



Antifungal potential of Clove essential oil (*Syzygium aromaticum* L.) in the post-smoking preservation of mackerel (*Trachurus trachurus*) in Benin

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Abstract

The present work aims to study the effectiveness of essential oil of *Syzygium aromaticum* (L.) in the post-smoking preservation of mackerel (*Trachurus trachurus*) in Benin. A total of sixty-seven (67) processors selected on smoking zone of Djidje (South of Benin) were surveyed. This survey was coupled with the sampling of smoked fish. Microbiological analyses were performed to identify potential sources of microbial contamination and investigate the bioactivity potential of essential oil extracted from leaves of *Syzygium aromaticum* as antifungal agent in the post-smoking preservation of mackerel (*Trachurus trachurus*). Results obtained showed that smoked fishes collected are contaminated by microorganisms, such as coliforms, *Staphylococcus* spp, yeasts and molds. Results of *in vitro* antifungal tests revealed that the essential oil has strong antifungal activity with the Minimum Inhibitory Concentrations (MIC) of 10 μ l/ml against fungi strains isolated from smoked fishes. Results of microbial count on smoked fish preserved with essential oil of *S. aromaticum* indicated that after five days of storage, no visible growth of bacteria and fungi was detected in samples of smoked fish preserved by adding the essential oil using adjunction method in all essential oil concentrations (0.25, 0.5 and 1ml/70g) tested. However, with the injection method, only the essential oil concentration of 1ml/70g has totally inhibited the bacteria and fungi growth. In opposition, major signs of tampering were noted in control smoked fish after 5 days. The preservation of smoked fish by incorporation essential oil increased the shelf life of the product. However, this protection is not for a long time due to the volatile property of the essential oil.

Keywords: Smoked fish, *Trachurus trachurus*, microbiological quality, essential oils.

Introduction

Fish and fish products have an important place in the diet of the people of West Africa¹. In Benin, fishing has an important place in the national socio-economic balance as it sustains some 500,000 people and accounts for 3% of GDP². However, the conservation of fish is very difficult due to the lack of adequate conservation system and post-harvest losses are estimated at about 20%³. To limit losses, smoking is the primary method of preserving fish in Benin and is still made in the traditional way⁴. Smoking of fish is one of the first traditional processing techniques in Benin. It concerns both the imported frozen fish as fresh fish from the local fishing. Smoked fishes are used for local consumption with a small amount for export to neighboring countries such as Togo, Nigeria, Burkina Faso and Niger⁵. During the process, the smoke is a major factor and its components perform actions on smoked fish, including its organoleptic qualities and the spoilage microbial flora of fish. Smoked mackerel is a widely consumed in Benin because of its availability and accessibility. This food is produced by women in ethnic who specially practice fishing activities in Benin. However, despite the many efforts in the conservation of fish through smoking, the problem still remains because of the perishable nature of smoked fish due to microbial growth. Thus,

the problems mainly related to the preservation during a long period of smoked fish, forcing traders to perform further periodic smoking of the fish already smoked, especially in cases of poor sales. The nutritional quality of these fishes smoked in several times is altered and the exposure to smoke for a long time, often leads to the presence of toxic chemical residue in flesh of the fish. These products constitute serious threats to the health of consumers⁶. The restrictions imposed by the food industry and regulatory agencies on the use of some synthetic food additives have led to renewed interest in searching for alternatives, such as natural antimicrobial compounds, particularly those from plants⁷. Essential oils (EOs) as well as derived compounds possess a wide range of activities of which the antimicrobial activity is most studied⁸⁻¹⁰. Their applications as preservatives in food or antiseptics and disinfectants have been widely investigated¹¹. The biological activities of Eos depend on the qualitative and quantitative characteristics of their components, which are affected by the plant genotype, plant chemotype, organ of plant, geographical origin, season, environmental, agronomic conditions, extraction method, and storage conditions of plants and the essential oils¹². The importance of alternative indigenous products to control alteration fungi are urgently needed¹³. Thus, the present study aims to evaluate the quality of smoked mackerel fish throughout

the processing chain, to identify potential sources of microbial contamination and investigate the bioactivity potential of essential oil extracted from leaves of *Syzygium aromaticum* as antifungal agent in the post-smoking preservation of mackerel (*Trachurus trachurus*) in Benin.

Material and Methods

Collection of plant leaves: Plant materials used for essential oil extraction were dried flowerbuds of *Syzygium aromaticum*. Plants were identified at the Benin national herbarium, where voucher specimens are deposited.

Essential oil extraction: Essential oil tested was extracted by the hydro-distillation method using Clevenger-type apparatus. Oils recovered was dried over anhydrous sodium sulphate and stored at 4°C until it was used¹⁴.

Gas chromatography-mass spectrometry analysis: The essential oil were analyzed by gas chromatography (PerkinElmer Auto XL GC, Waltham, MA, USA) equipped with a flame ionisation detector, and the GC conditions were EQUITY-5 column (60 m x 0.32 mm x 0.25 µm); H₂ was the carrier gas; column head pressure 10 psi; oven temperature programme isotherm 2 min at 70°C, 3°C/min gradient 250°C, isotherm 10 min; injection temperature, 250°C; detector temperature 280°C. Gas chromatography-mass spectrometry (GC-MS) analysis was performed using Perkin Elmer Turbomass GC-MS. The GC column was EQUITY-5 (60 m x 0.32 mm x 0.25 µm); fused silica capillary column. The GC conditions were injection temperature, 250°C; column temperature, isothermal at 70°C for 2 min, then programmed to 250°C at 37°C/min and held at this temperature for 10 min; ion source temperature, 250°C. Helium was the carrier gas. The effluent of the GC column was introduced directly into the source of MS and spectra obtained in the EI mode with 70 eV ionisation energy. The sector mass analyzer was set to scan from 40 to 500 amu for 2 s. The identification of individual compounds is based on their retention times, retention indices relative to C₅-C₁₈ n-alkanes and matching spectral peaks available with the published data¹⁵.

Sampling of fishes: In order to evaluate the quality of fish from receiving fresh fish until smoked fish and their sale, four specific points were selected for sampling. These sampling points are, in first, the fresh fish at the reception. The second sampling is made after pretreatment operations. The third sampling is made after smoking process and finally after three (03) hours of exposure of smoked fish in the sale conditions. Each sample consists of twenty (20) units of fish. Two sites of smoking were investigated. Site selection was done taking into account both the presence of large processors and secondly the fact that these sites represent the large areas of smoking in southern Benin. In addition, the mackerel is one of the most species fish smoked on these sites. The sampling was performed under aseptic conditions: sterile latex gloves are used to protect

hands during the sampling; fish samples are collected and packaged in sterile bags and packaged in a portable cooler. The entire sampling equipment is sterilized beforehand with alcohol at 90°C.

Microbiological analysis: For microbiological analysis, 25 g of each sample and 225 ml of peptone water was added and homogenized. From the initial concentration, appropriate decimal dilutions were prepared and aliquots were plated in duplicates on various media. Plate count agar was used for the total bacterial count. Plates were incubated at 30°C for 72 h. Desoxycholate was used for the total coliforms count and plates were incubated at 30°C for 24 h. Desoxycholate was also used for the faecal coliforms count. In this case, plates were incubated at 44°C and the identification was made using Eosine methylene blue (EMB) medium. Tryptone sulfite neomycin agar was used for anaerobic sulfite-reducer (ASR) count, and tubes were incubated at 37°C for 24 h. The method used for detection of *Salmonella sp.* is that specified by the standard NF V 08-052. After incubation, the number of colonies was tracked, using a colony counter. The number of bacteria expressed as Colony Forming Units per gram (CFU/g) was then determined by calculation, bearing in mind the factors of dilution. All media used for microbiological analysis were prepared as indicated by the manufacturer.

Fungal isolation and identification: The isolation of fungi from samples was performed using dilution plating method. 10 g of each smoked fish sample were added separately to 90 ml of sterile water containing, 0.1% peptone water. This was thoroughly mixed to obtain the 10⁻¹ dilution. Further, 10-fold serial dilutions up to 10⁻⁴ were made. 1 ml volume of each dilution were separately placed in Petri dishes, over which, 10 to 15 ml of potato dextrose agar amended with 60 µg/ml chloramphenicol (PDAC) was poured. The plates were incubated at 28 ± 2°C for 7 days. Fungal isolates from PDAC were sub-cultured on malt extract agar (MEA), and identification was carried out by using a taxonomic schemes primarily based on morphological characters, using the methods described by Singh et al.¹⁶.

Antifungal assay: Antifungal assay was performed by the agar medium assay¹⁴. Yeast Extract Sucrose (YES) medium with different concentrations of essential oil (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 or 8.0 µL/mL) were prepared by adding appropriate quantity of essential oil and Tween 20 to melted medium, followed by manual rotation of Erlenmeyer to disperse the oil in the medium. About 20 ml of the medium were poured into glass Petri-dishes (9 cm). The moulds grown on PDAC for 48h 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 YES and essential oil at different concentrations. Control plates (without essential oil) were inoculated following the same procedure. Plates were incubated at 25°C for 8 days and the mycelial growth was appreciated every day by measuring the average of two

perpendicular diameters passing by the middle of the disc^{17, 18}. The percentage inhibition (PI) of fungal growth was evaluated by the following equation: $PI = [1 - (d/dc)] \times 100$ ¹⁹. d: the diameter of growth zone in the test plate; dc: the diameter of growth zone in the control plate (Petri dish without essential oil).

Test of determination of the fungistatic or fungicidal activity: With the experimental concentrations where neither growth nor germination was observed, the fungistatic or fungicidal activity was tested. This assay consisted by 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 essential oil. If the mycelial growth is always inhibited, the plant extract is fungicidal at this concentration and lead to determinate the Minimal Fungicide Concentration (MFC). In the contrary case, it becomes fungistatic activity which is in relation with the Minimal Inhibitory Concentration (MIC)¹⁸.

Application tests: Application tests are designed to assess, in a semi-controlled environment, the effect of the essential oil in protecting smoked fish against of microbial alterations. To do this, five (21) kilograms of fresh mackerel fish were collected and smoked in standard smoking conditions and according to the diagram shown in figure 1. For the incorporation of essential oil on the smoked fish, two methods were used: incorporation using adsorption methods and incorporation by injection method. The injection of the essential oil was performed using sterile syringes at four body parts of smoked fish. An injection in the gill area, another injection at each of the two side faces of the fish and finally, a third injection point is the area at the tail of the fish. Periodic microbial analyses were performed in order to evaluate the quality of smoked fish during the storage.

Statistical analysis: Experiments were performed in triplicate, and data analyzed are mean ± SE subjected to one-way anova. Means are separated by the Tukey's multiple range tests when Anova was significant (P < 0.05) (SPSS 10.0; Chicago, IL, USA).

Results and Discussion

Result of microbial analysis of smoked fish indicated that samples are contaminated by microorganisms. The main microorganisms counted are shown in tables 1, 2. The results shown that the incidence of spoilage flora and the germs indicators of contamination in fish collected are in relation to the different stages of the process smoking. Indeed, the initial Total flora (Aerobic Mesophilic germ) of $9.4 \cdot 10^6$ CFU/g decreases at 6.710^6 CFU/g after pretreatment and $5.4 \cdot 10^6$ CFU/g after smoking of fish. These results indicate the effectiveness of various treatments on the microbial flora of fish. According to Diouf²⁰, the enumeration of the total flora, in the technological process, can be used to assess the impact of various operations.

Same observations are made with rates of total coliforms ($1.5 \cdot 10^1$ CFU/g to 0.0 CFU/g), yeasts (6.310^3 CFU/g to 1.110^3 CFU/g) and molds (2.110^3 CFU/g to 1.10^1 CFU/g). These results showed that the pretreatment and the smoking, reduced significantly (p < 5%) the spoilage flora and the various contamination germ indicators. The contamination of fish by thermo-tolerant coliforms may be a public health problem because these germs can cause the production of histamine, a biogenic amine resistant to heat and toxic to humans²⁰. The results of the enumeration of *Staphylococcus aureus* in the samples are consistent with those obtained by Djinou²¹ and Goueu²². This compliance obtained could be explained by the fact that the species of fish used (*Trachurus trachurus*) is supervised by regulations during importation.

Analysis of the results also showed that smoking has a more pronounced effect on coliforms and fungal flora. The strong antibacterial activity could be related to the temperature of smoking, the germicidal effect of the smoke or a synergistic action. Indeed, during smoking, smoke particles absorbed by the fish, especially stopped bacterial growth on the surface of the product. The heat of the fire dries fish or meat, and if the temperature is high enough, the fish or meat cooked. This prevents spoilage by bacteria and enzymes. During this treatment, two simultaneous phenomena are observed: dehydration coupled with a flavoring-coloring of the fish and the antiseptic effect of the smoke²³.

Table-1
Spoilage and flora (ufc/g) isolated from smoked fishes collected at different points of production chain

	Total Flora	Totals Coliforms	Fecals Coliformes	<i>E.coli</i>	<i>Staphylococcus aureus</i>	Yeasts	Moulds
Réception	9.4 10 ⁶ a	1.5 10 ¹ a	-	-	-	6.3 10 ³ a	2.1 10 ³
Pretraitment	6.7 10 ⁶ b	-	-	-	-	1.5 10 ⁴ b	10 ¹
Smoking	5.4 10 ⁶ c	-	-	-	-	1.1 10 ³ c	10 ¹
Exposure	2.4 10 ⁶ d	1.4 10 ³ a	1.5 10 ¹	4.0	-	7.6 10 ³ d	3.2 10 ²
microbiological Criteria (AFNOR 2000)	10³	10	10	10	10	10	10

Values are mean (n = 3). The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests. - : Absence.

These observations are similar to those of Degnon et al.²⁴, who also noted a decrease in the microbial load for smoking shrimp (*Penaeus spp.*). The absence of *E. coli* and *Staphylococcus aureus* in fresh fish samples and smoked fish and their presence after exposure in sale conditions, indicated a re-contamination of samples due to the conditions of sale of these fish in the markets. These results are also confirmed by the increase in total and fungal flora in these samples flora. This recontamination of sample is a result of low level of hygiene. The same results are also obtained in food sold in the streets, and germs identified in these foods are mostly Enterobacteriaceae and staphylococci. According to WHO²⁵, epidemiological data in hospitals showed a prevalence of 19% of diarrheal disease and 70% are foodborne. The causes are mainly related to poor processing and sale²⁶. The absence in samples of potentially pathogenic bacteria, including *Salmonella spp.* (table 2) is consistent with results reported by Djinou²¹ Goueu²² and Oulaï et al.²⁷. This is justified by the control made on the fish used (*Trachurus trachurus*) before entering on the territory of Benin. These results show that the consumption of smoked fish does not present a major health risk and are of satisfactory microbiological quality compared to the standard requirements (legislative and regulatory guide French, No. 8155 of 12 December 2000). Indeed, *Salmonella* bacteria mainly *S.tiphy* and *S. paratiphy* have many virulence factors: the presence of pili, toxin production, ability to survive in macrophages and the presence of virulence plasmid²⁸ and are primarily associated with food-borne infections (TIAC). According to the level of anaerobes sulfite-reducers bacteria, their destruction after smoking, confirming the effectiveness of smoking process. However, poor exposure of the food conditions could cause recontamination and microbial proliferation because of the nutritional potential of fish. The high level of fungal contamination (100%) is not only an important factor of change in the quality of the product, but also constitutes health risks to the consumer because of the toxigenicity of certain molds. These factors (mold) are increasingly taken into account in the development of antimicrobial products to maintain product quality.

Table-2
Pathogenic flora (ufc/g) isolated from smoked fishes collected at different points of production chain

	Anaérobic Sulfite Réducteurs	<i>Salmonella spp.</i>
Réception	2.0a	-
Pretraitment	2.0a	-
smoking	-	-
Exposure	1.0a	-
microbiological Criteria (AFNOR 2000)	02	Absence /25g

Values are mean (n = 3). The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests. - : Absence.

The identification of the mycoflora associated with smoked fish, using the mycological identification method of Filtenborg et al.

²⁹ based on the appearance of vesicles, metula, phialides and the ontogénicity of the spores, lead to mainly identify two strains of fungi: *Aspergillus candidus* and *Penicillium camemberti* (table 3). Based on epidemiological studies, *Aspergillus candidus* has been demonstrated as an emerging fungal agent of toenail onychomycosis and Ahmad et al.³⁰ report a case of a toenail infection caused by *A. candidus* in a healthy 60-year-old woman, based on macroscopic and microscopic characteristics of the culture as well as nucleotide sequencing of 28S region. *Penicillium camemberti* is used in the production of Camembert and Brie cheeses, on which colonies of *P. camemberti* form a hard, white crust. It is responsible for giving these cheeses their distinctive taste. However, their presence on smoked fishes could induct risks of undesired fermentation which can affect the quality of smoked fishes.

Table-3
Fungi isolated from smoked fishes collected at different points of production chain

Fungi	Number of case of isolate (N=20)	Occurrence (%)
<i>Aspergillus candidus</i>	06a	30a
<i>Penicillium camemberti</i>	11b	55b
Other	04c	15c

Values are mean. The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests

Results of *in vitro* antifungal tests made in order to investigate the bioactivity potential of essential oil extracted from *Syzygium aromaticum* revealed that the essential oil has strong antifungal activity with the Minimum Inhibitory Concentrations (MIC) of 10 µl/ml against both fungi strains investigated (table 4). This strong antifungal activity of the essential oil results from its chemical composition. Indeed, chemical analysis by GC and GC-MS of essential oil of *Syzygium aromaticum* (L.) enabled the identification of 12 components, (table 4) representing 98.7% of the essential oil. The chemical composition was characterized by eugenol (91.3%), *trans*-β-caryophyllene (4.4%) and eugenyl acetate (2.4%) (table 5).

Table-4
Inhibition Percentage of fungi with the essential oil of *S. aromaticum*

Concentrations of Eo	<i>Aspergillus candidus</i>	<i>Penicillium camemberti</i>
5 µl/ml	51.44 ± 0,5a	43.11 ± 0,3a
7.5 µl/ml	76.0 ± 0,2b	56.88 ± 0,7b
10 µl/ml	100 ± 0,00c	100 ± 0,00b

Values are mean. The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

Table-5
Major components identified as constituents of essential oil of *Syzygium aromaticum*

Components	IR	[%]
6-methyl hept-5-en-one	981	Tr
hept-2-yl acetate	1036	Tr
nonan-2-one	1087	Tr
Linalool	1095	0.1
Perillene	1103	Tr
benzyl acetate	1173	Tr
ethyl benzoate	1173	Tr
Eugenol	1359	91.3
trans-β-caryophyllene	1417	4.4
α-humulene	1452	0.5
eugenyl acetate	1521	2.4
caryophyllene oxide	1582	Tr
Total		98.7

The bioefficacy of the essential oil may be due to the presence of some highly fungitoxic components in the oil such as terpenoids. Indeed, *Syzygium aromaticum* (L.) oil has chemical compositions characterized by terpenoids (eugenol) as main chemical group. Terpenoids are a large group of antimicrobial compounds that are active against a broad spectrum of microorganisms³¹. Their antimicrobial activities are linked to their functional groups and it has also been reported that the hydroxyl group of phenolic terpenoids and the presence of delocalized electrons are important for the antimicrobial activity³¹. A range of essential oil components (linalool, thymol, eugenol, carvone, cinnamaldehyde, vanillin, carvacrol, citral, and limonene) have been accepted by the European Commission for their intended use as flavorings in food products³².

Table 6 presents the results of microbial count on smoked fish preserved with essential oil of *S. aromaticum* comparatively to the control (without essential oil). Results indicated that after five days of storage, no visible growth of bacteria and fungi was detected in samples of smoked fish preserved by adding the essential oil using adjunction method in all essential oil

concentrations (0.25, 0.5 and 1ml/70g) tested. However, with the injection method, only the essential oil concentration of 1ml/70g has totally inhibited the bacteria and fungi growth. In opposition, major signs of tampering were noted in control smoked fish after 5 days. These results indicated that the preservation of smoked fish by essential oil using adjunction method is more efficient than the method of preservation of smoked fish by essential oil using injection method. This efficiency may be due to the protective effect of bioactive substances present in the essential oil which is an effective barrier when coated on the surface, causing adsorption of bioactive molecules.

After ten days of storage, the results of microbiological analyzes indicate a novel presence of bacteria in samples preserved by the addition of essential oil, particularly the total flora and coliforms. However, no visible growth was counted at the fungal flora and staphylococci. These results indicate that despite the pronounced bioactive and protective activities of the essential oil, it provides effective protection against spoilage flora during an average period of five days. After this period, the bioprotection potential of the essential oil decreases gradually. This decrease may be due to the volatile property of the essential oils. Their exposure during a long time causes a gradual departure of volatile molecules that mix with the air.

Conclusion

The present study revealed that the microbial flora associated with smoked fish are coliforms, staphylococci and molds such as *Penicillium camemberti* and *Aspergillus candidus*, which is related to the low level of hygiene applied in the production of that food. *In vitro* antimicrobial tests indicated that essential oil of *Syzygium aromaticum* has a pronounced antimicrobial activity against spoilage flora. The preservation of smoked fish by incorporation essential oil increased the shelf life of the product. However, this protection is not for a long time due to the volatile property of the essential oil.

Table-6
Microbial Flora of smoked fishes preserved with the Essential oil of *Syzygium aromaticum*

Duration	Microbia	Control (without essential oil) *	Adjunction method			Injection method		
			Concentration of Eo (ml/70g of smoked fished)			Concentration of Eo (ml/70g of smoked fished)		
			0.25	0.5	1	0.25	0.5	1
5 days	Total Flora	>300	-	-	-	3.3 10 ¹	2.4 10 ¹	-
	Coliforms	1.5 10 ²	-	-	-	2.3 10 ¹	1.7 10 ¹	-
	<i>Staphylococcus spp</i>	2.6 10 ²	-	-	-	08	07	-
	Fungi and Yeasts	>300	-	-	-	-	-	-
10 days	Total Flora	>300	3.0 10 ¹	1.3 10 ¹	07	12.6 10 ¹	5.9 10 ¹	2.9 10 ¹
	Coliforms	4.5 10 ²	08	04	01	4.9 10 ¹	3.7 10 ¹	1.1 10 ¹
	<i>Staphylococcus spp</i>	7.6 10 ²	15	07	-	5.4 10 ¹	1.4 10 ¹	04
	Fungi and Yeasts	>300	-	-	-	08	08	06

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