**In vitro Antifungal activities of Essential oils extracted from Fresh Leaves of *Cinnamomum zeylanicum* and *Ocimum gratissimum* against Foodborne pathogens for their use as Traditional Cheese Wagashi conservatives**

Sessou Philippe¹², Farougou Souaibou¹, Azokpota Paulin³, Youssao Issaka¹ and Sohounhloué Dominique²

¹Laboratoire de Recherche en Biologie Appliquée, Ecole Polytechnique d’Abomey-Calavi, Université d’Abomey-Calavi, Cotonou, BENIN
²Laboratoire d’Etude et de Recherche en Chimie Appliquée, Ecole Polytechnique d’Abomey-Calavi, 01 BP 2009 Cotonou, BENIN
³Laboratoire de Microbiologie et de Biotechnologie Alimentaire, Faculté des Sciences Agronomiques, 01 BP 526 Cotonou, BENIN

Available online at: [www.isca.in](http://www.isca.in)

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**Abstract**

Alternative natural additives are needed in order to guarantee food safety in preservation against foodborne pathogens. Aromatic plants are traditionally employed for seasoning and prolongation of shelf life of food. The majorities of their properties are due to the essential oils produced by their secondary metabolism. The objective of this study was to assess in vitro antifungal activity of essential oils extracted from fresh leaves of *Cinnamomum zeylanicum* and *Ocimum gratissimum* obtained by hydrodistillation with Clevenger apparatus against spoilage and pathogens moulds Aspergillus terreus, Aspergillus usus, Aspergillus niger, Aspergillus aculeatus, Penicillium brevicipactum and Scopulariopsis brevicaulis both isolated from wagashi. The screening of their antifungal activity was carried out by determination of antifungal activity parameters as mycelial growth inhibition, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). Results obtained from this study showed that *Ocimum gratissimum* essential oil was the most effective as antifungal agent among the essential oils tested due probably to its prominent concentration in phenolic compound thymol with MIC ranged from 600 to 800 mg/L. *Scopulariopsis brevicaulis* and *Aspergillus terreus* were the most sensible strains with minimum fungicidal concentration (MFC) varying from 600 to 1000 mg/L while *Aspergillus aculeatus* was the most resistant mould of all to essential oil of cinnamon. With the rise of this study, it is shown that *Cinnamomum zeylanicum* and especially *Ocimum gratissimum* essential oils could be regarded as a very promising preservative for wagashi in order to prevent the mycelia growth responsible of its deterioration.

**Keywords:** *Cinnamomum zeylanicum, Ocimum gratissimum,* essential oils, wagashi, Benin, antifungal activity.

**Introduction**

The microbiological quality of a food constitutes one of the essential bases of its aptitude to satisfy the safety of the consumer. A food, exposed to deterioration by the fungus can have a decreasing in its sensory, nutritive and medical characteristics. Fungi are also responsible for the formation of taste and the production of allergenic compounds and mycotoxins¹. Traditional cheese locally called wagashi obtained without ripening in Benin and often colored by red sorghum² is an important source of animal proteins. Due to its proteins content, wagashi could efficacy contribute to the resolution of nutritional problems due to the deficiency of proteins³. However, wagashi is produced and preserved using rudimentary methods under unsanitary conditions which may lead to the contamination of the product by toxingogenic or pathogenic microorganisms especially fungi⁴. The contamination of this product by fungi may contribute to the loss of its quality and safety. In fact, the fungal growth may result in several kinds of cheese spoilage: off-flavours, toxins, mycolytic enzymes and rotting⁵. Furthermore, fungi produce allergenic compounds and toxic metabolites which may penetrate the cheese and affect the consumer’s health⁶,⁷. A better control measures to prevent spoilage of wagashi is necessary to avoid its contamination by mycoflora and minimize public health hazards. The use of synthetic fungicides to control cheese spoilage moulds has been discouraged due to their effects on cheese, carcinogenicity, teratogenicity, high and acute residual toxicity, long-term degradation⁸. One of the major problems related to the use of these chemicals is that the fungi develop resistance⁹,¹⁰. The use of higher concentrations of chemicals, to overcome the microbial resistance further enhances the risk of high level toxic residues in the products¹¹. Alternative natural additives are therefore needed in order, to guarantee food safety in preserved wagashi. Aromatic plants are traditionally employed for seasoning and prolongation of shelf life of food. The majority of their properties are due to the essential oils produced by their secondary metabolism¹². Essential oils (EOs) as antimicrobial agents are recognized as safe natural substances to their user and for the environment and they have been considered at low risk for resistance development by pathogenic microorganisms¹³. Among the aromatic plants, *Ocimum gratissimum* and *Cinnamomum zeylanicum* used as a food spices had shown strong antimicrobial activity¹⁴,¹⁵,¹⁶,¹⁷,¹⁸. Their efficacy on cheese...
mycoflora has been few studied. The efficacy of these essential oils on several moulds isolated from wagashi must be verified in order to measure their potential biopreservation for the valorization of this product. According to our studies, the main components of *Ocimum gratissimum* essential oil were thymol, γ-terpinene and p-cymene while that of *Cinnamomum zeylanicum* essential oil were cinnamyl acetate, cinnamaldehyde and benzyl benzoate.19-21 The objective of this research was to assess in vitro antifungal activity of essential oils of *Cinnamomum zeylanicum* and *Ocimum gratissimum* against six moulds, *Aspergillus terreus*, *Aspergillus ustus*, *Aspergillus niger*, *Aspergillus aculeatus*, *Penicillium brevicompactum* and *Scopulariopsis brevicaulis* both isolated from wagashi produced in Benin for their potential use as wagashi biopreservatives.

### Material and Methods

**Plants material and extraction of the essential oils:** Fresh leaves of *Ocimum gratissimum* and *Cinnamomum zeylanicum* were collected in Abomey-Calavi area (06°27'0.00 N and 2°21'0.00'' E) at University of Abomey-Calavi in Republic of Benin at October-November 2011 and were identified by Dr Yedomohan of National Herbarium of Benin. They were hydrodistilled for about 3 hours, using a Clevenger apparatus. Oil recovered in a dark sterile glass was dried over anhydrous sodium sulfate and stored at +4 °C until it was used.

**Strains of filamentous fungi tested:** The fungi used in this study were: *Aspergillus terreus*, *Aspergillus ustus*, *Aspergillus niger*, *Aspergillus aculeatus*, *Penicillium brevicompactum* and *Scopulariopsis brevicaulis*. They have been isolated and identifying from a traditional cheese wagashi collected near its vendors. Colonies of these moulds isolated from DBRC medium [Rose-Bengal Chloramphenicol Agar Base (CM0549 Oxoid, LTD Basingstoke, Hampshire, England), Chloramphenicol supplement (SR0078E, Oxoid), Dichloran (FLUKA Analytical Lot # SZB8239XY Sigma-ALORICH Product of Germany)] by dilution method were purified by streaking onto Malt Extract Agar and then three point inoculated onto MEA (LAB 37, United Kingdom) and Czapek Yeast autolysate Agar (Czapek-Dox Liquid medium modified (CM 0095 Oxoid LTD Basingstoke, Hampshire, England), NaNO₃ (Qualikems, Laboratory reagent Product No 506112), KCl (Qualikems, Laboratory reagent Product No P021112), MgSO₄.7H₂O (Qualikems, Laboratory reagent Product No M009112), Yeast extract (LP 0021 Oxoid), KH₂PO₄ (Pro analyis, Merk Darmstadt Germany A830673), Sucrose (AnalaR Lot K26990786 009 BDH Laboratory supplies, England), Agar bacteriological (LP 001 Oxoid) before identification based both on macroscopic characters (colony growth, colony diameter) and microscopic characters using the identification schema of Samson et al.23 and Pitt and Hocking.

**Antifungal assay:** The test was performed by the agar medium assay.25 Potato dextrose agar (PDA) medium with different concentrations of essential oil (200, 400, 600, 800 or 1000 mg.L⁻¹) were prepared by adding appropriate quantity of essential oil to melted medium, followed by addition of Tween 80 (100 µL to 100 mL of medium) to disperse the oil in the medium. About 20 ml of the medium were poured into glass Petri-dishes (9 cm x 1.5 cm). Each Petri-dish was inoculated at the centre with a mycelial disc (6 mm diameter) taken at the periphery of a fungus colony grown on PDA for 48 h. Positive control (without essential oil) plates were inoculated following the same procedure. Plates were incubated at 25°C for 8 days 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. The MGI (Mycelia Growth Inhibition) percentage was calculated according to the equation:

\[
MGI = \frac{dc-dt}{dc} \times 100
\]

where dc = mean diameter for control – 6 mm and dt = mean diameter for treated mycelium – 6 mm.

The minimal fungicidal concentration (MFC) values were determined by the method described by Angelini et al. This was done by subculturing the inhibited fungal discs at MICs on PDA medium without essential oil. Observations were recorded after 7 days of incubation at 25°C. Fungal growth on the seventh day was indicative of a fungistatic nature, while the absence of fungal growth denoted a fungicidal action of the oil.

**Statistical analysis:** Data from three independent replicate trials were subjected to statistical analysis using statistica version 6.0. Differences between means were tested using Z-test.

### Results and Discussion

Recently, the scientific interest in biological properties of essential oil (EO) has been increased. New researches about biological active secondary compounds present in EO of plants have been seen as a potential way to control fungal contamination. Our study has assessed potential antifungal activity of essential oils *Cinnamomum zeylanicum* and *Ocimum gratissimum*. The MGI, fungistatic and fungicidal activities values of these two essential oils against the tested fungi are reported in table-1. The results showed that the percentages of mycelial growth inhibition are significantly (p < 0.05) influenced by incubation time and essential oils concentrations. According to figures-1 and 2, mycelia growth was reduced with increasing concentration of essential oil while their growth increased with incubation time. Essential oil of *Ocimum gratissimum* had significant fungistatic activity against all the species investigated with MIC values ranged from 400 to 1000 mg/L and fungicidal activity against *Aspergillus terreus* (MFC = 1000 mg/L) and *Scopulariopsis brevicaulis* (MFC = 600 mg/L) whereas that *Cinnamomum zeylanicum* had total inhibited effect only on *Aspergillus terreus* (MIC = 400 mg/L and MFC = 600 mg/L), *Scopulariopsis brevicaulis* (MIC = 600 mg/L and MFC = 800 mg/L) and *Penicillium brevicompactum* (MIC = 1000 mg/L).
mg/L). Aspergillus aculeatus was the most resistant strain to Cinnamomum zeylanicum essential oil with MGI equal to 59.52% whereas Aspergillus terreus and Scopulariopsis brevicaulis were the most sensible on which the two volatiles extracts tested had fungicidal activity with MFC ranged from 600 to 1000 mg/L. Essential oil of Ocimum gratissimum was the most promising extract as natural agent for biopreservation of tested such as A. niger and A. ustus. The antifungal activity expressed by the two oils on species tested is thought to depend on specific toxicity of their single main active constituents or by their synergic effect. Furthermore, the biological activity of these oils is probably due their prominent concentration in thymol for Ocimum gratissimum and cinnamaldehyde for Cinnamomum zeylanicum.

The more interesting fungistatic activity of essential oil of clove basil oil than that of cinnamon oil is in relation to its richness in phenolic compound, the thymol. Generally, the essential oils possessing the strongest antimicrobial properties against foodborne pathogens contain a high percentage of phenolic compounds such as carvacrol, eugenol and thymol. An important characteristic of thymol is its hydrophobicity, which enables it to partition in the lipids of the fungal cell membrane, disturbing the structures and rendering it more permeable and leakage of ions and other cell contents can then occur.

Many studies have assessed antifungal activities of essential oil of Ocimum gratissimum against different foodborne pathogens. It was reported that volatile oil of O. gratissimum has been reported to have significant antimicrobial effects against both fungi and bacteria. Prakash et al. have reported that clove basil oil can be used as antimicrobial agent against fungal and aflatoxin B1 contamination of spices. According to Nguefack et al., Ocimum gratissimum essential oil detains good antifungal properties against mycotoxinogenic fungi. Essential oil Ocimum gratissimum studied by Faria et al. have showed that essential oil of clove basil is active against phytopathogenic fungi.

The less antifungal activity of cinnamon oil compared to Ocimum gratissimum essential oil could be due to its high content in cinnamyl acetate and benzyl benzoate which are no reported to possess antifungal activity in literature to our knowledge. Therefore, it becomes evident that there is a relationship between the average presence of cinnamaldehyde in essential oil of cinnamon and the moderate antifungal activity of this oil. Indeed, Simic et al. have reported that cinnamon oil had antifungal activity on many fungal species in relation to its content of cinnamaldehyde as major agent of inhibition of fungal growth. Ooi et al., Shahverdi et al. and Unlu et al. have also confirmed this activity of cinnamaldehyde, and could justify this activity against fungi recorded due to the possession of their polar hydrophilic functions (OH, COOH, NH2, and NO2) according to Diallo. Our study concerned cinnamon oil is contrary to those of literature where cinnamon oil possessed high antifungal activity. This difference of reaction of cinnamon oils could be due to their differences in chemical composition.

Conclusion

The present work has assessed and compared the antifungal activities of two essentials oils extracted from Ocimum gratissimum and Cinnamomum zeylanicum against fungi isolated from traditional cheese wagashi. Results obtained indicate that the essential oils of Ocimum gratissimum and Cinnamomum zeylanicum have an interesting antifungal activity. Meanwhile, essential of Ocimum gratissimum was the most active against all the tested strains. These oils especially Ocimum gratissimum essential oil could be used as natural antimicrobial agent in the fight against moulds species responsible for wagashi contamination. For the practical use of these oils as novel fungal-control agents of wagashi, further research is needed on safety issues for human health and this product acceptability when treated with clove basil oil.

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### Table-1

<table>
<thead>
<tr>
<th>Essential oil (mg/L)</th>
<th><em>A. aculeatus</em></th>
<th><em>A. niger</em></th>
<th><em>A. terreus</em></th>
<th><em>A. ustus</em></th>
<th><em>P. brevcompactum</em></th>
<th><em>S. brevicaulis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. zeylanicum</em></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td>0</td>
<td>9.58 ± 0.90b</td>
<td>0</td>
<td>57.69 ± 0.23a</td>
<td>0</td>
</tr>
<tr>
<td>400</td>
<td>0</td>
<td>0</td>
<td>100a (FS)</td>
<td>17.85 ± 0.71d</td>
<td>92.30 ± 0.76b</td>
<td>100a (FS)</td>
</tr>
<tr>
<td>600</td>
<td>35.71 ± 0.43c</td>
<td>7.14 ± 0.28e</td>
<td>100a (FS) (FC)</td>
<td>84.61 ± 0.54b</td>
<td>69.04 ± 1.72c</td>
<td>100a (FS)</td>
</tr>
<tr>
<td>800</td>
<td>50.00 ± 0.20d</td>
<td>45.23 ± 0.81e</td>
<td>100a (FS) (FC)</td>
<td>53.57 ± 0.14c</td>
<td>96.15 ± 0.38b</td>
<td>100a (FS)</td>
</tr>
<tr>
<td>1000</td>
<td>59.52 ± 0.81c</td>
<td>79.76 ± 1.90b</td>
<td>100a (FS) (FC)</td>
<td>79.76 ± 1.79b</td>
<td>100a (FS)</td>
<td>100a (FS)</td>
</tr>
<tr>
<td><em>O. gratissimum</em></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>200</td>
<td>14.28 ± 0.57c</td>
<td>0</td>
<td>42.46 ± 2.05a</td>
<td>0</td>
<td>30.76 ± 0.92b</td>
<td>29.76 ± 1.94b</td>
</tr>
<tr>
<td>400</td>
<td>59.52 ± 0.38d</td>
<td>57.14 ± 0.85e</td>
<td>80.82 ± 1.19b</td>
<td>16.07 ± 0.14f</td>
<td>67.94 ± 0.87c</td>
<td>100a (FS)</td>
</tr>
<tr>
<td>600</td>
<td>96.42 ± 1.55b</td>
<td>85.71 ± 2.8d</td>
<td>90.41 ± 1.36d</td>
<td>92.85 ± 0.71c</td>
<td>79.48 ± 1.42e</td>
<td>100a (FS)</td>
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<tr>
<td>800</td>
<td>100a (FS)</td>
<td>95.23 ± 0.93b</td>
<td>100a (FS)</td>
<td>98.80 ± 0.59a</td>
<td>94.87 ± 0.25b</td>
<td>100a (FS)</td>
</tr>
<tr>
<td>1000</td>
<td>100a (FS)</td>
<td>100a (FS)</td>
<td>100a (FS)</td>
<td>100a (FS)</td>
<td>100a (FS)</td>
<td>100a (FS)</td>
</tr>
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</table>

FS: fungistatic activity; FC: Fungicidal activity; Data in the line followed by different letters are significantly different (p < 0.05). The values are means of three repetitions ± standard deviation
a) Effect of different concentrations of *Cinnamomum zeylanicum* essential oil on *Aspergillus aculeatus* growth

b) Effect of different concentrations of *Cinnamomum zeylanicum* essential oil on *Aspergillus niger* growth

c) Effect of different concentrations of *Cinnamomum zeylanicum* essential oil on *Aspergillus terreus* growth
d) Effect of different concentrations of *Cinnamomum zeylanicum* essential oil on *Aspergillus ustus* growth

e) Effect of different concentrations of *Cinnamomum zeylanicum* essential oil on *Penicillium brevicompactum* growth
f) Effect of different concentrations of *Cinnamomum zeylanicum* essential oil on *Scopulariopsis brevicaulis* growth

**Figure-1**

Effect of different concentrations of essential oil of *Cinnamomum zeylanicum* against moulds investigated
a') Effect of different concentrations of *Ocimum gratissimum* essential oil on *Aspergillus aculeatus* growth

b') Effect of different concentrations of *Ocimum gratissimum* essential oil on *Aspergillus niger* growth
c') Effect of different concentrations of *Ocimum gratissimum* essential oil on *Aspergillus terreus* growth
d') Effect of different concentrations of *Ocimum gratissimum* essential oil on *Aspergillus ustus* growth
e') Effect of different concentrations of *Ocimum gratissimum* essential oil on *Penicillium breviforme* growth

f') Effect of different concentrations of *Ocimum gratissimum* essential oil on *Scopulariopsis brevicaulis* growth

Figure-2

Effect of different concentrations of essential oil of *Ocimum gratissimum* against moulds investigated