

Chemical Composition, Antioxidant and Antibacterial Properties of Essential Oil from *Ocotea Auriculiformis* Kost. (Lauraceae) Leaves, Endemic of Madagascar

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Abstract

Plants constitute an important source of secondary metabolites in which essential oils are well-known for their use in various domains such as pharmacy, therapeutic, cosmetology and foods. *In vitro* antimicrobial and antioxidant properties of *Ocotea auriculiformis* Kost. (Lauraceae) leaves essential oil is demonstrated and its chemical composition is reported in the present study. The essential oil from *Ocotea auriculiformis* Kost. (Lauraceae) leaves, an endemic plant of Madagascar was extracted by hydrodistillation method. Chemical composition using GC, GC/ MS and NMR¹³C methods showed that the essential oil contained around 47 products in which 93.95% were identified. Known compounds are constituted by 74.7% of hydrocarbons and 19.25% of oxygenated products. The essential oil is rich in sesquiterpene and monoterpene. *In vitro* antibacterial capacity of the essential oil was assessed by disc method against human and food pathogens. *Bacillus cereus* and *Streptococcus pneumoniae* were very sensitive to the essential oil with 11 mm and 25 mm of inhibition zone

respectively. The MIC of the essential oil was 1mg/mL for *Bacillus cereus* and 0.25 mg/mL for *Streptococcus pneumoniae*. MBC values were 2.5 mg/mL and 0.5 mg/mL, respectively. The ratio MBC/MIC for both strains was inferior to 4 concluding hence that the essential oil has bactericidal effect against the two sensitive strains. *In vitro* antioxidant capacity of the essential oil was performed according to qualitative (TLC) and quantitative (measure of DPPH radical scavenging) methods. The essential oil showed antioxidant activity with IC₅₀ value of 0.35 mg/mL

Keywords: Essential oil, Antioxidant, Antibacterial, *Ocotea auriculiformis*, Madagascar

Introduction

Malagasy flora is unique for its richness, its diversity and its endemism. Fourteen thousand species are recorded on 592000 km² of area. Eight botanical families, 25% of genera and 85% of species are only found in Madagascar (Pernet and Meyer, 1957; Rasoanaivo, 1999). In spite of several studies conducted on vegetal biodiversity, many species are not yet subjected to fundamental studies. Actually, many researchers pay their attention on endemic flora of Madagascar to extend scientific knowledge on the one hand and to safeguard endangered species on the other hand.

Therapeutic properties of medicinal plants and essential oils are well known (Turbide, 2010). In developing countries such Madagascar, these natural products constitute the base of traditional medicine. On the other side, chemical or synthetic preservatives used in food industries represent one part of techniques panoply and alternatives for insuring consumers' safety, extending foodstuffs conservation and reducing alteration caused by oxidation. Moreover, bacteria are mostly known to cause more intoxication cases and their treatment by chemical agents conducted to a regular selection of resistant bacterial strains.

Therefore, researchers and scientists have been resolving to use efficient and accessible alternatives from natural products which actually know a renewed interest. Essential oils could be represented and proved as relevant choice against contamination risk and for the reduction or the substitution of chemical or synthetic preservative agents.

Several studies have been demonstrated that essential oils have a large inhibition spectrum against pathogen bacteria and fungi (Remmal *et al.*, 1993b). In some works, thyme, organ, cinnamon and other aromatic plants essential oils were demonstrated to present inhibitor effect on bacteria and fungi development (Beuchat, 1976; Madhyasta and Bhat, 1984; Beraoud, 1990; Beraoud *et al.*, 1991; Bilgrami *et al.*, 1992).

These observations develop the curiosity to exploit Malagasy aromatic and endemic plant, *Ocotea auriculiformis* Kost. which is used anarchically in the collect site. Besides, no investigation was recorded for this specie. This work was, thus, undertaken with the purpose to determine the chemical composition and to test antioxidant and antibacterial potentials of essential oil from *Ocotea auriculiformis* Kost. leaves.

Materials and methods

Plant sampling

Plant material was harvested on July 2013 in the humid dense forest of Mandraka, Analamanga, Province of Antananarivo, Madagascar (S 18° 54' 290 of latitude / E 47° 55' 201 of longitude, altitude 1300 m). Voucher specimens were deposited at the herbarium of botanical Department of the Faculty of Sciences Antananarivo, Madagascar under the reference DBEV-15090601. Freshly harvested leaves were dried in a dry and ventilated local for 20 to 30 days.

Essential oil extraction

Essential oil was extracted by hydrodistillation method for 8 h using Clevenger equipment (Clevenger, 1928). Extracted essential oil was then dehydrated with dry sodium sulfate, put into an opaque vial and kept at -20°C to protect its chemical properties.

Chemical analyzes

Analytical GC

The GC analysis was carried out with a Clarus 500 Perkin-Elmer apparatus equipped with two flame ionization detectors (5FID), and fused capillary columns (50 m x 0.22 mm i.d., film thickness 0.25 micron), BP-1 (polymethylsiloxane) and BP-20 (polyethylene glycol). Carrier gas hydrogen; linear velocity, 0.8 mL/min. the oven temperature was programmed from 60°C to 220°C at 2°C/min and then held isothermal (20 min). Injector temperature was 250°C. (Injectionmode: split 1/60). Detector temperature: 250°C.

GC/MS Analysis

The oil was analyzed with a Perkin Elmer Turbo Mass detector (quadrupole), directly coupled to a Perkin Elmer Autosystem XL equipped with two fused-silica capillary columns (60 m x 0.22 mm i.d., film thickness 0.25 micro m) Rtx-1, polydimethylsiloxane and Rtx –wax, polyethylene glycol). Carrier gas helium at 1mL/min; split 1/80; injector temperature, 280°C; oven temperature programmed from 60°C to 230°C at 2°C/min and then held isothermal (35 min). The ion source temperature was 150°C; the

energy ionization was 70 eV, and the electron ionization (EI) mass spectra were acquired over the mass range of 35-350 Da. The oil injected volume was 0.2 μ L.

NMR¹³C analysis

NMR spectra were recorded on a Bruker AVANCE 400 fourier transform spectrometer operating at 100.13 MHz for ¹³C, equipped with a 5 mm probe, in deuterated chloroform (CDCl₃), with all shifts referred to internal tetramethylsilane (TMS). ¹³C NMR spectra were recorded with the following parameters: pulse width 5PW), 4 μ s (flip angle 45°); acquisition time, 2.7 s for 128K data table with a spectral width (SW) of 24000Hz (240 ppm) ; CPD mode decoupling; digital resolution 0.183 Hz/pt. The number of accumulated scans was 3000 for each sample (50-60 mg of EO in 0.5 mL of CDCl₃).

Identification of the compounds

For GC, the identification of the components was based on their retention indexes on apolar and polar columns which were evaluated in comparison with the standard curve of alkanes and those of reference components.

For GC/MS, the identification of the components was performed using AMDIS software and the NIST MS databases.

Comparison of the chemical shifts of carbons in the ¹³C NMR data library with those of reference spectra compiled in laboratory and literature made NMR data library.

Antioxidant activity

In vitro antioxidant capacity of the essential oil from *Ocotea auriculiformis* Kost. leaves was assessed according to two methods: qualitative evaluation by TLC method (*n*-hexane 9 mL / diethylique Ether 1 mL) summarized in the figure 1 and quantitative method by the measure of DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavengening.

For the qualitative method, the constituents of the essential oil showing antioxidant activity appeared in yellow-white on violet stain of DPPH solution.

Quantitative analysis was performed according to the methods described by Wang *et al.*, 2002 and Kivrak *et al.*, 2009. For that, 20 μ L of serial dilutions of essential oil in methanol (0.039 to 5 mg/mL) were mixed with 2 mL of DPPH; after 30 min. of incubation in dark, the absorbance was measured at 517 nm. Ascorbic acid was used as positive control and the essential oil was tested in triplicate. The inhibition of free DPPH radical in percentage (P %) was calculated as follows (Sharififar *et al.*, 2007) :

$$P \% = (A \text{ blank} - A \text{ sample} / A \text{ blank}) \times 100$$

With A blank: Absorbance of DPPH methanolic solution),

A sample: Absorbance of the extract.

The IC₅₀ value was calculated by linear extrapolation of the data that lay on the either side of the 50 % inhibition level from the three tests of the equation $P (\%) = f (C)$.

With C: concentration of the essential oil in the extract.

Antimicrobial activity

Antimicrobial activity of the essential oil was performed on three Gram positive bacteria: *Bacillus cereus* (ATCC 13061), *Staphylococcus aureus* (ATCC 11632), *Streptococcus pneumoniae* (ATCC 6301); five Gram negative bacteria: *Escherichia coli* (ATCC 70032), *Klebsiella oxytoca* (ATCC 700325), *Salmonella enteridis*, *Enterobacter cloacae* (ATCC 700323), *Pseudomonas aeruginosa* (ATCC 9207); one yeast: *Candida*, from microbial strains collection of the Laboratory of Environmental Microbiology/ National Center of Environmental Research, Antananarivo, Madagascar. These test-microorganisms were selected for their high frequency to contaminate foodstuffs and their pathogenicity.

Disc method was used to test antimicrobial activity of the essential oil (Hayes and Markovic, 2002). Sterile discs (6 mm in diameter) soaked with 10 µL of essential oil were put into Mueller Hinton agar plates previously inoculated with 10⁶cells/mL of test-bacteria. After incubation for 24 h at 37°C, antimicrobial activity was evaluated by the measure of the diameter of inhibition zone around the discs. A disc of sterile distilled water was used as negative control and antibiotic discs as positive controls (Netilmycine 30 µg, Spectinomycine 30 µg). All tests were done in triplicate. The results were interpreted according to pathogens sensibility: the pathogen is resistant if the diameter of inhibition zone (X) is inferior to 8 mm, sensitive: 9 mm < X < 14 mm, very sensitive: 15 mm < X < 19 mm, extremely sensitive: X > 20 mm (Ponce *et al.*, 2003).

The minimum inhibitory concentration (MIC) of the essential oil on two most sensitive bacteria was determined according to the method described by Remmal *et al.* (1993a) and Satrani *et al.* (2001). Two fold serial dilutions of the essential oil in Mueller Hinton broth added with 0.2 % of agar (1/10 to 1/800) were prepared. 1.5 mL of each dilution were, then, poured into test-tubes containing 13.5 mL of Mueller Hinton broth to obtain final concentrations from 1/100 to 1/8000 (v/v). Thereafter, the test-tubes were shaken and incubated at 37°C for 24 h. The MIC is defined as the lowest concentration of the essential oil where no bacteria growth is visible (Hansen *et al.*, 1994; Yajko *et al.*, 1995). Each tube content was streaked on Mueller Hinton agar media and incubated at 37°C during 72 h. The MBC,

defined as the lowest concentration of the essential oil allowing to kill 100 % of previous bacteria (Drugeon *et al.*, 1991), was then deduced.

Thus, bactericidal or bacteriostatic effect of the essential oil was determined by the ratio MBC/MIC. An antibacterial product is considered as bactericidal if $MBC/MIC \leq 4$ and bacteriostatic if $MBC/MIC > 4$ (Bouharb *et al.*, 2014). Mueller Hinton broth with 0.2% of agar was used as negative control and the test was realized in triplicate to minimize experimentation error.

Results and discussion

Essential oil extraction yield

Extraction yield of the essential oil from *Ocotea auriculiformis* Kost. leaves was 0.47%. It is relatively high in comparison with the ten species of Monteverde *Ocotea*, Costa Rica (Sayaka *et al.*, 2007) exploited as source of essential oils where the yields vary from 0,024% to 0,287%. Therefore, essential oil yield, physico-chemical properties and chemical composition are influenced by many factors such species, environmental conditions, extraction technique, drying technique, period, harvest site and age of plant material (Aberchane M. *et al.*, 2001 ; Bourkhiss *et al.*, 2011).

Chemical composition of essential oil

GC, GC/MS and NMR¹³C analyzes of *Ocotea auriculiformis* Kost. leaves essential oil revealed about 47 products in which 93.95% were identified (Table 1). 74.7% of the components are hydrocarbons and 19.25%, oxygenated derivatives. Among identified components, 77.35% are sesquiterpenes (59.2% of sesquiterpene hydrocarbon and 18.15% of oxygenated sesquiterpenes); 16.6% are monoterpenes (15.5% of monoterpene hydrocarbon and 1.1% of oxygenated monoterpenes). Majority components are α -humulene (42.6%), β -pinene (8.5%), (*E*)- β -caryophyllene (7.8%), α -pinene (6.4%), oxyde d'humulène II (4.8%), β -eudesmol (3.1%), guaïol (3%) and β -selinene (2%).

Table 1: Chemical composition of essential oil from *Ocotea auriculiformis* Kost. leaves

N°	Name	RI _a	RI _p	P (%)	Identification
1	α -pinene	929	1.017	6.4	RI, SM, NMR- ¹³ C
2	camphene	942	1.066	0.1	RI, SM
3	β -pinene	969	1.112	8.5	RI, SM, NMR- ¹³ C
4	β -myrcene	979	1.056	0.4	RI, SM
5	limonene	1.020	1.201	0.2	RI, SM
6	1,8-cineol	1.020	1.211	tr	RI, SM
7	terpinolene	1.077	1.283	tr	RI, SM
8	Linalol	1.082	1.546	0.1	RI, SM
9	α -terpineol	1.171	1.696	0.3	RI, SM

10	eugenol	1.326	2.166	0.1	RI, SM
11	δ -elemene	1.333	1.468	0.1	RI, SM
12	<i>a</i> -cubebene	1.347	1.455	0.3	RI, SM
13	<i>a</i> -ylangene	1.369	1.480	0.5	RI, SM
14	<i>a</i> -copaene	1.374	1.489	0.9	RI, SM, NMR- ¹³ C
15	β -bourbonene	1.381	1.516	0.9	RI, SM, NMR- ¹³ C
16	β -elemene	1.386	1.587	0.1	RI, SM
17	(E)- β -caryophyllene	1.415	1.594	7.8	RI, SM, NMR- ¹³ C
18	α -guaiene	1.445	1630	0.3	RI, SM
19	<i>a</i> -humulene	1.449	1.667	42.6	RI, SM, NMR- ¹³ C
20	γ -Muurolene	1.468	1.684	0.4	RI, SM
21	germacrene-D	1.473	1.705	0.9	RI, SM, NMR- ¹³ C
22	β -selinene	1.479	1.715	2	RI, SM, NMR- ¹³ C
23	<i>a</i> -selinene	1.488	1.719	1.1	RI, SM, NMR- ¹³ C
24	<i>a</i> -muurolene	1.491	1.719	0.2	RI, SM
25	<i>a</i> -bulnesene	1.497	1.711	0.2	RI, SM
26	γ -cadinene	1.504	1.753	0.2	RI, SM
27	cis-calamenene	1.507	1.828	0.1	RI, SM
28	δ -cadinene	1.512	1.753	0.9	RI, SM, NMR- ¹³ C
29	élémicine	1.516	2.224	0.3	RI, SM
30	<i>a</i> -calacorene	1.526	1.912	0.3	RI, SM
31	β -elemol	1.532	2.076	0.3	RI, SM
32	germacrene B	1.546	1.823	tr	RI, SM
33	oxyde caryophyllene	1.567	1.978	0.9	RI, SM, NMR- ¹³ C
34	guaïol	1.582	2.085	3	RI, SM, NMR- ¹³ C
35	humulol	1.585	2.155	0.5	RI, SM
36	oxyde humulene II*	1.592	2.034	4.8	RI, SM, NMR- ¹³ C
37	sequithuriferol*	1.592	2.117	1.6	RI, SM, NMR- ¹³ C
38	cubenol	1.608	2060	0.4	RI, SM
39	τ -cadinol*	1.614	2163	0.24	RI, SM, NMR- ¹³ C
40	τ -muurolool*	1.614	2181	0.36	RI, SM, NMR- ¹³ C
41	γ -eudesmol	1.626	2.262	tr	RI, SM
42	β -eudesmol	1.632	2.224	3.1	RI, SM, NMR- ¹³ C
43	<i>a</i> -cadinol	1.635	2221	0.95	RI, SM, NMR- ¹³ C
44	α -eudesmol	1.639	2.215	0.4	RI, SM
45	γ -bisabolol	1.647	2262	0.2	RI, SM
46	bulnesol	1.649	2.205	1.1	RI, SM, NMR- ¹³ C
47	juniper camphor	1.675	2.292	0.1	RI, SM
	TOTAL (%)			93.95	

P:Percentage; RI_a: apolar Retention Index ; RI_p : polar Retention Index; * : identification of Retention Index polar; tr:trace <0.1

The nine common compounds (α -pinene, β -pinene, β -elemene, β -caryophyllene, α -humulene, germacrene-D, γ -cadinene, δ -cadinene, and α -

adinol) had been previously reported in *Ocotea angustifolia* (Torres *et al.*, 2005) and *Ocotea brenesii* (Chaverri and Ciccio, 2005) leaves essential oils. These nine compounds are apparently common of the genus *Ocotea* and the following genera *Beilschmiedia*, *Cinnamomum*, *Laurus*, *Lindera*, *Nectandra*, and *Persea*. *Beilschmiedia alloiophylla*, *Beilschmiedia tilaranensis* (Setzer and Haber, 2007), *Laurus azorica* (Pedro *et al.*, 2001), and *Nectandra membranacea* (Wu *et al.*, 2006) leaves essential oils presented all the nine compounds. Furthermore, *Ocotea comoriensis* bark essential oil (Menut *et al.*, 2002) contained eight of the nine common compounds found in *Ocotea* species; β -elemene was not reported. However, *Ocotea auriculiformis* Kost. leaves essential oil in this study presented the nine common compounds found in the genus *Ocotea*. The difference of chemical composition observed between *Ocotea* could be explained by biotic and abiotic factors adaptation such as climate of the region, geographic factors (altitude, nature of the soil) which determine the synthesis of specific products (Brada *et al.*, 2007 ; Ghanmi *et al.*,2007). Then, chemical composition of essential oils doesn't depend only to plant organ and extraction method, but also to the origin of the species (Adams *et al.*, 1996; Koukos *et al.*, 2000; Hojjati *et al.*, 2009).

Antioxidant activity

The following figure shows the results of qualitative test and the measure of inhibition percentage of DPPH radical.

A: Ascorbic acid

B: Essential Oil from *Ocotea auriculiformis* Kost. leaves

1-2-3-4: Antioxidant activity

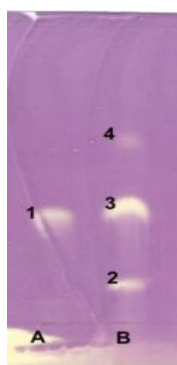
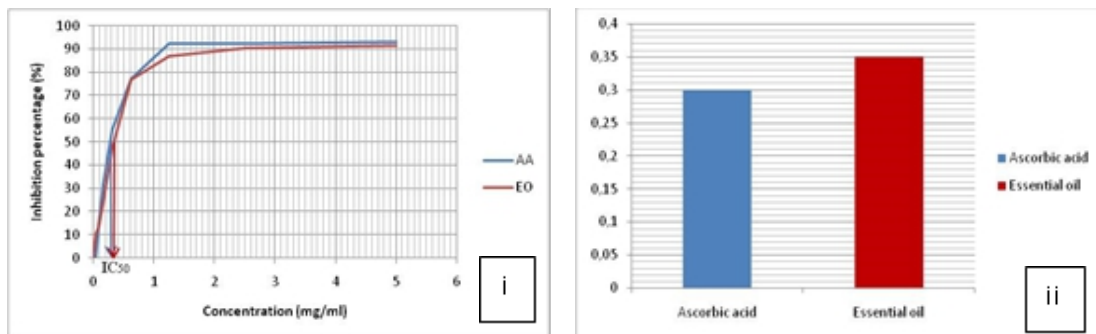


Figure 1: Antioxidant Activity of Essential Oil from *Ocotea auriculiformis* Kost. leaves by TLC method

Tested essential oil displayed antioxidant activity. Silica gel plate colored in violet by DPPH was discolored in yellow-white in the bands showing antiradical activity of the essential oil.



IC₅₀: Inhibition Concentration (50 %); HO: Essential oil;
AA: Ascorbic acid.

Figure 2: Antioxidant Activity of Essential Oil from the Leaves of *Ocotea auriculiformis* Kost., by the measure of DPPH (i) and IC₅₀ values (ii)

For quantitative test, the percentage of DPPH radical inhibition increased with the concentration either for ascorbic acid or for essential oil of *Ocotea auriculiformis* Kost. leaves. It would be noted that this inhibition percentage was comparable for the two substances for all concentrations tested.

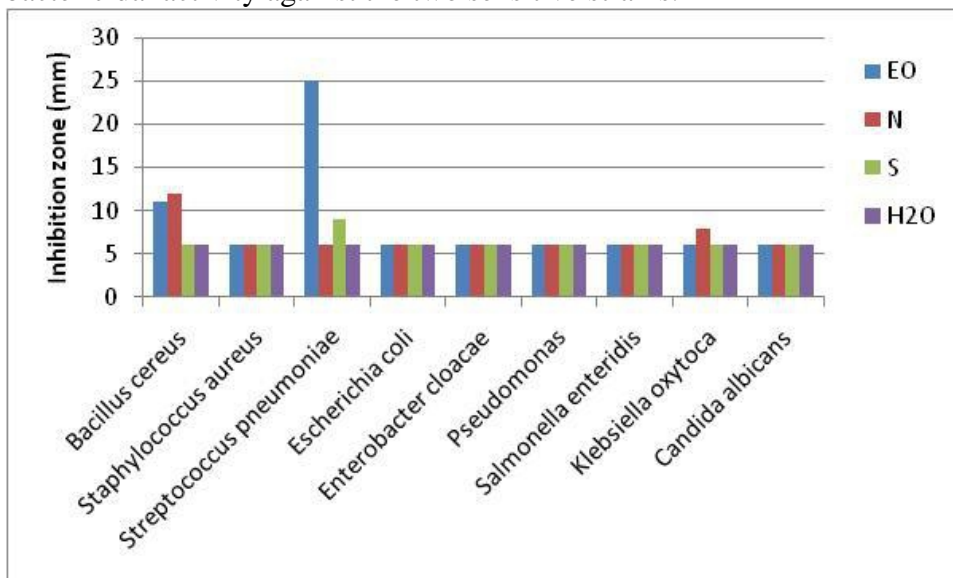
According to the results obtained essential oil of *Ocotea auriculiformis* Kost. leaves exhibited comparable antioxidant activity than ascorbic acid with IC₅₀ values of 0.35 mg/mL and 0.3 mg/mL respectively (Figure 2). This activity is due to phenol compounds in the essential oil which the main role is the reduction of free radicals (Villano *et al.*, 2007). It would be emphasized also that for essential oils, biological property is not only from majority components but minority compounds could show synergic or antagonistic activity to form efficient system against free radicals (Sidi and Ziane, 2003).

Antimicrobial activity

The essential oil of *Ocotea auriculiformis* Kost. leaves showed antibacterial activity especially against Gram + bacteria: *Streptococcus pneumoniae* and *Bacillus cereus* at a concentration of 100 µg/disc. The diameters of inhibition zone were 25 mm for *Streptococcus pneumoniae* and 11 mm for *Bacillus cereus* (Figure 3). According to Ponce *et al.*'s criteria, *Streptococcus pneumoniae* was extremely sensitive to the essential oil and *Bacillus cereus*, sensitive. MIC values for the two strains were 0.25 mg/mL and 1 mg/mL, respectively and MBC values were 0.5 mg/mL and 2.5 mg/mL, respectively. MIC values are comparables to those of *Ocotea bofo* and *Ocotea quixos* essential oils. *Ocotea bofo* essential oil MIC were ranged from 0.16 to 0.32 mg/mL for *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* (Alessandra, 2006). Those of *Ocotea quixos* were, however,

from 0.12 to 0.24 mg/mL for *Staphylococcus aureus* and *Enterococcus faecalis* (Renato, 2003).

MIC and MBC indicate the nature of the compounds activity against microorganisms. In this study, the ratio MBC/MIC was inferior to 4; *Ocotea auriculiformis* Kost. leaves essential oil was then qualified to possess bactericidal activity against the two sensitive strains.



EO : Essential oil ; N : Netilmycine ; S: Spectinomycine; H₂O: Water distilled

Figure 3: Antimicrobial activity of the essential oil from leaves from *Ocotea auriculiformis* Kost.

Antimicrobial activity of the essential oil from *Ocotea auriculiformis* Kost. leaves could be explained by its chemical profile rich in terpenes, especially α -pinene and β -pinene. This component displays several biological activities as antibacterial, anti-inflammatory, antiviral, expectorant, sedative, herbicide and insecticide (Ghanmi *et al.*, 2007). Moreover, because of the complexity of the chemical composition of the essential oils, antimicrobial activity may be due to the interaction between the different constituents.

Conclusion

Our results show that essential oil from leaves of *Ocotea auriculiformis* Kost. species is rich in sesquiterpenes and monoterpenes. Antibacterial activity of the essential oils based on MIC against common food-borne pathogens and strong antioxidant activity suggests its possible use in food industries as a new potential source of natural antibacterial and antioxidant agents. However, *in vivo* studies are recommended to determine the toxicity profile of the essential oil.

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