Effect of *Plectranthus glandulosus* and *Ocimum gratissimum* Essential Oils on Growth of *Aspergillus flavus* and Aflatoxin B_1 Production

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Summary

Essential oils of Ocimum gratissimum *and* Plectranthus glandulosus *leaves were extracted by steam distillation and analysed by GC-MS, and their effects on growth and aflatoxin* B_1 *production by* Aspergillus flavus *were tested at five levels (i.e 200, 400, 600, 800 and 1000 mg/l) using SMKY agar medium. The main components of* O. gratissimum *were thymol (47.7%) and β-terpinene (14.3%) whereas those of* P. glandulosus *were represented by β-terpinene (30.8%) and terpinolene (25.2%). After 8 days of incubation on essential oil-supplemented medium, growth of* A. flavus *was totally inhibited by 800 mg/l of* O. gratissimum *essential oil and by 1000 mg/l of* P. glandulosus *essential oil. The effect of essential oils on aflatoxin* B_1 *synthesis was evaluated in SMKY broth. The medium supplemented with different essential oil concentrations, was inoculated with* A. flavus *mycelium and incubated at 25 °C. At 2, 4, 6 and 8 days, aflatoxin* B_1 *concentrations in the supernatant were estimated using Enzyme Linked Immuno-Sorbent Assay (ELISA). Results showed that aflatoxin* B_1 *synthesis was inhibited by 1000 mg/l of both essential oils of* O. gratissimum *and* P. glandulosus *after 8 days of incubation. Results obtained in the present study indicate the possibility of exploiting* O. gratissimum *and* P. glandulosus *essential oils in the fight against strains of* A. flavus *responsible for biodeterioration of stored food products.*

Résumé

Effet des huiles essentielles de *Plectranthus glandulosus* et d'*Ocimum gratissimum* sur la croissance et la production d'aflatoxine B_1 par *Aspergillus flavus*

*Les huiles essentielles des feuilles d'*Ocimum gratissimum *et de* Plectranthus glandulosus *ont été extraites par hydrodistillation et analysées par CPG/SM et leurs effets sur la croissance et la production d'aflatoxine* B_1 *par* Aspergillus flavus *testées à cinq concentrations (200, 400, 600, 800 et 1000 mg/l en milieu gélosé SMKY). Les composés majoritaires de l'huile essentielle d'*O. gratissimum *furent le thymol (47,7%) et le β-terpinene (14,3%) tandis que ceux de* P. glandulosus *étaient représentés par le β-terpinene (30,8%) et le terpinolène (25,2%). Après 8 jours d'incubation sur un milieu supplémenté à l'huile essentielle, la croissance d'*A. flavus *a été totalement inhibée par 800 mg/l d'huile essentielle d'*O. gratissimum *et par 1000 mg/l de celle de* P. glandulosus*. L'effet des huiles essentielles sur la synthèse de l'aflatoxine* B_1 *a été évalué dans le milieu SMKY. Le milieu supplémenté avec différentes concentrations d'huile essentielle a été inoculé avec le mycélium d'*A. flavus *et incubé à 25 °C. Après 2, 4, 6 et 8 jours d'incubation, les concentrations en aflatoxine* B_1 *ont été estimées dans le surnageant par la technique ELISA. Les résultats ont montré que la synthèse de l'*AFB_1 *est inhibée par 1000 mg/l d'huile essentielle d'*O. gratissimum *et de* P. glandulosus *après 8 jours d'incubation. Les résultats obtenus dans la présente étude signalent une exploitation possible des huiles essentielles d'*O. gratissimum *et de* P. glandulosus *dans la lutte contre les souches d'*A. flavus *responsables de la biodétérioration d'aliments conservés.*

Introduction

The saprophytic mold *Aspergillus flavus* Link ex Fries is known for its ability to grow on a wide range of organic substrates and to alter the nutritional and organoleptic qualities of stored food products (14). Their impact on health through production of harmful metabolites is also well known. In this respect, *A. flavus* has been reported to produce aflatoxins and other mycotoxins (26, 33). Aflatoxin contamination of foods and feeds is a serious problem worldwide (6) resulting either in improper storage or preharvest contamination of corn, peanuts, cottonseed and tree nuts amongst others. In Africa, there is ample evidence of the direct and negative effects of aflatoxin on human health through increased incidence of liver cancer and because of its potential synergistic effect on hepatitis B (23). Moreover, recent studies have pointed out the immunosuppressive properties of aflatoxin (14, 23, 28).

Some food preservation measures, such as addition of chemicals, are used to prevent fungal growth in stored grain. However, the use of these protectants meets with several drawbacks such as (a) they are generally toxic to mammals and, (b) they leave residues in the product. Because of these limitations, there is a growing interest to use natural antifungal compounds like essential oils as an alternative way to protect food from fungus attacks or fungal essential oils are complex volatile compounds produced by plants. They are known to have various functions in plants including pest and disease resistance (10).

A number of studies have focused on inhibition of *Aspergillus* spp. growth and aflatoxin production using essential oils of higher plants (2, 25, 27). In this respect, *Ocimum gratissimum* and *Plectranthus glandulosus* (both Lamiaceae) occurring in Cameroon and other tropical areas have been shown to be good sources of essential oils (17, 18, 22).

In Cameroon, *O. gratissimum*, a shrub that grows up to 3 m

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high, is used for flavouring a local well-known dark fish sauce called "*Bongo Tjobi*". The infusion of the leaves is used as a pulmonary anti-septic, anti-tussive and anti-spasmodic, whereas the leaves essential oil is used externally to keep mosquitoes away (24). Similarly, *P. glandulosus* is a medicinal plant growing in the savannah area of Cameroon and used against influenza, cough and chest complaints (24). Ethnobotanic studies and preliminary surveys revealed that these two plant species are also used to preserve food products. In this respect, fresh leaves are used to preserve stored cowpea and maize from insect and fungus damage (15).

In a recent study, using agar disk diffusion method, thirteen essential oils obtained from aromatic plants of Cameroon were tested for their effectiveness against *Asperillus flavus*. Results obtained revealed that essential oils from fresh leaves of *O. gratissimum* and *P. glandulosus* were the most effective (30). In order to gather more information on the inhibition potential of these essential oils, their effect on aflatoxin B_1 needs to be further investigated.

The aim of this study was to determine the effects of essential oils from *O. gratissimum* and *P. glandulosus* leaves on the growth of *A. flavus* and aflatoxin B_1 production. Results obtained might yield significant information as to whether essential oils of these two plants can be used as food preservatives to fight *A. flavus*.

Materials and methods

Fungal strain and production of conidia

A. flavus Lab, strain obtained from the Microbiology Laboratory of the National School of Agro-Industrial Sciences (University of Ngaoundere, Cameroon), was used as test microorganism. It was grown on Sabouraud dextrose agar (Difco, Detroit, MI) at 30 °C for 5 days. Ten ml of 1% tween 20 were added for spore collection. Conidia were harvested by centrifugation at 1,000 g for 25 min and washed with 10 ml of sterile distilled water. The spore suspension was stored in sterile distilled water at 4 °C until used.

Essential oils

Plant material used for essential oil extraction were fresh leaves from *O. gratissimum* and *P. glandulosus*. Plants were collected from a wild population near the University of Ngaoundere and identified at the national herbarium of Yaounde (Cameroon), where voucher specimens are deposited. They were steam-distilled for about 8 hours using a Clevenger apparatus. Oils recovered were dried over anhydrous sodium sulphate and stored at 4 °C until they were used (5).

Analysis of essential oils

Gas chromatography

Essential oil (10 µl) was dissolved in pentane (100 µl) and 2 µl of the solution was injected into a GC 17A fitted with Flame Ionization Detector (FID) and integrator C-R6A-chromatopac (Shimadzu Co, Japan). The column used was SUPELCOWAX fused silica (film thickness: 0.2 µm, Supelco USA, 60 m x 0.25 mm). Column temperature was programmed at 40 to 250 °C with a rate of 6 °C/min. Injector and detector temperatures used were 220 and 280 °C, respectively. Carrier gas was hydrogen. Quantification was carried out by % peak area calculations (GC/FID using a non-polar column). Identifications were made by comparison of the retention time of each compound with that of known compounds (8, 11, 16, 19, 20).

Gas chromatography – Mass spectrometry

For GC/MS analysis, a GC–17A with a QP5050 mass spectrometer (Shimadzu Co, Japan) was used. The carrier gas was helium; injector temperature was 250 °C, interface heating was 280 °C, ion source-temperature was 200 °C, EI–mode was 70 ev whereas mass ranges were between 41 to 550 amu. Mass spectra correlations were carried out with Wiley, NBS, NIST and private aroma library spectra.

Antifungal assay

Antifungal assay was performed by the agar disc diffusion method (9). SMKY agar medium (sucrose 200 g, magnesium sulphate 0.5 g, potassium nitrate 3.0 g, agar 15 g and yeast extract 7.0 g/l), with different concentrations of essential oils (200, 400, 600, 800 or 1000 mg/l) were prepared by adding appropriate quantity of essential oil to melted SMKY agar, 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 x 1.5 cm). Each Petri–dish was inoculated at the centre with a mycelial disc (6 mm diameter) taken at the periphery of an *A. flavus* colony grown on SMKY agar for 48 hours. Control plates (without essential oil) were inoculated following the same procedure. Plates were incubated at 25 °C 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. Percentage of mycelial growth inhibition was calculated using the following formula (9):

$$PI = \frac{Gc - Go}{Gc} \, x \, 100,$$

where G_c= mean diameter for control – 6 mm and G_o= mean diameter for treated mycelium – 6 mm.

Growth and Aflatoxin B_1 assay

Culture technique

Spore suspension of *A. flavus* Lab was added to 50 ml SMKY broth to obtain approximately 10^3 spores/ml. Erlenmeyer flasks were incubated for 72 h at 25 °C on a rotary shaker (150 rpm). Mycelia were then collected on cheesecloth, rinsed with sterile 0.85% KCl and transferred to a sterile blender jar (Sartorius, Germany) containing 450 ml of 0.85% KCl. After homogenisation at high speed, mycelium was recollected on cheesecloth and rinsed with 0.85% KCl. It was transferred to 1.0 litre Erlenmeyer flask containing 500 ml of SMKY medium and incubated for 24 hours at 25 °C on rotary shaker. Mycelium was collected and pooled on cheesecloth, rinsed with 0.85% KCl and transferred in 3.0 g portions to 125 ml erlenmeyer flasks containing 25 ml of GMKY medium (which had the same composition as SMKY medium except that sucrose was replaced by glucose on a per gram basis) with different concentrations of essential oils: 200, 400, 600, 800 or 1000 mg/l. Three replicates of each treatment were prepared. Erlenmeyer flasks were then incubated at 25 °C in the dark with shaking. Tween 80 was used to emulsify the essential oil and was also added to the control.

Aflatoxin B_1 analysis

After 2, 4, 6 and 8 days, triplicates of each essential oil concentration were removed and mycelia were collected on filter paper (Whatman n° 1) and their dry weights were determined after washing and drying to constant weight at 60 °C (2). Aflatoxin B_1 was extracted in each supernatant using chloroform (4). Briefly, 5.0 ml portion of the supernatant was transferred to a 25 ml conical flask and 5.0 ml chloroform was added. After mixing, layers were allowed to separate and chloroform layer transferred to a 25 ml conical flask. Extraction was repeated twice. Extract was evaporated in a water bath at 45 °C and recuperated in 2 ml of methanol.

Quantitative estimation of aflatoxin B_1 was made using Enzyme Linked Immuno–Sorbent Assay (ELISA). Immuno enzymatic kits (Transia Plate Aflatoxin B_1, Art n° AF0496, Batch N° 13X131) were obtained from Diffchamb SA (Lyon, France).

Statistical analysis

Data from three independent replicate trials were subjected to statistical analysis using SPSS Statistical package (29). Differences between means were tested using Duncan Multiple Range Test.

Results

Essential oils analysis

Essential oils were obtained by steam distillation for about 8 hours each with a yield of 0.5% for *O. gratissimum* and 0.4% for *P. glandulosus*. Main components of *P. glandulosus* and *O. gratissimum* essential oils and their percentages are presented in tables 1 and 2. Main constituents were found to be β-thujone (30.8%), terpinolene (25.2%) and piperitenone oxide (10.9) for *P. glandulosus* and thymol (47.7%), β-terpinene (14.3%) and p-cymene (8.5%) for *O. gratissimum*.

Effect of *O. gratissimum* and *P. glandulosus* essential oils on mycelia growth of *A. flavus* Lab

Inhibitory effects of these essential oils on growth of *A. flavus* Lab were determined in SMKY agar at 25 °C (Figures 1 and 2). At 800 mg/l and 1000 mg/l, *O. gratissimum* and *P. glandulosus* essential oils caused 100% inhibition. Percentage of growth inhibition of the two essential oils were significantly ($P< 0.05$) influenced by incubation time and essential oil concentration. Mycelia growth was considerably reduced with increasing concentration of essential oils while their growth increased with incubation time. 7%, 28% and 37% inhibition of mycelia growth were observed at 200, 400 and 600 mg/l, respectively, with *O. gratissimum* essential oil, whereas 10%, 15%, 15% and 46% inhibition (Figures 1 and 2) were obtained at respectively 200, 400, 600 and 800 mg/l with that of *P. glandulosus* after 8 days of incubation. These results show that *O. gratissimum* essential oil was more active in inhibiting mycelia growth of *A. flavus* Lab than that of *P. glandulosus*.

Effects of *O. gratissimum* and *P. glandulosus* essential oils on production of AFB_1 by pregrown culture of *A. flavus* Lab

Exposure of mycelium of *A. flavus* Lab to different concentrations of essential oils of *O. gratissimum* and *P. glandulosus* at 25 °C for a period of 0 to 8 days caused varying degrees of inhibition of aflatoxin B_1 synthesis (Table 3). In essential oil-free cultures, aflatoxin B_1 (AFB_1) was detected after 24 hrs of incubation and reached a maximum at 72 hours. In the presence of 1000 µl/ml, both essential oils maintained complete inhibition of AFB_1 production for the whole duration (8 days) of the experiment.

In GMKY supplemented with 600 mg/l or 800 mg/l of *O. gratissimum* essential oil, aflatoxin B_1 synthesis by culture of *A. flavus* Lab was delayed by 4 days. After 8 days of incubation, the amounts of aflatoxin B_1 secreted in the medium were respectively 2.5 and 3.7 times lower than those of the control at 600 mg/l and 800 mg/l. The addition of 400 mg/l essential oil concentration delayed synthesis of AFB_1 for 2 days whereas AFB_1 synthesis was inhibited by 22.5% after 8 days of incubation. Concentration of 200 mg/l inhibited AFB_1 synthesis by only 24% after 8 days of incubation. On the other hand, the addition of 600 mg/l or 800 mg/l of *P. glandulosus* essential oil to GKMY broth prevented AFB_1 production for 2 days. Substantial amounts of AFB_1 were detected after 8 days of incubation (2.11 and 1.33 µg/ml respectively at 600 and 800 mg/l).

Fungal growth in SMKY broth was low between 2 to 8 days of incubation and differences between oil-treated and oil free-culture were relatively small. However, there was a gradual increase in toxin production from 2 and 8 days of incubation, indicating that fungal growth is not correlated to toxin production.

Table 1
Chemical composition of essential oil of *Ocimum gratissimum*

Component	Percentage
Hexanal-2	0.5
Sabinene	0.7
Myrcene	0.8
β -Phellandrene	1.9
β-3-Carene	0.4
β-Terpinene	14.3
p-Cymene	8.5
Dehydro-p-Cymene	1.3
Limonene	2.6
Trans- β-Ocimene	0.9
Terpinolene	2.3
1,8-Cineole	1.6
β-Thujone	0.5
Linalol	0.6
Carvacrol	0.9
Terpinen -4-ol	0.5
3,9-Epoxy-p-mentha-1,8-diene	1.1
β-Terpineol	1.4
Thymol	47.7
Bisabolene	0.5
β-Copaene	0.5
β-Caryophyllene	1.3
β-Cubebene	0.5
β-Farnesene	0.8
Caryophyllene oxide	0.5

Table 2
Chemical composition of essential oil of *Plectranthus glandulosus*

Component	Percentage
β-Pinene	0.6
Myrcene	2.2
β-Phellandrene	0.7
β-3-Carene	1.5
β-Terpinene	0.8
Limonene	3.2
Trans- β-Ocimene	0.6
Terpinolene	25.2
β-Thujone	30.8
Neral	0.8
Fenchol	1.5
Trans-p-Menth-2-en-ol	0.5
Borneol	0.5
p-Cymene-8-ol	3.6
Oxide cis-piperitone	3
Oxide trans-piperitone	0.5
Thymol	0.4
Trans-2-Hydroxypiperitone	0.6
4-Hydroxypiperitone	0.7
Piperitenone	1.3
Piperitenone Oxide	10.9
Isopulegone	1.8
Germacrene D	1.4

Discussion

Growth of *A. flavus* and aflatoxin B_1 production in some foodstuffs are considered as health hazards. With increasing consumer demand for naturally preserved food, examination of essential oils for antimicrobial properties has become attractive to researchers and food processors (1, 13, 21). *In vitro* results obtained in the present study suggest that essential oils of *O. gratissimum* and *P. glandulosus* might be useful agents for control of *A. flavus* growth and AFB_1 synthesis.

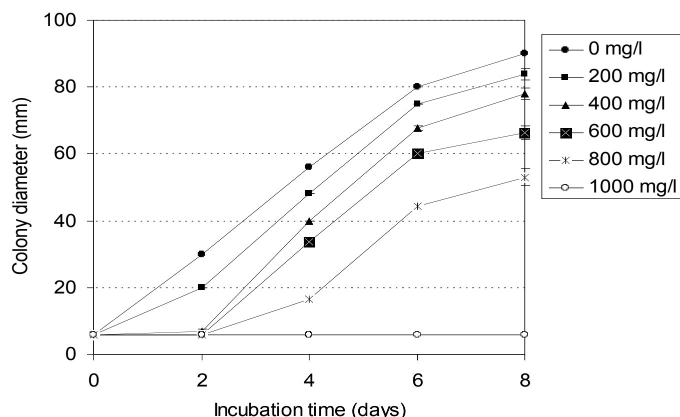

Figure 1: Effect of different concentrations of *P. glandulosus* on *A. flavus* growth.

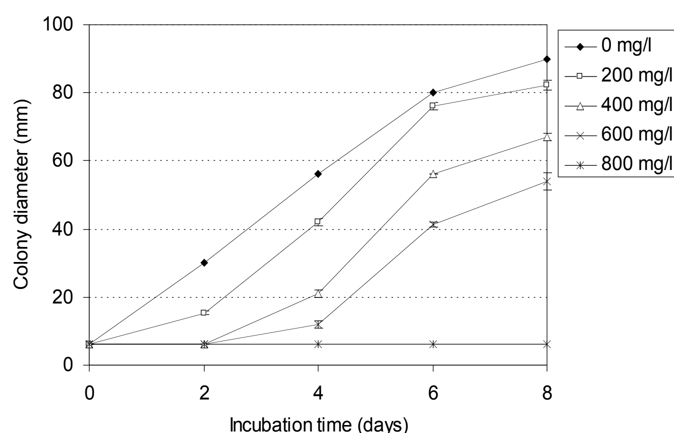

Figure 2: Effect of different concentrations of *O. gratissimum* on *A. flavus* growth.

Results obtained show that *O. gratissimum* appears to be more toxic against *A. flavus* Lab than *P. glandulosus* in the test conditions. MIC of *O. gratissimum* was 800 mg/l whereas that of *P. glandulosus* was 1000 mg/l. The different effect of these two oils may be due to their different compositions. According to literature (5, 31), there are two chemical types of *O. gratissimum* as defined by the main compounds in the essential oils from their leaves, i.e. the thymol type and the eugenol type. Results obtained in the present study indicate that *O. gratissimum* from Cameroon belongs to the thymol type.

MIC value of *O. gratissimum* essential oil obtained against *A. flavus* (800 mg/l) is different from those reported in literature, i.e. 2500 mg/l (3) and 1250 mg/l (1). Direct comparison between our own and other results may not be appropriate since the origin of plant material and experimental conditions were different and this can influence essential oil composition and the level of antimicrobial activity as previously reported (7, 10, 25). In other experiments, researchers used microdilution method while in our own study disc diffusion method was used.

It was also observed that MICs of the two essential oils varied with incubation time. It ranged from 400 mg/l, after 2 days of incubation, to 800 mg/l after 8 days for *O. gratissimum*, and 1000 mg/l for *P. glandulosus* respectively. This could be due to the fact that during a relatively long incubation period some volatile components in these oils may evaporate from the media, leading to decrease in their concentration.

Not much is known about the basic mechanisms by which these essential oils inhibit *A. flavus* growth. Research work has been carried out with some essential oils in order to

Table 3

Effect of essential oils of *O. gratissimum* and *P. glandulosus* on growth and aflatoxin B1 synthesis by *Aspergillus flavus* Lab

Oil concentration (mg/l)	Incubation time (days)							
	2		4		6		8	
	MDW	AFB_1	MDW	AFB_1	MDW	AFB_1	MDW	AFB_1
Control	522.5 ± 5.6^{a}	3.40 ± 0.1^{a}	532.0 ± 4.2^{a}	4.66 ± 0.0^{a}	540.5 ±3.5^{a}	4.89 ± 0.1^{a}	571.5 ± 4.9^{a}	5.09 ± 0.4^{a}
O. gratissimum								
200	511.5 ± 3.5^{b}	1.49 ± 0.0^{d}	521.0 ± 4.2^{b}	3.71± 0.07^{b}	522.5 ± 0.7^{b}	3.75 ± 0.07^{c}	529 ± 2.82^{b}	3.80 ± 0.09^{c}
400	508.0 ± 4.2^{b}	1.37 ± 0.0^{d}	512.5 ± 2.1^{c}	3.17 ± 0.1^{c}	514.5 ± 0.7^{c}	3.21 ± 0.12^{d}	512 ± 4.24^{c}	3.32 ± 0.14^{e}
600	506.0 ± 2.8^{b}	0.00 ± 0.0^{g}	516.5 ± 0.7^{c}	2.04 ± 0.09^{d}	513.5 ± 3.53^{c}	2.05 ± 0.07^{e}	513.5 ± 3.53^{c}	2.13 ± 0.14^{g}
800	506.5 ± 6.3^{b}	0.00 ± 0.0^{g}	506.5 ± 6.3^{d}	1.39 ± 0.1^{e}	506.0 ± 0.00^{c}	1.42 ± 0.1^{f}	505 ± 4.24^{d}	1.46 ± 0.08^{h}
1000	502.0 ± 0.0^{b}	0.00 ± 0.0g	502 ± 0.0^{d}	0.0 ± 0.0^{f}	502 ± 0.0^{c}	0.0 ± 0.0^{g}	503 ± 1.41^{d}	0.00 ± 0.00^{i}
P. glandulosus								
200	504.5 ± 0.7^{b}	2.53 ± 0.1^{b}	507.5 ± 0.7^{d}	3.87 ± 0.07^{b}	506.5 ± 3.53^{c}	4.11 ± 0.14^{b}	509.5 ± 2.12^{d}	4.14 ± 0.03^{b}
400	506.0 ± 2.8^{b}	1.69 ± 0.1^{c}	507.5 ± 0.7^{d}	3.35 ± 0.08^{c}	508 ± 1.41^{c}	3.38 ± 0.09^{d}	506.5 ± 0.7^{d}	3.54 ± 0.21^{d}
600	506.5 ± 0.7^{b}	1.14 ± 0.0^{e}	507 ± 2.82^{d}	2.00 ± 0.04^{d}	511.5 ± 0.7^{c}	2.16 ± 0.07^{e}	510.5 ± 2.12^{d}	2.41 ± 0.14^{f}
800	502.0 ± 0.0^{b}	0.43 ± 0.1^{f}	503 ± 4.24^{d}	1.34 ± 0.06^{e}	503.5 ± 2.12^{c}	1.36 ± 0.07^{f}	504.5 ± 2.12^{d}	1.62 ± 0.07^{h}
1000	502.0 ± 0.0^{b}	0.00 ± 0.0^{g}	503 ± 1.41^{d}	0.00 ± 0.00^{f}	502.5 ± 0.7^{c}	0.0 ± 0.00^{g}	503.0 ± 1.41^{d}	0.00 ± 0.00^{i}

Values in each column followed by the same letter do not differ significantly in Duncan Multiple Range Test ($P < 0.05$).
MDW: Mycelial dry weight (mg) AFB_1: Aflatoxin B_1 (µg/ml)

elucidate their mechanism of action. It is reported that essential oils damage a variety of enzyme systems of yeasts, thus affecting structural component synthesis and energy production (5). Studies on effects of *Cymbopogon nardus* on *Aspergillus niger* revealed that the plasma membrane of *A. niger* was seriously damaged with concentrations of essential oil between 200 to 1000 mg/l (7). These reasons may apply to the present study.

As compared to the inhibitory effects of *O. gratissimum* and *P. glandulosus* on fungal growth, no data to our knowledge are available concerning their effects on the production of mycotoxins by toxinogenic fungi. Inhibition of growth and aflatoxin production by *Aspergillus* spp. may represent separate effects (4). This justifies the investigation of these two aspects in the present study. Since aflatoxins are secondary metabolites and because their synthesis is dependent on the achievement of high degree of growth, the inhibition of mycelial growth makes it difficult to directly study the effect of essential oils on aflatoxin synthesis. To avoid this problem, the mould was first cultured in an essential oil-free medium. The mycelium was then transferred into medium containing either no essential oil or different concentrations of essential oils. The results obtained clearly indicated the inhibition of aflatoxin B_1 production by *A. flavus* at concentrations ranging from 200 to 1000 mg/l of both essential oils. These results suggest that inhibition of AFB_1 production cannot be attributed to growth insufficiency. The mechanism by which this inhibition occurs is not very clear. Aflatoxins are synthesized extramitochondrially from acetylcoenzyme A during a period of rapid glucose utilisation (12). Essential oils of *O. gratissimum* or *P. glandulosus* may restrict carbohydrate catabolism in *A. flavus* by acting on some key enzymes and this may result in the decrease of its ability to synthesize AFB_1.

Conclusion

The results of this study indicate that essential oils of *O. gratissimum* and *P. glandulosus* can be potent fungicides for the control of *A. flavus*. For the practical use of these oils as novel fungal-control agent, further research is needed on safety issues for human health. Other areas requiring attention are formulations to improve the fungicidal potency and stability.

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