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# Chemical Composition and Antimicrobial Activities of Volatile Extracts from Fresh Leaves of *Lippia multiflora* Moldenke against Pathogenic and Adulterated Microorganisms Isolated from Foods Sold Near Roads in Benin Republic

**Yehouenou Boniface[1], Sessou Philippe[1,2], Dahouenon-Ahoussi Edwige[1], Koudoro Yaya[1], Alitonou Guy Alain[1] and Sohounhloue Dominique[1]***

[1]*Laboratory of Study and Research in Applied Chemistry, University of Abomey-Calavi, Polytechnic School of Abomey-Calavi, Cotonou, Benin*

[2]*Laboratory of Research in Applied Biology, University of Abomey-Calavi, Polytechnic School of Abomey-Calavi, Cotonou, Benin*

***Corresponding Author**: Sohounhloue Dominique, Laboratory of Study and Research in Applied Chemistry, University of Abomey-Calavi, Polytechnic School of Abomey-Calavi, Cotonou, Benin.

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## Abstract

The present work has focused on the chemical composition of essential oils extracted from the leaves of *Lippia multiflora* from Pèrèrè and Savalou, a northern and a center regions of Benin Republic. The essential oil of Pèrèrè was essentially composed of oxygenated monoterpenes (linalool 91.9%) and the one of Savalou was diversely composed of oxygenated monoterpenes (1-8 cineole 50% and α-terpinol 13.8%) and hydrogenated monoterpenes (sabinene 14.4%). Antibacterial activities of the two essential oils were not so relevant against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. However, these oils displayed fungicidal activity against *Fusarium oxysporum* and fungiostatic activity against *Aspergillus ochraceus* and *A. parasiticus*. These oils can be used fungi control.

***Keywords:*** *Essential Oils; Lippia multiflora; Chemical Composition; Antifungal Activity*

## Introduction

People living under climate influences of Equator sustain unfortunately some disadvantages in relation with moderate temperature and permanent humidity favouring degradation of organic matter. This might create an environment suitable for the development of microorganisms which contaminate many foods sold near roads in Benin. Some of these strains were previously documented throughout informations collected from Ministry of Health in medicine blocks and pediatric pavilion of hospital centers in relation with unexpected occurring of digestive diseases and some associated affections, emphasizing implication of food toxic-infections in the etiology of general public health problems these last decades [1]. The main microorganisms isolated from common foods were clinically relevant and stood to the basis of specific affections. *Escherichia coli* and other pathogenic enterobacteria like *Enterobacter cloacae*, *Shigella dysenteriae*, *Salmonella typhi* and *paratyphi* which inducted stomachal affections such as diarrhea, typhoid fever and gastroenteritis, *Staphylococcus aureus* implicated in post-operative wounds infections, urinary tract infections, pneumonia and displayed a strong resistance to many drugs [2] and also *Aspergillus* and *Fusarium* which are mycotoxins producers mainly aflatoxins, fumonisins and tricothecenes [3] were tested among the major causes of food toxic-infections. However, some active molecules extracted from aromatic plants like essential oils, could constitute a credible alternative to fight against these microorganisms when applicated to foods with controlled concentrations.

They were designed GRAS (Generally Recognized as Safe) because they are toxicless, biodegradable, costless and detained antibacterial, fungicidal, insecticidal, acaricidal and cytotoxic properties [4].

*Lippia multiflora* Moldenke, belonging to Verbenaceae family of perennial herbaceous plants is a shrubby aromatic plant, growing up to 1.2 m with whitish flowers on cone-like heads in a terminale panicle and nearly 12 mm long, widely distributed in west and Central Tropical Africa [5,6]. This plant has been used in traditional and herbal medicine to treat bronchial inflammation, malaria fever, conjunctivitis, gastro-intestinal disturbance, enteritis, cough and colds and possessed fatigue-relieving and diuretic properties. It's cooked and used to relieve stress and enhance sleep. Traditionally, it has been used as a substitute for tea and as a mouth disinfectant and its oil shown marked antimicrobial activity [7].

The essential oil composition of *Lippia multiflora* was largely diversified and characterized by high terpenoids content in particular 1-8 cineole, linalool, thymol and thymol-acetate, geranial and neral, sabinene, α-terpineol, ipsidienone (Z) and (E) ocimene, α-phellandrene, myrcene, limonene, γ-terpinene, (E)-caryophyllene, p-cymene, myrtenol, (E) et (Z)-tagetone and β-farnesene.

## Purpose of the Study

The purpose of this study was to investigate the chemical composition and potential antimicrobial activity of *Lippia multiflora* essential oil from two regions of Benin Republic.

## Materials and Methods

### Collect and identification of plant material

Leaves of *Lippia multiflora* were collected on October 2014 in Savalou Department of Collines and in Pèrèrè Department of Borgou. The botanical material was identified by Professor Akoegninou and a voucher specimen was deposited in the Herbarium of Botanic Garden of Department of Vegetal Biology (University of Abomey-Calavi).

### Microorganisms

Antimicrobial tests were conducted in LERCA Polytechnical School of Abomey-Calavi (LERCA/EPAC/UAC) using *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and the fungi *Fusarium oxysporum*, *Aspergillus ochraceus* and *Aspergillus parasiticus*. All microorganisms were supplied by the Laboratory of Food Safety and Water quality of Ministry of Health and controlled with API System (Apparatus and Identification Procedures La Balme-les Grottes Cedex 2 France).

### Extraction of essential oils

The essential oil was extracted from fresh leaves (250g) by hydrodistillation during 3 hours using Clevenger apparatus in LERCA/Polytechnic School of Abomey-Calavi, University of Abomey-Calavi. Oil recovered was dried over anhydrous sodium sulphate and stored at 4°C until it was used. The extraction yields were estimated at 1.36 ± 0.01 for Pèrèrè essential oil and at 1.07 ± 0.03 for Savalou one.

### Analysis of essential oils

Obtained essential oils were packed in an amber vial and freeze storage. A sample of each oil was diluted in dichloromethane (1 mg/ml) and was subjected to analysis by gas chromatography coupled to flame ionization detector (GC/FID) and by gas chromatography coupled with mass spectrometry (GC/MS). Analysis parameters by GC/FID were column DB-5 (25m x 0.25 µm) temperature programming from 60 to 240°C with increase of 3°C.mn$^{-1}$ using hydrogen and synthetic air as carrier gases, with a flow rate of 1.0 ml.mn$^{-1}$. The identification of chemical components was carried out by GC and GC quadruple mass spectrometry (SM).

### Biological assays

### Minimum Inhibitory Concentration (MIC)-broth microdilution method

The determination of the MIC broth microdilution method was proposed by Bajpai., *et al*. [8] and reported by Yehouenou., *et al* (2010). The microdilution on 96 well plates was settled up with Mueller-Hinton Broth (MHB) added with 0.02 g/L phenol red and MHB consti-

tuted the negative control and the positive one was a suspension of tested bacteria strain diluted in MHB (1.5 x $10^8$ cfu/mL McFarland scale 0.5). The microplates were incubated at 37 ± 1°C for 24h covered with parafilm paper.

### Minimum bactericidal concentration (MBC)

The MBC was determined by the method proposed by Oussou., *et al*. [9] and reported by Kpadonou., *et al* [10]. Each microliter plate content 50 µL in which no color change occurred was isolated on sterile Mueller Hinton Agar poured in Petri dishes. These plates were incubated at 37°C for 24h. The MBC is the lowest concentration of essential oil which 99.9% of the microorganisms were killed. The tests were carried out in triplicate.

### Antifungal activity

Preparation of the culture medium 11.5g agar of yeast extract (Yeast extract AGAR) and 10g of anhydrous glucose were mixed with 500 ml of distilled water for the preparation of culture medium. After sterilization and addition of oxytetracycline (0.1%) 5 ml, this medium was cast in limp of Petri dish 9 cm in diameter at a rate of 17 ml.

### Detection of the moulds

A quantity of food largely consumed in Benin (Adoyo, meat soup, refreshed drink called "Adoyo" and cooked maize flour) was diluted in sterile peptone water in order to detect fungi responsible of their deterioration. 30 minutes after homogenizing each sample, 0.1 ml of the inocula was spread out on the sterilized mould medium Yeast Extract Glucose Agar (YEGA) and uniformly. The pre-sent limp was incubated at 25 ± 1°C five days awarded from day light. The moulds were identified by microscopy according to keys of Samson., *et al* [11].

### Transplantation and mycelia growth

The moulds detected after examination and identification then, are transplanted (subcultured) using a disc of 6 mm in diameter which carries spores from the anamorph mould on the surface of Petri dish containing the former medium YEGA containing tested essential oils at different concentrations or no (positive control). The moulds subcultured were incubated at 25 ± 1°C. The mycelial growth was appreciated every day by measuring the average of two perpendicular diameters passing by the middle of the disc, from the first day till the seventh one at, least 7 days [12] cited by Koudoro., *et al* [13]. The antifungal activity was evaluated by the following equation: I= [1-(d/dc)] × 100 [14].

I: Index antifungal;

d: Diameter of growth of Petri dish treated out of essential oil;

dc: Diameter of growth of the control (witness) [Petri dish without essential oil].

### Test of determination of the fungiostatic or fungicidal activity

With the experimental concentrations where neither growth nor germination was observed, the fungiostatic or fungicidal activity was tested. This test consisted in taking the mycelial disc not germinated at the end of the incubation of the Petri dish and reintroducing it in a new culture medium (former one) without natural extract. If the mycelial growth is always inhibited, the fungicidal activity of the natural extracts was noted and in the contrary case, it's spoken about fungistatic activity.

## Results and Discussion

*Lippia multiflora* essential oil of Savalou was in majority constituted of oxygenated monoterpenes (1-8 cineole 50.6%, α-terpineol 13.8%, linalool 0.3%, camphene 6-ol 0.1%, terpinen-4-ol 0.3%), hydrogenated monoterpenes (sabinene 14.4%, α-pinene 3.7%, myrcene 2.9%, β-pinene 1.9%, γ-terpinene 0.3%, δ-cadinene 0.2%), hydrogenated sesquiterpenes 6.8% and oxygenated sesquiterpenes 0.1%.

*Lippia multiflora* essential oil of Pèrèrè was constituted of 14 identified components in which 92.1% of oxygenated monoterpenes (linalool 91.9% and 1-8 cineole 0.2%), hydrogenated monoterpenes (sabinene 0.1% and myrcene 0.1%), hydrogenated sesquiterpenes

(germacrene D 2.9%, α-caryophyllene 1.1%, δ-cadinene 0.4%, trans-β-ocimene 0.3%, α-copaene (0.2%, aromadendrene 0.9%), oxygenated sesquiterpenes ((E)-nerolidol 0.5% and caryophyllene oxide 0.1%). The most varied composition in oxygenated and hydrogenated monoterpenes of *Lippia multiflora* essential oil of Savalou seemed to be more relevant on *Escherichia coli* ATCC25922, *S. aureus* ATCC 25923 with MIC respectively 0.64 mg/mL and 0.64 mg/mL than *Lippia multiflora* essential oil of Perere with composition in oxygenated monoterpenes in majority linalool in high proportion (91.9%) of which MIC for the two strains *Escherichia coli* and *Staphylococcus aureus* were respectively 1.92 mg/mL and 1.92 mg/mL. However, the two essential oils possessed a weak antimicrobial power against *Staphylococcus aureus* ATCC 25923 and relative and differential fungicidal power against *Aspergillus ochraceus*, *A. parasiticus* and *Fusarium oxysporum*. The two essential oils stood fungistatic on *Aspergillus* strains (*ochraceus* and *parasiticus*) but *Lippia multiflora* essential oil of Savalou was fungicidal on *Fusarium oxysporum* at a concentration of 2000 ppm from the 4$^{th}$ day of contact. *Lippia multiflora* essential oil of Pèrèrè was composed of fourteenth molecules of which the major one was linalool (91.9%) and the one of *Lippia multiflora* of Savalou displayed twenty components of which the major was the 1-8 cineole (50.6%), α-terpinol (13.7%) and sabinene (14.4%). The chemotype of Pèrèrè *Lippia multiflora* essential oil was linalool-germacrene D and the one of Savalou was 1-8 cineole-sabinene. The chemotype of *Lippia multiflora* essential oil of Perere was exceptional because showed a chemotype previously described by Kanko in 1999 and classified in the group 1, the one of acyclic monoterpenes with linalool discovered in Nigeria, Kenya, Benin and Togo. The chemotype of Savalou *Lippia multiflora* essential oil was classified according to Kanko [15] in the group III, the one of cyclic or bicyclic represented by the 1-8 cineole and associated to other components such as the α-terpinol and sabinene expressed by this chemotype. This one was quite the same previously identified by Avlessi [16] and Alitonou [17]. Only *Lippia multiflora* essential oil of Savalou analyzed possessed 0.3% of linalool so that in the two compositions described before by Avlessi and Alitonou in Benin, linalool was identified in relative proportions equal to 10.90%. None of two chemotypes was identified in the one of Perere.

| KI | Components identified | % |
|---|---|---|
| 964 | Sabinene | 0.1 |
| 977 | Myrcene | 0.1 |
| 1024 | 1,8-cneol | 0.2 |
| 1037 | Trans-β-ocimene | 0.3 |
| 1101 | Linalol | 91.9 |
| 1375 | α-copaene | 0.2 |
| 1388 | Aromadendrene | 0.1 |
| 1422 | α-caryophyllene | 1.1 |
| 1446 | Allo-aromadendrene | 0.9 |
| 1484 | Germacrene D | 2.9 |
| 1520 | δ-cadinene | 0.4 |
| 1553 | (E)-nerolidol | 0.5 |
| 1577 | Caryophyllene oxide | 0.1 |
| 1588 | NI | 0.1 |
| 1639 | α-muurolene | 0.1 |
| | Hydrogenated monoterpens | 0.2 |
| | Oxygenated monoterpens | 92.1 |
| | Hydrogenated Sesquiterpens | 5.7 |
| | Oxygenated Sesquiterpens | 0.6 |
| | Total | 98.6 |

***Table 1:*** *Chemical composition of Pèrèrè Lippia multiflora essential oil.*

| KI | Components identified | % |
|---|---|---|
| 920 | **α**-thujene | 0.3 |
| 929 | **α**-pinene | 3.7 |
| 969 | sabinene | 14.4 |
| 973 | **β**-pinene | 1.9 |
| 981 | myrcene | 2.9 |
| 1033 | 1,8-cineol | 50.6 |
| 1040 | **γ**-terpinene | 0.3 |
| 1063 | NI | 0.8 |
| 1084 | Terpinolene | 0.2 |
| 1092 | Linalol | 0.3 |
| 1094 | NI | 0.6 |
| 1123 | Camphen-6-ol | 0.1 |
| 1162 | Pinocarvone | 0.2 |
| 1164 | NI | 0.9 |
| 1175 | Terpinen-4-ol | 0.3 |
| 1192 | **α**-terpinol | 13.8 |
| 1378 | **α**-cubebene | 0.1 |
| 1426 | NI | 1.8 |
| 1450 | Aromadendrene | 4.2 |
| 1487 | Germacrene - D | 2.1 |
| 1519 | Isoledene | 0.2 |
| 1524 | **δ**-cadinene | 0.2 |
| 1556 | (E) - nerolidol | 0.1 |
| | Hydrogenated monoterpens | 23.7 |
| | Oxygenated monoterpens | 65.3 |
| | Hydrogenated sesquiterpens | 6.8 |
| | Oxygenated sesquiterpens | 0.1 |
| | Total | 95.9 |

***Table 2:*** *Chemical composition of essential oil of Lippia multiflora from Savalou.*

The antimicrobial screening of *Lippia multiflora* essential oil neither that from Pèrèrè nor the one from Savalou in this study demonstrated any antibacterial activity against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 (MIC respectively for *Escherichia coli* 1920 $\mu g.mL^{-1}$/640 $\mu g.mL^{-1}$ and for *Staphylococcus aureus* ATCC 25923 1920 $\mu g.mL^{-1}$/640 $\mu g.mL^{-1}$). This is in contrast to the results reported by Oussou., *et al.* [18] and those of Bassole., *et al.* [19] which may be attributed to geranial and high content of thymol respectively in their samples. The phenolic compounds such as thymol are supposed to be at the basis of the antibacterial activity detected in the sample of Burkina-Faso [19]. It's surprising that Ivory Coast essential oil of *Lippia multiflora* with a chemotype 1-8 cineol-geranial displayed an antibacterial activity against *Escherichia coli* ATCC 25922. However, *Lippia multiflora* essential oil of Savalou was more relevant than the one of Pèrèrè because of the wide variation in its chemical profile. Concerning the moulds, this essential oil from *Lippia multiflora* of Savalou is fungicidal at a concentration of 2 $\mu L.mL^{-1}$ from the 4$^{th}$ day on *Fusarium oxysporum* but stayed fungistatic at the same concentration on *Aspergillus parasiticus* and *Aspergillus ochraceus* (Figure 1 to 6) [20,21].

| Strains tested | MIC (µg.mL$^{-1}$) | | MBC (µg.mL$^{-1}$) | |
|---|---|---|---|---|
| | EO Savalou | EO Perere | EO Savalou | EO Perere |
| *Escherichia coli* ATCC 25922 | 640 | 1920 | 5120 | 15360 |
| *Staphylococcus aureus* ATCC 25923 | 640 | 1920 | 1280 | 3840 |

***Table 3**: Minimal Inhibition Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of essential oils of Lippia multiflora from Savalou and Perere on S. aureus and E. coli.*
*EO: Essential Oil.*

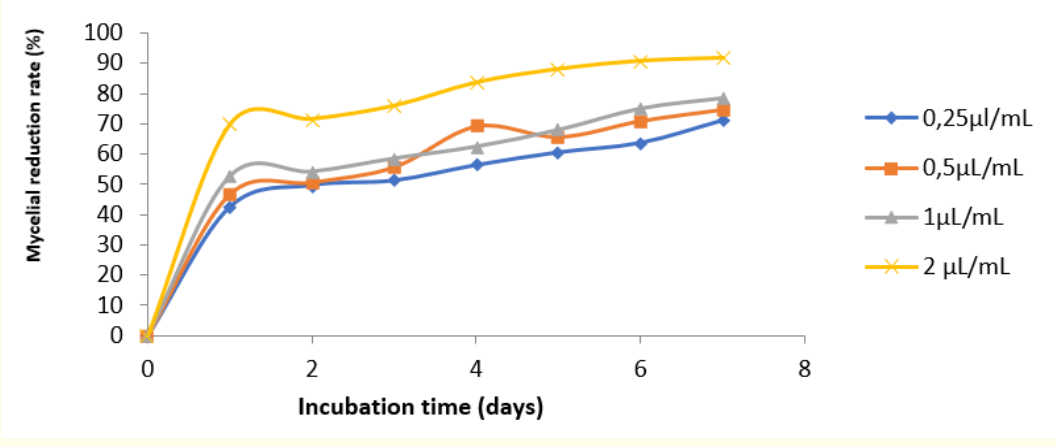


***Figure 1:** Mycelial growth reduction on Fusarium oxysporum in function of different concentrations of Lippia multiflora essential oil of Savalou.*

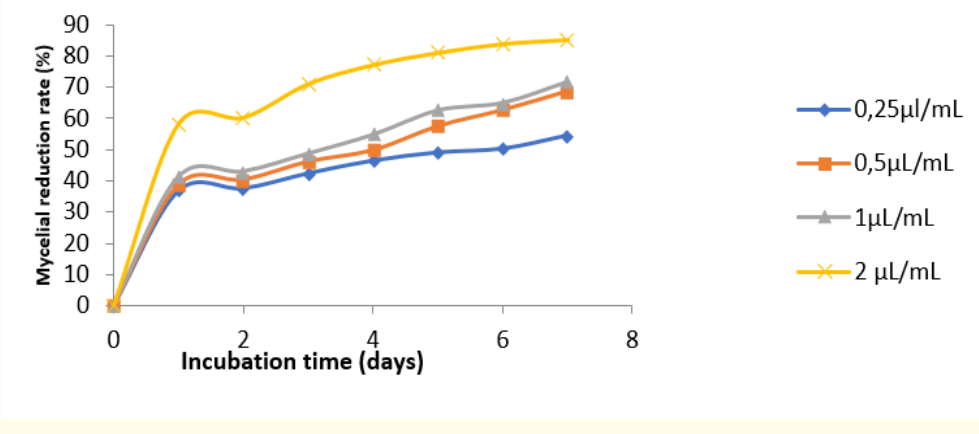


***Figure 2:** Mycelial growth reduction on Aspergillus ochraceus in function of different concentrations of Lippia multiflora essential oil of Savalou.*

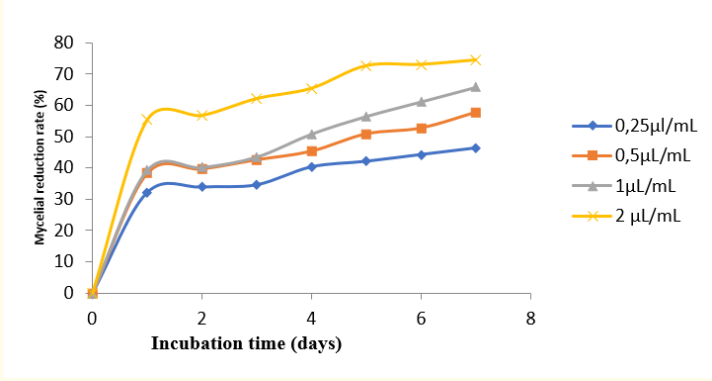


***Figure 3:*** *Mycelial growth reduction on Aspergillus parasiticus in function of different concentrations of Lippia multiflora essential oil of Savalou.*

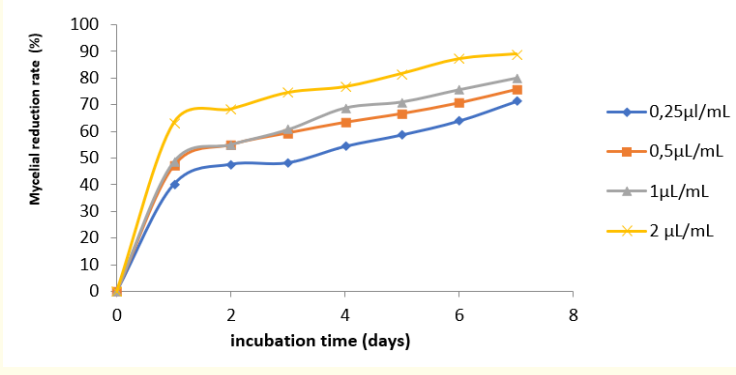


***Figure 4:*** *Mycelial growth reduction on Fusarium oxysporum in function of different concentrations of Lippia multiflora essential oil of Pèrèrè.*

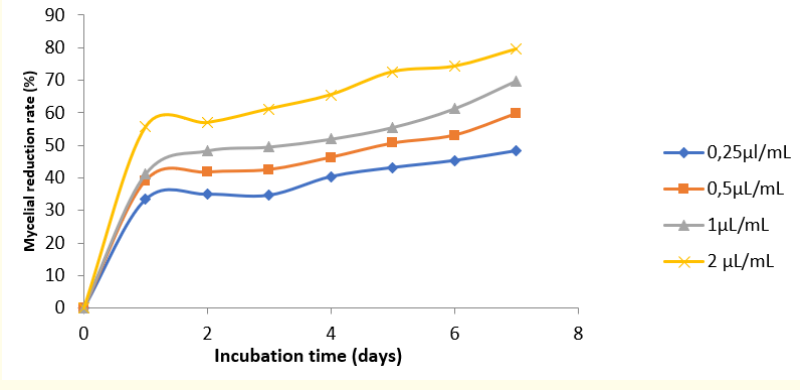


***Figure 5:*** *Mycelial growth reduction on Aspergillus parasiticus in function of different concentrations of Lippia multiflora essential oil of Savalou.*

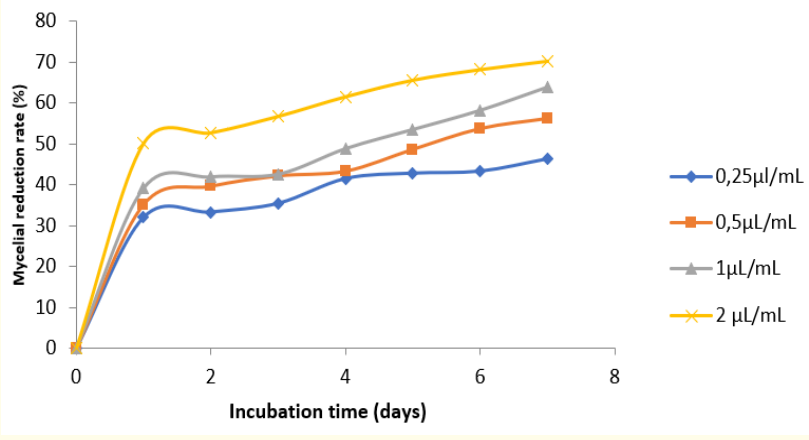


***Figure 6:*** *Mycelial growth reduction on Aspergillus parasiticus in function of different concentrations of Lippia multiflora essential oil of Pèrèrè.*

## Conclusion

Essential oils of *Lippia multiflora* from Benin (Pèrèrè and Savalou) analyzed in this study presented two chemotypes different from each other. The one of Pèrèrè was dominated by Linalol content (chemotype Linalol 91.9%- Germacrene D 2.9%) and the one from Savalou was composed of 1-8 cineole 50.6%, sabine 14.4% and α-terpinol 13.8% (chemotype EO cineole-sabinene). Concerning their antimicrobial activities, neither the one of Pèrèrè nor that from Savalou display any antibacterial activity against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. However, *Lippia multiflora* essential oil from Savalou because of its wide variation in its chemical profile detained strong fungicidal activity against *Fusarium oxysporum*. This reveals its biological activity for the preservation of foods against moulds.

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**Volume 16 Issue 3 March 2020**