–15 **(2007)**

3. Santos C.C.M., Lopes M.R.V. and Kosseki S.Y., Ocorreˆncia de aflatoxinas em amendoim e produtos de amendoim comercializados na regia~o de Sa~o Jose´ de Rio Preto/SP, Revista do Instituto Adolfo Lutz, **60(2),** 153–157 **(2001)**

4. IARC, Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins, IARC monographs on the evaluation of carcinogenic risks to humans, *56 International Agency for Research on Cancer*, 489–521 **(1993)**

5. Rasooli I. and Abyaneh M.R., Inhibitory effects of thyme oils on growth and aflatoxin production by *Aspergillus parasiticus, Food Control*, **15**, 479–483 **(2004)**

6. Atanda S.A., Aina J.A., Agoda S.A., Usanga O.E. and Pessu P.O., Mycotoxin Management in Agriculture: a Review, *J. Anim. Sci. Adv*., **2**, 250-260 **(2012)**

7. Rasooli I., Fakoor M.H., Yadegarinia D., Gachkar L., Allameh A. and Rezaei M.B., Antimycotoxigenic characteristics of Rosmarinus officinalis and Trachyspermum copticum L. essential oils, *Int. J. Food Microbiol*., **122**, 135–139 **(2008)**

8. Burt S., Essential oils: their antibacterial properties and potential applications in foods-a review, *Int. J. Food Microbiol.,* **94**, 223–253 **(2004)**

9. Martins A.P., Salgueiro L., Vila R., Tomi F., Canigueral S., Casanova J., Proenca-da-Cunha A. and Adzet T., Composition of the essential oils of *Ocimum canum, Ocimum gratissimum and Ocimum minimum, Planta Med*., **65**, 187–189 **(1999)**

10. Adams R.P., Identification of Essential Oil Components by Gas Chromatography ⁄ Mass Spectrometry, Carol Stream, IL: Allured Publishing Corporation **(2007)**

11. N'guyen M.T., Identification des espèces de moisissures potentiellement productrices de mycotoxines dans le riz commercialisé dans cinq provinces de la région centrale du Vietman: Etude des conditions pouvant induire la production de mycotoxines, Institut National Polytechnique de Toulouse (INPT), Thèse de doctorat, 147 **(2007)**

12. Davis N.D., Iyer S.K. and Diener U.L., Improved method of screening for aflatoxins with coconut agar medium, *Appl. Environ. Microb.*, **53**, 1593-1595 **(1987)**

13. Attanda O.O., Development of diagnostic medium for direct visual determination of aflatoxin and its control using traditional spices, PhD thesis, University of Agriculture, Department of microbiology, Abaokuta, Nigeria, 217 **(2005)**

14. Adjou E.S., Yehouenou B., Sossou C.M., de Souza C.A. and Soumanou M.M., Occurrence of mycotoxins and associated mycoflora in peanut cakes products (kluiklui) marketed in Benin, *Afr. J. Biotechnol.,* **(2012)**, *in press*

15. de Billerbeck V.G., Roques C.G., Bessière J.M., Fonvieille J.L. and Dargent R., Effect of Cymbopogon nardus (L) W. Watson essential oil on the growth and morphogenesis of *Aspergillus niger*, *Can. J. Microbiol.*, **47**, 9–17 **(2001)**

16. Kumar R., Mishra A.K., Dubey N.K. and Tripathi Y.B., Evaluation of *Chenopodium ambrosioides* oil as a potential source of antifungal, antiaflatoxigenic and antioxidant activity, *Int. J. Food Microbiol*., **115**, 159–164 **(2007)**

17. Yin M.C. and Tsao S.M., Inhibitory effect of seven Allium plants upon three Aspergillus species, *Int.J. Food Microbiol*., **49**, 49–56 **(1999)**

18. Attanda O.O., Ogunrinu M.C. and Olorunfemi F.M., A neutral red desiccated coconut agar for rapid detection of aflatoxigenic fungi and visual determination of aflatoxins, *World Mycotox. J.*, **4(2),** 147-155 **(2011)**

19. Adjou E.S., Dahouenon-Ahoussi E., Degnon R., Soumanou M.M. and Sohounhloue D.C.K., Investigations on bioactivity of essential oil of *Ageratum conyzoides* L., from Benin against the growth of fungi and aflatoxin production, *Int. J. Pharm. Sci. Rev. Res.,* **13(1),** 143-148 **(2012)**

20. Voda K., Boh B., Vrta-cnik M. and Pohleven F., Effect of the antifungal activity of oxygenated aromatic essential oil compounds on the white-rot *Trametes versicolor* and the brown-rot *Coniophora puteana*, *Int. Biodeterior. Biodegrad.*, **51**, 51–59 **(2003)**

21. Marotti M., Dellacecca V., Piccaglia R. and Giovanelli E., Agronomic and chemical evaluation of three varieties of *Foeniculum vulgare* Mill, Presented at First World Congress on Medicinal and Aromatic Plants for Human Welfare, Maastricht, The Netherlands, 19–25 **(1992)**

22. Suhr K.I. and Nielsen P.V., Antifungal activity of essential oils evaluated by two different application techniques against rye bread spoilage fungi, *J. Appl. Microbiol.,* **94(4),** 665–674 **(2003)**

23. Caballero B., Trugo L.C. and Finglas P.M., *Encyclopedia of Food Sciences and Nutrition*, Amsterdam: Academic Press **(2003)**

24. Bagamboula C.F., Uyttendaele M. and Debevere J., Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and p-cymene towards *Shigellason- nei* and *S. flexneri. Food Microbiol.,* **21**, 33–42 **(2004)**

25. Dorman H.J.D. and Deans S.G., Antimicrobial agents from plants: antibacterial activity of plant volatile oils, *J. Appl.Microbiol.,* **88**, 308–316 **(2000)**

26. Ultee A., Bennik M.H.J. and Moezelaar R., The phenolic hydroxyl group of carvacrol is essential for action against the foodborne pathogen *Bacillus cereus*, Appl. *Environ. Microbiol.,* **68**, 1561–1568 **(2002)**

27. Ben Arfa A., Combes S., Preziosi-Belloy L., Gontard N. and Chalier P., Antimicrobial activity of carvacrol related to its chemical structure, *Lett. Appl. Microbiol.,* **43**, 149–154 **(2006)**

28. Veldhuizen E.J.A., Tjeerdsma-Van T., Bokhoven J.L.M., Zweijtzer C., Burt S.A. and Haagsman H.P., Structural requirements for the antimicrobial activity of carvacrol, *J. Agric. Food Chem.,* **54**, 1874–1879 **(2006)**

29. Rhayour K., Bouchikhi T., Tantaoui-Elaraki A., Sendide K. and Remmal A., The mechanism of bactericidal action of oregano and clove essential oils and of their phenolic major components on *Escherichia coli* and *Bacillus subtilis*, *J. Essent.Oil Res.,* **15**, 356–362 **(2003)**

30. Bassolé I.H.N., Lamien-Meda A., Bayala B., Tirogo S., Franz C., Novak J., Nebié R.C. and Dicko M.H., Composition and antimicrobial activities of *Lippia multiflora* Moldenke, *Mentha* x *piperita* L. and *Ocimum basilicum* L. essential oils and their major monoterpene alcohols alone and in combination, *Molecules,* **15**, 7825–7839 **(2010)**

31. Bakkali F., Averbeck S., Averbeck D. and Idaomar M., Biological effects of essential oils – a review, *Food Chem. Toxicol.*, **46**, 446–475 **(2008)**

32. Prakash B., Shukla R., Singh P., Mishra P.K., Dubey N.K. and Kharwar R.N., Efficacy of chemically characterized *Ocimum gratissimum* L. essential oil as an antioxidant and a safe plant based antimicrobial against fungal and aflatoxin B1 contamination of spices, *Food Res. Int.*, **10**, 128 -132 **(2010)**

33. Sessou P., Farougou S., Azokpota P., Youssao I. and Sohounhloué D., In vitro antifungal activities of Essential oils extracted from Fresh Leaves of *Cinnamomum zeylanicum* and *Ocimum gratissimum* against foodborne pathogens for their use as traditional cheese wagashi conservatives, *Res. J. Recent Sci.*, **1(9),** 67-73 **(2012)**

34. Sessou P., Farougou S., Alitonou G., Djenontin T.S., Yèhouénou B., Azokpota P., Youssao I. and Sohounhloué D., Chemical composition and antifungal activity of essential oil of fresh leaves of *Ocimum gratissimum* from Benin against six mycotoxigenic fungi isolated from traditional cheese *wagashi, I. Res. J. Biological Sci.* **1(4),** 22-27**(2012)**

35. Avlessi F., Alitonou G.A., Djenontin T.S., Tchobo F., Yèhouénou B., Menut C. and Sohounhloué D., Chemical composition and biological activities of the essential oil extracted from the fresh leaves of *Chromolaena odorata* (L. Robinson) growing in Benin, *ISCA J. Biological Sci.* **1(3),** 7-13 **(2012)**