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Biocontrol of *Aspergillus flavus* Strains Isolated from Bambara Groundnut (*Vigna subterranea* (L.) Verdc.) Seeds Using Essential Oils of *Lippia multiflora* Moldenke, *Ocimum americanum* L. (Lamiaceae) and *Eucalyptus cameldulensis* Dehnh

Mahamadi Nikiema[1,2], Amidou S. Ouili[1*], Cheik Omar Tidiane Compaoré[1], Assiètta Ouattara[1], François Palenfo[1], Aboubakar Sidiki Ouattara[1]

[1]Laboratoire de Microbiologie et de Biotechnologies Microbiennes, Université Joseph KI-ZERBO, Ouagadougou, Burkina Faso
[2]Institut Supérieur de Développement Durable (ISDD), Université Yembila Abdoulaye Toguyeni, Fada N'Gourma, Burkina Faso
Email: *amidou.s.ouili@gmail.com

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Abstract

In nature, plant extracts play a crucial role in defending plants against biotic and abiotic stressors. Moreover, the use of plant-based products, such as plant extracts, represents a promising alternative to synthetic fungicides, which pose potential health risks to consumers. In this study, the antifungal activity of the essential oils (EOs) of *Lippia multiflora*, *Eucalyptus camaldulensis* and *Ocimum americanum* was evaluated against two strains of *Aspergillus flavus* via the agar dilution method. These two *Aspergillus flavus* fungi was isolated from Bamabra groundnut seeds. *Lippia multiflora* essential oil (EO) showed the best results compared with the other oils, with a minimum inhibitory concentration (MIC) of 9000 µg·mL^{-1}. The MIC for *Eucalyptus camaldulensis* and *Ocimum americanum* EOs was 10,800 µg·mL^{-1}. In view of their antifungal properties, these EOs could be used to develop a new, safe antifungal agent for food preservation.

Keywords

Aspergillus flavus, Essential Oil, Antifungal, Bambara Groundnut

1. Introduction

The overuse of chemicals in food preservation poses health risks to consumers. For this reason, it is important to prioritize research and the use of bio-pesticides during crop storage. Essential oils are increasingly studied for their ability to inhibit microorganisms and extend food shelf life due to their antimicrobial properties, including antibacterial, antiviral, and antifungal activities. They exhibit significant antibacterial activity against foodborne pathogens such as *Salmonella* and *Escherichia coli*, with oils like thyme (*Thymus vulgaris*) and cinnamon (*Cinnamomum zeylanicum*) reducing bacterial loads in meats and dairy products [1]. Certain essential oils, such as lemon oil (*Citrus limon*) and eucalyptus oil (*Eucalyptus globulus*), have demonstrated antiviral properties, with lemon oil inhibiting specific foodborne viruses [2]. The incorporation of essential oils like garlic (*Allium sativum*) and rosemary into food packaging can prolong shelf life by slowing bacterial growth [3]. The control of fungal growth in food using local plant extracts, particularly EOs, could be an effective and healthy solution. Indeed, several EOs have the capacity to produce a wide range of antifungal metabolites [4], [5] that can inhibit fungal growth, preserve food quality and safety and extend food shelf life [6]. Due to their natural origin, EOs are more prized by consumers than chemical agents. These oils are also recognized by the Food and Drugs Administration (FDA) as non-dangerous [7]. EOs of *Lippia multiflora* tested against strains of *Aspergillus flavus* isolated from wheat bran showed a 100% inhibition rate at a concentration of 2.5% [8].

In addition, Tiendrebeogo *et al.* [9] reported that *Lippia multiflora* EO had the ability to inhibit the mycelial growth of *Bipolaris oryzae*, *Pyricularia oryzae* and *Fusarium moliniformes* strains isolated from rice by up to 100% at concentration ranged between 100 to 600 ppm. Etienne *et al.* [10] showed that the essential oil of *Lippia multiflora* was able to completely inhibit the growth of strains of *Aspergillus* sp., *Rhizopus* sp. and *Fusarium* sp. isolated from potatoes at a concentration of 0.666 µL/mL after 7 days incubation. EOs obtained from *Ocimum* species, such as *Ocimum gratissimum*, inhibited the growth of several fungi, including the plant pathogens *Botryosphaeria rhodina*, *Rhizoctonia* sp. and two strains of *Alternaria* sp. EOs of *Ocimum americanum* inhibited the growth of strains of *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida krusei* and *Candida parapsilosis* [11]. The work of Gakuubi *et al.* [12] showed that *Eucalyptus camaldulensis* EO was able to completely inhibit the mycelial growth of several *Fusarium* species, including *F. oxysporum*, *F. solani*, *F. verticillioides*, *F. proliferatum* and *F. subglutinans* at concentrations ranged between 7 and 8 µL/mL after five days of incubation. Several studies have shown that the EO of *Eucalyptus cameldulensis* has the ability to inhibit the growth of a wide range of fungi, such as *Aspergillus niger*, *Chaetomium globosum*, *Rhizopus oryza*e, *Thanatephorus cucumeris* and *F. oxysporum* [13]. Somda *et al.* [14] also reported that the EO of *Eucalyptus camaldulensis* had antifungal activity against strains of *Glomerella graminicola*, *Phoma sorghina* and *F. moniliforme* isolated from the soil.

EOs are natural sources of biomolecules that deserve to be explored because, as well as having the ability to inhibit fungal growth, they are also able to reduce the synthesis of mycotoxins in foods [12] [13] [15] [16]. Recent studies demonstrated the inhibitory effect of *Cymbopogon citratus* and *Cymbopogon schoenanthus* EOs on the synthesis of aflatoxin B_1 [17] and, the possibility of controlling *Aspergillus flavus* and *Aspergillus parasiticus* on corn, using two formulations based on EOs of *Cymbopogon giganteus* and *Eucalyptus camaldulensis* [18].

Ouili *et al.* [19] recently isolated two mycotoxin-producing strains of *Aspergillus flavus* from Bambara groundnut seeds. The objective of this study is to evaluate the antifungal activity of essential oils from *Lippia multiflora*, *Eucalyptus camaldulensis*, and *Ocimum americanum* against these two aflatoxin-producing fungal strains.

2. Material and Methods

2.1. Essential Oils

The EOs of *Lippia multiflora*, *Ocimum americanum* and *Eucalyptus cameldulensis* used for the tests in our study were obtained from the Institut de Recherche en Sciences Appliquées et Technologies (IRSAT) of Burkina Faso (**Figures 1-3**). These EOs were extracted for 3 h using the hydrodistillation method in a clenvenger-type apparatus. Anhydrous sodium sulphate was used to dry the EO, which was then stored in an airtight, glass container at 4˚C in a refrigerator.

2.2. Antifungal Screening of EOs

Antifungal activity was tested using the two strains of *Aspergillus flavus* (AVBF26, AVBF66) isolated from Bambara groundnut. Previous studies have shown that these two strains are able to produce aflatoxins B_1 and B_2 when inoculated onto rice [19].

For the antifungal screening, various concentrations (112.5, 225, 450, 900, 1800 et 3600, 9000, 10,800, 12,600 μg·mL^{-1}) of EOs from each plant (*Lippia multiflora*,

Figure 1. Plant of *Lippia multiflora.*

Figure 2. Plant of *Ocimum americanum.*

Figure 3. Plant of *Eucalyptus camaldulensis.*

Ocimum americanum and *Eucalyptus cameldulensis*) were incorporated into molten Potato Dextrose Agar (PDA) (approximately 44˚C) containing 0.1% Tween-20. Approximately 20 ml of each mixture (PDA agar + EO) obtained was distributed in Petri dishes (90 mm), and after solidification of the culture medium, plugs of a 7-day fungal culture (4 mm), taken from the actively grown region of the colonies, were placed in the center of the Petri dishes and incubated at room temperature. Fungal colony diameters were measured after seven days of incubation. Each test was carried out in triplicate. PDA agar without EO was used as a negative control and the antifungal agent Calthio C infused into the PDA agar (final concentration 2.5 mg/l) was used as a positive control. The inhibition rates of fungi using EOs were calculated using the following formula:

$$\text{Growth Inhibition (GI) (\%)} = \frac{dc - dt}{dc} \times 100$$

where d*c* = Mean mycelial growth diameter in the negative control plates (PDA

without EOs).

d*t* = Mean mycelial growth diameter in the treatment.

The MIC was defined as the lowest concentration of EO that caused 100% inhibition. For the minimum fungicidal concentration (MFC) determination, fungal discs whose growth had been completely inhibited in the MIC experiment were transferred to new PDA plates previously prepared without EO. These plates were then incubated at room temperature for 5 days. The MFC is the lowest EO concentration at which fungal inhibition is irreversible in the absence of the inhibitor (EO).

2.3. Statistical Analysis

The data were analyzed using XLSTAT software and the mean separation was done by LSD at P = 0.05.

3. Results and Discussion

Antifungal Activity of EOs

The inhibition rates of the three EOs (*Lippia multiflora*, *Ocimum americanum* and *Eucalyptus cameldulensis*) tested against the strains of *Aspergillus flavus* (AVBF26 and AVBF66) are presented in Table 1.

Table 1. Growth inhibition (%), MIC and MFC of EOs.

EOs (µg·mL^{-1})	Growth inhibition (GI)	
	A. flavus (AVBF26)	*A. flavus* (AVBF66)
Ocimum americanum		
112.5	5.46 ± 3.9^{gg}	2.29 ± 0.81^{eh}
225	16.94 ± 2.4^{ff}	8.62 ± 3.72^{eg}
450	36.06 ± 2.68^{ee}	28.73 ± 3.25^{df}
900	38.79 ± 2.79^{ee}	36.78 ± 2.15^{de}
1800	54.09 ± 1.34^{dd}	48.85 ± 0.81^{cd}
3600	63.38 ± 0.77^{cc}	55.74 ± 2.15^{cc}
9000	83.06 ± 2.79^{bb}	82.75 ± 4.22^{bb}
10,800	100.00 ± 00^{aa*}	100.00 ± 00$^{aa*°}$
12,600	100.00 ± 00$^{aa°}$	100.00 ± 00^{aa}
Eucalyptus camaldulensis		
112.5	17.48 ± 1.55^{ff}	10.34 ± 1.41^{ef}
225	18.03 ± 1.34^{ff}	12.06 ± 1.41^{ef}
450	48.63 ± 2.4^{ee}	39.08 ± 1.61^{de}
900	60.1 ± 2.4^{dd}	41.37 ± 1.41^{dd}
1800	61.20 ± 2.4^{dd}	57.47 ± 0.81^{cc}

Continued

3600	67.21 ± 1.34^{cc}	59.19 ± 0.81^{cc}
9000	74.86 ± 0.77^{bb}	75.28 ± 0.81^{bb}
10,800	100.00 ± 0.00^{aa*}	100.00 ± 00$^{aa*°}$
12,600	100.00 ± 0.00$^{aa°}$	100.00 ± 00^{aa}
Lippia multiflora		
112.5	34.42 ± 2.32^{fg}	28.16 ± 1.63^{gg}
225	51.36 ± 1.55^{ef}	47.12 ± 0.81^{ff}
450	67.76 ± 2.79^{de}	63.21 ± 2.15^{ee}
900	73.22 ± 5.41^{cdd}	68.96 ± 1.41^{dd}
1800	81.42 ± 2.04^{bcc}	74.13 ± 1.41^{cc}
3600	87.97 ± 0.77^{bb}	89.08 ± 0.81^{bb}
9000	100.00 ± 00$^{aa*°}$	100.00 ± 00$^{aa*°}$
10,800	100.00 ± 00^{aa}	100.00 ± 00^{aa}
12,600	100.00 ± 00^{aa}	100.00 ± 00^{aa}

Means with the same letter(s) show no significant difference (multivariate analysis, Fisher's protected LSD at $p \leq 0.05$). °MFC; *MIC.

It can be seen that the sensitivity of fungi (AVBF26, AVBF66) increases when the concentrations of EOs increase (Figure 4, Figure 5).

Lippia multiflora EO, showed the best inhibition rates compared to the rest of the EOs, with a MIC and MFC of 9000 µg·mL^{-1} (Table 1). The MIC of *Ocimmum americanum* and *Eucalyptus cameldulensis* EOs on the fungi tested was 10,800

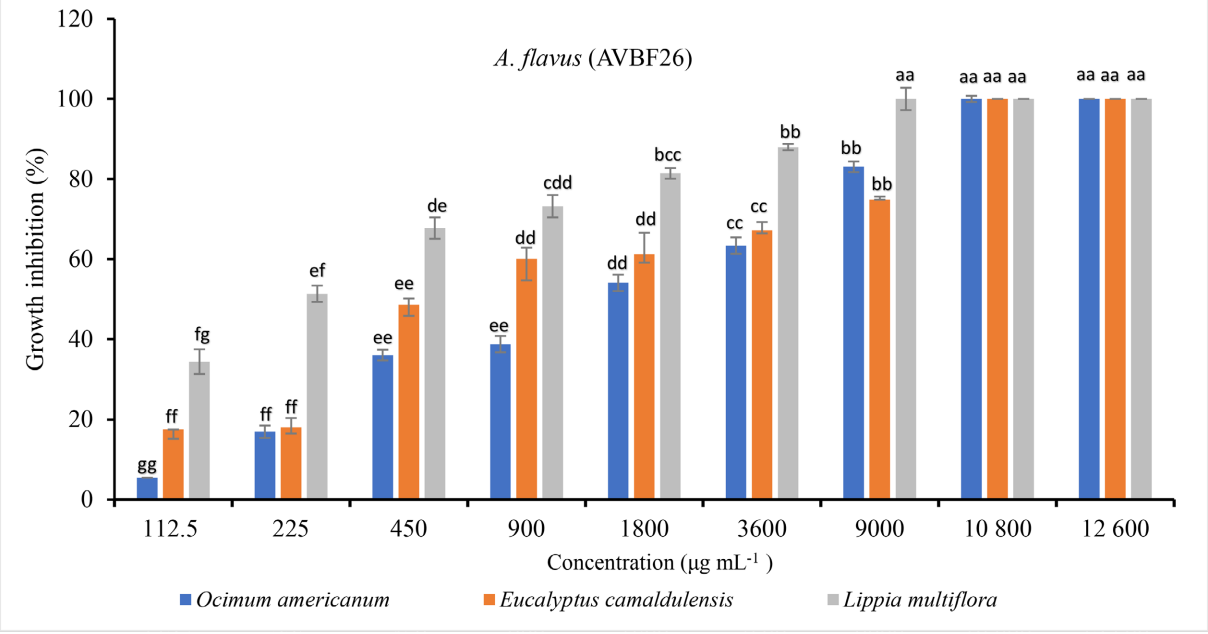

Figure 4. Average growth inhibition of the EOs at different concentrations on *A. flavus* (AVBF26).

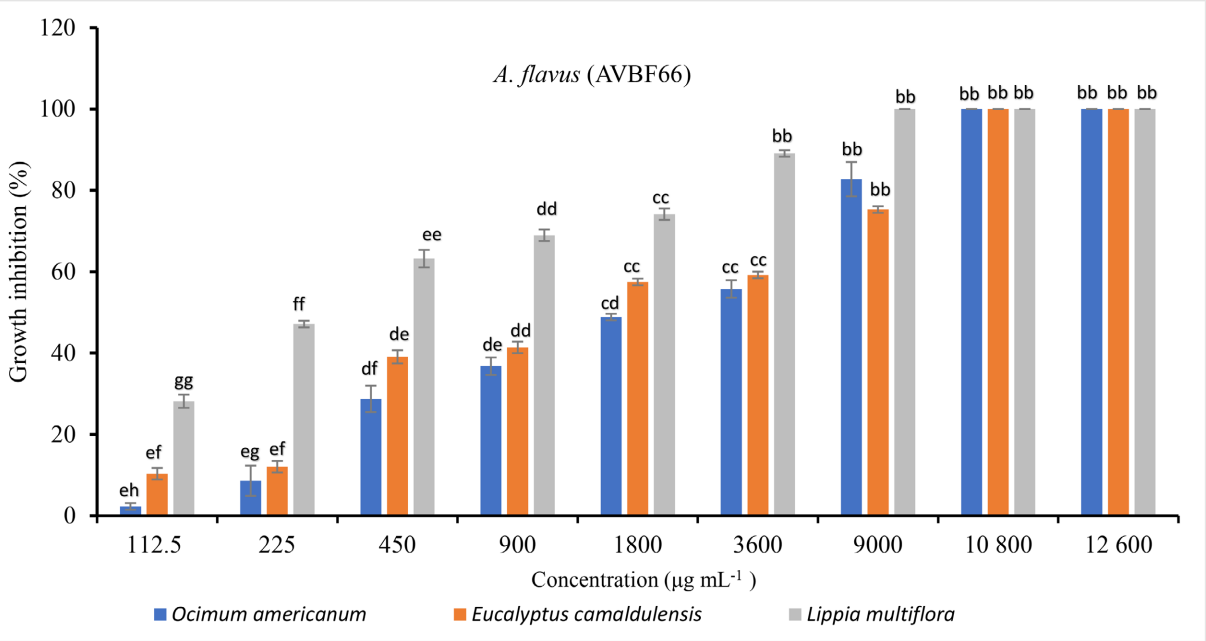

Figure 5. Average growth inhibition of the EOs at different concentrations on *A. flavus* (AVBF66).

µg·mL^{-1} and their MFC ranged between 10,800 and 12,600 µg·mL^{-1}. The lowest concentrations (112.5, 225, 450, 900, 1800 and 3600 µg·mL^{-1}) of *Lippia multiflora* EO showed inhibition rates between 34.42% and 87.97% on AVBF26 and 28.16% and 89.08% on AVBF66 (**Table 1**). Those (112.5, 225, 450, 900, 1800 and 3600, 9000 µg·mL^{-1}) of *Ocimum americanum* showed inhibition rates between 5.46 and 83.06% on AVBF26 and 2.29% and 82.75% on AVBF66 (**Table 1**). The EO of *Eucalyptus cameldulensis* showed inhibition rates between 17.48 and 74.86% on AVBF26 and 10.34 and 75.28% on AVB66 for the same concentrations (**Table 1**). These results show that the essential oils of *Lippia multiflora*, *Ocimum americanum* and *Eucalyptus cameldulensis* have fungicidal properties and can be used instead of synthetic fungicides. Numerous studies have shown that EOs are made up of several compounds capable of inhibiting the proliferation of bacteria and fungi in vitro [12] [17] [19] [20]. Antimicrobial and antifungal compounds generally found in EOs are terpenes, alkaloids, lactones, phenolic compounds, flavonoids and naphthoquinones [21]. The main components found in *Lippia multiflora* EO are c-terpinene, p-cymene, thymyl acetate, thymol and b-caryophyllene; those found in *Eucalytus cameldulensis* are a-pinene, eucalyptol and limonene. Camphor, 1, 8-cineol, a-pinene camphor, (Z)-methyl cinnamate and trans a-bergamotene are mainly encountered in the EO of *Ocimum americanum* [8] [21] [22] [23]. The synergistic effect of the antifungal compounds of each EO used in our study could explain the inhibition of the growth of the fungal strains tested. In fact, essential oils (EOs) exhibit inhibitory effects on *Aspergillus flavus* primarily through several well-established mechanisms. Upon contact, they alter the permeability of fungal cell membranes, leading to leakage of essential internal components and loss of cellular integrity. Recent studies have shown that volatile

compounds such as terpenes and phenols present in EOs, including clove oil (*Syzygium aromaticum*) and thyme oil (*Thymus vulgaris*), disrupt fungal cell membranes by damaging membrane lipids [24]-[27]. EOs are also effective in inhibiting spore germination and mycelial growth, which are crucial steps for food colonization. For example, cinnamon oil (*Cinnamomum verum*) and rosemary oil (*Rosmarinus officinalis*) have been shown to reduce spore germination and inhibit mycelial growth of *Aspergillus flavus* [28]. Some EOs induce oxidative stress by generating free radicals and reactive oxygen species, thereby damaging fungal cellular structures. Phenolic compounds and EOs such as clove and oregano oil (*Origanum vulgare*) increase the production of these oxidative agents, leading to cell death in *Aspergillus flavus* [28]. Additionally, EOs can interfere with fungal metabolic pathways by inhibiting essential growth enzymes. Recent studies have demonstrated that peppermint oil (*Mentha piperita*) and citronella oil (*Cymbopogon citratus*) affect protein synthesis and enzymatic activities, disrupting the biological processes of *Aspergillus flavus* [29]. Some EOs modify the local acidity around the fungi, creating an environment unfavorable for their growth. Lemon oil (*Citrus limon*) is known to lower the pH in the environment, which limits the growth of *Aspergillus flavus* [30].

Essential oils offer promising benefits for food preservation due to their antimicrobial and antioxidant properties, providing a natural alternative to synthetic preservatives and contributing to improved food safety. However, their use must be carefully regulated to avoid excessively high concentrations that could affect the taste or quality of the food. The effects of essential oils may vary depending on the type of food and the target microorganism. While their effectiveness has been demonstrated under controlled conditions, further studies are needed to evaluate their performance in large-scale food production environments.

4. Conclusion

The EOs evaluated in this study were all capable of inhibiting the strains of *A. flavus* tested. *Lippia multiflora* oil showed the best inhibition rates on fungal strains with a MIC of 9000 µg·mL^{-1}. It is followed by those of *Eucalytus cameldulensis* and *Ocimum americanum* which showed the same MIC (10,800 µg·mL^{-1}). These EOs are natural sources of biomolecules that can be used for the formulation of new antifungal agents for the preservation of Bambara groundnut seeds. To do this, additional studies are necessary to evaluate the combined effect of these EOs against mycotoxinogenic fungi and their phytotoxicity on Bambara groundnut seeds.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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