

Research Article

The Essential Oil of *Cymbopogon Citratus* Can Significantly Inhibit the Mycelia Growth, Conidia Germination of Mycotoxigenic *Aspergillus* Isolated from Smoked Fish (*Ethmalosa Fimbriata*) and Protect Against Mold Contamination

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Abstract

Ethmalosa fimbriata is a fish recognized for its high nutritional value and its beneficial effect on the health of consumers. In order to ensure a permanent supply throughout the year, this fish is dried or smoked and store. Despite that, *Ethmalosa fimbriata* is often colonized by molds especially mycotoxigenic *Aspergillus spp.* To reduce contamination, peoples commonly use chemicals that however have a negative environment impact and may be harmful to human and animal health. The aim of this study was to evaluate the bio activity of *Cymbopogon citratus* essential oil against mycotoxigenic *Aspergillus* from smoked *Ethmalosa fimbriata* and to protect this fish against mold. Fish samples were purchased in Douala markets, fungi isolated by dilution plate method and identified on the basis of their macroscopic and microscopic characteristics. Oil was extracted by Hydro-distillation and analyzed by gas chromatography coupled with mass spectrometry. The antifungal activity was evaluated by the agar incorporation method and microdilution. As results, the oil yield obtained was of 0.45% with 32 compounds among which geranial (39.66%), neral (30.77%) and α -fenchene (14.14%) were major components. Three mycotoxigenic fungi were isolated and identified as *Aspergillus parasiticus*, *Aspergillus glaucus* and *Aspergillus flavus*. *C. citratus* essential oil completely inhibited the mycelial growth at 595 ppm, 1195 ppm and 1295 ppm respectively, for these mycotoxigenic *Aspergillus*. Moreover, the total conidia germination of these fungi was achieved at 100 ppm. Whether it be preventive or curative test, fish on which essential oil was applied did not show any visible signs of contamination after 18 days of incubation compare to control fish on which visible mycelia grow and conidia were observed just after 3 days of incubation. Any attempt to reisolate fungi from treated fish failed especially for preventive test. These results show that essential oil of *Cymbopogon citratus* could be used to formulate a mycobiocide for smoked *Ethmalosa fimbriata* preservation.

Keywords: *Cymbopogon citratus*; Essential oil; *Aspergillus*; Anti-fungal activity; Mycotoxins; Smoked fish

Abbreviations: C.c: *Cymbopogon citratus*; CEA: Coconut Extract Agar; DMSO: Di Methyl Sulphur Oxide; EO: Essential Oil; GPS: Global Positioning System; GS-MS: Gas Spectrometry-Mas Spectrometry; MFC: Minimal Fungicidal Concentration; MIC: Minimal Inhibitory Concentration; PDA: Potatoes Dextrose Agar; PDB: Potatoes Dextrose Broth; SDA: Sabouraud Dextrose Agar; UV: Ultra-Violet

Introduction

Ethmalosa fimbriata is a fish known as Bonga. It is an important source of animal proteins, vitamins, minerals and essential fatty acids [1]. The farming of this fish is a common occupation of peoples living in the coastal areas and along major river banks in Cameroon where fish consumption was around 20 kg/habitant/year [2-4]. Despite the high importance, the post-fishing losses are estimated at 27% and represented 3,4 billion/year [5]. In fact, like other fishes *Ethmalosa fimbriata* is a rapid perishable product and to reduce losses, peoples use traditional conservation processes. In Cameroon for instance, 75 to 80% of collected fish is smoked or dried [6]. Despite these treatments, smoked dried fish is frequently altered by many fungi such as members of the genus *Aspergillus* especially when stored in an unsuitable environment [7]. Previous survey carried out in some local markets in Cameroon revealed that *Aspergillus* species where the most associated fungi with fish deterioration [8]. These fungi are well known to decrease nutritional and commercial value of fish [9] and to their capacity to produce mycotoxins who can cause multiple health problems to human and animals, including digestive cancers, immunologic and allergic responses [10]. To reduce smoked fish contamination, peoples commonly use chemical synthesized organic acids like methyl bromide and phosphine [11]. However, these chemicals have a negative environment impact and may be harmful to human and animal health [12]. Therefore, additional ecofriendly control methods of this spoiling mold are essential. In this respect, plants and their essential oils have been evaluated as natural sources of compounds for food preservatives due to their antimicrobial and antioxidant effects [13]. Cameroon has a very rich flora with many aromatic plants which possess various biological activities [14]. Among them *Cymbopogon citratus* is used for many purposes in African medicine [15]. In certain households in Cameroon, leaves of these plants are used to protect stored food products including smoked fish. The aim of this study was to evaluate the antifungal activity of *Cymbopogon citratus* essential oil against mycotoxigenic *Aspergillus* from smoked *Ethmalosa fimbriata* and to protect this fish against mold.

Materials and Methods

Plant Material and Fish Samples

Fresh leaves of *Cymbopogon citratus* were collected early at morning time in a farm located in Yassa (GPS coordinates: 4.03151, 97214). They were identified at the National Herbarium of the institute of agricultural sciences for development under the number SK 2158/13.

Smoked samples of *Ethmalosa fimbriata*, used in this study were purchased on August 2021 in four different Douala markets: Dakar, Deido, Central market and Sandaga. These markets were chosen base on their high flow of peoples and of fish distribution. Samples were transported in sterile plastic bags to the laboratory where fishes were ground into powder.

Essential Oil Extraction

Fresh leaves of *Cymbopogon citratus* essential oil was obtained by hydro distillation during four hours using Clevenger type apparatus. Essential oil recovered was dried over anhydrous Na_2SO_4 , sealed vials and stored in refrigerator at 4°C until use. The yield of extracted essential oil was calculated by the formula: yield (%) = [essential oil obtained (g) / vegetal material used (g)] × 100.

GC-MS Analysis of Essential Oil

GC-MS analyses were carried out using a Hewlett Packard 5570 GC-MS system operating in the electron impact mode at 70 eV, equipped with a HP 1 fused silica column (30 m x 0.25 mm, film thickness 0.25 µm) and interfaced with a quadrupole detector. The initial temperature of the column was 70°C, followed by heating to 200°C with a 10°C/min. rate. Carrier gas was He (flow rate 0.6 ml/min). The identification of the components was based on the comparison of their mass spectra with those of Wiley, NIST, Libraries and those described by Adams [16], as well as by comparison of their retention indices with literature values.

Fungal Isolation and Morphological Identification

Fungi were isolated by using dilution plating method. Briefly, 10 g of smoked fish powder were added to 90 ml of sterile peptone water (0.1%) and thoroughly mixed. Further, 10-fold serial dilutions up to 10 were made. One ml volume of each dilution was separately poured in Petri dishes containing 15 ml of Potato Dextrose Agar (PDA) – Chloramphenicol; Difco, USA. Plates were incubated at 28±2°C and monitored daily for any mycelia grow [17,18]. Fungi were then purified by successive transfers. The suspected *Aspergillus* isolates were subcultured and transferred on to differential media; malt extract agar, czapek yeast extract agar and glycerol agar for species identification using macro morphological characteristics. Fungal slides were prepared from pure cultures on PDA media for microscopic characteristics [19].

Screening of Isolates for Aflatoxins Production

The method of Ouattara-Sourabie [20] was used to screen the ability of fungal isolates to produce aflatoxins. For intense fungal isolates were plated on Coconut Extract Agar (CEA) and incubated at 28°C ±2°C during 7 days. After incubation period, fluorescence under Ultraviolet (UV) radiation was assessed using wood lamp at wavelength of 365 nm. The fluorescence and characteristic color of fluorescence were used to evaluate the ability of each isolate to produce aflatoxins. Aflatoxin diffusion areas shows a blue radiation around the isolate under UV light.

Antifungal Assay

The antifungal activity of the essential oil was evaluated by the agar incorporation method [21]. The test was carried out in 90 mm Petri dishes containing PDA-chloramphenicol medium. The oil was first diluted with Di Methyl Sulphur Oxide (DMSO) (ratio1:9). The essential oil was added aseptically into the medium at an appropriate volume to produce various concentrations ranging from 500 to 2000 ppm. PDA-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 *Aspergillus spp.* Each treatment consisted of triplicate plates incubated at 28°C ±2°C for seven days in the dark. Mycelia growth was monitored by measuring the growth diameter following two perpendicular lines going through the center of the dish. The inhibition percentage of mycelia growth was calculated according to the formula: %I = [(D_c - D_t) / D_c] × 100, where D_c is the diameter of microbial colony in the control and D_t 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.

Inhibition of Conidia Germination

The effect of the EO on the conidia germination was tested using a liquid dilution method in Potatoes Dextrose Broth (PDB). Firstly, to obtain conidia suspension the surface of 7 days old *Aspergillus* PDA plate was washed with 2 mL of sterile distilled water containing few drops of Tween 80. This solution was centrifuged at 2000 rpm for 5 min and the spore suspension (supernatant) recovered and adjusted to 10⁵ spores/mL using the Malassez cell. For the test, oil was added separately into microcupules containing 100 µl PDB in serial dilution following a geometrical progression with a common ratio of two in order to obtain the final concentrations of 2000, 1000, 500, 250, 125 and 62.5 ppm for the *Cymbopogon citratus* essential oil. After that aliquot (100 µl) of the spore suspension of each *Aspergillus* was introduced in microcupules [22]. Tests were carried out in triplicates and incubated during five days at 28 ± 2°C. The minimal sporidial concentration was observed visually and under optical microscopic (40×).

In Situ Test

Smocked *Ethmalosa fimbriata* collected was disinfected with ethanol 70% for 3 min, rinsed with sterilized distilled water cut into pieces and distributed in sterile Petri dish in triplicate for each test. For preventive test, 1mL of EO (MIC) solution was first spray on fish and spore suspension (100 µl) four hours later while for curative test conidia suspension was spray and EO added four hours later. Petri dish containing fish pieces were stored at 28± 2°C and monitored daily to detect visual contamination. After 18 days, an attempt to re-isolate *Aspergillus* from infected fish tissue was done as previously described.

Statistical Analyses

Experiments were performed in triplicate, and data obtained as mean ± standard deviation were analyzed by ANOVA test using IBM SPSS Statistics 20.1 (Chicago, IL, USA), version 10.0. Means are separated by the Tukey's multiple range test when ANOVA was significant (p<0.05).

Results

Hydrodistillation of fresh leave of *Cymbopogon citratus*

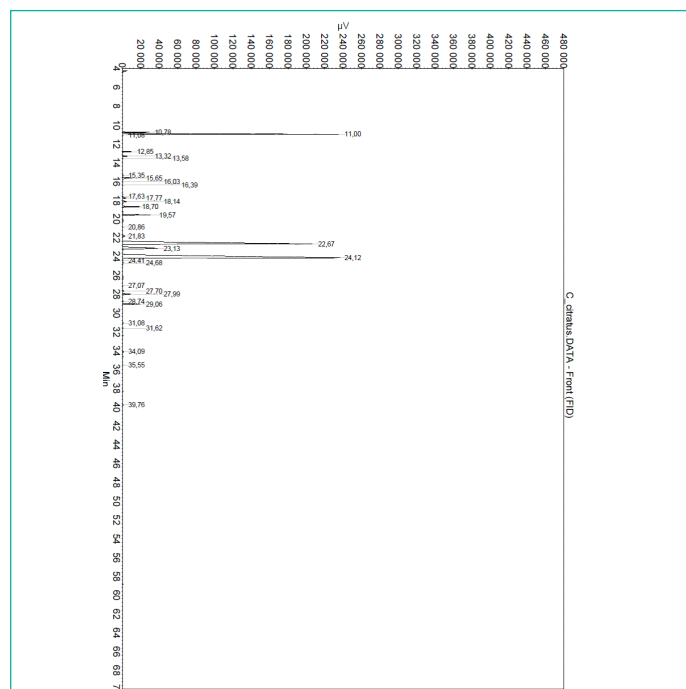


Figure 1: GC-MS mass spectra of *Cymbopogon citratus* essential oil.

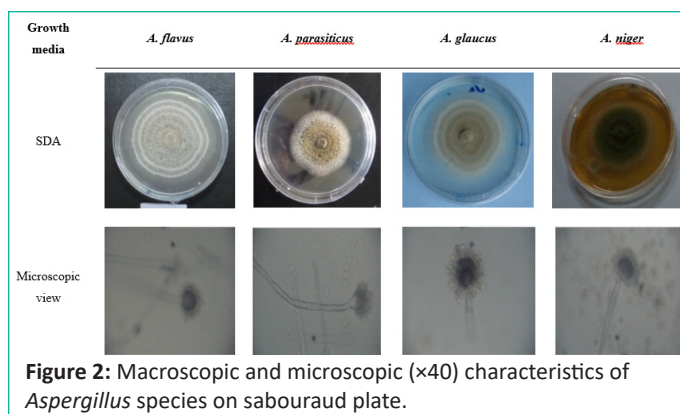


Figure 2: Macroscopic and microscopic (×40) characteristics of *Aspergillus* species on sabouraud plate.

Table 1: Macroscopic and microscopic characters of *Aspergillus* species.

| Species | Surface | Color | Shape | Vesicle serration |
|-----------------------|-----------|--------------|-----------|-------------------|
| <i>A. flavus</i> | Spherical | Pale brown | Ellipsoid | Uniseriate |
| <i>A. parasiticus</i> | Finely | Yellowish | Pyriform | Uniseriate |
| <i>A. glaucus</i> | Spherical | Green orange | Small | Biseriate |

shown a yellowish essential oil with a yield of 0.45%. GC-MS mass spectra of the oil is shown on Figure 1. Analytical results shown thirty-two components. The major components were geranial (39.66%), neral (30.77%) and α-fenchene (14.14%). Many *Aspergillus* fungi were isolated from smocked *Ethmalosa fimbriata* samples. Three of them could produce mycotoxins on coconut extract agar plate base on the observations under UV light microscope (Figure 3). They were identified as *Aspergillus parasiticus*, *Aspergillus glaucus* and *Aspergillus flavus* on the basis of their macroscopic and microscopic characteristics (Table 1 and Figure 2). The antifungal activity shows that *Cymbopogon citratus* essential oil caused a dose dependent inhibition of mycelial growth on the three tested mycotoxigenic *Aspergillus* (Figure 4). The minimal inhibitory concentrations of their radial growth were (ppm) 1295, 1195, 595 respectively, for *Aspergillus parasiticus*, *Aspergillus glaucus*, *Aspergillus flavus*. These MIC were also the fungicidal concentrations. In the same line the conidia of all the tested *Aspergillus* were inhibited with the same MIC and MIF 100 ppm. Base on that concentration the preservative

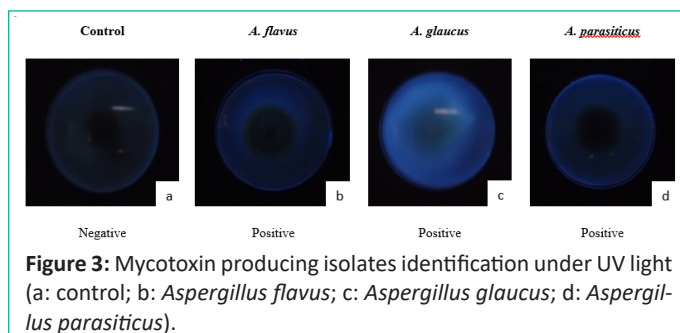


Figure 3: Mycotoxin producing isolates identification under UV light (a: control; b: *Aspergillus flavus*; c: *Aspergillus glaucus*; d: *Aspergillus parasiticus*).

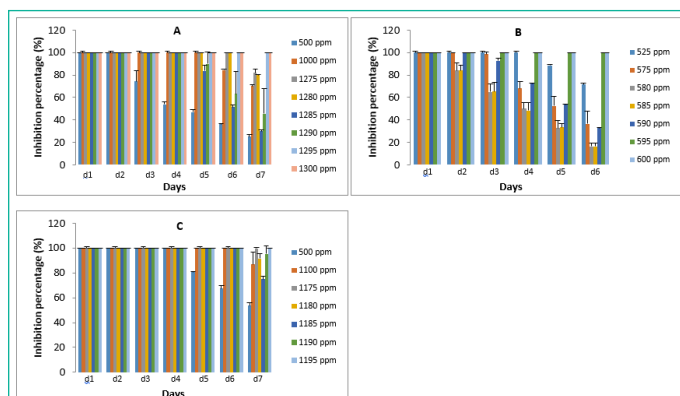


Figure 4: Inhibition percentage of essential oil on fungal: (A) *Aspergillus parasiticus*, (B) *Aspergillus flavus* and (C) *Aspergillus glaucus*.

Table 2: Chemical composition of *Cymbopogon citratus* essential oil.

| N° | Compound | Retention Index | Percentage (%) | Method of identification |
|----|----------------------------|-----------------|----------------|--------------------------|
| 1 | α -pinene | 941 | 1.63 | RI, GC-MS |
| 2 | α -fenchene | 947 | 14.14 | RI, GC-MS |
| 3 | Camphene | 949 | 0.05 | RI, GC-MS |
| 4 | α - phellandrene | 994 | 0.53 | RI, GC-MS |
| 5 | α -terpinene | 1006 | 0.33 | RI, GC-MS |
| 6 | Δ -3-carene | 1011 | 0.04 | RI, GC-MS |
| 7 | γ -terpinene | 1050 | 0.08 | RI, GC-MS |
| 8 | Thiophene | 1056 | 0.74 | RI, GC-MS |
| 9 | Octanol | 1065 | 0.07 | RI, GC-MS |
| 10 | Hydrate trans-sabinene | 1073 | 0.03 | RI, GC-MS |
| 11 | Thujanol | 1100 | 0.06 | RI, GC-MS |
| 12 | Thujone | 1103 | 0.29 | RI, GC-MS |
| 13 | Campholenal | 1110 | 0.34 | RI, GC-MS |
| 14 | Oxyd limonene | 1121 | 1.15 | RI, GC-MS |
| 15 | Terpinen-1-ol | 1139 | 1.94 | RI, GC-MS |
| 16 | Borneol | 1165 | 0.06 | RI, GC-MS |
| 17 | Myrtenol | 1185 | 0.30 | RI, GC-MS |
| 18 | Neral | 1202 | 30.77 | RI, GC-MS |
| 19 | Geraniol | 1212 | 5.08 | RI, GC-MS |
| 20 | Geranial | 1232 | 39.66 | RI, GC-MS |
| 21 | B-citronellol | 1238 | 0.07 | RI, GC-MS |
| 22 | Cinamaldehyde | 1244 | 0.08 | RI, GC-MS |
| 23 | Thymol | 1293 | 0.07 | RI, GC-MS |
| 24 | Acetate of terpinyl | 1306 | 0.16 | RI, GC-MS |
| 25 | Acetate of myrtenyl | 1312 | 0.60 | RI, GC-MS |
| 26 | Menthol | 1328 | 0.07 | RI, GC-MS |
| 27 | Acetate of citronellyl | 1335 | 1.31 | RI, GC-MS |
| 28 | α -copaene | 1378 | 0.09 | RI, GC-MS |
| 29 | β -cubebene | 1390 | 0.07 | RI, GC-MS |
| 30 | α -guaiene | 1444 | 0.06 | RI, GC-MS |
| 31 | α -humulene | 1477 | 0.06 | RI, GC-MS |
| 32 | Germacrene-B | 1576 | 0.09 | RI, GC-MS |
| | Monoterpene hydrocarbons | | 17.60 | |
| | Oxygenated Monoterpenes | | 82.03 | |
| | Sesquiterpene hydrocarbons | | 0.37 | |

test was done with direct application of essential oil by spraying method. Whether it be preventive or curative test, fish on which essential oil was applied did not show any visible signs of contamination after 18 days of incubation compare to control fish on which visible mycelia grow and conidia were observed just after 3 days of incubation (Figure 5). Moreover, attempt to

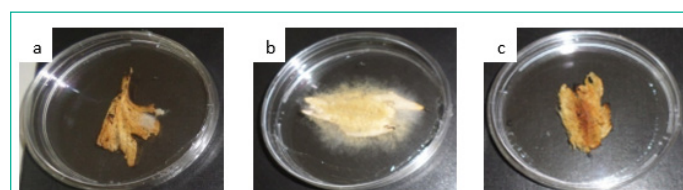


Figure 5: *In situ* preservative test of smoked *Ethmalosa fimbriata* at minimal inhibitory concentration. (a: not treated fish after 3 days; b: not treated fish after 8 days c: treated fish after 18 days).

reisolate fungi from treated fish failed especially for preventive test.

Discussion

Essential oils are natural mixtures of hydrocarbons and oxygen such as alcohols, aldehydes, ketones, carboxylic acids, esters and lactones containing organic substances of plants. They contain three or four major components while the others exist as trace amounts [23].

They have a long history of application as antimicrobial agents in food preservation [24]. In this study the main components found in *Cymbopogon citratus* essential oil was geranial (39.66%), neral (30.77%) and α -fenchene (14.14%). However, many studies revealed quantitative and qualitative variations in the composition of this oil. Therefore, previous studies showed that neral (41.3%), myrcene (33.0%), and geraniol (10.4%), were the major compounds in oil extracted from *Cymbopogon citratus* of southern Benin [25]. The difference between the chemical compositions of essential oils could be explained by the extrinsic and intrinsic factors including age of the plant and agroecological factors such as locality, climatic and seasonal conditions [26]. In this study, three mycotoxicogenic *Aspergillus* species were isolated from smoked *E. fimbriata* namely *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus glaucus*. Previous studies in southern Benin showed that many *Aspergillus* species were associated with the deterioration of smoked *E. fimbriata* including these species [27]. The efficacy of plant essential oils to inhibit growth of different post-harvest food pathogenic fungi has been demonstrated [28,29]. Our finding revealed the effectiveness of *Cymbopogon citratus* essential oil to inhibit the mycelia growth and conidia germination of *Aspergillus spp.*

This inhibition significantly varied with essential oil concentration as well as with the fungal species. Different changes in the antifungal activity against *Aspergillus spp* may be attributed to the lipolytic variation in the composition of cellular membrane of the target microorganisms and their genetic constitution. The efficiency of *Cymbopogon citratus* essential oil as antifungal agents, inhibitory and fungicidal activities against mycelial growth and conidia germination was previously reported in many studies and be attributed to the presence of some interactions between the major and the minor compounds. Indeed, the antifungal activities of *Cymbopogon citratus* oil could be associated with its major components such as geranial, neral, and α -fenchene as well as the minor one present in the oil. They may act together synergistically to induce disturbance in several enzymatic systems that are involved in the energy production and structural component synthesis. Once the phenolic compounds contained in the oil cross the cellular membrane, the membrane enzymes and proteins could cause an opposite flow of protons thereby affecting the cellular activity [31-31]. Moreover, the antifungal activity of *Cymbopogon citratus* may be in part attributed to the presence of an aromatic nucleus and a phenolic hydroxyl (OH) group that are known to be reactive and can form hydrogen bonds with the -SH groups of the active sites of the targeted enzymes, resulting in the deactivation of the enzymes in the fungi [32]. *In situ* tests revealed that essential oil from *C. citratus* inhibited the colonization of *Ethmalosa fimbriata* by *Aspergillus* species at a certain concentration after many days of storage. These results corroborate the finding from previous studies on the potential of *Cymbopogon citratus* essential oil to preserved food stuff against microorganisms. In this line *Cymbopogon citratus essential oil* was used to control

contamination of stored rice and had the potential to be an alternative to synthetic fungicides in the food industry [33].

Conclusion

These findings revealed *in vitro* and *in situ* biological activities effects of *Cymbopogon citratus* essential oil against three mycotoxicogenic *Aspergillus spp* and their preservative potential to protect smoked *Ethmalosa fimbriata* against mold contamination. However, its use for formulation of organic fungicide needs more investigation on post-harvest fish process, essential oil optimization and impact of this essential oil on fish organoleptic properties.

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