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Chemical composition and antifungal activity of essential oil of fresh leaves of *Cinnamomum zeylanicum* from Benin against six mycotoxigenic fungi isolated from a traditional cheese wagashi

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ABSTRACT

The use of essential oils in the food industry, 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 *Cinnamomum zeylanicum* essential oil from Benin against *Aspergillus* (*flavus* and *tamari*), *Fusarium* (*poae* and *verticillioides*) and *Penicillium* (*citrinum* and P. *griseofulvum*) species isolated from traditional cheese wagashi. The chemical composition of the essential oil extracted from fresh leaves was studied by GC-FID and GC-MS and showed (E) ethyl cinnamate with (E)-cinnamaldehyde and benzyl benzoate (39.9, 25.0 and 20.5% respectively) as major compounds. The evaluation of fungal activity showed a significant fungistatic activity against both F*usarium* species and *Penicillium griseofulvum* with a MIC ranged from 800 to 1000 mg/L due probably to the prominent concentration in (E)-cinnamaldehyde of *C. zeylanicum* essential oil. Results obtained in the present study indicate the possibility of exploiting *Cinnamomum zeylanicum* essential to preserve wagashi from *Fusarium and Penicillium* contamination.

Introduction

Traditional cheese locally called wagashi obtained without ripening in Benin is an important source of animal proteins especially for rural populations. It is widely consumed not only by rural poor people but also in the main cities of Benin. Due to its proteins content, wagashi could efficacy contribute to the resolution of nutritional problems due to the deficiency of proteins (Kèkè et al., 2008). 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, discolouration, mycolytic enzymes and rotting (Filtenborg *et al*., 1996). Furthermore, fungi produce allergenic compounds and toxic metabolites (mycotoxins) which may penetrate the cheese and affect the consumer's health (Nasser, 2001). It was reported that occurrence of Aflatoxin M1 in cheese could probably increase the risk of developing cancer (Elkak et al., 2012) or toxic and carcinogenic effects (WHO-IARC, 2002). Moreover, fungi have been reported by Aissi et al. (2009) 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, long-term degradation, and other side-effects in humans (Barkat and Bouguerra, 2012). One of the major problems related to the use of these chemicals is that the fungi develop resistance. The use of higher concentrations of chemicals, to overcome the microbial resistance further enhances the risk of high level toxic residues in the products (Angelini et al., 2006).

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 (Wang and Huang, 2010). The majority of their properties are due to the essential oils produced by their secondary metabolism (Rashid et al., 2010). Great interest is given to these oils by the industry and scientific research for two reasons: on the one hand, their antimicrobial activity (Burt, 2004), on the other hand, essential oils are recognized as substances GRAS, which make them useful as natural preservatives in food industries (Nguefack et al., 2004; Gachkar et al., 2007). Among the aromatic plants, a spice *Cinnamomum zeylanicum* native of Sri Lanka and South India and commonly

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known as cinnamon is used in the food flavour and pharmaceutical industries (Bakkali et al., 2008; Leela, 2008). The oil has shown strong antimicrobial (Ranasinghe et al., 2002; Suhr and Nielsen, 2003; Dusan et al., 2006; Carno et al., 2008; Unlu et al., 2010), acaricidal (Fichi et al., 2007), insecticidal (Yang et al., 2005) activities.

To our Knowledge, there are no published reports on chemical composition and its antifungal activity of essential oil extracted from fresh leaves of *Cinnamomum zeylanicum* acclimated in Benin. The objective of this research was to assess *in vitro* antifungal activity of essential oil of *Cinnamomum zeylanicum* against six mycotoxigenic fungi, *Aspergillus flavus*, *A. tamarii*, *Fusarium poae*, *F. verticillioides*, *Penicillium citrinum* and *Penicillium griseofulvum* both isolated from a traditional cheese wagashi produced in Benin for its potential use as wagashi conservative.

Material and Methods

Plant material and extraction of the essential oil

Fresh leaves of *Cinnamomum zeylanicum* were collected in Abomey-Calavi area (06°27'0.00 N and 2°21'0.00'' E) at Cocotomey in Republic of Benin at October 2011 and were identified by 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 (Yèhouenou et al., 2010).

Identification of chemical components of *Cinnamomum zeylanicum* essential oil

Quantitative and qualitative analyses of the essential oil of fresh leaves of *Cinnamomum zeylanicum* was carried out by gas chromatography/flame ionization detection (GC/FID) and gas chromatography/mass spectrometry (GC/MS) using two columns HP-5 and CABOWAX. The identification of individual compounds was based on the comparison of their relative retention times with those of authentic samples on the HP-5 and CABOWAX columns and by matching the linear retention indices and mass spectra of peaks with those obtained from authentic samples Adams (2007).

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 (ISO 21527-1: 2008) 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 Samson et al (1995) and Pitt and Hocking (2009).

Antifungal assay

The test was performed by the agar medium assay (Tatsadjieu et al., 2009). 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 = (dc-dt)/dc x 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. (2006). 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 (Statistica, 2010). Differences between means were tested using Z-test.

Results and Discussion

Chemical composition of *Cinnamomum zeylanicum essential oil*

The chemical composition of *Cinnamomum zeylanicum* with yield equal to 1.58 ± 0.02 % is presented in Table 1. Thirty nine components which represented 99.4 % of the total oil were identified in the essential oil. The main components were (E)-ethyl cinnamate (39.9%), (E)-cinnamaldehyde (25.0%) and benzyl benzoate (20.5%). The other minor compounds in significant percent were linalool (1.9%), α-pinene (1.8%), camphene (1.1%), sabinene (0.9%), dihydrocinnamyle acetate (0.9%) and oxyde caryophyllene (0.8%). Its chemotype is (E)-ethyl cinnamate - (E)-cinnamaldehyde. In all, 61.6% of components are oxygenated sesquiterpens, 28.5% of oxygenated monoterpens, 6.3% of hydrogenated monoterpens, 2.1% of aromatic components, 0.6% of Hydrogenated sesquiterpens and 0.3% of other components.

The major components found in our essential are quite different compared with data reported by Kamaliroosta et al. (2012) for Tehran specimen; they have found a prominent composition in cinnamic aldehyde 62.09%, para methoxy cinnamic aldehyde 11.56%, alpha copaene 6.98%). This composition is also different compared with data provided by Schmidt et al. (2006) on *Cinnamomum zeylanicum* from Sri Lanka where eugenol 74.9%, followed by caryophyllene 4.1%, benzyl benzoate 3.0%, linalool 2.5%, eugenyl acetate 2.1% and cinnamyl acetate 1.8% are major components. Vernin et al. (1994) analyzed the essential oil extracted from *C. zeylanicum* leaves by GC and GC-MS and have identified p-cymene (21.35%) and eugenol (16.7%) as major components. Essential oils from cinnamon leaf grown in Bangalore and Hyderabad (India) have showed eugenol (81.4-84.5%) as the main component (Mallavarapu et al., 1995). Jirovetz et al. (2001) reported linalool (85.7%) as predominant component of essential oil of *Cinnamomum zeylanicum* collected from South India whereas Raina et al. (2001) had identified eugenol (76.6%) and linalool (8.5%) as major compounds of essential of cinnamon leaves from Little Andaman (India). Suhr and Nielsen (2003) reported that eugenol (71.92%), benzyl benzoate (5.53%) and Cinnamaldehyde (1.88%) were the main components of essential oil of cinnamon leaf. These major compounds obtained are therefore few different of those (trans-cinnamaldehyde

62.79%, limonene 8.31% and cinnamaldehyde propylene 5.55%) obtained by Simic et al. (2004) for the same plant of Belgrade, and those ((E)-cinnamaldehyde 68.95%, benzaldehyde 9.94%, (E)-cinnamyl acetate 7.44%) from *C. zeylanicum* bark essential oil of Turkey (Unlu et al., 2010). The essential oil studied contains less quantity of benzyl benzoate comparatively of essential oil of the same plant from Nigeria which contained 74.8% of benzyl benzoate (Tira-Picos et al., 2009) and from Brahmaputra Valley, India cinnamon leaf which contained 65.4% of benzyl benzoate as main component of essential oil according to studies of Nath et al. (1996).

These differences noticed in the chemical composition of essential oil of *Cinnamomum zeylanicum* studied compared to those of literature is probably due to the difference of regions, soil, climate and period of harvest.

Biological activities of *Cinnamomum zeylanicum* essential oil

The MGI and fungistatic activity values of the essential oil of cinnamon 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). The radial growth of *Penicillium griseofulvum* specie was totally inhibited by essential oil of *Cinnamomum* at 800 mg/L whereas those of *Fusarium* species were inhibited by the same oil at 1000 mg/L. *Penicillium griseofulvum* was the less resistant moulds with MIC equal to 800 mg/L whereas *Aspergillus* species were the least sensitive to cinnamon oil with MGI value inferior to 38% (Table 2).The MIC value of cinnamon against *Fusarium* species moulds assayed were 1000 mg/L. In sum, essential oil of cinnamon had a fungistatic activity against only *Fusarium* species and *Penicillium griseofulvum* investigated. *Cinnamomum zeylanicum* 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 (Lopez-Reyes et al., 2010). It was reported that *Cinnamomum zeylanicum* essential oil is active against the postharvest pathogens such as *Rhizopus nigricans*, *Aspergillus flavus* and *Penicillium expansum*, the causal agents of jujube or orange fruit (Xing et al., 2010).

The biological activity of this oil is linked to it richness in oxygenated sesquiterpens mainly to cinnamaldehyde. Indeed, Simic et al. (2004) have showed that cinnamaldehyde completely inhibit fungal growth and aflatoxin production. 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. Cinnamaldehyde displays a linkage property with the main lipid of fungi cellular membrane: the ergosterol. According to Murray et al. (2003), antifungal agents such as essential oils kill the fungal cell via binding primarily to ergosterol, the major sterol found in fungal cellular membrane. This binding destroys the osmotic integrity of the membrane, followed by leakage of intracellular potassium, magnesium, sugars, and metabolites which lead to cellular death. The less inhibition of *Aspergillus* species by essential oil of *C. zeylanicum* studied is probably due to its high content in (E)-ethyl cinnamate which doesn't possess antifungal activity on *Aspergillus* species particularly *Aspergillus fumigatus* at concentration of 1000 µg/g (Dubey et al., 2000) and could interfere negatively with the active component cinnamaldehyde.

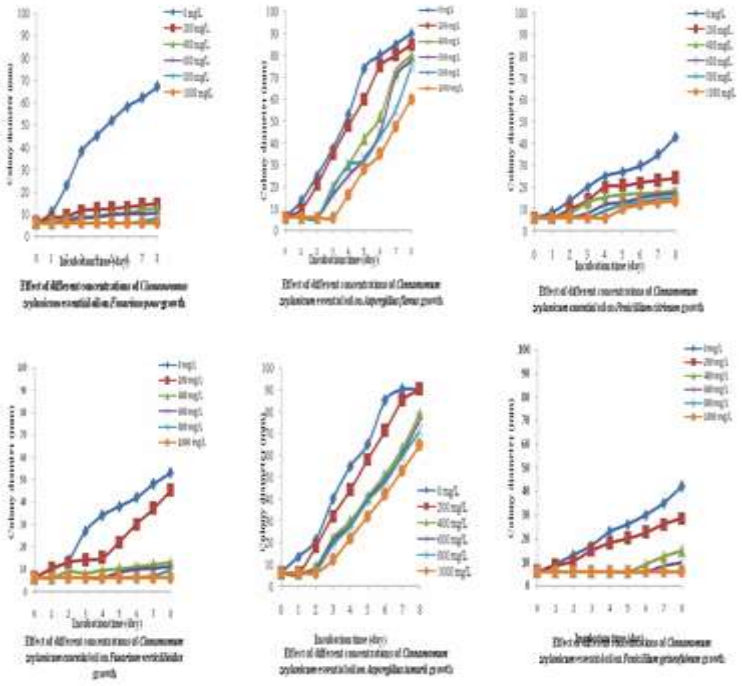

Figure 1. Effect of different concentrations of essential oil of *Cinnamomum zeylanicum* against mycotoxinogenic moulds investigated

Conclusion

Essential oil extracted from fresh leaves of *Cinnamomum zeylanicum* with (E) ethyl cinnamate (39.9%), (E)-cinnamaldehyde (25.0%) and benzyle benzoate (20.5%) as major compounds had high effect on the radial growth of *Fusarium poae, Fusarium verticillioides and Penicillium griseofulvum* isolated from wagashi produced in Benin one the one hand. Moreover, this oil exhibited a weakness antifungal activity mainly against *Aspergillus* species tested. Cinnamon essential oil could be used as natural antimicrobial agent in the fight against strains of *Fusarium and Penicillium species* responsible for biodeterioration of stored wagashi. For the practical use of this oil as novel fungal-control agent, further research is needed on safety issues for human health.

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Table 1 : Chemical composition of essential oil extracted from fresh leaves of *Cinnamomum zeylanicum*

N°	Noms des composés	RI (HP-5)	RI (Adams)	RI (Carbowax)	(%)
1	α-pinene	936	932	1031	1.8
2	camphene	952	946	1077	1.1
3	benzaldehyde	962	952	1533	0.5
4	β-pinene	981	974	-	t
5	sabinene	976	969	1118	0.9
6	myrcene	991	988	1164	0.2
7	α-phellandrene	1007	1002	1171	0.2
8	para-cymene	1026	1020	1276	0.3
9	limonene	1031	1024	1205	0.7
10	β-phellandrene	1032	1025	1216	0.6
11	alcool benzylique	1034	1026	1841	0.2
12	trans-β-ocimene	1047	1044	1253	0.3
13	Linalool	1101	1095	1542	1.9
14	(E)-but-1-enyl benzene	1142	1135	-	t
15	Camphre	1150	1141	-	0.2
16	3-phenyl prop-3-en-1-ol	1165	1165	1886	0.6
17	hydrocinnamaldehyde	1169	-	1789	0.1
18	Ethyl benzoate	1172	1169	1682	0.7
19	terpinen-4-ol	1180	1174	1605	0.1
20	α-terpineol	1193	1186	1696	0.1
21	3-phenyl pro-2-enal	1222	1222	-	0.2
22	M=134	1263	-	-	0.1
23	(E)-cinnamaldehyde	1283	1267	2035	25.0
24	(E)-alcool cinnamique	1307	1303	2258	0.2
25	eugénol	1359	1356	-	0.1
26	dihydrocinnamyle acetate	1372	1388	-	0.9
27	α-copaene	1381	1374	1498	0.2
28	β-cubebene	1390	1387	-	t
29	(E)-β-caryophyllene	1427	1421	1608	0.4
30	cinnamyl acetate	1439	1443	2144	t
31	(E)- ethyle cinnamate	1459	1465	2052	39.9
32	M =222	1462	-	-	0.1
33	caryophyllene oxide	1594	1582	1897	0.8
34	humulene epoxyde II	1609	1608	-	t
35	caryophylla-4(12),8(13)-dien-5-ol	1642	1644	-	0.1
36	M= 218	1646	-	-	0.1
37	benzyl benzoate	1782	1759	-	20.5
38	cinnamyle benzoate	2000	-	-	0.2
39	acide hexadecanoique	2227	1859	-	0.1
	Hydrogenated monoterpens				6.3
	Oxygenated monoterpens				28.5
	Hydrogenated sesquiterpens				0.6
	Oxygenated sesquiterpens				61.6
	Aromatic components				2.1
	Others components				0.3
TOTAL					99.4
t (traces) ≤ 0.05%; RI: Retention Indice					

Table 2: Mycelial growth inhibition and fungistatic activity of essential oil of *Cinnamomum zeylanicum* on tested fungi

Essential oil (mg/L)	Mycelia growth inhibition (%)					
	Aspergillus flavus	*Aspergillus tamarii*	*Fusarium poae*	*Fusarium verticillioides*	*Penicllium citrinum*	*Penicillium griseofulvum*
200	5 .95 ± 0.20 e	0.00 ± 0.00 f	86.06 ± 0.48 a	17.02 ± 0.23 d	51.35 ± 1.07 b	37.50 ± 2.20 c
400	11.90 ± 0.12 f	13.09 ± 0.03 e	89.34 ± 0.06 a	85.10 ± 1.24 b	66.21 ± 0.45 d	75.00 ± 1.02 c
600	14.88 ± 0.40 c	16.67 ± 1.60 c	92.62 ± 1.30 a	89.36 ± 0.35 a	70.27 ± 0.26 b	88.89 ± 0.32 a
800	17.86 ± 0 .60 e	22.62 ± 0.22 d	96.72 ± 0.24 a	92.55 ± 0.25 b	75.68 ± 1.30 c	100 Fs a
1000	35.7 1± 1.20 c	30.36 ± 0.45 d	100 Fs a	100 Fs a	79.73 ± 0.21 b	100 Fs a

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