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Growth inhibition of the stored fish (*Ethmalosa fimbriata*) fungus *Aspergillus flavus*, exposed to extracted essential oils from *Callistemon citrinus* and *Ocimum canum*

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The aim of this study was to evaluate the antifungal effects of essential oils from *Callistemon citrinus* and *Ocimum canum* against *Aspergillus flavus*. Major components in the oil of *C. citrinus* were 1,8-cineole (60.6%), α-pinene (18.5%), limonene (5.0%) and α-terpineol (5.0%). The oil of *O. canum* was mainly composed of 1.8-cineole (20.8%), linalol (14.3%), eugenol (11.9%), terpinen-4-ol (7.4%) and germacrene D (4.9%). Inhibition of the mycelia growth of *A flavus* increased significantly ($p < 0.05$) with the essential oils concentrations. Positives correlations were observed between inhibition percentages and the concentration of *C. citrinus* ($p < 0.001$; $r = 0.873$) and *O. canum* oils ($p< 0.001$; $r = 0.768$). *O. canum* oil was fungicide at 325 ppm while *C. citrinus* was fungistatic at all the tested concentrations with the highest inhibition percentage means of 89.74 %. Antifungal activity of the essential oil of *O. canum* was significantly ($p < 0.001$) higher than that of *C. citrinus*. Antioxidant activity of *O. canum* (49.3 mol/l) was significantly ($p < 0.001$) higher than that from *C. citrinus* (5.0 mol/l). These observations suggest the possible exploitation of the oils from *O.canum* and *C.citrinus* as potential approach for smoked *Ethmalosa fimbriata* preservation against *A. flavus.*

Key words: Antifungal effects, *Aspergillus favus*, *Ethmalosa fimbriata,* essential oils.

INTRODUCTION

Ethmalosa fimbriata, a fish commonly known as "Bonga", belongs to the family Clupeidae. In Cameroon, the farming of this fish is a common occupation of people living in the coastal areas and along major river banks. It is one of the best sources of proteins, vitamins, essential fatty acids and minerals. It contains essential nutrients required for supplementing both infant and adult diets (Stansby, 1987; Abdullahi et al., 2001). Various brands of

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oils are also extracted from the flesh and the fluids of this fish. Due to its high susceptibility to microbial spoilage, autolysis, oxidation and hydrolysis of fats, fish is a highly perishable product (Frazier and Westhoff, 1978). Care is therefore required in handling as well as its preservation for food. The commonly used post-harvest method of preserving *E. fimbrata* in Cameroon is smoke drying. This process can increases the shelf life of fish by eliminating microorganisms or prevent their growth (Falioye et al., 2002; Ali et al., 2011). However, despite this treatment, smoke dried fish is frequently altered by many fungi such as members of the genus *Aspergillus* especially when stored in an unsuitable environment (Falioye et al., 2002; Gram, 2010). Our preliminary survey in some local markets in Cameroon showed that *Aspergillus flavus* was the most commonly occurring fungus on stored *E. fimbriata*. This fungus is well known to decrease the commercial and nutritive value of the fish and produce a number of toxic metabolites including aflatoxin (Edema and Agbon, 2010). Additional control methods of this spoiling mold are essential. In this respect, natural products could be developed for reducing losses during storage. Selected plants and their essential oils have been evaluated as natural sources of compounds for food preservatives due to their antimicrobial and antioxidant effects (Tasadjieu et al., 2009; Prakash et al., 2015).Their main components show antioxidant activities and antimicrobial activity against a wide range of microorganisms including filamentous fungi (Hyldgaard et al., 2012; Sameza et al., 2014). Variable results have been observed depending on the origin of biological substances; testing conditions and target microorganisms (Delaquis et al., 2002; Hyldgaard et al., 2012). Cameroon flora is very rich with aromatic plants which have various biological activities (Amvam et al., 1998). Among these, *Callistemon citrinus* and *Ocimum canum* are used as spice, condiment, ornamental plants and African medicine. Previous works showed that, essential oils extracted from *Ocimum sp* and *Callistemon sp.* had many biological activies including antifungal and antioxidant. These activities were related to the main component 1,8 cineole (Kumar et al., 2011; Shukla et al., 2012; Alves Silva et al., 2013). In certain households in Cameroon, leaves of these plants are used to protect stored food products. The aim of this study was to evaluate the antioxidant potential and antifungal activities of essential oils from *C. citrinus* and *O*. *canum* against *Aspergillus flavus*, one of the most occurring spoiling fungi of stored smoke dried *E. fimbriata* in Cameroon.

MATERIALS AND METHODS

Plant material and essential oil extraction

C. citrinus and *O. canum* plants were harvested in March 2011 in Douala, Cameroon and dried during 3 days at room temperature (28 ± 2°C). Herbarium/plants were identified at the National Herbarium of Cameroon. Leaves of *C. citrinus* and *O. canum* were steam-distilled for 4 h using a Clevenger apparatus. Each oil recovered was dried over anhydrous Na_2SO_4, stored in an amber-colored flask and kept at 4°C until use.

Essential oils analysis

Essential oils obtained were analyzed by Gas chromatography (GC). It was performed on a Varian-HP 5890 with flame ionization detector fitted with a fused silica capillary column (30 m x 0.25 mm coated with DB-1, film thickness 0.25 mm). The temperature program was set to 60°C-246°C at 3°C/min, with injector temperature set at 200°C, detector temperature set at 200°C carrier with N_2 gas set to 1 ml/min. The linear retention indices of the components were determined relatively to the retention times of series of n-alkanes and the percentage composition were obtained from electronic integration measurements without taking into account relative response factors. Gas chromatography coupled with mass spectrometry analyses were performed using a Hewlett-Packard GC 5890 equipped with an HP-1 (cross-linked methyl siloxane) fused column (30 m x 0.25 mm, film thickness 0.25 µm) and interfaced with a quadrupole detector (model 5970); temperature programmed at 70°-200°C (10°C/min); injector temperature, 220°C; temperature of connection parts, 180°C; carrier gas, helium at a flow rate of 0.6 ml/min; injection type, split, 1:10 (1µl of a 10:100 pentane solution); ionization voltage, 70 eV; electron multiplier, 1400 eV; mass range, 35-300; scan rate, 2.96 scan/seconde. The identification of the constituents was assigned based on comparison of their retention indices and their mass spectra with those published by Adams (2007).

Aspergillus flavus

The *Aspergillus flavus* used in this study was isolated from fresh smoked fish samples of *E. fimbriata* purchased from a local market (Douala Cameroon). Fish tissue segments (2-4 mm) from flesh and gills were cut using a sterile scalpel and seeded aseptically on *A. flavus* selective medium (Griffin and Garren, 1974). After 3-4 days of incubation at 30°C, the mycelia emerging from the tissues were transferred to fresh medium. Colonies of *A. flavus* developing from tissue segments were transferred to Czapez-Dox Agar for confirmation of identity according to the criteria of Raper and Fennell (1965). The cultures were stored at 4°C on Sabouraud Dextrose Agar (SDA)-chloranphenicol slants and sub-cultured once a month.

Antifungal assay

The agar incorporation method (Lahlou, 2004) was used to evaluate the antifungal activity of the essential oils. The test was carried out in 90 mm Petri dishes containing SDA-chloramphenicol medium. The oils were first diluted with Di Methyl Sulphur Oxide (DMSO) (ratio1: 9). These essential oils were added aseptically into the medium at an appropriate volume to produce various concentrations ranging from 100 to 800 ppm. SDA-chloramphenicol medium supplemented only with DMSO was used as negative control. After solidification, the media were inoculated with 5 mm discs obtained from the edge of 3-days old mycelia culture of *A. flavus*. Each treatment consisted of triplicate plates incubated at 30°C in the dark. Mycelia growth was monitored by measuring the growth diameter following two perpendicular lines going through the centre of the dish. These measurements were made daily for 7 days. The inhibition percentage of mycelia growth was calculated by comparing them with those in the blank dish without essential oil using the formula below: %I = (Dc - Dt) / Dc, where Dc is the diameter of microbial colony in the control and Dt the diameter of

the colony in the treated plate. The fungicidal or fungistatic activity was determined by transferring the discs from the Petri dishes with no apparent growth into non-supplemented medium.

Antioxidant activity

The antioxidant potential of the essential oils was determined using the FRAP (Ferric Reducing Antioxidant Power) method adapted from Benzie and Strain (1996). FRAP reagent contained 4 ml of 10 mM TPTZ (tripyridyltriazine) in 40 mM HCl plus 4 ml of 10 mM $FeCl_3$,$6H_2O$ and 40 ml of 300 mM acetate buffer (pH 3.6). Tenµl of essential oil was diluted in methanol at different concentrations then 75 µl of essential oil-methanol were mixed with 2 ml of FRAP reagent daily prepared. The absorbance was recorded after 12 min incubation at the room temperature with a spectrophotometer UV V-1100 at 593 ηm. FRAP values were obtained by comparing with standard curves created by ascorbic acid solutions concentrations ranging from 50-800 µM. The Equivalent Concentration (EC) was defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mM $FeCl_3$, $6H_2O$. EC was calculated as the concentration of antioxidant giving an increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mM of ascorbic acid solution, determined by using the corresponding regression equation.

Statistical analysis

Data were analyzed using Sigmastat version 2.03 (Synstat Sofware Inc). Results are presented in term of means ± standard deviation. Multiple comparisons of mean values were set up using one-way parametric ANOVA when the normality and equal variance test passed. When these conditions were not matched, we used the non-parametric Krustcall-Wallis test.

RESULTS AND DISCUSSION

Yields and chemical composition of the essential oils

The extraction yields of the essential oils from *C. citrinus* and *O. canum* were 0.8 and 0.3%, respectively (W/W). The yield obtained from *C. citrinus* is lower than that obtained by Oyedeji et al. (2009) (1.2%) and Silva et al. (2010) (1.1%) from South Africa and Brazil, respectively. Similarly value from the *O. canum* was less than the result of Hassane et al. (2011) who analyzed *O. canum* from two different regions of Reunion Islands and obtained 2.04 and 1.4%. Representative GC map of the oils are presented in Figures 1 and 2. Table 1 lists the components identified in the essential oils with their percentage composition and relative retention indices. Twenty-nine constituents were identified and classified in the oil of *C. citrinus,* representing 98.8% of the total oil. The major components were 1.8-cineole (60.6%), α-pinene (18.5%), limonene (5.0%) and α-terpineol (5.0%).The abundance of 1,8-cineole in the essential oil of *C. citrinus* makes it similar to those obtained in all the previous studies from Hymalayas and Reunion Island samples (Srivastava et al., 2001; Mya et al., 2002). However, a key difference in the oils lies in the relative quantities of α-pinene and α-terpineol. In the oil of *O. canum*, fifty components (96.7%) were identified and quantified (Table 1). The major compounds were 1.8-cineole (20.8%), linalol (14.3%), eugenol (11.9%), terpinen-4-ol (7.4%) and germakrene D (4.9%). The results on the analysis of the essential oils of *O. canum* growing in two regions of Comores Island also showed abundant of 1,8-cineole (Hassane et al., 2011). However quantitative and qualitative differences between our results and these oils were noticed. The Maweni-Dimani region from Comores essential oil had 1,8-cineole (48.88%), camphor (14.98%), α-pinene (5.71%), β-pinene (4.66%) and γ-elemene (3.91%) as predominate constituents while the Ivoini-Mitsamihouli sample was mainly composed by 1,8-cineole (34.22%), camphor (13.69%), isopropyl propanoate (9.13%), γ-elemene (5.43%) and α-pinene (3.83%). The differences in yield and constituents of the oils could be attributed to difference in genetic of the plants and geographical/environmental conditions (Amvam et al., 1998; Bakkali et al., 2008).

Effects of essential oils on fungal growth

Results showed that, the inhibition of the mycelia growth of the fungus increased significantly ($p < 0.05$) with the essential oils concentrations (Table 2). Total inhibition occurred at 325 ppm with the essential oil from *O. canum*. For *C. citrinus* the highest inhibition percentage means was 89.74%. *O. canum* oil was fungicide at 325 ppm while *C. citrinus* was fungistatic at all the tested concentrations. Results showed that from 200 ppm, the activity of the essential oil of *O. canum* was significantly ($p < 0.001$) higher than that of *C. citrinus*. There was a positive correlation between inhibition growth of *A. flavus* and the concentration of *C. citrinus* oil ($p < 0.001$; $r = 0.873$) and *O. canum* oil ($p < 0.001$; $r = 0.768$). Essentials oils from various sources exhibit broad-spectrum antimicrobial activity and their biological properties have been related to their chemical composition (Nguefack et al., 2012; Djenane et al., 2013). Indeed, compounds such as 1.8-cineole, pinene, limonene, terpineol and eugenol are present in both essential oils analyzed and have been shown to exert various biological activities including antifungal (Isman, 2000; Dayan et al., 2009). It has been demonstrated that essential oils inhibit postharvest pathogens mainly due to their direct effect on the mycelia growth by affecting the cellular metabolism of the pathogen (Serrano et al., 2005; Regnier et al., 2010). The hydrophobicity of the oils and their components allows them to partition in the lipid layer of the fungal cell membranes and result in disruption in membrane structure and cell membrane integrity (Beckman 2000). In addition, the inhibiting activities of these essential oils may not only be attributable to their major components but, to a synergistic effect of individual minor and/or major compounds (Nguefack et al., 2012; Sivakumar and

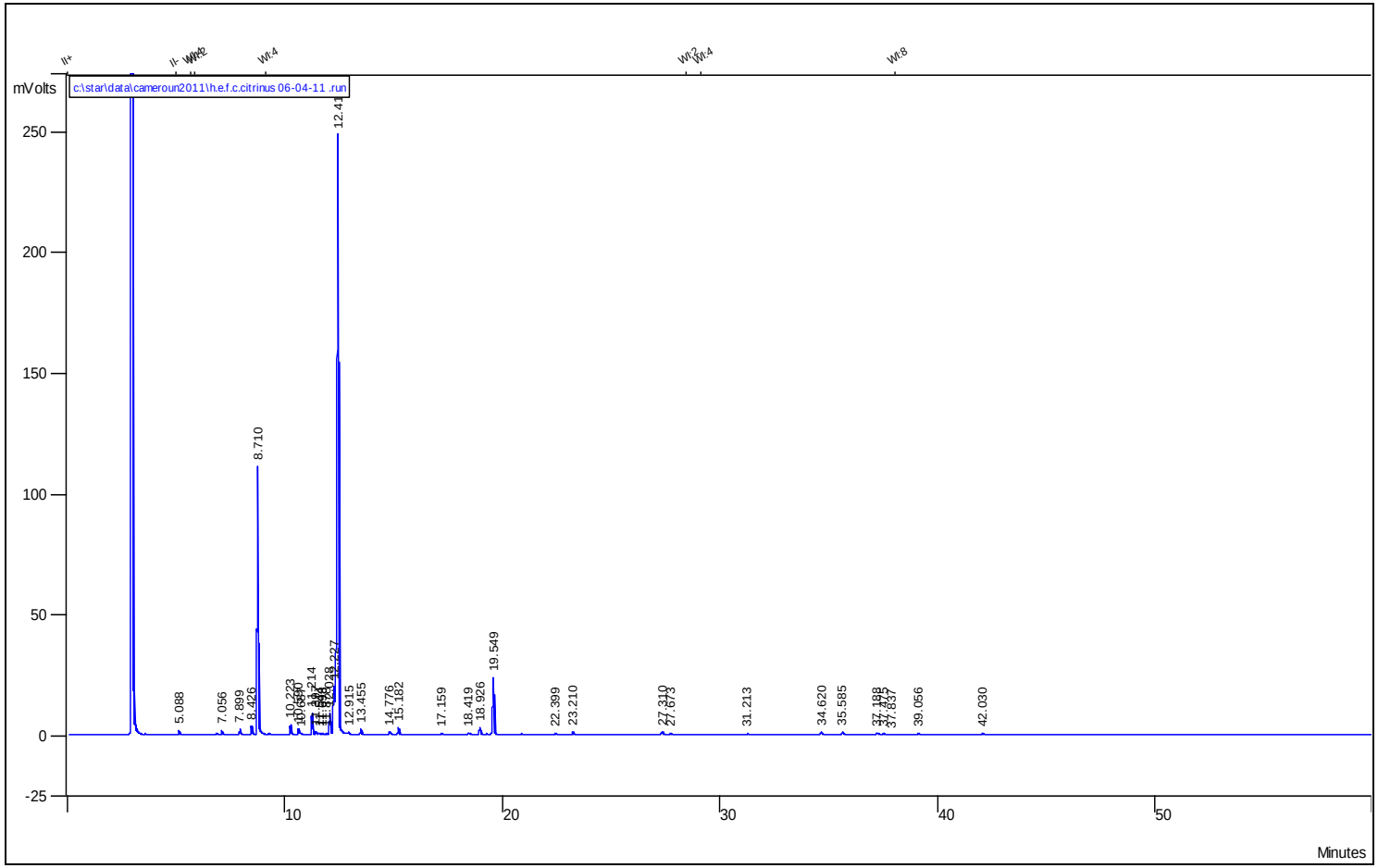

Figure 1. Chromatogram of chemical analysis of *C. Citrinus* essential oil.

Bautista-Banos, 2014).

Antioxidant activities of the essential oils

Mean values of equivalent concentration of antioxidant activities showed that the antioxidant potential of *O. canum* and *C. citrinus* essential oils were 49.3 and 5.0 mol/l respectively (Table 3). It was shown that, the activity of the oil from *O. canum* is significantly ($p < 0.001$) higher than that from *C. citrinus.* Previous studies of Prakash et al. (2011) reported that the essential oils of *Ocimum* sp. had antioxidant activities. Antioxidants retard oxidation and are sometimes added to meat and poultry products to prevent or slow oxidative degradation of fats. Antioxidant agents are effective due to different mechanisms such as free radical scavenging, chelating of pro-oxidant metal ions or quenching singlet-oxygen formation (Lopez-Luzt et al., 2008). These activities could

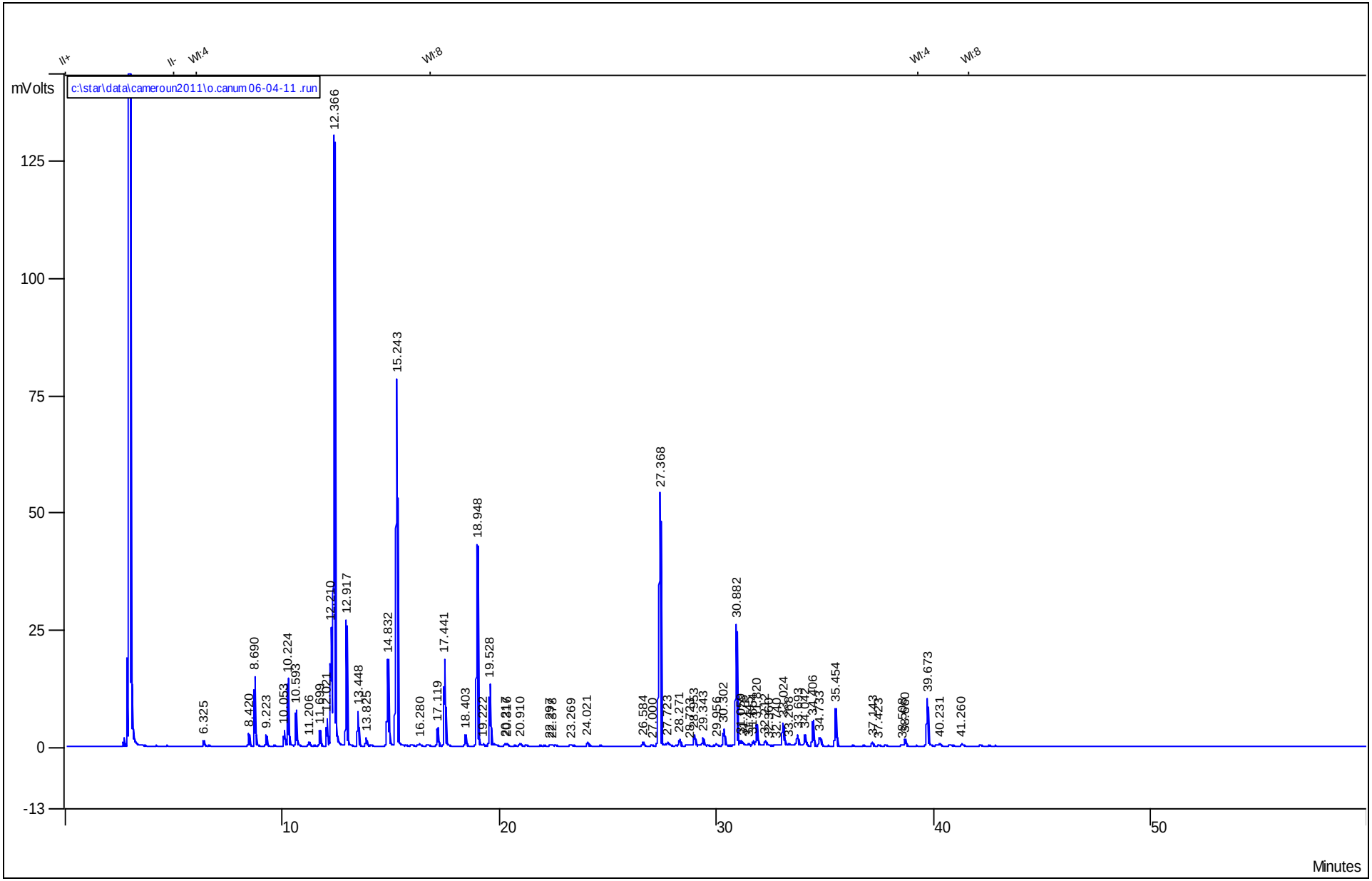

Figure 2. Chromatogram of chemical analysis of *O. canum* essential oil.

Table 1. Yields and chemical composition of essential oils of *Callistemon citrinus* and *Ocimum canum* from Cameroon.

Components	Percentage of constituents		
	RI on DB-1	*C. citrinus*	*O. canum*
Linear aliphatic compounds		**0.7**	**0.2**
Isovaleric acid	828	0.2	-
Hexan-1-ol	865	-	0.2
5-Hydroxylpentanal	886	0.2	-
Isobutyl d'isobutyrate	910	0.3	-
Monoterpenes		**97.3**	**82.2**
Monoterpene hydrocarbons		**29.8**	**20.7**
α-Thujene	926	0.5	0.3
α-Pinene	934	18.5	1.9
Camphene	948	-	0.3
Sabinene	972	-	0.5
β-Pinene	977	0.7	2.1
Myrcene	988	0.4	1.1
α-Phellandrene	1005	1.7	0.2
δ-3-Carene	1009	0.3	-
p-Cymene	1016	Tr	0.5
β-Phellandrene	1024	2.0	0.9
Limonene	1028	5.0	4.3
(E)-β- Ocimene	1045	0.1	3.9
γ-Terpinene	1057	0.4	1.1
Terpinolene	1088	0.2	3.6
Oxygen-containing monoterpenes		**67.5**	**61.5**
1.8-Cineole	1033	60.6	20.8
Hydrate cis-sabinene	1066	-	0.3
Linalol	1099	0.6	14.3
trans-Pinocarveol	1140	0.1	0.6
Camphre	1146	-	3.2
Bornéol	1168	0.1	0.4
Terpinèn-4-ol	1179	0.7	7.4
α-Terpineol	1192	5.0	2.2
cis-Carveol	1221	-	0.1
Hydrate sabinene acetate	1253	0.1	Tr
Thymol	1287	-	0.2
Eugenol	1359	0.2	11.9

Table 1. Contd.

Components	Percentage of constituents		
	RI on DB-1	***C. citrinus***	***O. canum***
Carvyle cis- acetate	1366	0.1	0.1
Sesquiterpenes		**0.8**	**14.2**
Sesquiterpenes hydrocarbons		**0.3**	**8.2**
α-Cubebene	1342	-	0.1
α-Copaene	1379	-	0.2
α-Bergamotene	1407	-	0.3
(Z)-β- Farnesene	1437	-	0.1
α-Humulene	1455	-	0.7
Germacrene D	1484	-	4.9
α-Zingiberene	1492	-	0.1
γ-Cadinene	1518	-	0.1
δ-Cadinene	1525	0.3	0.9
α-Cadinene	1541	-	0.3
Germacrene B	1601	Tr	0.5
Oxygen- containing sesquiterpenes		**0.5**	**6.0**
Elemol	1548	0.2	Tr
Nerolidol	1563	-	0.1
Germacrene D-4-ol	1575	-	1.3
Caryophyllene oxide	1585	0.2	Tr
Viridiflorol	1591	0.1	-
Humulene II epoxide	1610	-	0.5
β-Eudesmol	1643	-	1.5
α-Bisabolol	1684	-	0.1
(2Z, 6E)- Farnesol	1721	-	0.3
(2E, 6E)-Farnesol	1746	-	1.9
(2 E, 6 E)- Methyl farnesoate	1786	-	0.1
Aromatic constituent		**0.0**	**0.1**
1, 3, 5-Trimethylbenzene	1122	-	0.1
Total		**98.8**	**96.7**
Yield of oils (%)		**0.8**	**0.3**

Table 2. Range and mean values of the percentages of growth inhibition of *A. flavus* at different concentrations of essential oils.

Conc. (ppm)	n	Min.	Max.	Mean ± SD	Conc. (ppm)	n	Min.	Max.	Mean ± SD	Comparison : Student t test
Callistemon citrinus $F_{(6,140)}$ = 83.516 ; p < 0.001***					***Ocimum canum*** $F_{(8,180)}$ = 303.146 ; p < 0.001***					
100	21	0.00	11.11	1.60 ± 3.31	100	21	0.00	26.09	3.40 ±7.00	t = -0.854 ; ddl = 40 ; p= 0.398^{ns}
200	21	0.00	22.22	4.06 ± 3.30	200	21	0.00	32.73	55.95 ± 20.09	t = 11.125 ; ddl = 40 ; p< 0.001
300	21	0.00	37.04	15.62 ± 15.63	300	21	0.00	66.97	86.98 ± 15.46	t = 14.924 ; ddl = 40; p< 0.001
-	-		-	-	325	21	100.00	100.00	100.00 ± 0.00	-
-	-		-	-	350	21	100.00	100.00	100.00 ± 0.00	-
-	-		-	-	375	21	100.00	100.00	100.00 ± 0.00	-
400	21	0.00	100.00	32.53 ± 32.70	400	21	100.00	100.00	100.00 ± 0.00	t = -9.456 ; ddl = 40 ; p< 0.001
500	21	22.58	100.00	57.07 ± 22.39	500	21	100.00	100.00	100.00 ± 0.00	t = -8.788 ; ddl = 40 ; p< 0.001
-	-		-	-	600	21	100.00	100.00	100.00 ± 0.00	-
700	21	60.22	100.00	81.28 ± 17.02						-
800	21	73.68	100.00	89.74 ± 12.16						-

Comparison of the activities of the two oils at different concentrations				
Concentration (ppm)	100	200	300	400
O. canum vs C. citrinus	q=0.741ns	q=16.039***	q=20.917***	q=15.187***

*** = Highly significant difference (p <0.001); ns = no significant difference.

Table 3. Variation of equivalent concentration (mol/l) of antioxidant of *Callistemon citrinus* and *Ocimum canum* essential oils.

Essential oil	N	Min.	Max.	Means ± SD	Essential oil	n	Min.	Max.	Means ± SD	Student t-test Comparaison :
C. citrinus	15	4.0	6 .5	5.0 ± 0.8	***O. canum***	9	42 .0	57.4	49.3 ± 6.8	t =-25.418 ; ddl = 22 ; p < 0.001

be related to the presence of compounds such as eugenol, thymol and 1-8 cineole known for their antioxidant properties (Mishra et al., 2013). We also identified these compounds in *O. canum* and *C. citinus* oil in noticeable amount (Table 1).

The essential oils from *O. canum* and *C. citrinus* exhibited antifungal activity since they were able to kill or inhibit the mycelia growth of *A. flavus.* They also had antioxidant activities. Through these properties, they could be used as an approach solution to preserve stored smoked *E. fimbriata* and other foodstuff. Nevertheless, detailed studies are required to study the preservative effect of these oils directly on the dried fish.

Conflict of Interests

The authors have not declared any conflict of interests.

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