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Chemical Composition and Antifungal activity of Essential oil of Fresh leaves of *Ocimum gratissimum* from Benin against six Mycotoxigenic Fungi isolated from traditional cheese *wagashi*

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Abstract

Aromatic plants are traditionally used for seasoning and prolongation of shelf life of food. The majority of their properties are due to the essential oils produced by their secondary metabolism. Essentials oils could guarantee food safety in preserved against foods pathogenic and adulterated microorganisms. Technological application of essential oils, as natural sanitizing agents, requires the definition of optimal conditions. The aim of the present work was to evaluate some antifungal activity parameters as mycelial growth inhibition, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of Ocimum gratissimum essential oil against Aspergillus (flavus and tamarii), Fusarium (poae and verticillioides) and Penicillium (citrinum and griseofulvum) species isolated from traditional cheese wagashi. The essential oil obtained by hydrodistillation of leaves of Ocimum gratissimum (Lamiaceae) collected in Abomey-Calavi (Atlantic, Southern Benin) was analyzed using capillary GC and GC/MS. The major compounds of the EO were thymol, γ-terpinene and p-cymene (28.1, 21.3 and 16.5% respectively). The evaluation of antifungal activity of this oil has shown a significant fungistatic activity against all species tested with a MIC ranged from 800 to 1000 mg/L due probably to the prominent concentration of thymol in this EO. The results have shown the possibility of exploiting Ocimum gratissimum essential oil in the fight against moulds species responsible for biodeterioration of stored wagashi.

Keys words: *Ocimum gratissimum,* volatile extract, chemical composition, *wagashi*, Benin, antifungal activity.

Introduction

Traditional cheese locally called *wagashi* obtained without ripening in Benin is an important source of animal proteins. It is widely consumed in Benin and could efficacy contribute to the resolution of nutritional problems due to the deficiency of proteins[1]. However, *wagashi* is produced and preserved using rudimentary methods under unsanitary conditions which may lead to the contamination of the product by toxinogenic or pathogenic microorganisms especially fungi. 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 rotting[2]. Furthermore, fungi produce allergenic compounds and toxic metabolites which may penetrate the cheese and affect the consumer's health[3]. It was reported that occurrence of Aflatoxin M1 in cheese could probably increase the risk of developing cancer or toxic and carcinogenic effects[4]. Moreover, fungi have been reported by Aissi et al.[5] to cause extensive deterioration of *wagashi* which affects its preservation and may lead of occurring of mycotoxins carcinoms, mutagenicity and liver cancer. 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, and long-term degradation[6]. One of the major problems in relation with the use of these chemicals is the development of resistance[7,8]. The use of higher concentrations of chemicals, to overcome the microbial resistance further enhances the risk of high level toxic residues in the products[9]. 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[10]. The majority of their properties are due to the essential oils produced by their secondary metabolism[6].

Essential oils (EOs) as antimicrobial agents are recognized as safe natural substances to their users and for the environment and they have been considered at low risk for resistance development by pathogenic microorganisms[11]. Among the aromatic plants, *Ocimum gratissimum* is used as a food spice[12-13] and in traditional medicine against pains such as urinary tract infections and respiratory diseases, diarrhea, bronchitis, liver disease and dysentery, cardiovascular disease, HIV1 infections[14]. Several authors have showed strong antimicrobial activities of the essential oil of this plant[13,15-20] but its efficacy on cheese mycoflora was weaker studied in the literature data. The efficacy of this essential oil on fungi isolated from *wagashi*

must be verified in order to measure its potential biopreservation for the valorization of this product.

The main of this research is to assess *in vitro* antifungal activity of essential oil of *Ocimum gratissimum* against six mycotoxigenic fungi, *Aspergillus* (*flavus and tamarii*), *Fusarium* (*poae and verticillioides*) *and Penicillium* (*citrinum and griseofulvum*) isolated from *wagashi* produced in Benin for its potential use as conservative agent.

Material and Methods

Plant material and Extraction of the essential oil: Fresh leaves of *Ocimum gratissimum* 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 2011 and were identified by National Herbarium of Benin. They were hydrodistilled during 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[21].

Chemical analysis of *Ocimum gratissimum* essential oil: Quantitative and qualitative analyses of the essential oil of *Ocimum gratissimum* were carried out by gas chromatography / flame ionization detection (GC/FID) and gas chromatography /mass spectrometry (GC/MS).

GC/FID analyses were performed using a Varian CP-3380 GC equipped with a DB1 (100% dimethylpolysiloxane) fitted with a fused silica capillary column (30 m x 0.25 mm, film thickness 0.25 µm) and Supelcowax 10 (polyethylene glycol) fused capillary column (30 m x 0.25 mm, film thickness 0.25 µm); temperature program 50°-200°C at 5°C/min, injector and detector respectively at 220°C and 250°C, carrier gas N_2 at a flow rate of 0.5 mL.min^{-1}. Diluted samples (10/100, v/v, in methylene chloride) of 2.0 µL were injected manually in a split mode (1/100). The percentage compositions were obtained from electronic integration measurements without taking into account relative response factors. The linear retention indices of the components were determined relatively to the retention times of a series of *n*-alkanes (C_9-C_{20}).

GC/MS analyses were performed using a Hewlett Packard apparatus equipped with a HP1 fused silica column (30 m x 0.25 mm, film thickness 0.25 µm) and interfaced with a quadruple detector (Model 5970). Column temperature was programmed from 70° to 200°C at 10°C/min; injector temperature was 220°C. Helium was used as carrier gas at a flow rate of 0.6 mL.min^{-1}, the mass spectrometer was operated at 70 eV. 2.0 µL of diluted samples (10/100, v/v, in methylene chloride) were injected manually in the split mode (1/100).

The identification of individual compounds was based on the comparison of their relative retention times with those of authentic samples on the DB1 column and by matching the linear retention indices and mass spectra of peaks with those obtained from authentic samples and/or the NBS75K.L and NIST98.L libraries and published data[22].

Strains of filamentous fungi tested: The fungi used in this study were: *Aspergillus flavus*, *A. tamari*, *Fusarium poae*, *F. verticillioides*, *Penicillium citrinum* and *P. griseofulvum*. They have been isolated and identifying from a traditional cheese *wagashi* collected near its vendors. Colonies of these moulds isolated from DBRC medium by dilution method[23] were purified by streaking onto Malt Extract Agar (MEA) and then three point inoculated onto MEA and Czapeck Yeast autolysate (CYA) agar before identification based both on macroscopic characters (colony growth, colony diameter) and microscopic characters using the identification schema of Pitt and Hocking[24].

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) for the dispersion. 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: $\mathrm{MGI} = \frac{dc-dt}{dc} X100$

Where: dc = mean diameter for control – 6 mm, dt = mean diameter for treated mycelium – 6 mm.

The Minimal Fungicidal Concentration (MFC) values were determined by the method described by Angelini et al. [9]. This was done by sub-culturing 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[26]. Differences between means were tested using Z-test.

Results and Discussion

Chemical composition of *Ocimum gratissimum essential oil:* The chemical composition of *Ocimum gratissimum* essential oil with yield equal to 0.78 ± 0.04 % is presented in table -1. Thirty five components which represented 98.9 % of the total oil were identified in the essential oil. The main components were thymol (28.1%), γ-terpinene (21.30%) and p-cymene (16.5%). The other minor compounds in significant percent were myrcene (7.2%), α-thujene (5.8%) and limonene (2.5%). Its chemotype is thymol-γ-terpinene. The different classes of

compounds were hydrogenated monoterpens (60.9%), oxygenated monoterpens (33.1%), hydrogenated sesquiterpens (2.4%), aromatic components (2.1%), and oxygenated sesquiterpens (0.2%). This composition is comparable with data reported previously by Sahouo et al [27] , Oussou et al. [15] for Ivory Coast specimen, where thymol was the prominent compound (70.8 and 34.6%, respectively). The main components of this oil were quite different of those isolated (eugenol 57.82% and (Z)-α-bisabolene (17.7%) by Aquinos Lemos et al [28] from similar oil in Brazil. Matasyoh[16] has found eugenol (68.81%) and methyl-eugenol (13.21%) as main components of essential oil of *Ocimum gratissimum* in Kenya. Saliu et al.[13] have reported that main components of *Ocimum gratissimum* essential oil in Nigeria were eugenol (61.9%) and cis-ocimene (8.2%). These differences in the chemical composition of essential oil of *Ocimum gratissimum* were probably due to the difference of regions, soil, climate and period of harvest.

Table -1
Chemical composition of essential oil of *Ocimum gratissimum*

RI*	Component	Percent Composition	Identification methods
992	**α-thujene**	**5.8**	MS, RI
934	α-pinene	1.2	GC, MS, RI
948	camphene	0.3	GC, MS, RI
969	sabinene	-	MS, RI
973	β-pinene	0.7	GC, MS, RI
986	**myrcene**	**7.2**	GC, MS, RI
998	α-phellendrene	0.7	GC, MS, RI
1010	α-terpinene	4.0	MS, RI
1018	**p-cymene**	**16.5**	GC, MS, RI
1023	limonene	2.5	GC, MS, RI
1025	1,8-cineole	2.1	GC, MS, RI
1042	(E)-β-ocimene	0.3	GC, MS, RI
1054	**γ-terpinene**	**21.3**	GC, MS, RI
1066	*trans*- linalool oxyde	-	GC, MS, RI
1071	*cis*- linalool oxyde	-	GC, MS, RI
1078	p-cymenene	2.1	MS, RI
1085	terpinolene	0.2	GC, MS, RI
1092	linalool	0.2	GC, MS, RI
1160	borneol	0.2	GC, MS, RI
1172	methyl butanoate	-	GC, MS, RI
1180	terpinen-4-ol	1.1	GC, MS, RI
1185	p-cymèn-8-ol	0.3	GC, MS, RI
1189	α-terpineol	0.1	GC, MS, RI
1235	thymol methylether	0.3	GC, MS, RI
1282	**thymol**	**28.1**	GC, MS, RI
1288	carvacrol	0.6	GC, MS, RI
1369	α-copaene	0.1	GC, MS, RI
1417	β-caryophyllene	1.2	GC, MS, RI
1429	*trans*-α-bergamotene	0.1	GC, MS, RI
1449	α-humulene	0.2	MS, RI
1478	germacrene D	0.1	MS, RI
1485	β-selinene	0.4	MS, RI
1590	α-selinene	0.2	MS, RI
1516	δ-cadinene	0.1	MS, RI
1613	oxyde de caryophyllène	0.2	MS, RI
	Hydrogenated monoterpens	**60.9**	
	Oxygenated monoterpens	**33.1**	
	Hydrogenated sesquiterpens	**2.4**	
	Oxygenated sesquiterpens	**0.2**	
	Aromatic components	**2.1**	
	Total	**98.9**	

RI*, Retention index relative to *n*-alkanes (C_9-C_{20}) on a DB1 capillary column (100% dimethylpolysiloxane); Identification methods: GC, identification based on retention times of authentic compounds, MS, identification based on computer matching of the mass spectra of peaks with NBS75K.L, NIST98.L libraries and published data[20], RI, tentative identification based on comparison of retention index of the compounds with published data[20]

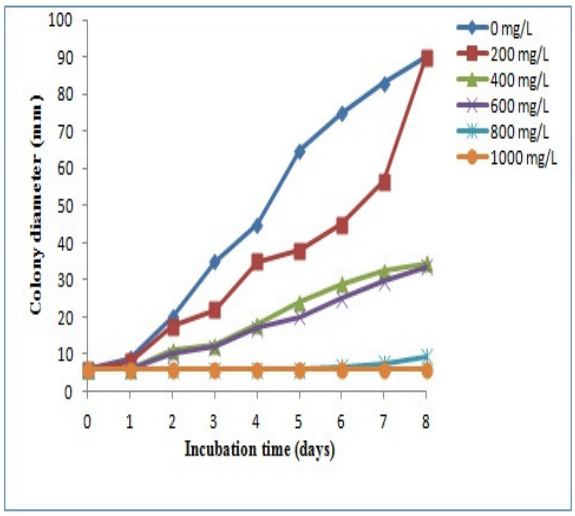
Biological activities of *Ocimum gratissimum* essential oil: The MGI and fungistatic activity values of the essential oil of *Ocimum gratissimum* against the tested fungi are reported in table -2.

The result show that the percentages of mycelial growth inhibition are significantly ($p < 0.05$) influenced by incubation time and essential oil concentrations. Mycelia growth was reduced with increasing concentration of essential oil while their growth increased with incubation time (figure -1). Essential oil of *Ocimum gratissimum* had significant fungistatic activity against all the species investigated with MIC values ranged from 800 to 1000 mg/L. *Penicillium* species and *Fusarium poae* were the most sensible to this essential oil with MIC equal to 800 mg/L. *Ocimum gratissimum* essential oil efficacy against these species isolated from *wagashi* is thought to depend on specific toxicity of its single main active constituents or by its synergic effect[29]. Furthermore, the biological activity of this oil is probably due to its prominent concentration in thymol which is a phenolic compound. Generally, the essential oils possessing the strongest antimicrobial properties against food borne pathogens contains a high percentage of phenolic compounds such as carvacrol, eugenol (2-methoxy-4-(2-propenyl) phenol) and thymol[11,30]. 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[11, 31]. Many studies have assessed antifungal activities of essential oil of *Ocimum gratissimum* against different food-borne pathogens. It was reported that volatile oil of *O. gratissimum* had significant antimicrobial effects against both fungi and bacteria[20]. Prakash et al.[17] have reported that clove basil oil can be used as antimicrobial agent against fungal and aflatoxin B_1 contamination of spices. According to Nguefack et al.[18], *Ocimum gratissimum* essential oil detains good antifungal properties against mycotoxinogenic fungi. Essential oil of *Ocimum gratissimum* studied by Faria et al.[19] has shown that essential oil of clove basil is active against phytopathogenic fungi. These different results testify once again the antimicrobial potential of clove basil essential oil.

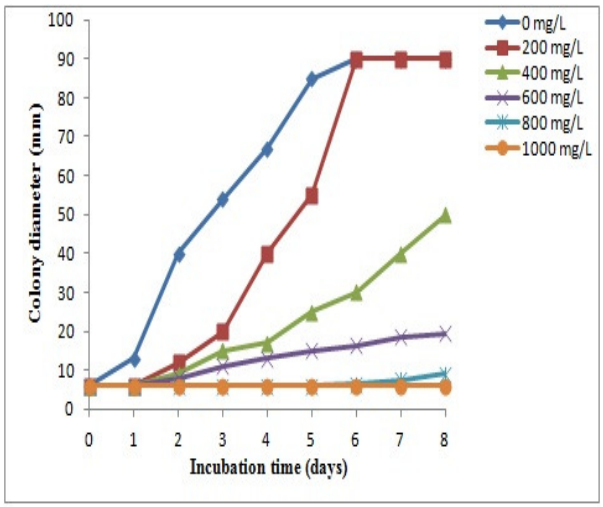
Table -2
Mycelial growth inhibition and fungistatic activity of essential oil of *O. gratissimum* on tested fungi

Essential oil (mg/L)	Mycelia growth inhibition (%)					
	Aspergillus flavus	***Aspergillus tamarii***	***Fusarium poae***	***Fusarium verticillioides***	***Penicllium citrinum***	***Penicillium griseofulvum***
200	0.00 e	0.00 e	59.3 ± 0.8 a	34.8 ± 0.1 c	14.9 ±1.3 d	55.3 ± 0.4 b
400	66.1 ± 1.9 d	47.6 ± 0.5 e	85.6 ± 0.2 a	76.78 ± 0.5 c	51.4 ± 2.2 e	82.9 ± 0.3 b
600	67. 3± 0.7 e	83.9 ± 1.3 c	88.1 ± 0.5 a	87.5 ± 1.4 ab	73.0 ± 2.5 d	85.5 ± 0.3 bc
800	95.8± 1.5 b	96.4± 0.2 b	100 a (Fs)	91.1 ± 0.9 c	100 a (Fs)	100 a (Fs)
1000	100 a (Fs)	100 a (Fs)	100 a (Fs)	100 a (Fs)	100 a (Fs)	100 a (Fs)

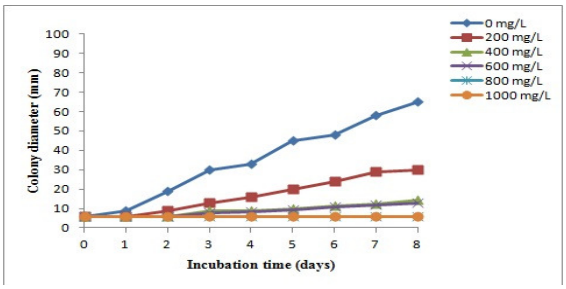
(Fs): fungistatic activity; Data in the line followed by different letters are significantly different ($p < 0.05$). The values are means of three repetitions ± standard deviation



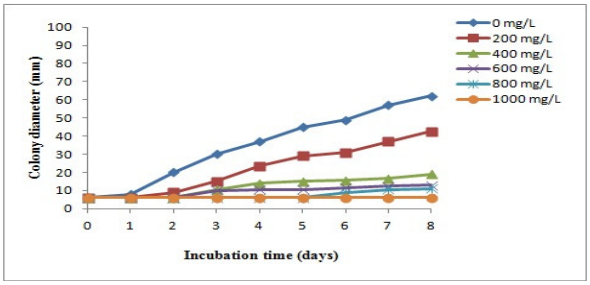
a) Effect of different concentrations of *Ocimum gratissimum essential* oil on *Aspergillus flavus* growth



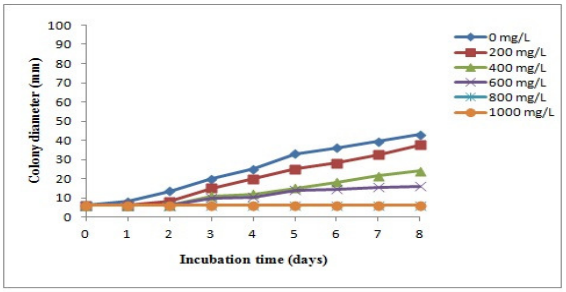
b) Effect of different concentrations of *Ocimum gratissimum essential* oil on *Aspergillus tamarii* growth



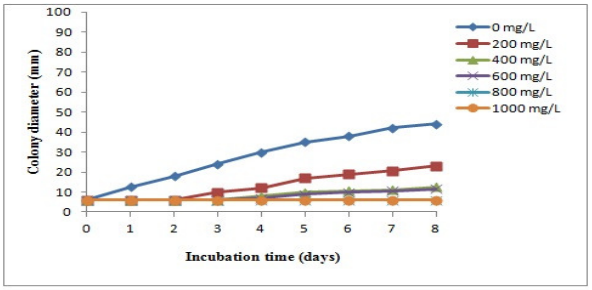
c) Effect of different concentrations of *Ocimum gratissimum essential* oil on *Fusarium poae* growth



d) Effect of different concentrations of *Ocimum gratissimum essential* oil on *Fusarium verticillioides* growth



e) Effect of different concentrations of *Ocimum gratissimum essential* oil on *Penicillium citrinum* growth



f) Effect of different concentrations of *Ocimum gratissimum essential* oil on *Penicillium griseofulvum* growth

Figure -1
Effect of different concentrations of essential oil of *Ocimum gratissimum* against mycotoxinogenic moulds investigated

Conclusion

Essential oil extracted from fresh leaves of *Ocimum gratissimum* with thymol (28.1%), γ-terpinene (21.30%) and p-cymene (16.5%) as major compounds had high effect on the radial growth inhibition of *Aspergillus*, *Fusarium and Penicillium* species isolated from traditional cheese *wagashi* produced in Benin at concentrations ranged from 800 to 1000 mg/L. This essential oil could be used as natural antimicrobial agent in the fight against moulds species responsible for biodeterioration of preserved *wagashi*. For the practical use of this oil as novel fungal-control agent of *wagashi*, further research is needed on safety issues for human health and this product acceptability when treated with clove basil oil.

Acknowledgements

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