Mycotoxicology Society of Nigeria

Mycotoxicology, 2016, 3: 15-23

Volatile oils of *Piper guineense* inhibit growth and aflatoxin production in *Aspergillus flavus* and *A. parvisclerotigenus*

[a,*]Ezekiel C. N., [a]Adetunji C. O., [a]Owolabi O. A., [b,c]Anokwuru C. P., [d,e]Amos-Tautua B. M. W., [a]Oyedele O. A., [f]Oni O. E., [g]Imafidon T. F., [a]Onumaegbu C. O., [h]Odumosu B. T. and [i]Oyedeji A. O.

[a]Department of Microbiology, Babcock University, Ilishan Remo, Ogun State, Nigeria.
[b]Department of Basic Sciences, Babcock University, Ilishan Remo, Ogun State, Nigeria.
[c]Department of Chemistry, University of Venda, Thouhoyandou, South Africa.
[d]Department of Chemistry, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria.
[e]Department of Applied Chemistry, University of Johannesburg, Doornfontein Campus, Doornfontein, Johannesburg, South Africa.
[f]Department of Microbial Ecophysiology, Faculty of Biology/Chemistry, University of Bremen, Bremen, Germany.
[g]Mycotoxin Laboratory, National Agency for Food and Drug Administration and Control, Oshodi, P.M.B. 21482, Ikeja, Lagos, Nigeria.
[h]Department of Microbiology, University of Lagos, Akoka-Yaba, Lagos, Nigeria.
[i]Department of Chemical and Physical Sciences, Walter Sisulu University, Mthatha, South Africa.
*Corresponding author: chaugez@gmail.com.

ABSTRACT

Growth inhibition and anti-aflatoxigenic activities of various extracts and steam distilled/essential oil of *Piper guineense* were assessed on *Aspergillus flavus* CBS 133921 and *A. parvisclerotigenus* CBS 135587 after determining the volatile compound profiles by gas chromatography/mass spectrometry. Twenty and 14 volatile compounds constituting 57.14% and 90.07% of the total extracted oils were identified in the hexane extract and hydro-distilled oil respectively. Sesquiterpene hydrocarbons and alcohols dominated the chemical profiles of both oils. The hexane extract had the highest (47.7–50.8%) significant ($p<0.05$) inhibitory activity against aflatoxigenic fungal growth while essential oils showed the highest significant ($p<0.05$) inhibitory activity (97.61–99.37%) against aflatoxin production in both strains. The overall pattern of aflatoxin inhibition in both aflatoxigenic species by the extracts or oil is hydro-distilled oils > hexane extract > methanolic extract > defatted methanolic extract. Volatile compounds of *P. guineense* inhibit aflatoxigenic fungal growth and aflatoxin biosynthesis. To our knowledge, this is the first systematic and comparative report evaluating antifungal and anti-aflatoxigenic activities of *P. guineense* extracts obtained via different techniques.

Keywords: Aflatoxin; *Aspergillus*; Essential oils; Food safety; Phytoinhibition; Spices.

1.0 INTRODUCTION

Aflatoxins are toxic secondary metabolites produced by diverse *Aspergillus* species (e.g. *A. flavus*, *A. parasiticus* and the minisclerotial species such as *A. parvisclerotigenus*) when they naturally contaminate foods and feed commodities including cereals, nuts and oilseeds, and spices (Strosnider et al. 2006). The presence of aflatoxins in the food chain is associated with significant decrease in food quality and increased economic losses. These losses can be quantified in terms of low agricultural outputs leading to loss of farmer income, creation of trade barriers and elevated risks of developing diseases in humans (e.g. cancers, immune-related disorders and growth faltering especially in children) and animals. Health effects of aflatoxins are prominent after dietary exposure to aflatoxin-contaminated foodstuffs (Strosnider et al. 2006). Huge setbacks associated with aflatoxin contamination of agricultural products necessitated the development of strategies for prevention of aflatoxigenic fungal growth and subsequent production of toxic metabolites in agricultural produce (Allameh et al. 2011). Among the many methods for aflatoxin control, the use of plants and plant products has recently gained most interest due to resistance of fungi to chemical preservatives and associated legal implications (Montes-Belmont and Carvajal, 1998; Soliman and Badeaa, 2002; El-Nagerabi et al. 2012; Gandomi et al. 2009). Plant extracts and essential oils (EO) from spices, herbs and other plants possess antifungal activity (Fan and Chen, 1999; Juglal et al. 2002; Soliman and Badeaa, 2002). Specifically, compounds such as phenols and volatile oils have been reported to inhibit toxigenic fungal growth as well as mycotoxin production in food systems (Hartung et al. 1973; Montes-Belmount and Carvajal, 1998; Bakan et al. 2003; Gowda et al. 2004; Rasooli and Abyaneh, 2004; Sanchez et al. 2004; Rocha Vilela et al. 2009; El-Nagerabi et al. 2012; Shukla et al. 2012; Passone et al. 2013). EOs are vola-

tile and complex compounds characterized by a strong odour and are produced from aromatic plants as secondary metabolites. They are usually obtained by steam or hydro-distillation and mostly contain aromatic organic compounds which have the potential to control aflatoxin production (Chatterjee, 1990). Our previous results showed that aqueous extracts of *P. guineense* inhibited as much as 99.7% of aflatoxin production in *A. flavus* and *A. parvisclerotigenus* without any significant effect on growth of several strains of both species (Ezekiel et al. *submitted*). However, it was unclear whether non-aqueous components of *P. guineense* would yield similar results. No report is available on the antifungal/aflatoxigenic potentials of volatile oils of *P. guineense* in spite of its wide cultivation in the African tropical forest zones and frequent use in African homes and beyond as condiments and food flavourings. Therefore, this study is aimed at evaluating the anti-aflatoxigenic activity of volatile components of *P. guineense* against *A. flavus* and *A. parvisclerotigenus*.

2.0 MATERIALS AND METHODS

2.1 *Aspergillus strains*

Two aflatoxigenic strains: *Aspergillus flavus* (CBS 133921) and *A. parvisclerotigenus* (CBS 135587); previously obtained from peanut cake and sesame seeds (Ezekiel et al. 2013, 2014b) respectively, were used in this study. The strains were deposited in the culture collection of the CBS-KNAW Fungal Biodiversity Centre, Netherlands. Pure cultures of both strains were also maintained at 4 ºC as 5/2 agar (5% V-8 juice and 2% agar, pH 5.2) plugs in sterile water in the Department of Biosciences and Biotechnology, Babcock University. Prior to the inhibitory studies, both strains were plated out on 5/2 agar for 5 days.

2.2. *Plant material and preparation of extracts and essential oils (EO)*

Fresh fruits (1.5 kg) of *P. guineense* were purchased from Ilishan market, Ogun State, Nigeria between November 2014 and January 2015. The fruits were air dried for 4 days and ground into fine powder using a Waring blender (Marlex Emerald UNIT III, Daman). The ground samples were divided equally into three groups: A–C. Briefly, group A samples were extracted by hydro-distillation for 4 h using a laboratory scale Clevenger-type apparatus (AOAC, 1980). Group B samples (500 g of AP powder) were soaked in 1000 ml hexane for 24 h and evaporated after filtration to obtain the hexane extract. Extraction of group C samples (500 g of AP powder) was carried out as described for group B but methanol was used as the extracting solvent. Defatted methanol (group D) extract was obtained by extracting the residue of group B with methanol. All extracts (B-D) were concentrated using a vacuum rotary evaporator (EYELA N-1001, Rikakikai Co. Ltd., Tokyo) at 40 ºC. Concentrates were transferred into glass vials and kept in the dark at 4 ºC until further analysis.

2.3. *Gas chromatography–mass spectrometric (GC-MS) analysis of EO and extracts*

The compound profiles of the EO and hexane extracts of AP fruits were determined by a GC-MS technique. Samples were analyzed on a 7890A GC system (Agilent Technology, USA) equipped with a 5974 MS detector (Agilent Technology, USA) and a capillary column (Hp 5 ms, 30.00 m × 0.32, 0.25 µm thickness, Agilent Technology, USA). Helium (99.9 % purity) served as carrier gas. Initial column temperature was 50 ºC to hold for 1 min to a final temperature of 250 ºC at 3.5 ºC/min. Samples (0.1 µl) were injected manually in the split mode. Mass spectra were recorded at 70 eV. Mass range was from m/z 50 to 550. The KI (Kovats of index) of EO and the hexane extracts relative to C5–C24 *n*-alkanes obtained on a non-polar DB-5MS column were established. Furthermore, the KI of EO and hexane extracts were compared with the KI provided in the literature by comparison of the mass spectra with those recorded by the NIST08 (National Institute of Standards and Technology) and Willey (ChemStation data system). Individual components of the samples were identified by retention indices and compared with those of same compounds known from literature (Adams, 2001).

2.4. *Antifungal and anti-aflatoxigenic assays*

2.4.1 *Inoculation of aflatoxigenic* Aspergillus *strains on extract/EO amended media*

The inhibitory effects of the extracts (hexane, methanolic and defatted methanolic) and EO of AP on growth and aflatoxin production by the aflatoxigenic strains of *A. flavus* and *A. parvisclerotigenus* were determined on neutral red desiccated coconut agar (NRDCA). A slight modification of the agar plate method of Matamoros-León et al. (1999) was performed for the assays. Briefly, 15 ml aliquots of freshly prepared NRDCA were dispensed into 20 ml vials, autoclaved and cooled to about 45ºC. Extracts (100 mg/ml and 200 mg/ml) or EO (200 µl and 400 µl) of AP were added to the vials and the mixture was carefully shaken. The extract- or EO-molten agar mix was emptied into 9 cm sterile Petri plates, allowed to solidify and centrally inoculated with each aflatoxigenic strain. Control plates (NRDCA without extract or EO) were set up for each aflatoxigenic strain. Negative control plates A and B [NRDCA with hexane (plate A) and methanol (plate B)] were also set up for each strain. All set ups were in triplicates to allow for assessment of variations in aflatoxin production. The inoculated plates were incubated in the dark at 30 ºC for 5 days.

2.4.2. ***Assessment of growth inhibition of aflatoxigenic fungi***

Radial growths of the fungal cultures were taken daily for 5 days in order to determine whether the extracts or EO inhibited fungal growth. Measurements were recorded in mm and percentage inhibition of fungal growth was calculated according to the equation of Pandey et al. (1982):I (%) = [(C – T) / C] × 100…… (1)

Where:

I is inhibition (%),

C is colony diameter on the control plate (mm),

T is colony diameter on the test plate (mm).

2.4.3. ***Determination of aflatoxins in inoculated media***

High performance liquid chromatographic (HPLC) analysis was carried out on control and extract/EO-amended NRDCA cultures of *A. flavus* and *A. parvisclerotigenus* to quantify the levels of aflatoxins, expressed as total aflatoxin (∑aflatoxins B1, B2, G1 and G2). The amounts of aflatoxins produced in the test cultures were compared to those in the control cultures and served as an index of measuring the inhibitory activities of the spice towards aflatoxin production. A modification of the AOAC (2000) method of aflatoxin extraction was adopted for this study. Two agar plugs (5 mm in diameter) were taken from the older part of each culture and extracted with 2.5 ml of 80% methanol on a ZHP-100 Intelligent Thermostatic Shake Cultivation Cabinet (Gallenkamp, England). Extracts were filtered using micro-filters and cleaned up by adding 6 ml hexane. The mixture was vigorously shaken and the lower methanol/toxin phase was collected into a polypropylene cup. The cleanup process was repeated and the pooled methanol/toxin phase was passed through a layer of anhydrous sodium sulphate to remove any water molecule. Purified aflatoxin extracts (200 µl) were analyzed with a 2484 Series HPLC System with fluorescent detector (Hitachi High Technologies Inc., USA). The mobile phase was water: methanol: acetonitrile (5:2:1 v/v/v), flow rate of 1 ml/min, excitation wavelength of 346nm and emission was 440nm. Post column derivatization system was performed on a KOBRA cell.

2.5. ***Statistical analysis***

Data evaluation and analysis was performed with SPSS® for windows v.14.0 (SPSS, Chicago, IL, USA). Means of percentage inhibition of fungal growth and aflatoxin production by the strains were separated by the Duncan's multiple range test, and further tested for significance by analysis of variance at $\alpha = 0.05$. In order to create a normal distribution in the figure for quantities of aflatoxins produced and percentage inhibition of aflatoxin production, aflatoxin concentrations were logarithm transformed using the formula, Log10 $(1+x)$; where x represents aflatoxin concentration in µg/kg.

3.0. RESULTS AND DISCUSSION

3.1 ***Chemical composition of extracts and EO***

Different mass spectral patterns were obtained for hexane oil extract and hydrodistilled oil as shown in Figs. 1 and 2 respectively. In the aforementioned order, 20 (Table 1) and 14 (Table 2) individual volatile compounds were identified. The identified volatile compounds in the hexane extract constituted 57.14% of the total extracted oil while those in the steam distilled oil formed 90.07% of the total oil. Sesquiterpene hydrocarbons and aliphatic fatty acids were the predominant classes of volatile compounds identified in the hexane extract while sesquiterpene hydrocarbons predominated in the hydro-distilled oil. Specifically, the major compounds in the hexane extract were mono (-2-ethylhexyl) phthalate (a fatty acid, 16.83%) and β-caryophyllene (a sesquiterpene, 12.69%) while β-bisabolene (a sesquiterpene, 43.19%) was the main constituent in the hydrodistilled oil. Of the various chemotypes in Nigeria (Oyedeji et al. 2005), the volatile oil profiles in our study were relatively similar to the profiles from hydrodistilled oils of same spice reported by Ekundayo et al. (1988) and Olonisakin et al. (2006). The variations in volatile oil composition and quantities obtained in our study and those reported by the aforementioned studies may be due to several environmental and genetic differences (Oyedeji et al. 2005; Ghasemi Pirbalouti et al. 2013, 2015) as well as difference in the dryness of the spices used or hydrodistillation time [4 h in this study and 5 h in Olonisakin et al. (2006)]. For example, the compositional difference of β-caryophyllene and β-bisabolene observed in the hexane oil (Table 1) and EO (Table 2) suggests that the method of extraction impacted on the kinds and proportions of individual compound obtained from the spice. Similar to our study, El-Shazly et al. (2004) reported that hexane extracts of certain species of *Achillea* plants yielded more components compounds compared to fractions obtained via hydrodistillation. Our study presents the first report of volatile compound profiles from hexane extracts of *P. guineense*.

3.2. ***Inhibitory effects of extracts and EO on aflatoxigenic fungal growth***

All extracts and EO inhibited growth of the two aflatoxigenic *Aspergillus* strains at varying levels (Figs. 3 and 4). Growth inhibition of *A. flavus* by all the extracts and EO concentrations commenced after 48 h (Fig. 3) and after 24 h for the *A. parvisclerotigenus* strain (Fig. 4).

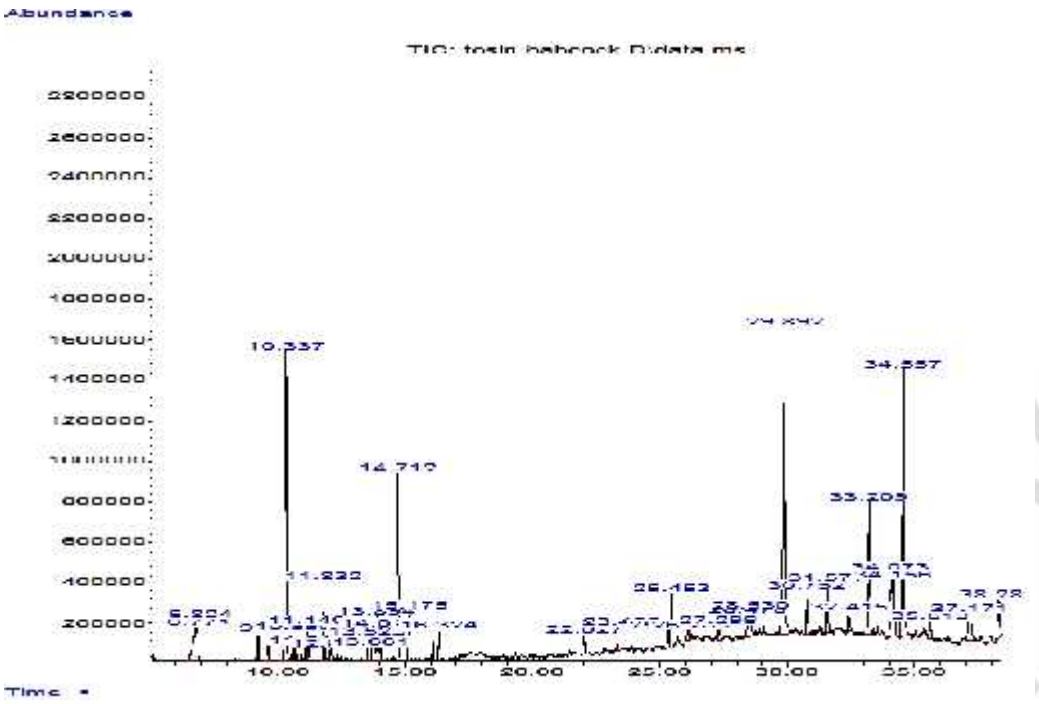

Fig. 1. Gas chromatogram of hexane extract of *Piperguineense*.

At all time intervals, the hexane extract exhibited the highest significant ($p<0.05$) activity against the growth of the two aflatoxigenic species (range for *A. flavus* = 31.3–47.7%; range for *A. parvisclerotigenus* = 3.4–50.8%) while the defatted methanolic extract showed the least inhibitory activity against *A. flavus* (range = 1.1–3.0%) and *A. parvisclerotigenus* (range = 2.4–3.2%) growths regardless of the tested volumes.

Table 1. Composition of volatile compounds in hexane extract of *Piper guineense*.

S/N.	RT[a] (mins)	Compounds	Content (%)	RI[b] (Kovats)
1.	9.235	α-Copaene	0.83	1379
2.	9.636	β-Elemene	1.06	1389
3.	10.339	β-Caryophyllene	12.69	1421
4.	10.648	γ-Elemene	0.86	1429
5.	11.833	Germacrene D	2.57	1479
6.	12.073	Selina-4,6-diene (δ-seliene)	0.56	1490
7.	13.521	Germacrene A	0.76	1503
8.	13.893	β-Bisabolene	1.54	1503
9.	14.019	δ-Cadinene	0.99	1524
10.	15.175	Elemol	1.80	1547
11.	16.084	Ledol	0.97	1565
12.	22.029	4-Methyl pentadecanoate	0.63	1884
13.	23.483	n-nonadecane	1.93	1900
14.	25.325	Methyl-9, 12-octadecandienoate	0.74	2077
15.	25.451	Methyl-cis-13-octadececenoate	1.72	2085
16.	26.207	Oleic acid	0.66	2141
17.	28.891	Mono(-2-ethylhexyl)phthalate	16.83	2162
18.	33.210	Diiso-octyl phthalate	4.98	2704
19.	34.154	Tritetracontane	2.53	4292
20.	37.170	Tetratetracontane	2.49	4395
Class composition				
Sesquiterpene hydrocarbons (1–9)			21.86	
Sesquiterpene alcohols (10 & 11)			2.77	
Aliphatic fatty acids (12–20)			32.51	
Total identified			57.14	

[a]Retention time

[b]Retention index

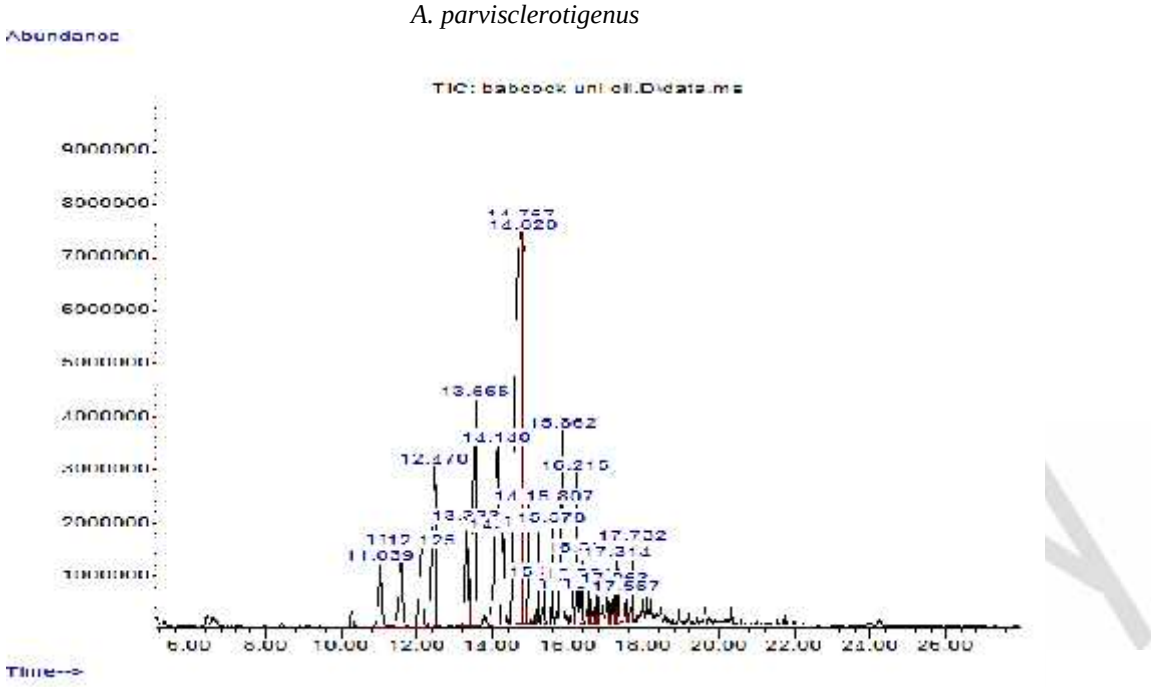

Fig. 2. Gas chromatogram of essential oil of *Piperguineense*.

For *A. flavus*, there was no significant ($p>0.05$) difference in the growth inhibitory pattern exhibited by the methanolic extract and EO at all time intervals (Fig. 3) while the higher concentration (200 mg/ml) of methanolic extract significantly ($p<0.05$) inhibited the growth of *A. parvisclerotigenus* at all time intervals than the EO (Fig. 4b). Essential oils and hexane extracts of diverse plants usually contain volatile compounds which exhibit antimicrobial activities commonly related to their chemical structures, the concentration in which they are present, and their interactions which can affect their bioactivity (Cabral et al. 2013). The high inhibition of fungal growth by the hexane extract could be associated with the composition of β-caryophyllene (12.69%) and mono (-2-ethylhexyl) phthalate (16.83%); two lipophilic low molecular weight compounds. Beta-caryophyllene has been reported as a major constituent of several plants, e.g., *Zingiber nimmonii* (Sabulal *et al.* 2006), *Alpinia galangal* (Mayachiew and Devahastin, 2008), *Spinanthera ordoratissima* (Galdino *et al*. 2012),

Table 2. Composition of essential oils from steam distillation of *Piper guineense*.

S/N.	RT[a] (mins)	Compounds	Content (%)	RI[b] (Kovats)
1.	11.038	α-Copaene	2.43	1379
2.	11.587	β-Elemene	3.75	1389
3.	12.125	α-Gurjunene	3.23	1413
4.	12.468	β-Caryophyllene	5.75	1421
5.	13.332	α-Humulene	3.49	1455
6.	13.567	β-cis Farnesene	9.45	1479
7.	14.139	β-Selinene	7.71	1486
8.	14.288	α-Selinene	2.80	1494
9.	14.757-14.820	β-Bisabolene	43.19	1503
10.	14.957	β-Sesquiphellandrene	1.33	1516
11.	15.581	Neroidol	1.36	1524
12.	15.810-15.861	Caryophyllene oxide	4.89	1582
13.	16.777	Humulene epoxide	0.35	1608
14.	17.566	α-Eudesmol	0.34	1652
Class composition				
Sesquiterpene hydrocarbons (1–10)			83.13	
Sesquiterpene alcohols (11 &14)			1.70	
Oxygenated sesquiterpenes (12 & 13)			5.24	
Total identified			90.07	

[a]Retention time
[b]Retention index

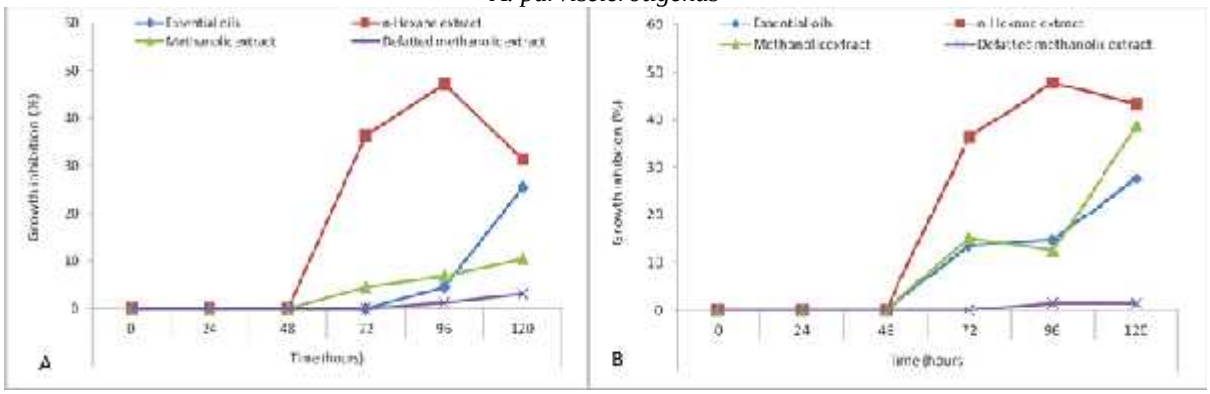

Fig. 3. Percentage inhibition of *A. flavus* CBS 133921 growth on neutral red desiccated coconut agar amended with four extracts from *Piper guineense* at two levels (A: 100 mg/ml of extracts and 200 µl of essential oil; B: 200 mg/ml of extracts and 400 µl of essential oil) for 5 days at 30°C.

Copaifera multijuga Hayne (Lucca *et al.* 2014), *Capaiba balsam*, *Syzigium aromaticum* (Pant et al. 2014), *Cajanus cajas* leaves (Qi et al. 2014), *Humulus lupulus* and *Eugenia caryophyllata* (Evrendilek, 2015). It is reported to be associated with a number of plant defense mechanisms and is inhibitory to fungal and bacterial strains (Reinsvold et al. 2011). Mono (-2-ethylhexyl) phthalate was among phthalate isolates in *n*-hexane extracts of *Azadirachta indica* that individually showed antifungal activity (Akpuaka et al. 2012). It is possible that these compounds permeated easily through the cell membranes and caused disruption to the fungal cell organization thus inhibiting growth (Shukla et al. 2012; Cabral et al. 2013). Although researchers have reported a relationship between chemical structures of dominant compounds and antimicrobial activity, minor compounds also have been linked to a synergistic effect between other compounds (Bluma and Etcheverry, 2008; Cabral *et al*. 2013).

3.3. *Inhibitory effects of extracts and EO on aflatoxin production*

Both strains of the *Aspergillus* species produced relatively high quantities of total aflatoxin in the extract-/EO-free NRDCA medium after 5 days of incubation. However, when NRDCA was amended with the *P. guineense* extracts and EO, varying degrees of concentration-dependent inhibition of aflatoxin biosynthesis were observed for both fungal species (Fig. 5). There were also significant ($p<0.05$) differences in aflatoxin inhibition levels of various extracts and EO at each extract concentration tested for both species except between the 100 mg/ml hexane extract and methanolic extract tested against *A. parvisclerotigenus*. As shown in Fig. 5, EO showed the highest significant ($p<0.05$) aflatoxin inhibitory activity in *A. flavus* (mean inhibition: 200 µl = 97.61%, 400 µl = 98.95%) and *A. parvisclerotigenus* (mean inhibition: 200 µl = 98.87%, 400 µl = 99.37%) while defatted methanolic extract had the least inhibitory activity in both species (mean inhibition in *A. flavus*: 100 mg/ml = 69.96%, 200 mg/ml = 81.80%; mean inhibition in *A. parvisclerotigenus*: 100 mg/ml = 49.73%, 200 mg/ml = 50.70%).

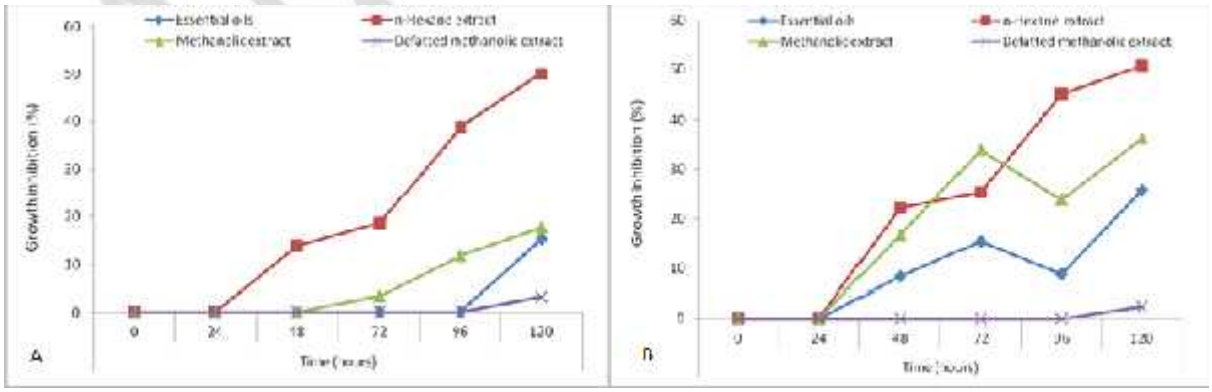

Fig. 4. Percentage inhibition of *A. parvisclerotigenus* CBS 135587 growth on neutral red desiccated coconut agar amended with four extracts from *Piper guineense* at two levels (A: 100 mg/ml of extracts and 200 µl of essential oil l; B: 200 mg/ml of extracts and 400 µl of essential oil) for 5 days at 30°C.

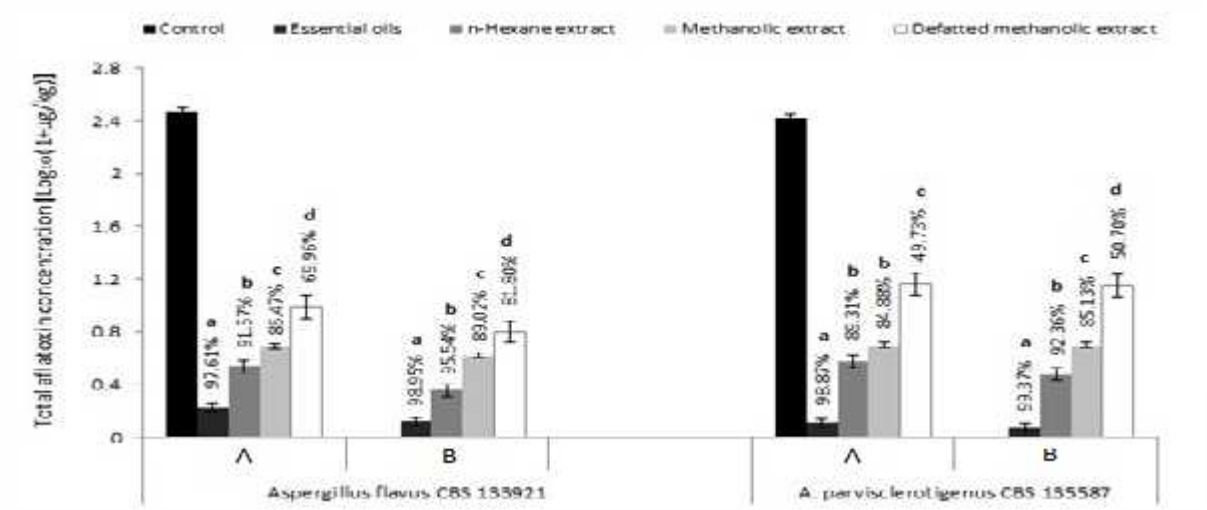

Fig. 5. Inhibition of aflatoxin production in *A. flavus* CBS 133921 and *A. parvisclerotigenus* CBS 135587 by two levels (A: 100 mg/ml of extracts or 200 µl of essential oil; B:200 mg/ml of extracts or 400 µl of essential oil) of steam distilled essential oils from *Piper guineense* in neutral red desiccated coconut agar after amended 5 days of incubation at 30°C. Lines on bars indicate the standard error of mean ($\alpha = 0.05$). Percentage inhibition values are shown above error bars. Values on bars with different alphabets are significantly different ($\alpha = 0.05$).

The very high and significant inhibition of aflatoxin production exhibited by the EO over other extracts may be attributed to the high quantities of sesquiterpene hydrocarbons (83%) in the EO than in the hexane extract. Beta-bisabolene has been reported as a major component of *Teucrium marum* (Ricci et al. 2005), *Alpinia galangal* (Mayachiew and Devahastin, 2008), *Daucus carota* L. subsp. *carota* (Maxia et al. 2009), *Piper capense* (Matasyoh et al. 2011) and *Ocimum gratissimum* (Silva et al. 2012). Although β-bisabolene, in contrast to hexane extracts, was detected (43.19%) in the EO in our study prompting its implication in the observed high antiaflatoxigenic activity of EO, synergistic interactions between several volatile constituents remains a possibility (Cabral et al. 2013). In our previous study (Ezekiel et al. *submitted*), aqueous extract exhibited profound inhibition against aflatoxin production. In this study, the volatile compounds have shown similar capacity to inhibit aflatoxin production. Hence AP contains a broad spectrum of antiaflatoxigenic compounds. The present study further supports the findings of a previous study by Ezekiel et al. (2014a) which showed that whole AP fruits when co-stored with maize for 56 days prevented aflatoxin formation better than AP powder due to loss of volatile oils over time.

CONCLUSION

This study showed that volatile compounds of *P. guineense* obtained via hydrodistillation and extraction with organic compounds (methanol and hexane) are inhibitory to growth and aflatoxin biosynthesis in aflatoxigenic fungi in contrast to the extraction solvents (negative controls) which did not show any inhibitory activity against mould growth or toxin production. Although more work still needs to be done to completely unravel the exact mechanisms of action of the bioactive compounds in *P. guineense* towards *Aspergillus* and aflatoxin inhibition, volatile oils are obviously useful food control products against aflatoxin-producing *Aspergillus* species that pose food safety threats in subtropical regions.

CONFLICT OF INTEREST

The authors have no conflict regarding this study.

ACKNOWLEDGEMENTS

The authors thank the Director General and Director, Laboratory Services of the National Agency for Food and Drug Administration and Control (NAFDAC) for supporting this research.

REFERENCES

Adams, R.P. (2001). Identification of essential oil components by gas chromatography/mass spectroscopy. Allured Publ. Corp, USA.

Akpuaka, A., Ekwenchi, M.M., Dashak, D.A. and Dildar, A. (2013). Biological activities of characterized isolates of n-hexane extract of *Azadirachta indica* A. Juss (Neem) leaves. *N. Y. Sci. J.*, 6: 119–124.

Allameh, A., Ziglari, T. and Rasooli, I. (2011). Phytoinhibition of growth and aflatoxin biosynthesis in toxigenic fungi. In: Torres-Pacheco, I. (Ed.) Aflatoxins - Detection, Measurement and Control. InTech, Croatia. pp. 283–316.

Association of Official Analytical Chemists (AOAC) (1980). Official methods of analysis, USA, 13th ed.

Association of Official Analytical Chemists (AOAC) (2000). Official methods of analysis, USA, 17th ed.

Bakan, B., Bily, A.C., Melcion, D., Cahagnier, B., Regnault-Roger, C., Philogene, B.J.R. and Richard-Molard, D. (2003). Possible role of plant phenolics in the production of trichothecenes by

Fusarium graminearum strains on different fractions of maize kernels. *J. Agric. Food Chem.* 51: 2826–2831.

Bluma, R.V. and Etcheverry, M.G. (2008). Application of essential oils in maize grain: Impact on *Aspergillus* section *Flavi* growth parameters and aflatoxin accumulation. *Food Microbiol.* 25: 324–334.

Cabral, L.C., Pinto, F.V. and Andrea, P.A. (2013). Application of plant derived compounds to control fungal spoilage and mycotoxin production in foods. *Int. J. Food Microbiol.* 166: 1–14.

Chatterjee, D. (1990). Inhibition of fungal growth and infection in maize grains by spice oils. *Lett. Appl. Microbiol.* 11: 148–151.

Ekundayo, O., Laakso, I., Adegbola, R.M., Oguntimein, B., Sofowora, A. and Raimo, H. (1988). Essential oil constituents of Ashanti pepper (*Piper guineense*) fruits (Berries). *J. Agric. Food Chem.* 36: 880–882.

El-Nagerabi, S.A.F., Al-Bahry, S.N., Elshafie, A.E. and Al-Hilali, S. (2012). Effect of *Hibiscus sabdariffa* extract and *Nigella sativa* oil on the growth and aflatoxin B_1 production of *Aspergillus flavus* and *Aspergillus parasiticus* strains. *Food Cont*. 25: 59–63.

EL-Shazly, A.M., Hafez, S.S. and Wink, M. (2004). Comparative study of the essential oils and extracts of *Achillea fragreantissima* (Forssk.) Sch. Bip. and *Achillea santolina* L. (Asteraceae) from Egypt. *Pharmazie* 59: 226–230.

Evrendilek, G.A. (2015). Empirical prediction and validation of antibacterial inhibitory effects of various plant essential oils on common pathogenic bacteria. *Int. J. Food Microbiol*. 202: 35–41.

Ezekiel, C.N., Sulyok, M., Babalola, D.A., Warth, B., Ezekiel, V.C. and Krska, R. (2013). Incidence and consumer awareness of toxigenic *Aspergillus* section *Flavi* and aflatoxin B_1 in peanut cake from Nigeria. *Food Cont*. 30: 596–601.

Ezekiel, C.N., Anokwuru, C.P., Amos, B.M.W., Ejiofor, E.E., Oriola, O.R., Obani, T. and Olajuyigbe, O.O. (2014a). Ashanti pepper (*Piper guineense*) reduces aflatoxin formation in poorly stored maize grains. *N. Y. Sci. J*. 7: 64–71.

Ezekiel, C.N., Udom, I.E., Frisvad, J.C., Adetunji, M.C., Houbraken, J., Fapohunda, S.O., Samson, R.A., Atanda, O.O., Agi-Otto, M.C. and Onashile, O.A. (2014b). Assessment of aflatoxigenic *Aspergillus* and other fungi in millet and sesame from Plateau State, Nigeria. *Mycology* 5: 16–22.

Ezekiel, C.N., Sulyok, M., Anokwuru, C.P., Amos-Tautua, B.M.W., Oni, O.E., Anyasor, G.N., Obonyo, M.A. and Krska, R. Preliminary screening of aqueous extract of *Piper guineense* against aflatoxin biosynthesis in *Aspergillus flavus* and *A. parvisclerotigenus*. *Submitted*.

Fan, J.J. and Chen, J.H. (1999). Inhibition of aflatoxin-producing fungi by Welsh onion extracts. *J. Food Protect*. 62: 414–417.

Galdino, P.M., Nascimento, M.V.M., Florentino, I.F., Lino, R.C., Fajemiroye, J.O., Chaibub, B.A., de Paula, J.R., de Lima, T.C.M. and Costa, E.A. (2012). The anxiolytic-like effect of an essential oil derived from *Spiranthera odoratissima* A. St. Hil. leaves and its major component, β-caryophyllene, in male mice. *Prog. Neuro-Psychopharmacol. Biol. Psych*. 38: 266–284.

Gandomi, H., Misaghi, A., Basti, A.A., Bokaei, S., Khosravi, A., Abbasifar, A. and Javan, A.J. (2009). Effect of *Zataria multifora* Bioss. essential oil on growth and aflatoxin formation by *Aspergillus flavus* in culture media and cheese. *Food Chem. Toxicol*. 47: 2397–2400.

Ghasemi, P.A., Hashemi, M. and Taherian, G.F. (2013). Essential oil and chemical compositions of wild and cultivated *Thymus daenensis* Celak and *Thymus vulgaris* L. *Ind. Crops Prod*. 48: 43–48.

Ghasemi, A., Bagheri, G.B., Mousavi, G.S.A. and Malekpoor, F. (2015). Chemical composition of essential oils from the aerial parts and underground parts of Iranian valerian collected from different natural habitats. *Ind. Crops Prod*. 63: 147–151.

Gowda, N.K.S., Malathi, V. and Suganthi, R.U. (2004). Effect of some chemical and herbal compounds on growth of *Aspergillus parasiticus* and aflatoxin production. *Anim. Feed Sci. Technol*., 116: 281–291.

Hartung, T.E., Bullerman, L.B., Arnold, R.G. and Heidelbagh, N.D. (1973). Application of low dose irradiation to a fresh bread system for space flight. *J. Food Sci*. 38: 129–132.

Juglal, S., Govinden, R. and Odhav, B. (2002). Spice oils for the control of co-occurring mycotoxin-producing fungi. *J. Food Protect*. 65: 683–687.

Lucca, L.G., de Matos, S.P., Borille, B.T., Dias, D.O., Teixeira, H.F., Veiga, V.F., Limberger, R.P. and Koester, L.S. (2014). Determination of β-caryophyllene skin permeation/retention from crude copaiba oil (*Copaifera multijuga* Hayne) and respective oil-based nanoemulsion using a novel HS-GC/MS method. *J. Pharm. Biomed. Anal*. 104: 144–148.

Matamoros-León, B., Argaiz, A. and López-Malo, A. (1999). Individual and combined effects of vanillin and potassium sorbate on *Penicillium digitatum*, *Penicillium glabrum*, and *Penicillium italicum* growth. *J. Food Protect*. 62: 541–543.

Matasyoh, J.C., Wathuta, E.M., Kariuki, S.T. and Chepkorir, R. (2011). Chemical composition and

larvicidal activity of *Piper capense* essential oil against the malaria vector, *Anopheles gambiae*. *J. Asia-Pacific Entomol*. 14: 26–28.

Maxia, A., Marongiu, B., Piras, C., Porcedda, S., Tuveri, E., Gonçalves, M.J., Cavaleiro, C. and Salgueiro, L. (2009). Chemical characterization and biological activity of essential oils from *Daucus carota* L. subsp. *carota* growing wild on the Mediterranean coast and on the Atlantic coast. *Fitoterapia* 80: 57–61.

Mayachiew, P. and Devahastin, S. (2008). Antimicrobial and antioxidant activities of Indian gooseberry and galangal extracts. *LWT-Food Sci. Technol*. 41: 1153–1159.

Montes-Belmont, R. and Carvajal, M. (1998). Control of *Aspergillus flavus* in maize with plant essential oils and their components. *J. Food Protect*. 61: 616–619.

Olonisakin, A., Oladimeji, M.O. and Lajide, L. (2006). Chemical composition and antibacterial activity of steam distilled oil of Ashanti pepper (*Piper guineense*) fruits (berries). *J. Appl. Sci*. 6: 2520–2522.

Oyedeji, O.A., Ajayi, A., Adeniyi, B. and Koenig, W.A. (2005). Essential oil composition of *Piper guineense* and its antimicrobial activity. A new chemotype from Nigeria. *Phytother. Res*. 19: 362–364.

Pandey, D.K., Tripathi, N.N., Tripathi, R.D. and Dixit, S.N. (1982). Fungitoxic and phytotoxic properties of the essential oil of *Caesulia axillaris* Roxb. (Compositae). *J. Appl. Bot*. 56: 256–257.

Pant, A., Saikia, S.K., Shukla, V., Asthana, J., Akhoon, B.A. and Pandey, R. (2014). Beta-caryophyllene modulates expression of stress response genes and mediates longevity in *Caenorhabditis elegans*. *Exp. Gerontol*. 57: 81–95.

Passone, M.A., Gorardi, N.S. and Etcheverry, M. (2013). Antifungal and antiaflatoxigenic activity by vapor contact of three essential oils, and effects of environmental factors on their efficacy. *LWT-Food Sci. Technol*. 53: 434–444.

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

Reinsvold, R.E., Jinkerson, R.E., Radakovits, R., Posewitz, M.C. and Basu, C. (2011). The production of the sesquiterpene β-caryophyllene in a transgenic strain of the *Cyanobacterium synechocystis*. *J. Plant Physiol*. 168: 848–852.

Ricci, D., Daniele, F.D., Giamperi, L., Anahi, B.A., Epifano, F., Burini, G. and Curini, M. (2005). Chemical composition, antimicrobial and antioxidant activity of the essential oil of *Teucrium marum* (Lamiaceae). *J. Ethnopharmacol*. 98: 195–200.

Rocha, V.G., de Almeida, G.S., Regitano D'Arce, M.A.B., Duarte, M.M.H., Brito, J.O., da Silva, M.F., Silva, S.C., de Stefano, P.S.M., Calori-Domingues, M.A. and da Gloria, E.M. (2009). Activity of essential oil and its major compound, 1,8-cineole, from *Eucalyptus globules* Labill., against the storage fungi *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare. *J. Stored Prod. Res*. 45: 108–111.

Sabulal, B., Dan, M., Anil, J.J., Kurup, R., Pradeep, N.S., Valsamma, R.K. and George, V. (2006). Caryophyllene-rich rhizome oil of *Zingiber nimmonii* from South India: chemical characterization and antimicrobial activity. *Phytochem*. 67: 2469–2473.

Sanchez, E., Heredia, N. and Garcia, S. (2004). Inhibition of growth and mycotoxin production of *Aspergillus flavus* and *Aspergillus parasiticus* by extracts of *Agave* species. *Int. J. Food Microbiol*. 161: 245–249.

Silva, L.L., Parodi, T.V., Reckziegel, P., Garcia, V.O., Bürger, M.E., Baldisserotto, B., Malmann, C.A., Pereira, A.M.S. and Heinzmann, B.M. (2012). Essential oil of *Ocimum gratissimum* L.: anesthetic effects, mechanism of action and tolerance in silver catfish, *Rhamdia quelen*. *Aquaculture* 350–353: 91–97.

Soliman, K.M. and Badeaa, R.I. (2002). Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. *Food Chem. Toxicol*. 40: 1669–1675.

Strosnider, H., Azziz-Baumgartner, E., Banziger, M., Bhat, R.V., Breiman, R., Brune, M.N., DeCock, K., et al. (2006). Workgroup report: public health strategies for reducing aflatoxin exposure in developing countries. *Environ. Health Perspect*. 114: 1989–1903.

Shukla, R., Singh, P., Prakash, B. and Dubey, N.K. (2012). Antifungal, aflatoxin inhibition and antioxidant activity of *Callistemon lanceolatus* (Sm.) Sweet essential oil and its major component 1,8-cineole against fungal isolates from chickpea seeds. *Food Cont*. 25: 27–33.

Qi, X.L., Li,T.T., Wei,Z.F., Guo,N., Luo,M., Wang,W., Zu,Y.G., Fu,Y.J and Peng,X. (2014). Solvent-free microwave extraction of essential oil from pigeon pea leaves [*Cajanus cajan* (L.) Millsp.] and evaluation of its antimicrobial activity. *Ind. Crops Prod*. 58: 322–328