International Research Journal of Biological Sciences ISSN 2278-3202
Vol. **1(6),** 18-25, October **(2012)** *I. Res. J. Biological Sci.*

Chemical composition and Antimicrobial activities of *Cinnamomum zeylanicum* Blume dry Leaves essential oil against Food-borne Pathogens and Adulterated Microorganisms

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Available online at: www.isca.in
Received 30th June 2012, revised 4th July 2012, accepted 5th July 2012

Abstract

Essential oils of cinnamon species have lot-of biological activities including antimicrobial, antioxidant and antifungal properties. Furthermore, cytotoxic and apoptotic activities of several constituents were identified throughout its biological properties. In the present paper, essential oil (EO) obtained by hydrodistillation of leaves of Cinnamomum zeylanicum Blume (Lauraceae) collected respectively at Cocotomey (Atlantique, Southern Benin) was analyzed using capillary GC and GC/MS. The aim of the present work was to evaluate antibacterial and antifungal activities of this Eo in relation with its chemical composition. Minimum inhibitory concentration (MIC) and mycelial growth inhibition were investigated on C. albicans, Aspergillus ochraceus, Aspergillus parasiticus, Fusarium oxysporum and Penicilium digitatum. The EO was mainly composed of (E) cinnamaldehyde (37.6%), cinnamyl acetate (23.7%), cinnamyl benzoate (16.4%) and other compounds. The oil have shown significant antibacterial properties against E. coli ATCC 25922 (MIC = 0.80mg.ml-1), S. aureus ATCC 25923 (MIC = 0.20mg.ml^{-1}) and fungicidal activity against C. albicans (MIC = 0.40mg.ml^{-1}), fungistatic activities against Aspergillus ochraceus, Aspergillus parasiticus and fungicidal against Fusarium oxysporum and Penicilium digitatum. Results obtained in the present study indicate the possibility of exploiting cinnamon essential oil in the fight against food-borne pathogens and adulterated microorganisms responsible for biodeterioration of stored foodstuffs.

Key-words: antimicrobial activities, *Cinnamomum zeylanicum*, MIC, fungistatic, fungicidal, activities.

Introduction

Cinnamomum zeylanicum Blume belongs to the botanical family of Lauraceae and was introduced in West Africa through the National herbarium of Ghana (University of Kumasi) in 1970. Rohwer et al[1] have reported that among the 2500 species which composed the Lauraceae family, *Cinnamomum zeylanicum* Blume grown over the world in Sri Lanka Madagasgar, Comor Islands, India and Indochine. In Benin, the plant was usually met in the area of Abomey-Calavi and Cocotomey, two agglomerations near Cotonou, the economic capital of Benin.

EO of *Cinnamomum zeylanicum* Blume has shown various therapeutic actions[2]. Some people from West Africa (Ghana, Togo) usually enhanced flavor of smoking fishes with *Cinnamomum zeylanicum* Blume leaves. Essential oil is used as additive in tomatoes and spices soups as preservative, and against unexpected fermentation of the mixture. Baratta et al[3] have reported antimicrobial and antioxidant properties of the EO and its potential use in foods preservation. Simic et al[4] have revealed its antifungal activities. Many studies revealed the apoptosis-inducing activity of some compounds near cinnamon like taxol[5,6].

Any previous work was devoted in our knowledge to the antimicrobial properties of *Cinnamomum zeylanicum* Blume EO in Benin. The aim of the present studies is to investigate antimicrobial activity of *Cinnamomum zeylanicum* EO in relation with its chemical composition, especially to check and confirm its potential use in common road foods as preservative.

Material and Methods

Collect and identification of plant material: Leaves of *Cinnamomum zeylanicum* were collected in October, 8th, 2011 on a plain area named Cocotomey far away fifteen kilometers from Cotonou. The botanical material was identified by Prof. Akoegninou and a voucher specimen was deposited in the Herbarium of the Botanic Garden of Department of vegetal Biology (University of Abomey-Calavi).

Microorganisms: Antimicrobial tests were conducted in LERCA, Polytechnic School of Abomey-Calavi (LERCA/EPAC/UAC) using Gram negative bacteria *E. coli* (ATCC 25922); Gram positive bacteria *S. aureus* (ATCC 25923); and the fungi, *Candida albicans* ATCC 10231, *Fusarium oxysporum*, *Penicillium digitatum*, *Aspergillus ochraceus* and Aspergillus *parasiticus*. All microorganisms

were supplied by the Laboratory of Food safety and water quality of Ministry of Health and controlled with API System (apparatus and identification Procedures La Balme-les-Grottes Cedex 2 France).

Extraction of essential oil: The essential oil was extracted from fresh leaves (150 g) by hydrodistillation during 3 hours, using a Clevenger apparatus, in LERCA/Polytechnic School of Abomey-Calavi, University of Abomey-Calavi (LERCA/EPAC/UAC). Oil recovered was dried over anhydrous sodium sulfate and stored at +4 °C until it was used. The extraction yield was estimated in 1.1 ± 0.2%.

Analysis of essential oils: Gas Chromatography : GC analyses were performed on a Varian gas chromatograph, model CP-3380, with flame ionization detector containing two silica capillary columns: HP5 J&W Agilent (5%-phenyl-methylpolysiloxane) capillary column (30 m x 0.25 mm i.d. x 0.25 µm film) and Supelcowax 10 (polyethylene glycol) fused capillary column (30 m x 0.25 mm i.d. x 0.25 µm film); N_2 was the carrier gas at 0.8 mL/min; injection type 1 µL 10:100 CH_2Cl_2 solution, split ratio 1:100; injector temperature 220°C, detector temperature 250°C; temperature program 60-220°C at 3°C/min, then kept at 220°C during 20 minutes. The linear retention indices of the components were determined relative to the retention times of a series of *n*-alkanes. The percentage composition of the essential oils was computed by the normalization method from the GC-FID peak areas on the HP5 capillary column, assuming an identical mass response factors for all compounds.

Gas Chromatography-Mass Spectrometry: GC/MS analyses were performed using a Hewlett–Packard GC 5890 series II equipped with a HP5 (5%-phenyl-methylpolysiloxane) fused silica column (30 m x 0.25 mm; film thickness 0.25 µm) and a DB-Wax fused silica column (30 m x 0.25 mm; film thickness 0.25 µm) interfaced with a quadrupole detector (model 5972) applying the same temperature programs; injector temperature, 220°C; MS transfer line temperature, 180°C; carrier gas, helium at a flow rate of 0.6 mL/min; injection type, split, 1:10 (1 µL 10:100 CH_2Cl_2 solution); ionization voltage, 70 eV; electronmultiplier 1460 eV; scan range 35-300 amu; scan rate, 2.96 scan/s.

The identification of the constituents was based on comparison of their relative retention times with either those of authentic samples or with published data in the literature[7] and by matching their mass spectra with those obtained by from authentic samples and/or the NBS75K and Wiley 7th NIST 98 EPA/NIH libraries spectra and literature data[7].

Biological assays: Minimum inhibitory concentration (MIC)-broth microdilution method: To determine the MIC, broth microdilution method proposed by Bajpai et al [8] and reported by Yèhouénou et al.[9,10] were used. The microdilutions on 96 well plates were used with MHB and 0.02 g/L phenol red. Essential oil and Mueller Hinton Broth (MHB) constitute the negative control. The positive one is bacteria strain added with MHB. The microplates were incubated at 37 ± 1°C for 24 hours, covered with a parafilm paper.

Minimum bactericidal concentration (MBC): MBC were appreciated by method proposed by Oussou et al.[11] reported respectively by Kpadonou et al[12]. To determine the MBC, each microliter-plate well content 50 µL in which no color change occurred, the mixture of essential oil and the strain was isolated on sterile MHA poured in Petri dishes. These plates were incubated at 37°C for 24 hours. The MBC is the lowest concentration of essential oil which 99.9% of the microorganisms were killed. The tests were carried out in triplicate.

Antibacterial activity of essential oils of *Cinnamomum zeylanicum:* *Effect of essential oils on viable counts of bacteria:* The two strains ATCC *Staphylococcus aureus* and *Escherichia coli* have been chosen and used on the basis of their sensibility to the essential oil, considering their MIC. Each tube which well had been used for the viable counts contains the bacteria suspension (10^7 CFU/mL) of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 in the medium MHB. They are inoculated with the MIC of the essential oil approximately 10 mL and kept at 37°C[13]. Samples for viable cell counts were taken out at 0, 20, 40, 80, 100 and 120 minutes time intervals. Enumeration of viable counts in Muller Hinton Agar (MHA) plates was monitored as following: 0.1 mL sample of each treatment was diluted into buffer peptone water there by diluting it 10-fold and spread on the surface of MHA. The colonies were counted after 24 hours of incubation at 37°C. The controls (strains of *Staphylococcus aureus* and *Escherichia coli*) were inoculated without essential oil for each bacteria strain under the same experimental condition[14].

Antifungal activity: Preparation of the culture medium: 11.5 g agar of yeast extract (yeast extract AGAR) and 10 g of anhydrous glucose are mixed with 500 ml of distilled water for the preparation of culture medium. After sterilization and addition of oxytetracycline (0.1 %) 5 mL, this medium was cast in limp of Petri dish 9 cm in diameter at a rate of 17 mL.

Detection of the moulds: A quantity of food largely consumed in Benin (meat soup, refreshed drink called "Adoyo" and cooked maize flour) was diluted in sterile peptone water in order to detect fungi responsible of their deterioration. 30 min after homogenizing each sample, 0.1 mL of the inoculate was spread out on the sterilized mould medium YEGA (yeast extract glucose agar) and uniformly. The present limp was incubated at 25°C ± 1°C five days awarded from day light. The moulds were identified by microscopy according to keys of Samson et al.[15].

Transplantation and mycelia growth: Antifungal activities of the essential oil were studied by agar well diffusion method[17]. The moulds detected after examination and identification then, are

transplanted (subcultured) using a disc of 6 mm in diameter which carries spores from the anamorph mould on the surface of Petri dish containing the former medium YEGA containing tested essential oils at different concentrations or no (positive control). The moulds subcultured were incubated at 25 °C ± 1 °C. The mycelial growth was appreciated every day by measuring the average of two perpendicular diameters passing by the middle of the disc, from the first day till the seventh one at, least 7 days (Khallil[16] cited by Koudoro et al.[17]).

The antifungal activity was evaluated by the following equation: I= [1-(d/dc)] x100[18].

Were, I: index antifungal; d: diameter of growth of Petri dish treated out of essential oil; dc: diameter of growth of the control (witness) (Petri dish without essential oil)

Test of determination of the fungistatic or fungicidal activity: With the experimental concentrations where neither growth nor germination was observed, the fungistatic or fungicidal activity was tested. This test consisted in taking the mycelial disc not germinated at the end of the incubation of the Petri dish and reintroducing it in a new culture medium (former one) without natural extract. If the mycelial growth was always inhibited, the fungicidal activity of the natural extracts was confirmed, and in the contrary case, it's spoken about fungistatic activity.

Antimicrobial assay: It concerned to test the sensitivity of the stock *E. coli* ATCC 25922 in the presence of certain specific antibiotic discs (diameter = 6mm) such as Ceftriazone 30 µg, Gentamycin 10 µg, Nalidixic acid 30 µg and Chloramphenicol 30 µg on the one hand and that of the stock *S. aureus* ATCC 25923 on the other hand using the antibiotic discs which are specific for it such as Lyncomycin and Erytromycin 15 µg and Tetracyclin 30 µg. The activity of essential oil was evaluated in comparison of those of antibiotics discs by measuring the diameters of inhibition of the stocks. The essential oil was been spread on sterile disc of 6 mm of diameter at same concentrations with the antibiotic disc.

Results and Discussion

Chemical composition of the EO: The yield of EO of *Cinnamomum zeylanicum* was relatively high (1.1 ± 0.3%). Table-1 gives the chemical composition of the EO of *Cinnamomum zeylanicum* Blume dry leaves. The main compounds of this EO was (E) cinnamaldehyde (37.6%), cinnamyl acetate (23.7%), cinnamyl benzoate (16.4%), benzyl benzoate (2.4%), α-pinene (2.7%), linalool (2.2%), camphene (1.5%), β-pinene (1.1%), limonene (1.0%), 3-phenyl-prop-3-en-1-ol (0.9%), ethyl benzoate (0.8%). This composition shows the noticeable amount of aromatic components, and the hydrogenated monoterpens were present at low percentage with the presence of linalool (2.2%) and eugenol (0.2%).

Antimicrobial activity of the EO: The minimal inhibitory Concentrations are ranged from 0.20 $mg.mL^{-1}$ to 1.6 $mg.mL^{-1}$, respectively 0.80 $mg.ml^{-1}$ on *E. coli* ATCC 25922, 0.20 $mg.mL^{-1}$ against *S. aureus* ATCC 25923 and 1.60 $mg.mL^{-1}$ against *Salmonella typhi* and *Klebsiella pneumoniae* and 0.40 $mg.mL^{-1}$ on *Candida albicans*. The Minimal Bactericidal Concentrations were respectively 3.20 $mg.mL^{-1}$, 0.80 $mg.mL^{-1}$, 6.40 $mg.mL^{-1}$ and 1.60 $mg.mL^{-1}$ against *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *Salmonella typhi* and *Klebsiella pneumoniae* and *Candida albicans* (table-2).

Antibiotical power of the EO: Due to the inhibition diameter (mm) observed, *E. coli* ATCC 25922 strain tested was sensitive to ceftriazone 30 µg (21.0 ± 0.1 mm), gentamycin (18.0 ± 0.1 mm), chloramphenicol (18.0 ± 0.2 mm) and resistant to nalidixic acid (0.0 mm) while the EO against the tested strain of *E. coli* stood with diameters respectively for ceftriazona (30.2 ± 0.2 mm), gentamycin (29.1 ± 0.1 mm), nalidixic acid (29.1 ± 0.1) and chloramphenicol (28.2 ± 0.2 mm).

S. aureus ATCC 25923 strains tested was sensitive both to the antibiotical standard discs and the EO respectively 24.0 ± 0.2 mm and 20.0 ± 0.1mm for Lymcomycin, 24.0 ± 0.2 mm and 21.0 ± 0.2mm for erythromycin, and 30.0 ± 0.2 mm and 30.0 ± 0.2 mm for tetracycline. No antibiotical activity was observed against *S. aureus* ATCC 25923 from oxacillin and the EO revealing that the strain *S. aureus* ATCC 25923 was similar to a BORSA (Boderline Oxacillin Resistant *Staphylococcus aureus*) previously described in the literature[19]. Thus EO extracted from the dry leaves of *Cinnamomum zeylanicum* possessed an antibiotical power against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 and this fact confirms theoretical calculate of antibiotical power ($MBC/MIC \leq 4$) (table-3).

Count of tested strains viability: The Essential Oil extracted from dry leaves of *Cinnamomum zeylanicum* has shown widely inhibitory activity against both bacteria and yeast. After 40min time of exposure with the three microorganisms, *S. aureus* more sensitive were reduced 28.6% of its quantum while *E. coli* was recorded only 15% of reduction. After 100min, *S. aureus* had lost 85.7% of its cell while *E. coli* had been reduced over 57.1% of its colonies (figure-1).

Antifungal activities of the EO against from moulds strains belonging to the *Deuteromyceta:* The Essential Oil revealed antifungal activities against the four tested strains (*F. oxysporum*, *P. digitatum*, *A. parasiticus* and *A. ochraceus*) according to the concentrations applied and the exposure time. We have also observed that at the concentrations less than 2000 ppm, the EO have shown a fungistatic action against the four moulds, but at the concentration equal to 2000 ppm, it displayed a fungicidal activity from the 5th and the 6th days respectively against *F. oxysporum* and *P. digitatum*, but stood nethertheless fungistatic at the same concentration and at the same time of exposure against *A. parasiticus* and *A. ochraceus* (figures-2a, 2b, 2c and 2d).

Table-1
Chemical composition of essential oil extracted from *Cinnamomum zeylanicum* Blume dry leaves of BENIN

N°	Noms des composés	RI (HP-5)	RI (Adams)	RI (Carbowax)	(%)
1	**α-pinene**	**935**	**932**	**1031**	**2.7**
2	camphene	**950**	946	1077	1.5
3	benzaldehyde	**961**	952	1533	0.7
4	sabinene	**974**	969	-	t
5	β-pinene	**979**	974	1118	1.1
6	myrcene	**990**	988	1164	0.1
7	α-phellandrene	**1006**	1002	1171	0.2
8	p-cymene	**1025**	1020	1276	0.3
9	limonene	**1030**	1024	1205	1.0
10	β-phellandrene	**1032**	1025	1216	0.8
11	benzyalcohol	**1033**	1026	1841	0.3
13	*trans*-β-ocimene	**1046**	1044	1253	0.4
14	**linalool**	**1100**	**1095**	**1542**	**2.2**
15	*trans*-pinocarveol	**1150**	1135	-	t
16	camphre	**1141**	1141	-	t
17	3-phenyl prop-1-en-3-ol	**1164**	-	1886	0.9
18	dihydrocinnamaldehyde	**1168**	-	1789	0.1
19	ethyl benzoate	**1172**	1169	1682	0.8
20	terpinen-4-ol	**1180**	1174	1605	0.1
21	α-terpineol	**1192**	1186	1696	0.1
22	(Z)-cinnamaldehyde	**1221**	1219	-	0.2
23	(Z)-cinnamic alcohol	**1263**	-	-	0.1
24	**(E)-cinnamaldehyde**	**1278**	**1267**	**2035**	**37.6**
25	(E)-cinnamic alcohol	**1307**	1303	2258	0.2
26	eugenol	**1359**	1359	-	0.1
27	dihydrocinnamyle acetate	**1372**	1388	-	1.0
28	α-copaene	**1381**	1374	1498	0.8
29	β-cubebene	**1389**	1387	-	t
30	(E)-β-caryophyllene	**1426**	1421	1608	1.2
31	**cinnamyl acetate**	**1439**	**1443**	**2144**	**23.7**
32	(E)-ethyl cinnamate	1455	1465	2052	0.2
33	caryophyllene oxyde	**1593**	1582	1897	1.0
34	humulene epoxide II	**1608**	1608	1608	0.1
35	caryophylla-4(12),8(13)-dien-5-ol	**1641**	-	-	0.1
36	nd	**1665**		-	0.1
37	nd	**1771**		-	0.1
38	**benzyl benzoate**	**1779**	**1759**	**-**	**2.4**
39	**cinnamyl benzoate**	**1935**	**-**	**-**	**16.4**
	Monoterpene hydrocarbons				**8.1**
	Oxygenated monoterpenes				**2.4**
	Sesquiterpene hydrocarbons				**2.0**
	Oxygenated sesquiterpenes				**1.2**
	Aromatic components				**84.9**
	nd= not detcted				**0.2**
	Total				**98.6**

t (traces) ≤ 0,1%

Table-2
Antibiotical sensitivity of EO extracted from dry leaves of *Cinnamomum zeylanicum* Blume of BENIN

Strains tested	***Escherichia coli ATCC 25922***				***Staphylococcus aureus ATCC 25923***			
antibiotic	Ceftria-zone 30 µg	Genta-mycin 10 µg	Nalidixic Acide 30 µg	Chloram-phenicol 30 µg	Lynco-mycin 15 µg	Erythro-mycin 15 µg	Tetracy-clin 30 µg	Oxacilline 30 µg
Family	Cephalosporin	Aminoside	Quinolone	Phenicole	Macrolide	Macrolide	Tetracyclin	Penicillin
Diameter (mm)	21.1 ± 0.1	18.1 ± 0.1	-	18.2 ± 0.2	24.2 ± 0.2	24.2 ± 0.2	30.1 ± 0.1	-
Cinnamomum zeylanicum (Lauraceae)	30.2 ± 0.2	29.1 ± 0.1	29.1 ± 0.1	28.2 ± 0.2	20.1 ± 0.1	21.2 ± 0.2	30.2 ± 0.2	-

Table-3
Minimal Inhibitory concentration (MIC), Minimal Bactericidal Concentration (MBC) and Antibiotic power

Parameters	***E. coli* ATCC 25922**	***S. aureus* ATCC 25923**	***C. albicans* ATCC 10231**	***Klebsiella pneumoniae***	***Salmonella typhi***
MIC	0.80	0.20	0.40	1.60	1.60
MBC	3.20	0.80	1.60	6.40	6.40
Antibiotic power	4.00	4.00	4.00	4.00	4.00

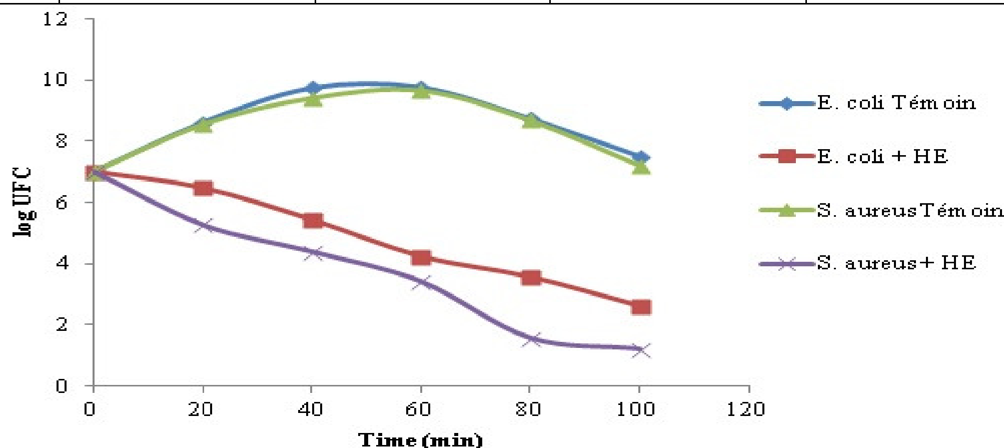

Figure- 1
Effect of essential oil extracted from *Cinnamomum zeylanicum* dry leaves (MIC) on viability of *Escherichia coli* and *Staphylococccus aureus* per unity of time

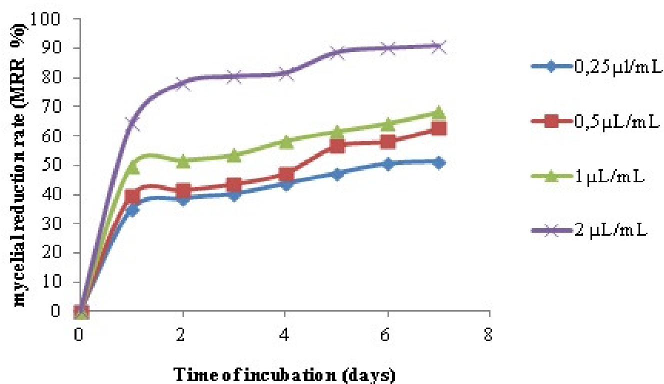

Figure -2a
Mycelial growth reduction of *Fusarium oxysporum* in function of different concentrations of essential oil of *Cinnamomum zeylanicum* applied

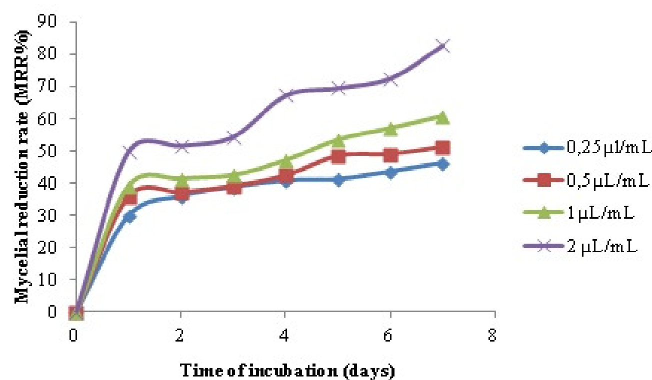

Figure-2b
Mycelial growth reduction of *Penicillium digitatum* in function of different concentrations of essential oil of *Cinnamomum zeylanicum* applied

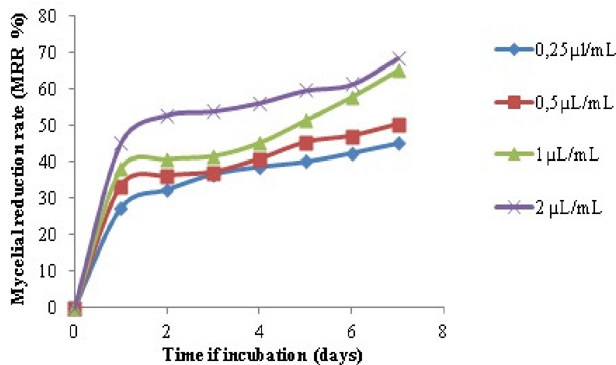

Figure-2c
Mycelial growth reduction of *Aspergillus ochraceus* in function of different concentrations of essential oil of *Cinnamomum zeylanicum* applied

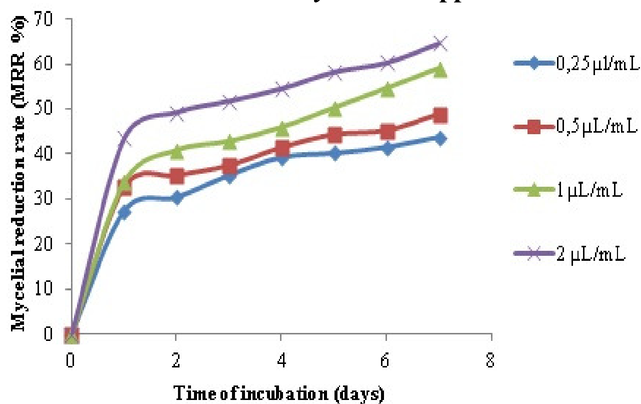

Figure-2d
Mycelial growth reduction of *Aspergillus parasiticus* in function of different concentrations of essential oil of *Cinnamomum zeylanicum* applied

This study has evaluated the chemical composition and antimicrobial activities of EO of *Cinnamomum zeylanicum* dry leaves commonly used in Benin and its neighboring countries such as Togo and Ghana as flavouring additive. The yield of the EO was 1.1%, comparable with data reported in the literature 0.93%[20].

The results of GC-MS analysis of the oil consigned in Table-1 showed that 39 compounds were identified representing 98.2% of the total oil. The major compounds were (E) cinnamaldehyde (37.6%), cinnamyl acetate (23.7%), cinnamyl benzoate (16.4%), aromatic components (84.9%), and hydrogenated monoterpènes (8.1%). Baratta et al.[3], Simic et al.[4], Yang et al.[21] and Unlu et al.[20] have previously identified in this oil cynnamaldehyde as main compound with a noticeable amount of cinnamyl benzoate, α-pinene, linalool and limonene. The antimicrobial activity recorded in this study could be attributed to the main aromatic compounds.

The EO revealed an antibiotical power against *C. albicans* ATCC 10231, *F. oxysporium* and *P. digitatum*. The previous studies of Mohamedi[22] and De Billerbeck et al.[23] allowed evaluating the antigungal properties of the EO. The mycelia reduction rate (MRR) observed in our study (≤80%) for the *Aspergilla* and more than 90% for *P. digitatum* and *F. oxysporum* have confirmed that antifungal activities of our sample are probably in relation with the oxygenated terpens which act alone and/or in synergy with another compounds as (E) cinnamaldehyde, cinnamyl acetate, cinnamyl benzoate, hydrogenated monoterpens and sesquiterpens. Bullerman et al.[24] as cited by Simic et al.[4] have reported this activity and have mentioned cinnamaldehyde and eugenol as major agents of inhibition of fungal growth. Freidman et al.[25], Ooi et al.[26], Shahverdi et al.[27], Shan et al. [28] and Unlu et al.[20] have also confirmed this activity of cinamaldehyde, and could justify both activities against bacteria and fungi recorded due to the possession of their polar hydrophilic functions (OH, COOH, NH_2, and NO_2) according to Diallo[29].

Antibacterial activities could be in relation with main composition of our sample cinnamaldehyde and cinnamyl compounds widely discussed in the literature data for their anti-infections properties[30]. Marino et al.[31] have insisted on the antimicrobial properties of essential oils extracted from aromatic plants due to aromatic compounds. The mechanisms of their action on the cytoplasm membrane and the cell wall are well known and previously reported by Burt[32] and Bajpai et al.[13]. But this antibacterial activity is selective and differential depending of the composition of cell wall of bacteria tested (Gram positive or Gram negative). The highest sensitivity of Gram+ (85% of reduction) with the oil, than Gram- (57% of reduction) could be justified by the nature of external membranous of the Gram negative constituted by hydrophobic lipopolysaccharides, which limit the oil penetration in the cells and also its action.

Conclusion

In the present study, we have shown that *Cinnamomum zeylanicum* essential oil is mainly composed in cinnamaldehyde, cinnamyl acetate and cinnamyl benzoate. This composition can justify the significant antimicrobial activities observed. This oil can be used as food preservative and this specie is promising for furthermore investigations including its anti cancer properties.

Acknowledgments

Mr. Dossevi Lordson, Professor of Nutrition and Dietetics at Polytechnic School of Abomey-Calavi for his scientific contributions and plant material supplying.

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