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In vitro Activity of *Syzygium aromaticum* against Food Spoilage Fungi and Its Potential Use as an Antiradical Agent

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Abstract The aim of the present study was to investigate the essential oil of *Syzygium aromaticum* from Cameroon for its chemical composition, antiradical and antifungal activities against some common fungi causing spoilage of stored food products. The essential oil, obtained by hydrodistillation of dry fruits, was analyzed by GC and GC/MS. The main components of the oil were eugenol (81.9%), α - elemene (7.7%) and δ - Cadinene (10.2%). Determination of antiradical activity of the oil was studied by the DPPH (diphenyl picrylhydrazyl) method. The antiradical activity of *Syzygium aromaticum* essential oil (SC50 = 23.17 mg/L) was higher than that of butylated hydroxy toluene (BHT), which was used as the reference compound (SC50 = 65.03 mg/L). The evaluation of the antifungal activity of the essential oil of *S. aromaticum* by the incorporation technique showed a strong antifungal activity with minimum inhibitory concentration (MIC) of 300 ppm against *A. niger* and *A. carbonarius*, 400 ppm against *A. flavus*, *A. versicolor* and *F. oxysporium*, and finally 500 ppm against *A. fumigatus*. *A. fumigatus* was the most resistant fungal strain to the essential oil of *S. aromaticum*. Results obtained in the present study indicate the possibility of exploiting *Syzygium aromaticum* essential oil to fight against strains of *A. niger* and *A. carbonarius*, *A. flavus*, *A. versicolor* and *F. oxysporium* responsible for biodeterioration of stored food products.

Keywords Essential oil, *S. aromaticum*, Chemical composition, Antioxidant activity, Antifungal activity

1. Introduction

The presence of molds in foodstuffs can lead to deterioration, with major consequences being the reduction of food value (deterioration of the nutrients) and the reduction of organoleptic qualities (color, taste, odor, texture). Thus, it is estimated that the uncontrolled growth of molds in food stuffs leads to 5-10% loss of food harvests in the world annually [1]. Under favorable conditions of temperature, moisture, pH and composition of substrate, a great number of molds species are able during their development on many foodstuffs, to synthesize and excrete toxic secondary metabolites known as mycotoxins. Among a hundred mycotoxins identified presently, about thirty is truly significant for human and animal health because of their contamination frequency or their toxicity [2]. The principal fungal species that produce mycotoxins belong to the genus *Aspergillus*, *Penicillium* and *Fusarium* [3].

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In addition, the free radicals produced naturally by oxidation of food substances in free radical chain reactions, constitute a major problem in the conservation and preservation of manufactured food. These free radicals can initiate in consumers the oxidation and hence destruction of many organic molecules of biological importance and they are the origin of many diseases [4].

In order to control the damage due to molds and the oxidation of food substances, food industries generally make use of chemical fungicides (benzoic propionates, acids and their salts) and synthetic antioxidants such as butyl hydroxytoluene (BHT) and butyl hydroxyanisole (BHA). However, although endowed with a great efficacy, the use of these synthetic chemical molecules in food preservation is increasingly being reduced in food industries worldwide, because of the enormous negative effects associated to their use such as risk of cancer [5]. On account of these negative effects of synthetic chemical molecules on the health of man, animals and quality of food, the research of alternatives methods of natural origin is very important for the control of these pathogenic molds, as well as free radicals in foodstuffs [6].

Essential oils and their components currently employed as

food flavorings are also known to have antimicrobial and antioxidant properties. Thus, they could be used as food preservatives, since a majority is classified as "safe" [7]. The use of essential oils as bio-conservatives became of great interest, especially for foodstuffs owing to the fact that consumers seek naturally preserved food [8]. These last years, much work has been done on the valorization of the properties of essential oils as natural preservatives [6].

In Cameroon, *Syzygium aromaticum* is largely exploited for its dry fruits, as spice and in traditional medicine (analgesics dental, antinevralgic, disinfectants, aromatic, stimulative, stomachic) [9]. Work carried out on the dry fruits of this plant showed that it was endowed with excellent antibacterial and antioxidant properties [10, 11].

As part of main study aimed on screening antifungal extracts of native plants from Cameroon, we evaluated the antifungal activity of plant used traditionally for several purposes including antimicrobial effects. The aim of the present study was to determine the effects of essential oil of *Syzygium aromaticum* fruits on the growth of *A. niger*, *A. carbonarius*, *A. flavus*, *A. versicolor*, *A. fumigatus* and *F. oxysporium* and to determine its antiradical activity. Results obtained might yield significant information as to whether essential oil of this plant can be used as food preservatives.

2. Materials and Methods

2.1. Fungal Strains

The fungal species used in this work were: a strain of *Aspergillus niger*, *Aspergillus carbonarius*, *Aspergillus fumigatus*, *Aspergillus versicolor*, *Aspergillus flavus* and a strain of *Fusarium oxysporium.* These strains were selected for their high frequency to contaminate foodstuffs and pathogenicity. All strains were offered by the Microbiology Laboratory of the National High School of Agro-Industrial Sciences, University of Ngaoundere, Cameroon.

2.2. Plant Material and Extraction of Essential Oil

The plant material used in this study consisted of the dry fruits of *S. aromaticum* collected in Bafoussam (West Cameroon) in July (2012). These plants were identified at the National Herbarium of Cameroon. The extraction of the essential oil was carried out by hydrodistillation with Clevenger apparatus. The recovered oil was stored at 4°C until use [12]. The extraction yields were calculated in percentage (v/w) relative to the starting plant material.

2.3. Chemical Analysis of Essential Oil

Essential oils obtained were analyzed by gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC/MS).

2.3.1. Gas Chromatography

The oil was analyzed on a Varian CP-3380 GC with flame ionisation detector fitted with a fused silica capillary column (30 m x 0.25 mm coated with DB5, film thickness 0.25 m); temperature program 50°-200°C at 5°C/min, injector temperature 200°C, detector temperature 200°C, carrier gas N_2 1ml/min. The linear retention indices of the components were determined relatively to the retention times of a series of n-alkanes and the percentage compositions were obtained from electronic integration measurements without taking into account relative response factors.

2.3.2. Gas Chromatography/Mass Spectrometry

GC/MS analyses were performed using a Hewlett-Packard apparatus equipped with an HP1 fused silica column (30 m x 0.25 mm, film thickness 0.25 m) and interfaced with a quadrupole detector (GC quadrupole MS system, model 5970). Column temperature was programmed from 70°-200°C at 10°C/min; injector temperature was 200°C. Helium was used as carrier gas at a flow rate of 0.6 ml/min. The mass spectrometer was operated at 70eV.

2.3.3. Identification of the Components by Their Retention Indices

Identification of the constituents was assigned on the basis of comparison of their retention indices and their mass spectra with those given in literature [13].

2.4. Antifungal Activity

Antifungal assay was performed using the agar disc diffusion [12]. Sabouraud dextrose agar (SDA) medium with different concentrations of essential oils (100 ppm, 200 ppm, 300 ppm up to 1000 ppm) were prepared by adding the appropriate quantity of essential oil to the melted medium, followed by manual rotation of the Erlenmeyer flask to disperse the oil in the medium. About 20 mL of the medium was poured into glass Petri–dishes (9 cm x 1.5 cm). Each Petri–dish was inoculated at the center with a mycelial disc (6 mm diameter) taken at the periphery of a fungal strain colony grown on SDA for 48 h. Control plates (without essential oil) were inoculated following the same procedure. Plates were incubated at 25 ± 2°C and the colony diameter was recorded each day. Minimal inhibitory concentration (MIC) was defined as the lowest concentration of essential oil in which no growth occurred. For each concentration, 3 tests were carried out.

2.5. Evaluation of Antiradical Activity

The antiradical activity was determined using 2,2-diphenyl-1- picrylhydrazyl (DPPH) free stable radical scavenger [14], which was dissolved in ethanol to give a 100 μM solution. To 2 mL of the ethanolic solution of DPPH was added 100 μL of a methanolic solution of an antioxidant reference (BHT) at different concentrations. The oil was tested using the same method. The control without antioxidant was represented by the DPPH ethanolic solution containing 100 μL of methanol. The decrease in absorption was measured at 517 nm after 1 h at room temperature. The actual decrease in absorption induced by

the test compound was calculated by subtracting that of the control. The concentration required for 50% reduction (SC_{50}) was determined graphically. All the spectrophotometric measurements were performed with a SAFAS UV-mc2 spectrophotometer, equipped with a multicell/multikinetics measuring system and with a thermostated cell-case.

2.6. Statistical Analysis

Data from three independent replicate trials were subjected to statistical analysis using the Statistica .06, Statistical package [15]. Differences between means were tested using Duncan Multiple Range Test.

3. Results

3.1. Chemical Analysis of Essential Oil

The essential oil obtained by hydrodistillation of the dry fruits of *S. aromaticum* presented a yield of 7.6% (v/w). The analysis of this essential oil by gas chromatography made it possible to identify 13 compounds as cited in table 1 in order of elution from the DB-5 column. As shown on the table, the chemical composition of the essential oil of *S. aromaticum* is dominated by eugenol (81.9%), followed by δ - Cadinene (10.2%) and finally β - elemene (7.7%). These 3 compounds represent approximately 99.8% of the total components of the essential oil of *S. aromaticum*. The other components of this essential oil are present in traces (<0.2%).

3.2. Antifungal Activities

During the incubation period, average values of daily measurements of the diameter of mycelial growth of the moulds were used to follow-up the growth pattern according to the concentration of essential oil.

3.2.1. Effect of *S. aromaticum* Essential Oil on All Fungal Strains

At initial concentrations of 0, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 ppm, the growth diameter of *Aspergillus niger, Aspergillus carbonarius, Aspergillus fumigatus, Aspergillus versicolor, Aspergillus flavus* and *Fusarium oxysporium* were recorded and are illustrated on figure 1.

From the figures it can be seen that mycelial growth increases with increase in incubation time. Statistical analyses showed that growth of the control of all fungal strain was significantly different per incubation day with a strong positive correlation coefficient ($p<0.05$, $r = 0.999$; $r = 0.984$; $r = 0.997$; $r = 0.999$; $r = 0.991$; $r = 0.995$ respectively for *A. niger; A. Carbonarius; A. flavus; F. oxysporium; A. versicolor* and *A. fumigatus*).

The test samples showed an inhibitory effect as growth decreases with increase in concentration of *S. aromaticum* essential oil. With respect to concentration, growth was statistically significant with negative correlation coefficients ($P>0.05$; $r = -0.990$, $r = -0.914$, $r = -0.963$, $r = -0.964$ respectively for *A. flavus, F. oxysporium, A. versicolor* and *A. fumigatus*), but except for *A. niger* and *A. carbonarius* ($P>0.05$; $r = -0.78$, $r = -0.82$ respectively), for which a faster growth was observed at 100 ppm compared to the control.

Essential oil of *S. aromaticum* totally inhibits the mycelial growth of *A. niger* and *A. carbonarius* as from 300 ppm; that of *A. flavus*, *A. versicolor* and *F. oxysporium* as from 400 ppm and finally *A. fumigatus* is completely inhibited at 500 ppm.

3.2.2. Minimum Inhibitory Concentration (MIC)

After noting the concentration at which minimum inhibition was observed from the preliminary tests, the MIC determined for the essential oil of *S. aromaticum* against all fungal strain are shown on table 2.

S. aromaticum essential oil exhibited the lowest MIC values with 300 ppm against *Aspergillus niger, Aspergillus carbonarius* and 400 ppm against *Aspergillus flavus, Aspergillus versicolor, Fusarium oxysporium. S. aromaticum* essential oil was less active against *Aspergillus fumigatus* with MIC value of 500 ppm.

Table 1. The chemical composition of the essential oil of *S. aromaticum* from Cameroon

Number	compounds	Percentage
1	α-Thujene	t
2	β-Pinene	t
3	γ-terpinene	t
4	Linalool	t
5	Terpinen-4-ol	t
6	α-terpineol	t
7	eugenol	81,9
8	β-elemene	7,7
9	β-caryophyllene	t
10	δ-Cadinene	10,2
11	α-Cadinol	t
12	myristic acid	t
13	oleic acid	t
Total Trace0.2%		**99.8%**
The components are listed in order of elution from the DB-5 column		

Table 2. MIC of the essential oil of *S. aromaticum*

Fungal species	MIC (ppm)
Aspergillus flavus	400
Aspergillus fumigatus	500
Aspergillus versicolor	400
Aspergillus niger	300
Aspergillus carbonarius	300
Fusarium oxysporium	400

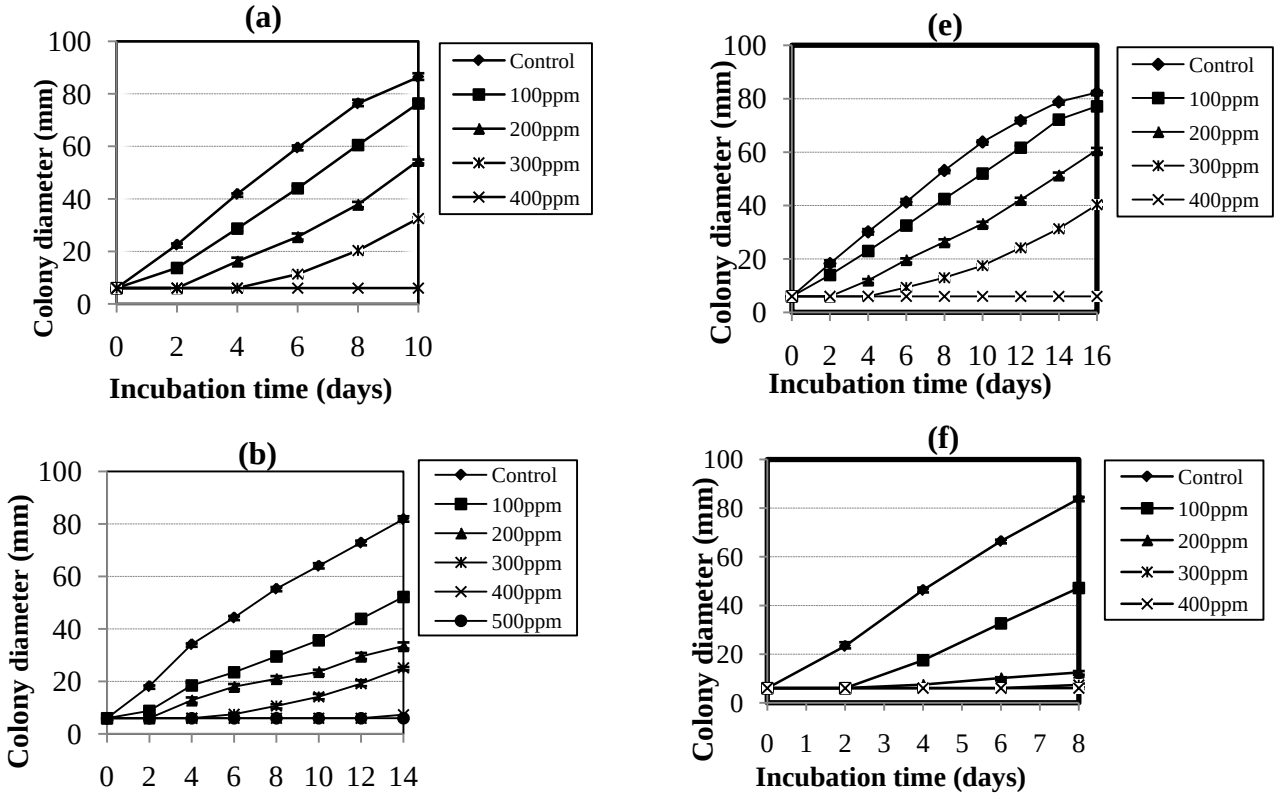

Figure 1. The effect of the different concentrations of the essential oil of *S. aromaticum* on the mycelial growth of: A. flavus (a); *A. fumigatus (b); A. carbonarius (c); A. niger (d); A. versicolor (e) and F. oxysporium (f)*

Table 3. Scavenging activity of *Syzygium aromaticum* essential oil and BHT on DPPH

Concentration (µg/mL)	Essential oil	BHT
	Inhibition percentage (%) [a]	Inhibition percentage (%) [a]
0.0	0.0	0.0
9.77	6.93	11.91
19.53	36.45	15.85
39.06	45.06	37.53
78.13	63.02	54.07
156.25	69.68	60.41
312.50	76.20	76.01
625	78.62	78.32
1250	80.99	79.24
2500	83.36	82.81
5000	85.36	83.26
10000	91.51	84.00
SC_{50} (µg/mL) [b]	23.17	65.03

[a] Mean values obtained from experiments performed in triplicate.
[b] Mean value determinated graphically

3.3. Antiradical Activities of Essential Oil

The results provided by DPPH- test made it possible to obtain the table 3. The concentration which provides 50 % of inhibition (SC_{50}) was used in order to compare the antiradical

activity of the essential oil of *S. aromaticum* with that of the commercial antioxidant molecule (BHT) used as preservative. The results obtained are given in table 3. Generally, it was observed that the scavenging capacity of the essential oil and BHT increases with their concentration in the reaction medium. The following results were obtained: SC_{50} (BHT) = 65.03 ± 0.99 µg/mL and SC_{50} (essential oil) = 23.17 ± 0.58 µg/mL. These results indicate that *S. aromaticum* essential oil is 2.8 times more active than the BHT.

4. Discussion

In the present study, *S. aromaticum* essential oil is rich in eugenol (81.9%), δ - Cadinene (10.2%) and β - elemene (7.7%). This chemical composition is different from that collected in other areas. Indeed, Viuda-Martos *et al.* [10] examined the chemical composition of essential oils of the dry fruits of *S. aromaticum* collected in the area of Ravetllat Aromatics, (Barcelona, Spain). They found 4 components with a prevalence of eugenol (85.5%), β - caryophyllene (10.54%) and the α - humulene (3.12%). This difference in composition is probably due to conditions such as the environment, the genotype, the geographical origin, the period of harvest, the place of drying, the temperature and the duration of drying, the parasites and the method of extraction [16].

Growth of *Aspergillus* sp. and *F. oxysporium* in some foodstuffs are considered as health hazards. With increasing consumer demand for naturally preserved food, examination of essential oils for antimicrobial properties has become attractive to researchers and food processors [17]. *In vitro* results obtained in the present study suggest that essential oil of *S. aromaticum* might be useful agents for control of *Aspergillus* sp. and *F. oxysporium* growth.

Results obtained show that *S. aromaticum* essential oil exhibited the lowest MIC values with 300 ppm against *Aspergillus niger, Aspergillus carbonarius* and 400 ppm against *Aspergillus flavus, Aspergillus versicolor, Fusarium oxysporium. S. aromaticum* essential oil was less active against *Aspergillus fumigatus* with MIC value of 500 ppm.

The significant bioactivity obtained in this present study with the essential oil of *S. aromaticum* could be in relation to its high percentage of eugenol (81.9 %) which is known for its strong antimicrobial activity [18]. Eugenol causes the morphological deformations of the mycelium while acting on the enzymes of the cell wall, such as the chitinases and glucanases [19]. In addition, some components that occur in lesser amount may also contribute to the antifungal activity of the oil, involving probably some type of synergism with the other active compounds.

The antifungal activity of essential oil used in the present study is different from those found by other authors who have used the same method. Viuda-martos *et al.* [20] showed that the essential oil of *Syzygium aromaticum* of Spain completely inhibits the mycelial growth of *A. niger* and *A. flavus* as from 330 ppm. Rana *et al*. [21] had a MIC of 10000 ppm on *Aspergillus* sp. and *F. oxysporum* with the essential oil of *S. aromaticum* collected in India. This difference in activity could be explained by the chemical profile of these essential oils, but also by possible synergistic interactions or antagonistic interactions between the various components present in each essential oil at the origin of an activity much more significant or weaker [22].

Results obtained also show that, the antifungal activity of *S. aromaticum* essential oil is not general for all fungal strains, for *A. niger* and *A. carbonarius*, the growth of the control was less than that of the sample with 100 ppm of *S. aromaticum* essential oil. This could be related to the presence of nutritive substances in essential oils such as fatty acids [23, 24]. *A. niger* and *A. carbonarius* produce the lipases which break down fatty acids, which could explain a faster growth compared to the control.

It was also observed that MICs of *S. aromaticum* essential oil against all fungal strains varied with incubation time. For example, it ranged from 200 ppm, after 2 days of incubation, to 300 ppm after 10 days for *A. niger* and 400 ppm for *A. flavus* respectively. This could be due to the fact that during a relatively long incubation period some volatile components in these oils may evaporate from the media, leading to decrease in their concentration [25].

Interest has increased considerably in finding naturally occurring antioxidant for use in foods to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity [26]. Several authors found that the natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases as well as retard lipid oxidative rancidity in foods [27]. The oil of *S. aromaticum* showed a greater radical scavenging capacity (SC_{50}) than the BHT. This greater SC_{50} of *S. aromaticum* essential oil could be explained by its chemical profile; mainly rich in eugenol (81.9%), but also by synergistic interactions between eugenol and the other minority components present in this essential oil [28]. The antiradical effectiveness of eugenol can be explained by the fact that it acts by three main mechanisms of action [14]: donation of hydrogen followed by the delocalization of the group substituted at the para position; dimerization between two phenoxylated radicals and complexation of $DPPH^{\cdot}$ with an aryl radical.

The results of this present study are in agreement with those of Muhammad *et al.* [11] which showed that the antiradical potential of *S. aromaticum* essential oil (SC_{50} = 4.56 ± 1.07 µg/mL) which originated from Pakistan was more effective than that of BHT (SC_{50} = 2.1 ± 0.92 µg/mL), but these results are contrary to those obtained by Khunkitti *et al.* [29].

5. Conclusions

The results of this study show a great antifungal activity of the essential oil of *S. aromaticum* on the mycelial growth of

A. flavus, A. fumigatus, A. niger, A. carbonarius, A. versicolor and *F oxysporium*, with minimum inhibitory concentration (MIC) about 300 to 500 ppm. Among all these moulds, *A. fumigatus* is the most resistant. The evaluation of the antiradical activity of the essential oil of the dry fruits of *S. aromaticum* in comparison with a reference antioxidant (BHT) showed that this essential oil has an antiradical activity of 2.8 times more active than that of BHT. These results show a possibility in exploiting the essential oil of *S. aromaticum* as an alternative solution to synthetic fungicides and antiradicals used for the conservation of agro-food products.

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