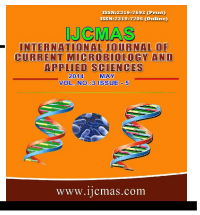
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 5 (2014) pp. 691-701**
http://www.ijcmas.com



Original Research Article

Antifungal Activities against some *Aspergillus* species of the Essential oils of *Canarium schweinfurthii* and *Aucoumea klaineana* growing in Cameroon

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Keywords

Essential oil, *Canarium schweinfurthii*, *Aucoumea klaineana*, antifungal, *Aspergillus spp.*

A B S T R A C T

Natural substances are looked upon as an alternative to synthetic chemical products as food preservatives. Evaluation of the antifungal activities of the essential oils of the resins of *Canarium schweinfurthii* harvested in Mbouda (West Region) and Lolodorf (South Region) and *Aucoumea klaineana* harvested in Lolodorf was the main objective of this study. After analyses by the incorporation technique, *Canarium schweinfurthii* (Lolodorf and Mbouda) were more active with minimum inhibitory concentrations (MIC) of 1800 ppm and 4500 ppm respectively against *Aspergillus flavus*, 2800 ppm and 3500 ppm respectively against *Aspergillus niger* and 1300ppm and 3800ppm respectively against *Aspergillus fumigatus*. Essential oil of *A. klaineana* inhibited fungal growth (% inhibition >50% at 5000 ppm). The MIC for the reference preservative, sorbic acid, was 1200 ppm, 250 ppm and 800 ppm respectively against *A. flavus*, *A. niger* and *A. fumigatus*. The three essential oils showed antifungal properties due to a possible synergy among their different compounds. These essential oils can thus be employed as a natural source for food preservatives.

Introduction

Growth in the human population has lead Filamentous moulds are sometimes harmful to man, not only by causing human diseases but also through contamination and eventual spoilage of human food. Food serves as a favourable medium for their growth and development. Several moulds, notably the genera *Aspergillus, Penicillium* and *Fusarium* are known to be contaminants of agricultural produce whether stored or still in the farm and/or for their capacity to produce toxic secondary metabolites or mycotoxins (Meyer et al., 2004).

Aspergillus is one of the most significant

components of the fungal flora of storage food in tropical conditions. The most frequent species implicated in food contamination include *A. flavus, A. niger, A. fumigatus* and *A. parasiticus.* The most dangerous mycotoxins noted for their teratogenic, mutagenic and carcinogenic effects include aflatoxin and ochratoxin produced respectively by *A. flavus* and *A. niger.*

In respect of the dangers which these moulds and their mycotoxins represent, the use of pesticides is regarded as the most effective technology for the protection of stored food. However in spite of their employment, considerable losses in the course of storage are still recorded on the one hand and the consequences such as resistance of the species, the residual level of the pesticides in food, non-degradation of these synthetic molecules, remain the major problems of their use. Thus there is a renewed interest in the search for natural alternative antimicrobial agents. It is well established that certain plants and their derivatives possess antimicrobial properties (Nguefack et al., 2004; Jazet et al., 2008).

Given that one of the principal means used in the research of bioactive substances is a systematic screening of the interaction between the microorganisms and the plant's extracts, the principal objective of this study was to test the antifungal properties of the essential oils of *Canarium schweinfurthii* and *Aucoumea klaineana* on *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus fumigatus* that frequently contaminate food, with specific objective being the determination of the Minimum Inhibitory Concentration (MIC) in order to establish their possible use as alternatives to the synthetic antifungal compounds.

Materials and Methods

- Essential oils of the resins of Canarium schweinfurthii Engl. harvested in Mbouda in the West Region of Cameroon
- Essential oils of the resins of Canarium schweinfurthii Engl. harvested in Lolodorf in the South Region of Cameroon
- Essential oils of the resins of Aucoumea klaineana Pierre harvested in Lolodorf in the South Region of Cameroon

The chemical composition of these essential oils were revealed by Jazet et al. (2010)

Fungal Material

The fungal species used in this work were: a strain each of *Aspergillus niger*, a strain of *Aspergillus flavus***,** and a strain of *Aspergillus fumigatus.*

All strains were offered by the Microbiology laboratory of ENSAI, University of Ngaoundere, Cameroon.

Method

Chemical analysis of essential oils

Essential oils obtained were analysed by gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC/MS).

***Gas chromatography*:** The oil was analysed on a Varian CP-3380 GC with flame ionisation detector fitted with a fused silica capillary column (30 m x 0.25 mm coated with DB5, film thickness 0.25μm); temperature program 50°-200 °C at 5 °C/min, injector temperature 200°C,

detector temperature 200 °C, carrier gas N_2 1ml/min. The linear retention indices of the components were determined relatively to the retention times of a series of n-alkanes and the percentage compositions were obtained from electronic integration measurements without taking into account relative response factors.

Gas chromatography/mass spectrometry

GC/MS analyses were performed using a Hewlett-Packard apparatus equipped with an HP1 fused silica column (30 m x 0.25 mm, film thickness 0.25 μm) and interfaced with a quadrupole detector (GC-quadrupole MS system, model 5970). Column temperature was programmed from 70°-200°C at 10°C/min; injector temperature was 200°C. Helium was used as carrier gas at a flow rate of 0.6 ml/min. The mass spectrometer was operated at 70eV.

Identification of the components by their retention indices

Identification of the constituents was assigned on the basis of comparison of their retention indices and their mass spectra with those given in literature (Adams, 2007).

Antifungal activities

The incorporation technique described by De Billerbeck (2001) was modified and used to evaluate the antifungal activity of our essential oils. This technique consists in observing mycelial growth of discs of microbial culture, inoculated at the centre of Petri dishes containing the culture medium supplemented by an essential oil at varying concentrations.

Preparation of Culture medium

A PDA (Potatoes Dextrose Agar) culture medium was used. Preparation was done as described by the producer. The medium was rendered selective to fungi by the addition of antibiotics (Ampicillin and Chloramphenicol).

Preparation of solutions of Essential oils

Each essential oil was mixed with dimethyl sulphoxide (DMSO) in a 1:9 proportion. The essential oil/DMSO solution obtained was incorporated in a given quantity of PDA medium so as to obtain the desired concentrations. The supplemented medium was then poured into three 90 mm Petri dishes, that is, 20 ml per petri dish, with regards to each microbe (*A. flavus, A. niger* and *A. fumigatus*). The dishes were then allowed at ambient temperature until solidification of medium. On a control dish, no essential oil was added.

Inoculation

After solidification, a 6 mm well was created at the centre of the solidified medium and a mycelial disc from a two–day old pre-culture of the respective microbe was deposited into the well. The dishes were incubated in a reversed position at 30^{o}C. Mycelial growth was evaluated by measuring the growth diameter until the control dish was completely covered**.**

Expression of Results

Percentage inhibition of fungal growth was calculated compared to the control sample where there was no essential oil using the formula:

$$\text{Percentage Inhibition } (\%\ \text{I}) = \frac{D_c - D_t}{D_c} \times 100$$

Where;

D_c (mm) = diameter of fungal growth in the control dish

D_t (mm) = diameter of fungal growth in the test dish

The antifungal activity of the essential oils was compared to that of a reference antifungal agent, sorbic acid. The experiment was repeated three times.

Statistical Analyses of Results

The dispositive used for *A. flavus* was a 3 x 6 x 10 factorial, used thus:

3 essential oils

6 concentrations: 0, 1000, 2000, 3000, 4000 and 5000ppm

10 measuring days: 0, 1, 2, 3, 4, 5, 6, 7, 8 and 9th day.

The dispositive used for *A. niger* was a 3 x 6 x 9 factorial, used thus:

3 essential oils
6 concentrations: 0, 1000, 2000, 3000, 4000 and 5000ppm
9 measuring days: 0, 1, 2, 3, 4, 5, 6, 7 and 8th day.

The dispositive used for *A. fumigatus* was a 3 x 6 x 9 factorial, used thus:
3 essential oils
6 concentrations: 0, 1000, 2000, 3000, 4000 and 5000ppm

7 measuring days: 0, 1, 2, 3, 4, 5 and 6th day.

Analysis of variances, multiple comparisons Duncan test and calculation of correlations were carried out with the software Statgraphics plus 5.0.

Results and Discussion

Chemical analysis

The results of chemical analysis are reported in table.1.

As shown on the table, the chemical composition of the three essential oils is dominated by monoterpenes hydrocarbon. As for the major compounds present, there is some qualitative similarity between the two *Canarium* (*p*-cymene, limonene and α-terpineol), although there is a difference in levels of these compounds. The essential oil of *A. klaineana* mark in this point of view a difference with the others with levels of 29.3%, 30.9% and 9.0% respectively for α-pinene, α-phellandrene and 1,8-cineole considered as their major compounds and relatively little in the *Canarium*. However, *p*-cymene is proving to be among the majority of the three extracts and appears to be the major qualitative relationship between these three species.

Antifungal activities

Within the incubation period, average values of daily measurements of the diameter of mycelial growth of the moulds were used to follow-up the growth pattern according to the concentration of essential oil.

Effect of Essential Oils on *Aspergillus flavus*

At initial concentrations of 0, 1000, 2000, 3000, 4000 and 5000ppm, the growth

diameter of *Aspergillus flavus* were recorded and are illustrated on figure 1.
From the figures it can be seen that mycelial growth increases with increase in incubation time. Statistical analyses showed that growth on the control was significantly different per incubation day with a strong positive correlation coefficient ($p<0.05$, $r = 0.9972$).

As for the test samples, growth increases with increase incubation day except for *Canarium schweinfurthii* (Mbouda) and *Canarium schweinfurthii* (Lolodorf) where there was total inhibition (no growth) at concentrations of 5000ppm and 2000ppm respectively. The test samples also showed an inhibitory effect as growth decreases with increase in concentration of essential oils was not statistically significant with a negative correlation ($P>0.05$; $r = -0.55$, $r = -0.52$, $r = -0.53$ respectively for *Canarium schweinfurthii* (Mbouda), *Canarium schweinfurthii* (Lolodorf) and *Aucoumea klaineana*.

Effect of Essential Oils on *Aspergillus niger*

Growth rate of *Aspergillus niger* at initial concentrations of 0, 1000, 2000, 3000, 4000 and 5000ppm, are illustrated on figure 2.

There was a general increase in growth with an increase in incubation day with a statistically significant increase in growth for the control, having a strong positive correlation coefficient ($p<0.05$; $r = 0.985$). *Canarium schweinfurthii* (Mbouda) totally inhibited growth of *A. niger* at 3000ppm until the ninth incubation day where there was a slight indication of growth. Meanwhile *Canarium schweinfurthii* (Lolodorf) inhibited growth totally from 3000ppm. As for *Aucoumea klaineana*, total inhibition was not observed; however, growth inhibition increases with an increase in essential oil concentration. The three samples showed a negative correlation between growth increase and increase in concentration ($p>0.05$; $r = -0.69$, $r = -0.67$ and $r = -0.53$ respectively for *Canarium schweinfurthii* (Mbouda), *Canarium schweinfurthii* (Lolodorf) and *Aucoumea klaineana*.

Effect of Essential Oils on *Aspergillus fumigatus*

Growth rate of *Aspergillus fumigatus* are illustrated on figures 3. Both the control and the test samples showed increase in growth with increase in incubation day. For the control, there was a statistically significant increase in mycelial growth, with a strong positive correlation coefficient ($p<0.05$; $r = 0.991$). As for the test samples, *Canarium schweinfurthii* (Mbouda) and *Canarium schweinfurthii* (Lolodorf) showed total inhibition at 4000ppm and 2000ppm respectively. The test samples also showed an inhibitory effect as growth decreases with increase in concentration of essential oils. With respect to concentration, growth was not statistically significant with negative correlation coefficients ($P>0.05$; $r = -0.65$, $r = -0.57$, $r = -0.60$ respectively for *Canarium schweinfurthii* (Mbouda), *Canarium schweinfurthii* (Lolodorf) and *Aucoumea klaineana*.

Generally, growth inhibition in the presence of an essential oil compared to the control can be explained by the fact that essential oils contain terpenoids having antifungal activities such as β-pinene, limonene, linalool, etc. These compounds either have an individual or synergistic effect (Tatsadjieu, 2003).

Percentage Inhibition

Though not all essential oil concentrations showed total inhibition, growth rate was different from that of the control. Each concentration of essential oil showed a certain degree of inhibition.

Case of *Aspergillus flavus*

The percentage growth inhibition of *Aspergillus flavus* by different concentrations of our essential oils is illustrated on figure 4A.

Percentage inhibition increased with increase in essential oil concentration. After ten incubation days, *Canarium schweinfurthii* (Lolodorf) and *Canarium schweinfurthii* (Mbouda) showed total inhibition at concentrations of 2000ppm and 5000ppm respectively, maximum inhibition for *Aucoumea klaineana* was 81.57±1.18%, which was statistically different from the others. There was a statistical significant difference ($p>0.05$) at 1000ppm between *Canarium schweinfurthii* (Lolodorf) and the other two essential oils, whereas inhibition was statistically not significant between *A. klaineana* and *Canarium schweinfurthii* (Mbouda). These statistical differences were maintained until at 5000ppm concentration where inhibition was total for the two *Canarium* species.

Specifically, the percentage inhibition for *Aucoumea klaineana* varies from 12.17±1.27 % to 81.73±2.56 % while that of *Canarium schweinfurthii* (Mbouda) varies from 11.51±1.59 and attained 100% at 5000ppm whereas the Lolodorf species of *Canarium schweinfurthii* showed an inhibition range from 51.98±1.43, attaining 100 % at 2000ppm.

On a general scale, the statistical difference of the inhibitory effect of *Canarium schweinfurthii* (Mbouda) was significant with *Canarium schweinfurthii* (Lolodorf) and non-significant with *Aucoumea klaineana*.

The case of *Aspergillus niger*

As illustrated on figure 4B, the higher the concentration, the greater the percentage inhibition, with 100% inhibition being attained at 3000ppm for *Canarium schweinfurthii* (Lolodorf) and 4000ppm for *Canarium schweinfurthii* (Mbouda). Maximum inhibition for *Aucoumea klaineana*, attained at 5000ppm, was 61.07±1.81%, a value whose difference is statistically significant ($p>0.05$) from the others. For each specific concentration, the two *Canarium* species did not show any statistical difference in inhibition throughout the experiment.

Case of *Aspergillus fumigatus*

Figure 4C illustrates the percentage inhibition of mycelial growth of *A. fumigatus* of the different essential oils. Inhibition, as in the case of the other *Aspergillus* species, increases with increase concentration of essential oils. Total inhibition was attained at 2000ppm and 4000ppm for *Canarium schweinfurthii* (Lolodorf) and *Canarium schweinfurthii* (Mbouda) respectively. The maximum inhibition of the essential oil of *Aucoumea klaineana* was 76.05±0.66 %, and was statistically different ($P>0.05$) from the *Canarium* species.

Aucoumea klaineana did not attain 100 % inhibition in all the series of concentrations tested, but its inhibition ranges from 5.72±0.69 to 76.05±0.66%. *Canarium schweinfurthii* (Mbouda)

inhibited from 17.92±1.13% and attained 100% from 3000ppm. As for *Canarium schweinfurthii* (Lolodorf), percentage inhibition ranges from 17.17±0.54% and 100% was attained as from 2000ppm.

Table.1 Chemical composition of different extracts (Jazet *et al.*, 2010)

IK	**Compounds** (In order of elution)	Essential oils composition (%)		
		C. schweinfurthii **(Lolodorf)**	***A. klaineana*** **(Lolodorf)**	***C. schweinfurthii*** **(Mbouda)**
Monoterpenes hydrocarbons		**61.9**	**82.9**	**73.4**
930	α-thujene	-	0.2	-
935	α-pinene	1.7	29.3	2.6
947	camphene	-	0.6	-
970	sabinene	2.0	0.3	0.2
977	β-pinene	0.4	0.8	1.2
983	myrcene	-	-	0.5
997	menthene	-	1.6	-
1002	α-phellandrene	1.1	30.9	4.1
1009	Δ^3 carene	0.3	2.3	-
1013	α-terpinene	0.5	2.4	2.7
1017	p-cymene	9.8	9.2	25.3
1031	limonene	42.7	0.4	36.6
1054	γ-terpinene	1.9	0.3	0.2
1084	terpinolene	1.5	4.6	-
Oxygenated monoterpens		**37.0**	**15.7**	**25.3**
1026	1,8-cineole	0.3	9.0	0.5
1090	linalool	-	-	0.3
1096	Camphor	-	0.8	-
1172	terpinen-4-ol	2.3	2.5	0.4
1185	α-terpineol	34.4	3.1	18.0
1225	carveol	-	0.3	1.4
1230	geraniol	-	-	0.5
1242	carvone	-	-	0.4
1253	piperitone	-	-	3.8

Table.2 MIC of essential oils and reference fungicide

	A. flavus	***A. niger***	***A. fumigatus***
Canarium schweinfurthii **(Mbouda)**	4500 ppm	3500ppm	3800ppm
Canarium schweinfurthii **(Lolodorf)**	1800 ppm	2800ppm	1300ppm
Aucoumea klaineana	> 5000ppm	> 5000ppm	> 5000ppm
Sorbic acid	1200ppm	250ppm	800ppm

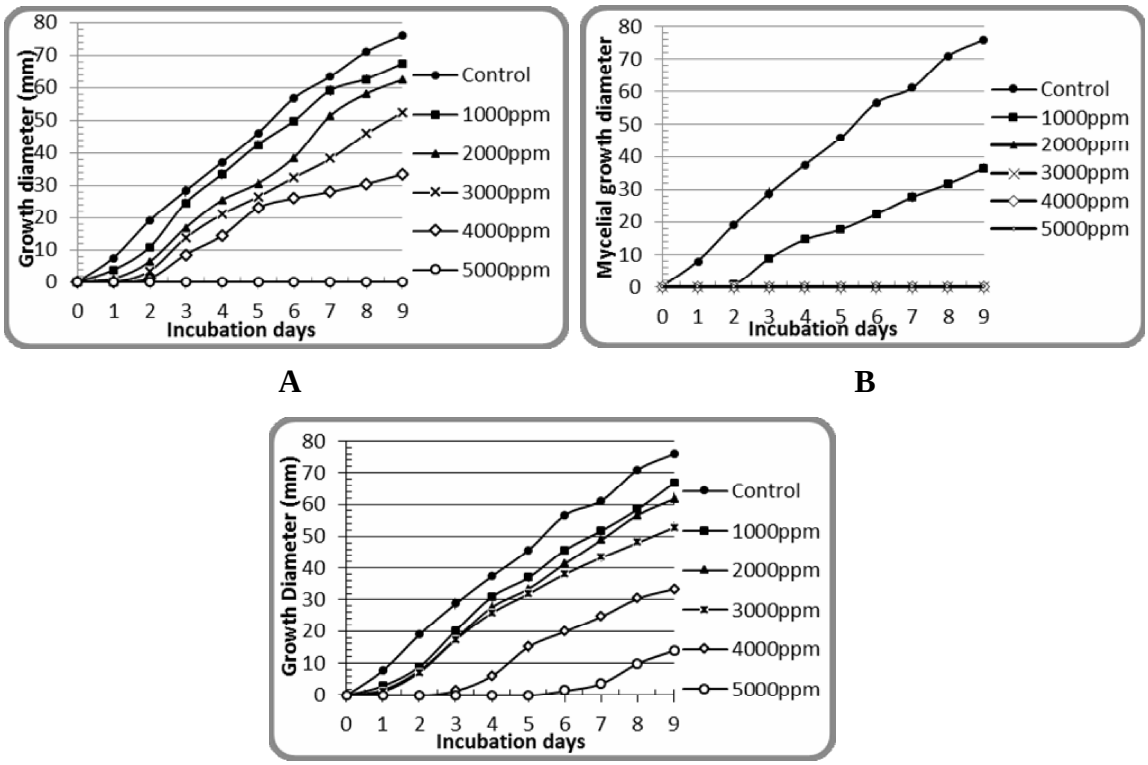

Figure.1 Effect on *A. flavus* of essential oils of **(A)** *C. schweinfurthii* (Mbounda), **(B)** *C. schweinfurthii* (Lolodorf) and **(C)** *A. klaineana*

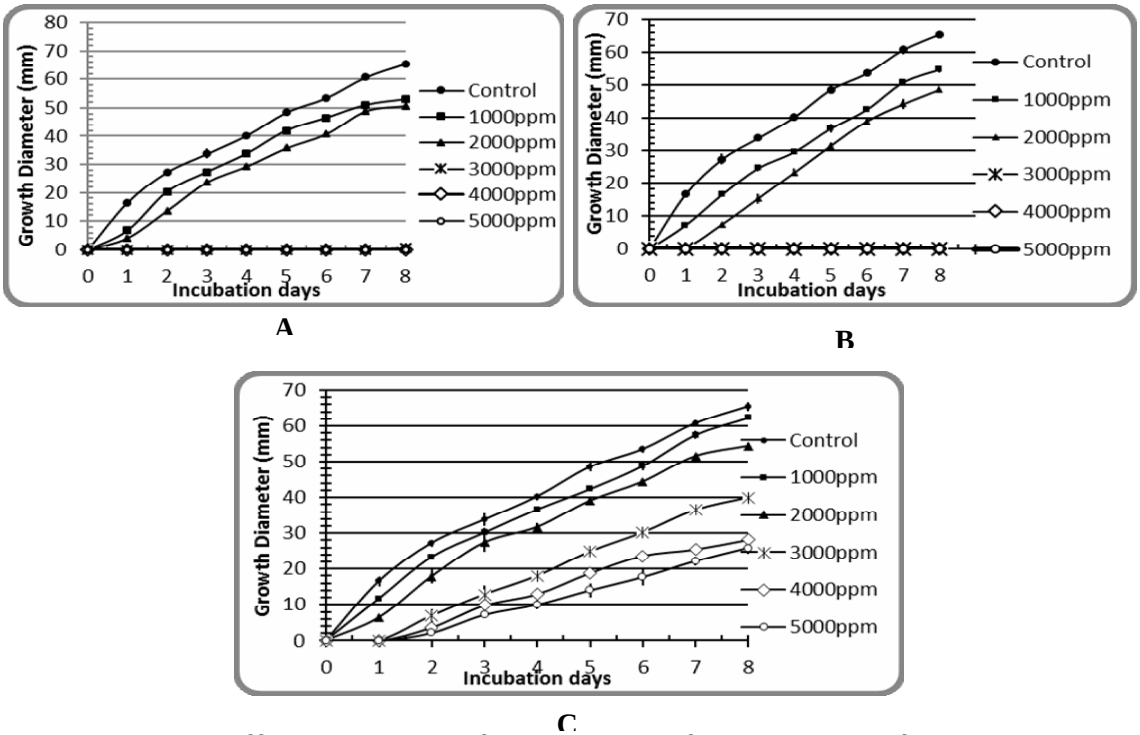

Figure.2 Effect on *A. niger* of essential oils of **(A)** *C. schweinfurthii* (Mbounda), **(B)** *C. schweinfurthii* (Lolodorf) and **(C)** *A. klaineana*

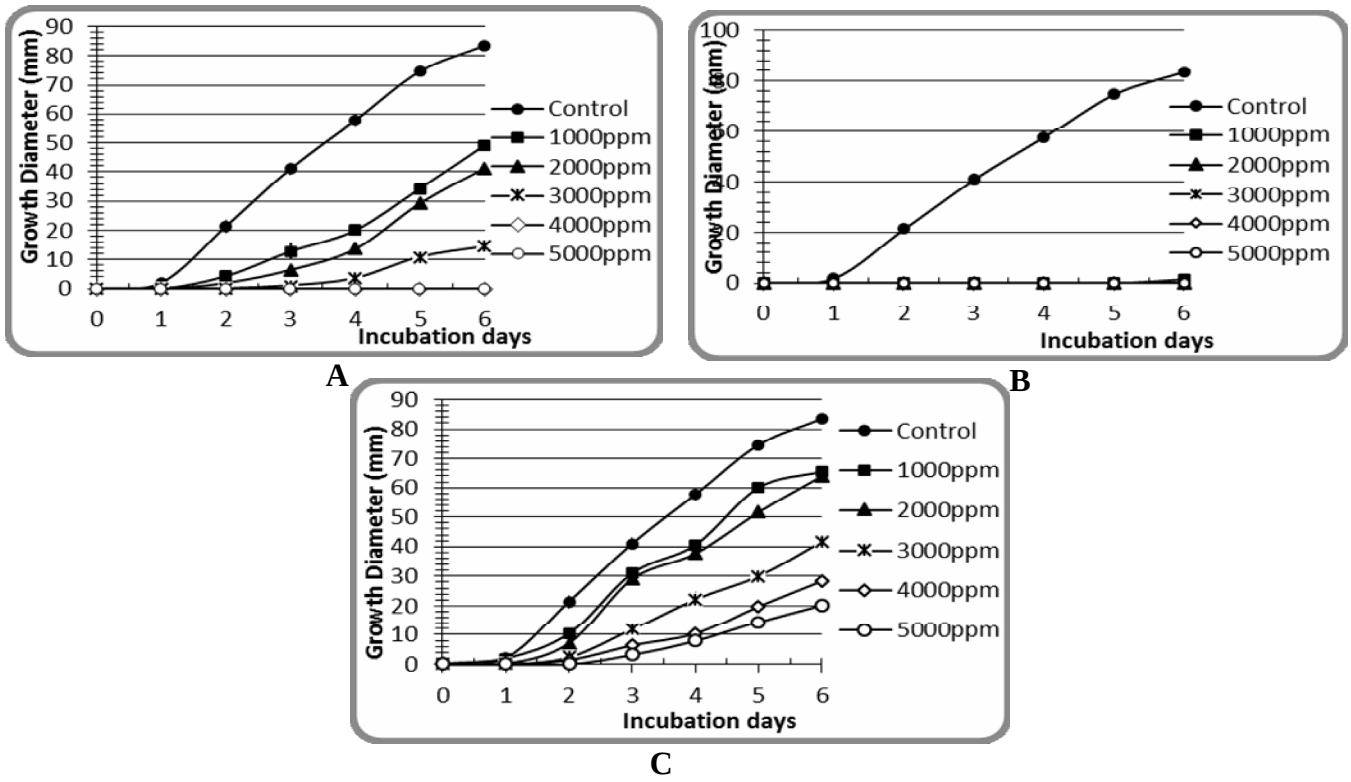

Figure.3 Effect on *A. fumigatus* of essential oils of **(A)** *C. schweinfurthii* (Mbounda), **(B)** *C. schweinfurthii* (Lolodorf) and **(C)** *A. Klaineana*

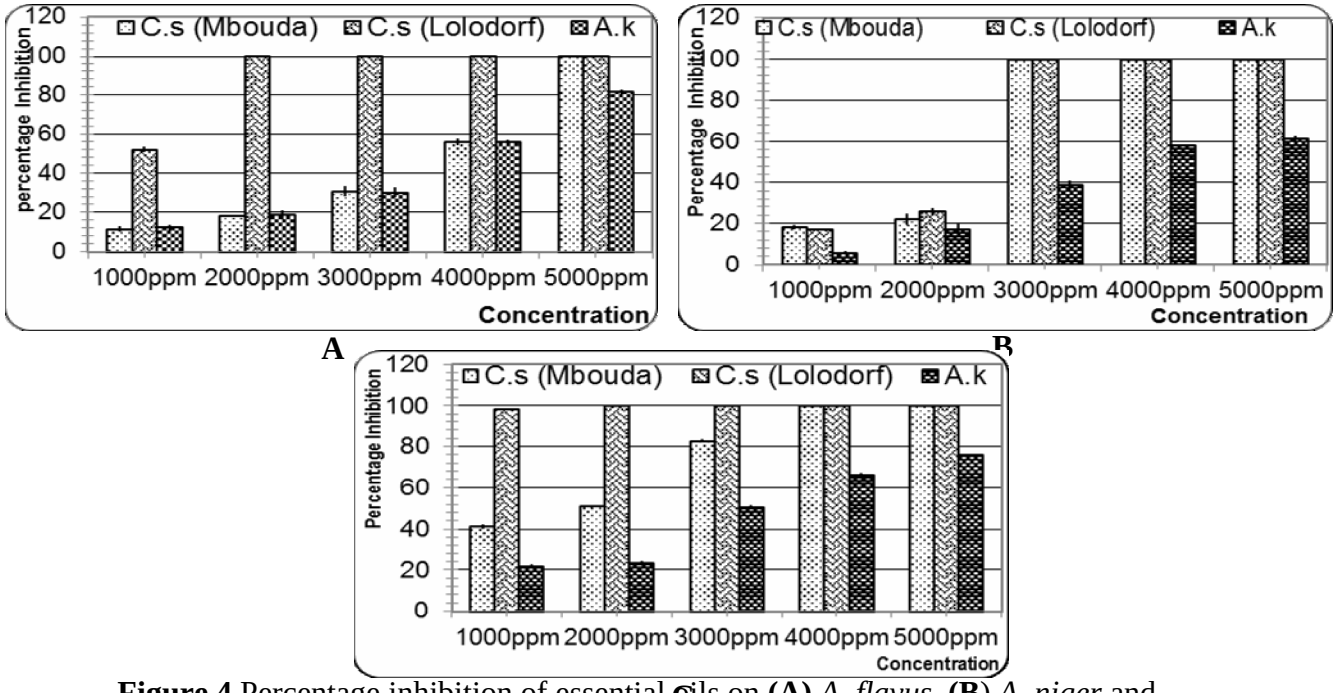

Figure.4 Percentage inhibition of essential oils on **(A)** *A. flavus*, **(B**) *A. niger* and **(C)** *A. fumigatus*

Minimum Inhibitory Concentration (MIC)

After noting the concentration at which minimum inhibition was observed from the preliminary tests, the MIC determined for the corresponding essential oils and reference fungicide used (sorbic acid) are shown on table 2.

Canarium schweinfurthii (Lolodorf) had the smallest MIC values of the three essential oils. From the preliminary tests, essential oil of *Aucoumea klaineana* did not show total inhibition and as such its MIC is greater than the maximum concentration used, that is, 5000ppm on all three *Aspergillus* species. Sorbic acid was the most effective for each of the *Aspergillus* species.

From the above tests, the essential oils can be classified statistically in terms of their antifungal activities; the most active being *Canarium schweinfurthii* (Lolodorf), then *Canarium schweinfurthii* (Mbouda) and lastly *Aucoumea klaineana*. The difference in activities of the essential oils could be attributed to the relative composition of oxygenated monoterpenes. *Canarium schweinfurthii* (Lolodorf) had 37.0%, *Canarium schweinfurthii* (Mbouda) 25.3% and *Aucoumea klaineana* 15.7%.

The MIC obtained for our samples against *A. flavus* are different from that obtained by Jazet *et al.* (2007) who worked on the essential oil of *Cinnamomum zeylanicum* and had an MIC value of 500ppm. Tatsadjieu *et al.* (2007) had an MIC of 600ppm for a combination of equal volumes of essential oils of *Ocimum gratissimum, Lippia rugosa* and *Xylopia aethiopica*. These differences in results could be as a result of the difference in composition of the essential oils.

The main objective of this part was to put to light the antifungal properties of the essential oils. Analyses revealed that all three samples of essential oils showed antifungal activities against *A. flavus, A. niger* and *A. fumigatus*.

From the minimum inhibitory concentration evaluated, essential oil of *C. schweinfurthii* (Lolodorf) was the most effective, followed by *C. schweinfurthii* (Mbouda). Even at the maximum concentration tested (5000ppm), *A. klaineana* did not show total inhibition of mycelial growth, though inhibition was greater than 50%.

Compared to the test antifungal of reference, sorbic acid, all samples of essential oils showed a lower activity for the corresponding *Aspergillus* species. Also, mycelial growth was barely inhibited by the essential oils at all tested concentrations. Despite their fungistatic nature and the lower activities of the essential oils than sorbic acid, they can still be employed as alternative antifungal agents thanks to their many other advantages (cheap, relatively available, natural, biodegradable ...).

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