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Efficacy of some essential oils in the fight against strains of mould extracted from smoked fish taken in South Benin

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Abstract
The use of plant extracts from aromatic plants in the fight against the alterative factors in foodstuffs is very much in vogue today. This study aims to prove the effectiveness of four (04) plants in the control of moulds isolated from smoked fish. The extraction yield, the majority composition of the oils and their effectiveness against moulds isolated from smoked fish was determined. From the results obtained, it appears that the essential oils of *Syzygium aromaticum* have the highest extraction yield. The determination of the composition of the essential oils showed that they are rich in compounds that suggest strong antifungal activity. The use of essential oils against mould isolated from smoked fish has shown that all oils are effective but that those from *Pimenta racemosa* and *Syzygium aromaticum* are the most effective.

Keywords: Fish, moulds, essential oils, effectiveness

1. Introduction
Fish and fish products provide income and livelihoods for many communities around the world [1]. In Benin, fish conservation and trade is the exclusive work of local women and those involved in redistribution. Smoking and drying are the main methods of preserving smoked fish in Benin and are still done in a traditional way [2]. However, the quality of smoked fish remains detrimental due to the development of microorganisms, particularly moulds. According to the work of [3], moulds are the main actors in the alteration of the marketable quality of smoked fish and thus contribute to their degradation. In addition to altering the marketable quality of fish, these moulds secrete toxins capable of causing serious disease. Since ancient times, men have used essential oils for their cosmetic, dietary and therapeutic needs [4]. Nowadays, many volatile compounds are common ingredients in pharmaceutical preparations. Thymol, for example, is used in dental care for its antiseptic properties or eugenol for its analgesic properties [5]. In an attempt to find new remedies for today's scourges, the scientific community has recently turned to the constituents of essential oils, as a significant number of volatile compounds, such as sesquiterpenes, have shown remarkable pharmacological activities against diseases such as cancer [6]. The work of [7] also showed that plant extracts rich in phenolic compounds and presenting a very marked antioxidant power could play an interesting role in the prevention of cancer because they are stabilizers of free radicals. Work by [8] reported the effectiveness of essential oils of *Mentha piperita* and *Cymbopogon citratus* in combating post-harvest peanut adulterating factors, as well as microorganisms that degrade the quality of local beers marketed in central Benin. Thus, the present study was conducted to evaluate the efficacy of certain essential oils (*Pimenta racemosa, Syzygium aromaticum, Lippia multiflora*, and *Ocimum gratissimu*) on fungal strains isolated from smoked fish in southern Benin.

2. Material and Methods
2.1 Materiels
The equipment used in this study consists of biological material and biological and chemical diagnostic equipment.

2.1.1 Biological materials

The plant material used consists of the leaves of four (04) food and medicinal plants acclimatized in Benin, namely, *Pimenta racemosa, Syzygium aromaticum, Lippia multiflora*, and *Ocimum gratissimum*.

Fig 1: Photo of *Syzygium aromaticum* flower buds **Common name:** Atinkin gbadota

Fig 2: Photo of *Ocimum gratissimum L*. Common name: Tchayo

Fig 3: P=hoto of *Pimenta racemosa* leaves **Common Name:** Laurier

Fig 4: Photo of *Lippia multiflora* leaves **Common Name:** Aglala

2.1.2 Chemical diagnostic equipment

The chemical diagnostic equipment consists of a clevenger-type hydrodistillation apparatus and a Gas Phase Chromatograph (GC), Mass Spectrometer (MS). The determination of the chemical composition of the different essential oils used was carried out at the Max Mousseron Institute of Biomolecules, Montpellier, France.

2.2 Methodology

The methodology followed in conducting this study can be grouped into three stages. The first stage consisted of the collection of plant material and the extraction of essential oils. The second stage consisted of determining the yield and chemical composition of the oils. The third stage consisted of carrying out biological tests and statistical analysis of the results.

2.2.1 Collection of plant material and extraction of essential oils

Fresh leaves of *Pimenta racemosa, Syzygium aromaticum, Lippia multiflora*, and *Ocimum gratissimum* were collected in Abomey-calavi. The essential oils (EO) were extracted using a Clevenger-type hydrodistillation apparatus. Indeed, one hundred (100) grams of fresh leaves are placed in the flask to which about 0.33L of water is added. The essential oil is heated to boiling for three (3) hours to obtain the essential oil.

2.2.2 Determination of the chemical composition of essential oils

2.2.3 Gas chromatographic analysis

The oils were analysed using a VARIAN CP.3380 gas chromatograph equipped with two capillary columns (apolar and polar) and connected to a Varian integrator (Model C-R4A°). The chromatographic analyses of the oils were carried out under the same conditions and the calculations of retention indices were carried out by comparing the retention times of the constituents with those of a mixture of alkanes (C9-C20 for DB-1 and C9-C26 for CWX 20M) obtained under the same experimental conditions.

2.2.4 Analysis by gas chromatography and mass spectrometry

Technical characteristics of the equipment

All the essential oils were analyzed by GPC / SM, a technique that results in the separation of the constituents of the mixture on a column mounted in series with a mass spectrometer. Each constituent leaving the column passes directly into the ionization field of the spectrometer. The molecule subjected to the electronic impact is fragmented. The mass spectra obtained for each compound are compared with data from the literature and the laboratory database [9]. The identification of compounds is based on their retention time, retention indices for C5-C18n alkanes, and matching spectral peaks available in published databases [9].

2.2.5 Evaluation of the biological properties of essential oils

It is mainly concerned the in vitro research of the antifungal power of essential oils (EO) of *Pimenta racemosa, Syzygium aromaticum, Lippia multiflora*, and *Ocimum gratissimum* against the moulds isolated from the different samples of fermented fish collected. Its purpose is to determine the minimum inhibitory concentration (MIC) of essential oils against these different strains of moulds. These tests were carried out on the solid medium Sabouraud agar with chloramphenicol and performed according to the method described by [10]. Different concentrations of essential oil (0.5; 1.0; 2.5 and 5.0 µL.mL-1) were tested by their addition to the culture medium. These plates were then incubated at 25°C and mycelial growth was monitored by measuring the mean

of two perpendicular diameters passing through the middle of the petri dish for 7 days [11]. The MIC was then the lowest concentration at which no fungal growth was observed.

2.2.6 Statistical analysis
To evaluate the effect of essential oils on the microbial flora, an analysis of variance with two classification criteria (essential oils and doses) was carried out. This was preceded by Shapiro and Leven tests which respectively allowed to verify the normality of the residues and the homogeneity of the variances. In case of the existence of a statistically significant difference, the SNK mean structuring test was carried out to compare the mean diameter of moulds according to oils and doses. Descriptive statistics (mean and standard error) were calculated. Also, trend curves were constructed to illustrate the variation of the average mould diameter over time. All statistical analyses were performed using R.3.6.0 software (R core Teem, 2019).

3. Results and Discussion
3.1 Results
3.1.1 Extraction yield of essential oils
The yields obtained after extraction of essential oils from the four (04) plants investigated were presented in Table 1. The analysis of these results showed that essential oil yields varied from one plant species to another. The essential oil yields of the plants studied ranged from 1.04 to 4.76%. Among the plants investigated, the seeds of *Syzygium aromaticum* (Figure 1) were very rich in essential oil with a yield of 4.76%. The leaves of *Pimenta racemosa* (Figure 3) gave a yield of 1.98%. The essential oil yield obtained from the leaves of *Ocimum gratissimum* (Figure 2) was 1.24%. *Lippia multiflora* (Figure 4) leaves yielded 0.96%.

Table 1: Yield in essential oils of the studied plants

Plants	Family	Yield (%)
Syzygium aromaticum	Myrtaceae	4,76 ± 0,11
Pimenta racemosa	Myrtaceae	1,98 ± 0,3
Lippia multiflora	Verbenaceae	1,04 ± 0,18
Ocimum gratissimum	Lamiaceae	1,24 ± 22

3.1.2 Results of the determination of the chemical composition of essential oils
The results of the chemical analysis by gas chromatography (GC) and GC/MS of the essential oil extracted from the leaves of *Ocimum gratissimum*, *Syzygium aromaticum*, *Pimenta racemosa* and *Lippia multiflora* harvested from the Abomey-calavi plateau are presented in Table 2. These results indicated that *Ocimum graticimum* is mainly de thymol (43.5%), p-cymene (12.3%) and γ- Terpinene (12.1%). As for the essential oil of *Syzygium aromaticum*, the analysis of the results reveals that the essential oil consists mainly of eugenol (91.3%), with low levels of hydrogenated monoterpenes (0.1%) and hydrogenated Sesquiterpenes (4.9%). The results also showed that the essential oil extracted from the leaves of *Lippia multiflora*, harvested in Abomey-calavi is mainly composed of 1,8-cineole (50.6%) and sabinene (13.8%), with a content of 23.7% hydrogenated monoterpenes and 65.3% oxygenated monoterpenes. With regard to the essential oil of *Pimenta racemosa*, the main chemical constituents present are: eugenol (60.7%), myrcene (13.4%) and chavicol (13.2%). This sample contains a high proportion of aromatic compounds (56.6%) followed by hydrocarbon monoterpenes (29.9%). Taking into account the chemical composition of these essential oils could predict an antimicrobial activity, given their high proportion of monoterpene alcohol [12] and oxygenated aromatic compounds [13].

Table 2: Majority composition of essential oils

Essentials oils	Composition
Lippia multiflora	1,8-cinéole (50,6%)
	sabinene (13,8%)
Pimenta racemosa	l'eugénol (60,7%)
	myrcène (13,4%)
	chavicol (13,2%)
Syzygium aromaticum	l'eugénol (91,3%)
	Trans-β-caryophyllene (4,4%)
Ocimum gratissimum	Thymol (31,79%)
	P-cymène (15,5%)
	γ-terpinène (12,34%)

3.1.3 Results of the antifungal activity of essential oils on the isolated fungal flora of collected fishes
The results of the antifungal activity of the essential oils on the fungal flora isolated from the collected fish are presented in Figures 5 and 6. Analysis of variance testing the effect of essential oils, dose, time and first order interaction on the diameter of the mills indicated a significant effect (p<0.05) of all these factors (essential oils, dose and time) as well as their interaction on the mean diameter of *A. flavus*, *A. ochraceus*, *A. ustus*, *A. niger*, *A. orizea*, *F. oxyporum* and *A thom*. This means that the variation in the diameter of these moulds from one plant extract to another depends on the doses applied on the one hand.

On the other hand, only the main factors time and essential oils and their interaction are significant on the diameter of *F. solani* and *A versicolor* whereas all the main factors (essential oils, doses and time) and their interaction significantly affected the average diameter of *A. wehener*. Moreover, these results showed that essential oils of *Syzygium aromaticum* and *Pimenta racemosa* had a more pronounced effect on the isolated strains than essential oils of *Lippia multiflora* and *Ocimum gratissimum*.

Tables 3, 4, 5 and 6 present the results of the minimum inhibitory and fungicidal concentrations of the essential oils tested on the strains. The results showed recoveries in *A. ochraceus* and *A. thom* treated with *S. aromaticum* at a dose of 0.125 µL/mL. In contrast, one repeat was found in *A. ochaceus*, *A. flavus* at 0.1 µL/mL and in *A. ustus* at 0.125 µL/mL for *P. racemosa*. However, the results also showed that at low concentrations, the essential oils of *S. aromaticum* and *P. racemosa* inhibited growth recovery of the majority of strains at low concentrations. Indeed, *S. aromaticum* exerted a fungicidal effect on *A. niger*, *A. orizea*, *A. versicolor* and *F. oxysporum* at a dose of 0.1 µL/Ml. It also had the same effect on *A. flavus*, *A.w. Wehenber*, *A. ustus* and *F. solani* at 0.125 µL/mL. However, it was fungicidal on *A. flavus*, *A.w. Wehenber*, *A. ustus* and *F. solani* at 0.125 µL/mL. *A. ochraceus* at 0.25 µL/mL. While *P. racemosa* was fungicidal on *A. orizea* at 0.075 µL/mL, it was fungicidal on *A. niger*, *A. wehenber*, *A. versicolor*, *F. oxysporum* and *F. solani* at 0.125 µL/mL. However, it was fungicidal at 0.25 µL/mL on *A. ochraceus*, *A. thom*, *A. flavus*, and *A. ustus*. It should be noted that these results showed that the essential oils of *O. gratissimum* and *L. multtiflora* also exerted a strong fungal activity on the strains but at high concentrations.

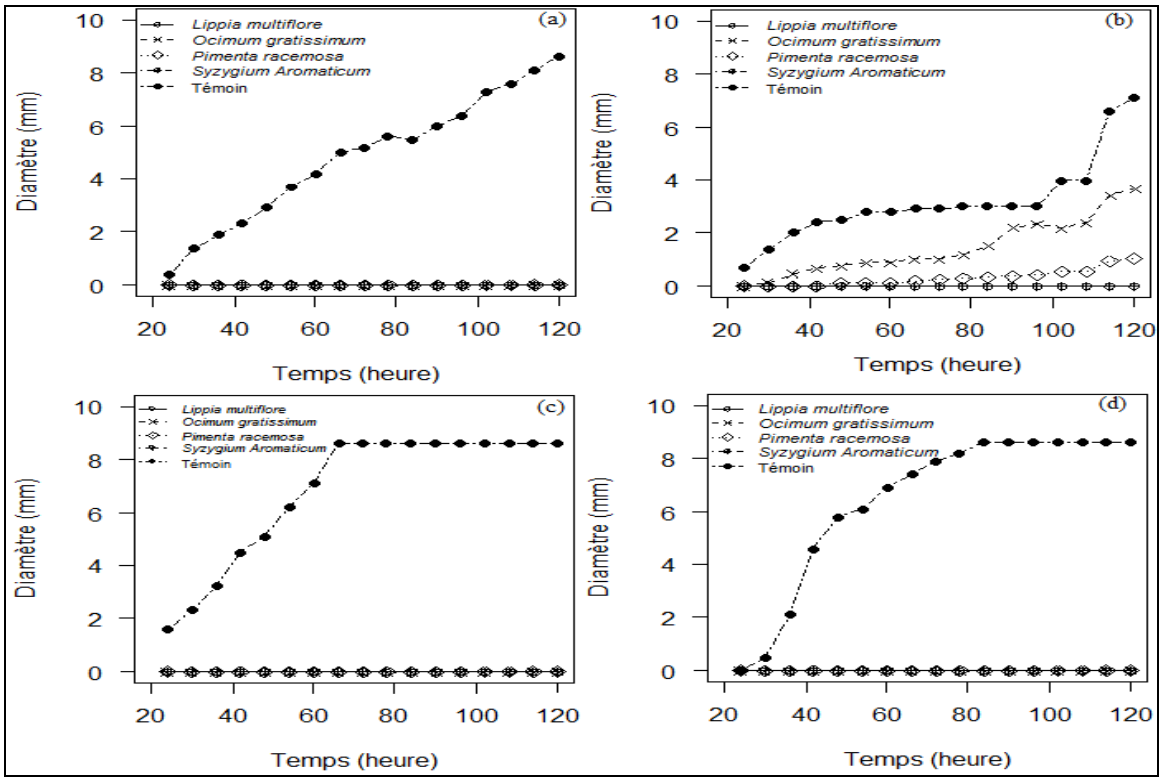

Fig 5: Variation in diameter of essential moulds (a: *F. Solani*, b: *A. Thom*, c: *A. Wehener*, d: *A. Versicolor*) according to oils

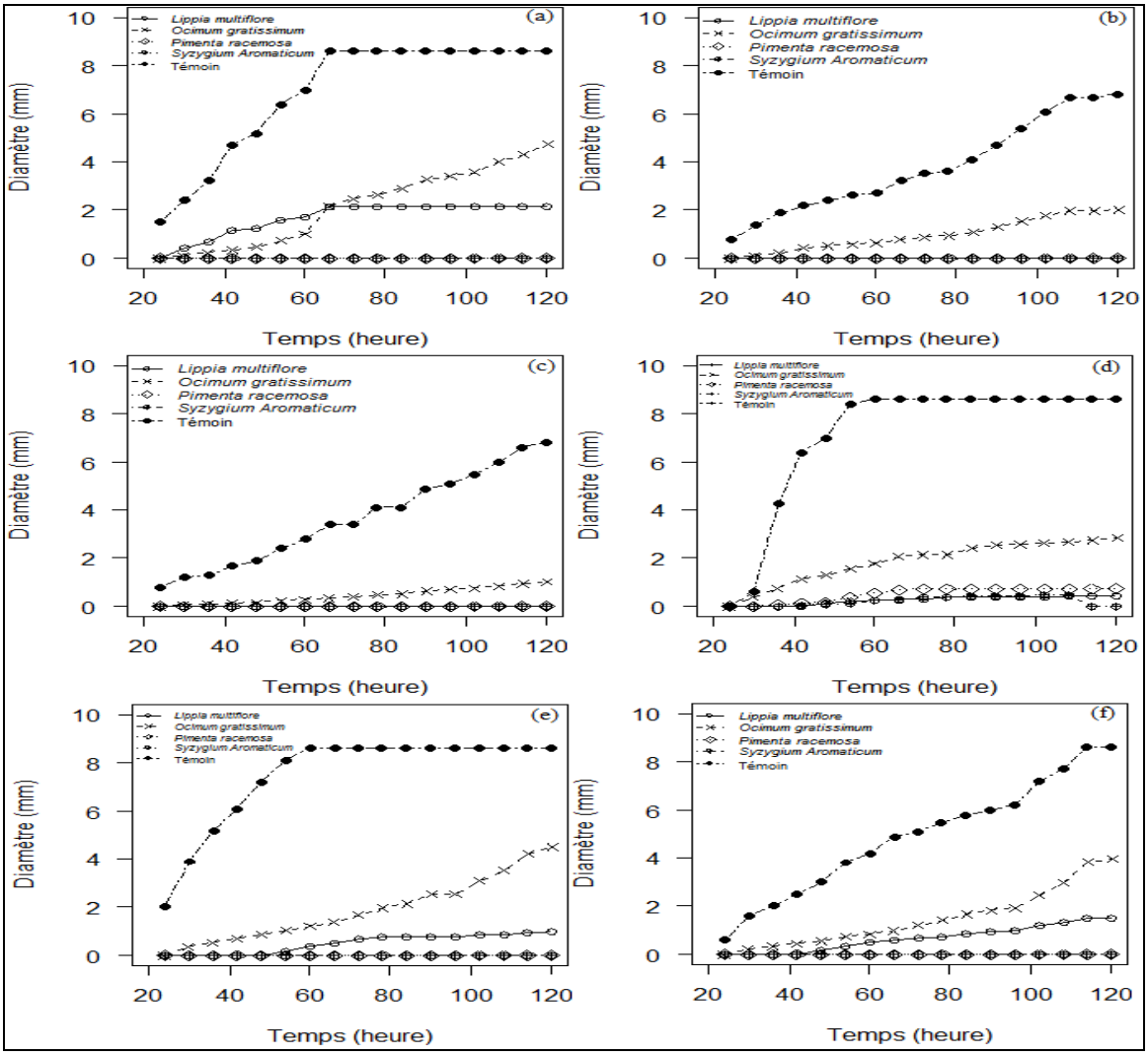

Fig 6: Variation in mould diameter (a: *A. flavus*, b: *A. ochraceus*, c: *A. ustus*, d: *A. niger*, e: *A. orizea*, f: *F. oxyporum*) according to essential oils (a: *F. solani*, b: *At. thom*, c: *Aw. wehener*, d: *A. versicolor*)

Table 3: Minimum inhibitory and fungicidal concentration of *Syzygium aromaticum*

Syzygium aromaticum										
	A. niger	***A. ochraceus***	***A.t. thom***	***A. flavus***	***A. ustus***	***A. orizea***	***A.w. wehenber***	***A. versicolor***	***F. oxysporum***	***F. solani***
CMF (µL/g)	0,1	0,25	-	0,125	0,125	0,1	0,125	0,1	0,1	0,125
CMI (µL/g)	-	0,125	0,125	-	-	-	-	-	-	-

- : Absence of activity in the tested concentration range

Table 4: Minimum inhibitory and fungicidal concentration of *Pimenta racemosa*

Pimenta racemose										
	A. niger	***A. ochraceus***	***A.t. thom***	***A. flavus***	***A. ustus***	***A. orizea***	***A.w. wehenber***	***A. versicolor***	***F. oxysporum***	***F. solani***
CMF (µL/g)	0,125	0,25	0,25	0,25	0,25	0,075	0,125	0,125	0,125	0,125
CMI (µL/g)	-	0,1	-	0,1	0,125	-	-	-	-	-

- : Absence of activity in the tested concentration range

Table 5: Minimum inhibitory and fungicidal concentration of *Lippia multiflora*

Lipia multiflora										
	A. niger	***A. ochraceus***	***A.t. thom***	***A. flavus***	***A. ustus***	***A. orizea***	***A.w. wehenber***	***A. versicolor***	***F. oxysporum***	***F. solani***
CMF (µL/g)	0,25	-	0,375	0,375	0,125	-	0,125	0,125	0,375	0,25
CMI (µL/g)	-	0,375	0,25	0,25		0,375	-	-	0,25	0,125

- : Absence of activity in the tested concentration range

Table 6: Minimum inhibitory and fungicidal concentration of *Ocimum graticimum*

	Ocimum graticimum									
	A. niger	***A. ochraceus***	***A.t. thom***	***A. flavus***	***A. ustus***	***A. orizea***	***A.w. wehenber***	***A. versicolor***	***F. oxysporum***	***F. solani***
CMF (µL/g)	-	0,5	0,375	0,375	0,25		-	0,125	0,5	0,25
CMI (µL/g)	0,5	0,375	-	-	-	0,5	0,125	-	-	0,125

- : Absence of activity in the tested concentration range

4. Discussion

The yield in essential oil of *Syzygium aromaticum* is close to that obtained by [14]. which is 4.74% from dry leaves of *Syzygium aromaticum* studied in Benin. For *Pimenta racemosa* essential oil, the yield is lower than that obtained by [15]. As for the essential oil of *Lippia multiflora*, its extraction yield is close to that obtained by [14]. which is 1.5%. These differences observed in yields could be related to the collection area, the nature of the soil, and the stage of development of the different plants.

The strong antifungal activity observed in the essential oil of *Pimenta racemosa*, could be due to the presence in this oil of molecules with strong antifungal activity such as Eugenol [12]. Indeed, Eugenol belongs to the group of terpenoids which are antimicrobials with a broad spectrum of action [16]. Their antimicrobial activity would be linked to their functional group, as the work of [16] showed that the hydroxyl group of phenolic terpenoids and the presence of delocalized electrons play a very important role in their antimicrobial power. Similarly, according to [17], phenolic compounds such as eugenol, anethole, carvacrol and thymol are mainly responsible for the antimicrobial properties of essential oils. The work of [13] also showed that among monoterpenoids, the most active are carvacrol, thymol, eugenol and dill. [18], also showed that three mechanisms of action could explain the antimicrobial activity of essential oils or their constituents such as thymol, carvacrol and Eugenol. Indeed, [19] reported that this activity is due to damage to cellular enzyme systems, especially those related to energy production and synthesis of structural compounds. [20], reported that this antifungal activity is also related to the inhibition of enzymes responsible for spore germination. Finally, [21] and [22] reported that in *A. parasiticus* and *A. flavus*, the antifungal activity of essential oils and their constituent is much more related to irreversible damage to the cell compartment, membrane and cellular organs.

5. Conclusion

The present work based on the evaluation of the antifungal properties of some plant extracts against the fungal alteration flora of smoked fish allowed us to find that *Syzygium aromaticum* has the highest extraction yield, i.e. 4.76%. The results of the antifungal activity of the essential oils on the fungal flora isolated from the collected fish showed that the essential oils of *P. racemosa* and *S. aromaticum* showed a more pronounced antifungal activity than the other oils with low minimum fungicidal and fungistatic concentrations.

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