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Chemical composition and biological activities of Ishpingo essential oil, a traditional Ecuadorian spice from *Ocotea quixos* (Lam.) Kosterm. (Lauraceae) flower calices

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Abstract

The essential oil of Ishpingo (*Ocotea quixos*, Lauraceae) fruit calices was analysed by GC (gas chromatography) and GC–MS (gas chromatography–mass spectrometry). Fourty-four compounds were identified. The main components detected were *trans*-cinnamaldehyde (27.9%), methylcinnamate (21.6%), 1,8-cineole (8.0%), benzaldehyde (3.6%), and β-selinene (2.1%). In vitro antioxidant properties of the essential oil, obtained by DPPH (1,1-diphenyl-2-picrylhydrazyl) and β-carotene bleaching assays, were also evaluated. The oil exerted a relatively good capacity to act as a non-specific donor of hydrogen atoms or electrons when checked by the diphenylpicrylhydrazyl assay, quenching 52% of the radical. On the other hand, it showed weak effects in inhibiting oxidation of linoleic acid when assayed by the β-carotene bleaching test. Antibacterial activity of the essential oil was also checked against gram positive (*Enterococcus foecalis, Staphylococcus aureus*) and gram negative strains (*Escherichia coli, Pseudomonas aeruginosa*). The oil also showed a dose-dependent antifungal activity against *Candida albicans*, *Saccharomyces cerevisiae*, phytopathogen *Pythium ultimum* and dermatophyte *Trichophyton mentagrophytes*.

Keywords: *Ocotea quixos*; Lauraceae; Essential oil; Antioxidant activity; Antibacterial activity; Antifungal activity; Cinnamaldehyde; Methyl cinnamate

1. Introduction

The expanding spice market is presently helping to focus attention on the various traditional spices from different areas of the World and great emphasis has recently been focused on the utilization of those spices and their essential oils as natural agents for food preservation (Baratta et al., 1998; Deans, 1991; Farag, Ali, & Taha, 1990; Farrell, 1990; Janssen, Scheffer, & Baerheim Svendsen, 1987). Due to consumer concerns regarding safety and adverse effects of synthetic food additives, spices are now not only evaluated for their seasoning and flavouring properties, but also for their functional characteristics as bacteriostatics, fungicides, antioxidants and nutrients (Hirasa & Takemasa, 1998).

Ocotea quixos (Lam.) Kosterm. (Lauraceae) is a medium sized tree native to Amazonian Ecuador and neighbouring countries (Jørgensen & León-Yánez, 1999), which is reputed to have known aromatic properties since the period of the Incas (Naranjo, 1969), but is not well known outside Ecuador. The tree produces biennial big and woody flower calices, locally called Ishpingo. Abscissic calices are harvested when felled from wild trees and are traditionally used, either fresh or dried, whole or crushed, by Amazonian indigenous people as a spice (Friedman et al., 1993).

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Recently, its use has become widespread also in non-rural areas under the name of *Flor de Canela*, due to its cinnamon-like perfume. It is traditionally used for flavouring cakes, beverages and infusions, besides being appreciated as an appetizer, eupeptic, a disinfectant and as a local anesthetic (Naranjo, Kijjoa, Giesbrecht, & Gottlieb, 1981). As commonly happens for many spices, a large part of the aroma and flavour of this spice is due to the presence of essential oils, some constituents of which, (cinnamaldehyde, cinnamic acid and methyl cinnamate), have been isolated (Naranjo et al., 1981). However, no extensive characterization of the Ishpingo oil has been performed so far and its food-related biological activities have never been assessed. Therefore, the aim of this paper is to determine the chemical composition of the essential oil from the Ecuadorian *O. quixos* flower calices and to provide a first report on its in vitro antioxidant, antibacterial and antifungal properties.

2. Materials and methods

2.1. Plant material

Ishpingo spice was obtained from entire dried *O. quixos* (Lam.) Kosterm. (Lauraceae) calices supplied by Fundacion Chankuap' (Macas, Ecuador) from three different stocks collected in January 2002 from wild trees on the outskirts of the Wasak'entsa reserve in eastern Ecuador (77° 15″ W/2° 35″ S). A voucher specimen of the spice was deposited in the Dipartimento delle Risorse Naturali e Culturali, University of Ferrara as ISH01.

2.2. Essential oil extraction

Essential oil was extracted by steam distillation of the entire flower calices with a commercial Clevenger apparatus (AOAC, 1990). After 8 h of steam distillation, 3.74 g of essential oil were obtained from 200 g of crude drug (yield 1.9±0.25%) The essential oil content was determined on a volume to dry weight basis. The values for essential oil content of the three replications were averaged and standard deviations calculated. The essential oil samples were stored in glass vials with Teflon-sealed caps at 2±0.5 °C in the absence of light.

2.3. Gas chromatography

Essential oil samples, from three separate distillations, were analysed and the relative peak areas for individual constituents averaged. Quantification was elaborated as the percentage contribution of each compound to the total amount present. The relative percentages were determined using a Fisons (Rodano, Milano, Italy) 9130-9000 series gas-chromatograph equipped with a Fisons EL980 processor, a FID detector and a MEGA SE52 (Mega, Legnano, Italy) poly-5% diphenyl–95%-dimethyl-siloxane bonded phase column (i.d. = 0.32 mm, length 30 m, film thickness = 0.15 μm). Operating conditions were as follows: injector temperature 280 °C; FID temperature 280 °C, Carrier (helium) flow rate 2 ml/min and split injection with split ratio 1:40. Oven temperature was initially 45 °C and then raised to 100 °C at a rate of 1 °C/min, then raised to 250 °C at a rate of 5 °C/min and finally held at that temperature for 10 min. 1 μl of each sample dissolved in CH_2Cl_2 was injected.

2.4. Gas chromatography/mass spectrometry analysis

Essential oil constituents were analyzed by a Hewlett Packard HP5890 series II plus gas chromatograph equipped with a HPMS 5989b mass spectrometer using electron impact and hooked to NBS75K. The constituents of the volatile oils were identified by comparing their GC (gas chromatography) retention times and the MS (mass spectrometry) fragmentation pattern with those of other essential oils of known composition, with pure compounds and by matching the MS fragmentation patterns with the above mentioned mass spectra libraries and with those in the literature (Adams, 1995). The GC conditions were the same as reported for GC analysis and the same column was used. The MS conditions were as follows: ionization voltage, 70 eV; emission current, 40 μA; scan rate, 1 scan/s; mass range, 35–300 Da; ion source temperature, 200 °C.

2.5. Biological activities

2.5.1. General

All the following biological activities of *O. quixos* (Ishpingo) essential oil were compared with those achieved with the commercial essential oil of thyme (*Thymus vulgaris*, Extrasynthese, Genay, France) in order to have a reference with a product reputed for its antioxidant (Dang, Takacsova, Nguyen, & Kristianova, 2000), antibacterial (Dorman & Deans, 2000) and antifungal properties (Zambonelli, Zechini D'Aulerio, Bianchi, & Albasini, 1996). Antioxidant activity was assessed through DPPH (1,1-diphenyl-2-picrylhydrazyl) and β-carotene bleaching tests, while antibacterial and antifungal activities of Ishpingo and thyme essential oil were performed on different classes of microorganisms. For antibacterial assays, gram positive (*Enterococcus foecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213) and gram negative (*Escherichia coli* ATCC 4350, *Pseudomonas aeruginosa* ATCC 17934) bacterial strains were employed. Antifungal activity was assessed on the yeasts *Candida albicans* ATCC 48274 and *Saccharomyces cerevisiae* ATCC 2365, on the phytopathogen *Pythium ultimum* Trow, kindly supplied by Professor G.

D'Ercole (Institute of Vegetal Pathology, University of Bologna, Italy) and the dermatophyte *Trichophyton mentagrophytes* var. *mentagrophytes* (C.P. Robin) R. Blanch CBS 160.66 (Centraal Bureau Voor Schimmelcultures, Baarn, the Netherlands). The culture media and conditions employed for ATCC strains were in accordance with American Type Culture Collections protocols (www.atcc.org). Phytopathogen and dermatophytic fungi were cultured on SDA (Sabouraud Dextrose Agar, Difco, Franklin Lakes, NJ, USA) at 26±1 °C. All the data collected for each assay are the averages of three determinations of three independent experiments.

2.5.2. Free radical scavenging activity: DPPH test

Free radical scavenging activity of essential oils was measured according to the procedure of Yamaguchi, Takamura, Matoba, and Terao (1998). An aliquot of essential oil (10 μl) was mixed with 900 μl of 100 mM Tris–HCl buffer (pH 7.4), 40 μl of ethanol and 50 μl of 0.5% (w/w) Tween 20 solution. 1 ml of 0.5 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma St. Louis, USA) ethanol solution was then added to the mixture. Tween 20 (Sigma) was used as an oil-in-water emulsifier (Choi, Song, Ukeda, & Sawamura, 2000). The mixture was shaken vigorously and immediately placed in a UV–Vis spectrophotometer (ThermoSpectronic Helios γ, Cambridge, UK) and the absorbance at 517 nm was monitored for 70 min, until the reaction reached a plateau. A blank was assessed, as in the solution assay described above without the essential oils, instead of which distilled water was employed. Trolox (1 mM) (Sigma) was used as a positive control. The radical scavenging activity of each sample was calculated according to the following formula for Inhibition percentage of DPPH: $Ip^{DPPH}\% = (A_B - A_A)/A_B \times 100$ (Yen & Duh, 1994), where A_A and A_B are the absorbance values of the test and of the blank sample, respectively, after 70 min.

2.5.3. Antioxidant activity: β-carotene bleaching test

Antioxidant activity of essential oils was determined using β-carotene bleaching test (Taga, Miller, & Pratt, 1984). Approximately 10 mg of β-carotene (type I synthetic, Sigma) was dissolved in 10 ml of $CHCl_3$. 0.2 ml of the solution was pipetted into a boiling flask containing 20 mg of linoleic acid and 200 mg of Tween 40. $CHCl_3$ was removed using a rotary evaporator (Büchi 461, Switzerland) at 40 °C for 5 min. 50 ml of distilled water were slowly added to the residue under vigorous agitation, to form an emulsion. 5 ml of the emulsion were added to a tube containing 0.2 ml of the essential oils solution prepared according to Choi et al. (2000). The absorbance was immediately detected at 470 nm and then it was checked every 15 min for 60 min, maintaining the test emulsion in a water bath at 50 °C. Positive controls consisted of butylated hydroxy anisole (BHA), while negative controls consisted of test emulsions in which the essential oils were substituted by equal amounts of distilled water.

2.5.4. Activity against bacteria and yeasts

The biological activity against these classes of microorganisms was determined by employing the standard disks diffusion technique (Benson, 1990; Okeke, Iroegbu, Eze, Okoli, & Esimone, 2001). Mother cultures of each microorganism were set up 24 h before the assays, in order to reach the stationary phase of growth. The tests were assessed by inoculating, from the mother cultures, Petri dishes with proper sterile media with the aim of obtaining a microorganism concentration of 10^6 cfu (colony forming units)/ml and 10^5 cfu/ml for bacteria and yeasts, respectively. An aliquot of dimethylsulfoxide (DMSO) was added to the essential oils in order to obtain a 0.01–0.5 mg/ml concentration range. Serial dilutions of the DMSO/essential oil solution were deposited on sterile paper disks (6 mm diameter, Difco) which were subsequently placed in the centre of the inoculated Petri dishes. The Petri dishes were then incubated at 37 °C for 24 h and the (bacterial growth) inhibition zone diameter (IZD) was measured to the nearest mm. The lowest concentration of each DMSO/essential oil solution deposited on the sterile paper disk showing a clear zone of inhibition was taken as the MIC (minimum inhibitory concentration) (Okeke et al., 2001). Controls were set up with DMSO in amounts corresponding to the highest quantity present in the test solution.

2.5.5. Activity against fungi

Antifungal activity on *P. ultimum* and *T. mentagrophytes* var. *mentagrophytes* was obtained by dissolving essential oils in DMSO and aseptically adding to sterile media at 45 °C in order to obtain the four concentrations of 50, 100, 200 and 500 μg/ml. The DMSO concentration in the final solution was adjusted to 0.1%. Controls were set up with equivalent quantities of DMSO. The cultures were obtained by transplanting mycelium disks (diameter 10 mm) from a single culture in the stationary phase. Then they were incubated at 26±1 °C on SDA (sabouraud dextrose agar) on thin cellophane sheets until the logarithmic growth phase was reached. Subsequently, the cultures were transferred to Petri plates with a medium containing the essential oil diluted to the above-mentioned final concentrations. From this moment on, the diametral development was measured at 24 h intervals for 7 days.

2.6. Statistical analysis

For each of the data collected, relative standard deviation are given. Analyses of variance (ANOVA)

followed by LSD post-hoc determinations, and *t*-test were then performed. All computations were done using the statistical software Statistica 6.0 (StatSoft Italia srl).

3. Results and discussion

3.1. Chemical composition

The flower calices of *O. quixos,* after having undergone steam distillation, yielded a pale brown oil with a warm, rather sweet, spicy, cinnamon-like odour in a yield of 1.9%. Table 1 shows *O. quixos* essential oil composition in terms of components and classes of compounds; 44 of the 55 components were identified, which constitutes more than 98% of the volatile fraction. The oil was rich in oxygenated monoterpenes (48.8%) with a high content of aldehydes (32.2%). The concentration of sesquiterpenes was low (13.7%), with important amounts of β-selinene (2.06%), β-caryophyllene (1.85%), α-humulene (1.79%) and copaene (1.26%). The most abundant chemicals, mainly responsible for the cinnamon-like taste of the spice, were *trans*-cinnamaldehyde and methyl cinnamate, which accounted for 27.9% and 21.7% respectively. Contrary to previous reports (Naranjo et al., 1981), the presence of *o*-methoxycinnamaldehyde was negligible, while cinnamic acid was not detected. Compounds involved in the biosynthetic phenylpropanoids pathway accounted for a global 54.4%. More than 350 species belong to the *Ocotea* genus, mainly distributed in the Americas and in Southern Africa, and are widely known as commercial sources of spicy essential oils. Ishpingo essential oil differs from commercially exploited *O. pretiosa* and *O. cymbarum* oils (Weiss, 1997) by the absence of both methyleugenol and safrole. In particular, the absence of safrole allows only minor constraints on the use of Ecuadorian Ishpingo essential oil (due to the recognized toxicity of this compound) (Ioannides, Delaforge, & Parke, 1981).

Table 1
Essential oil composition of Ecuadorian *Ocotea quixos* (Ishpingo) calyces

Peak	Compound[a]	ID method[b]	RT[c]	RA[d]
1	Styrene	MS	7.38	0.44
2	α-Thujene	MS	9.38	0.44
3	α-Pinene	MS, GC	9.72	3.17
4	Camphene	MS	10.57	0.19
5	Benzaldehyde	MS, GC	11.36	3.15
6	β-Pinene	MS	12.57	1.67
7	β-Myrcene	MS, GC	13.99	0.21
8	α-Phellandrene	MS	14.78	0.26
9	3-Carene	MS	15.26	0.45
10	α-Terpinene	MS	15.88	0.83
11	*p*-Cymene	MS	16.63	4.81
12	1,8-Cineole + Limonene	MS	17.27	8.09
13	γ-Terpinene	MS, GC	19.91	1.63
14	Linalool oxide	MS	21.43	0.1
15	Terpinolene	MS	22.89	0.33
16	Linalool	MS	24.76	3.2
17	Camphor	MS	28.62	0.15
18	Hydrocinnamic aldehyde	MS	31.12	0.74
19	Borneol	MS	31.84	0.10
20	4-Terpineol	MS, GC	33.01	2.19
21	α-Terpineol	MS	34.85	2.9
22	*cis*-Cinnamaldehyde	MS	37.88	0.39
23	*trans*-Cinnamaldehyde	MS, GC	45.17	27.91
24	α-Cubebene	MS	54.66	1.14
25	Copaene	MS	57.20	1.26
26	Cinnamic acid methyl ester	MS, GC	58.39	21.65
27	β-Elemene	MS	58.78	0.23
28	β-Caryophyllene	MS, GC	60.23	1.85
29	α-Santalene	MS	60.54	0.36
30	α-Bergamotene	MS	61.47	0.47
31	*trans*-Cinnamil acetate	MS, GC	61.85	0.09
32	α-Humulene	MS	62.08	1.79
33	*z*, β-Santalene	MS	62.61	0.11
34	Farnesene isomer	MS	62.72	0.1
35	γ-Muurolene	MS	63.32	Tr[e]
36	β-Selinene	MS	63.62	2.06
37	α-Selinene	MS	64.02	0.87
38	Unk. naphtalene type	MS	64.34	0.11
39	γ-Cadinene + β-bisabolene	MS	64.83	0.89
40	δ-Cadinene	MS	65.26	1.6
41	*o*-Methoxycinnamaldehyde	MS, GC	65.38	0.1
42	Unk. naphtalene type	MS	65.6	0.41
43	Caryophyllene oxide	MS	67.21	0.36
44	Benzyl benzoate	MS	72.25	0.05

[a] Compounds are listed in order of elution from a SE-52 column.
[b] MS, peaks identified on MS comparison with file spectra; GC/MS, peak identified on comparison with pure reference standards.
[c] RT, Retention time on a SE52 column in minutes.
[d] RA%, Relative area percentage (peak area relative to total peak area %).
[e] Tr, trace (<0.05%).

3.2. Antioxidant activity

The in vitro antioxidant activity of Ishpingo essential oil was assessed by both the DPPH test and the β-carotene bleaching test, which allow, respectively the primary and the secondary step of oxidation to be followed (Gordon, 1990). The results were compared to the activities of synthetic antioxidants and to *T. vulgaris* commercial essential oil, known for its antioxidant properties (Dang et al., 2000). This type of approach allows the antioxidant effectiveness of an essential oil to be defined, as it is almost impossible to express the antioxidant activity as an absolute value universally (Mantle et al., 1998; Ruberto, & Baratta, 2000; Zygadlo, Lamarque, Maestri, & Grosso, 1995). In the DPPH test, Ishpingo essential oil revealed a remarkable scavenging effect. In fact, the antiradical activity, expressed as DPPH inhibition percentage of Ishpingo oil, was between those of Trolox and thyme essential oil (Figs. 1 and 2). This result is particularly relevant,

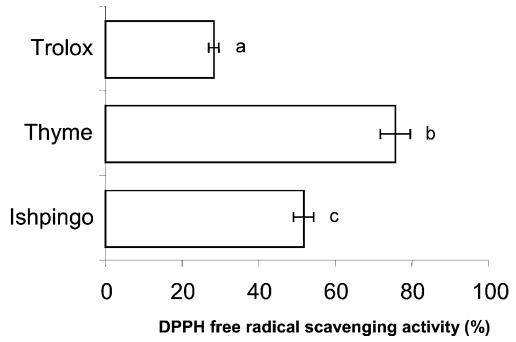

Fig. 1. Free radical scavenging activity percentage of *Ocotea quixos* (Ishpingo) essential oil evaluated by the DPPH assay and comparison with commercial *Thymus vulgaris* (Thyme) essential oil and reference compound (Trolox). Different letters mean significant differences ($P < 0.001$) among the DPPH scavenging activities based on LSD post-hoc tests.

given the fact that *T. vulgaris* essential oil is a highly reputed antioxidant among spice derived essential oils (Zygadlo et al., 1995). On the contrary, Ishpingo essential oil performed poorly in the β-carotene bleaching test, where its protective action against linoleic acid peroxidation was weak (Fig. 3).

Thus, these results showed different behaviour of the oil as a free radical scavenger and as an oxidation inhibitor. This discrepancy may be due to the different mechanisms involved in the different stages of oxidation and to the hydro/lipophilicity of the antioxidants. In fact, while the DPPH method is independent of the putative antioxidant polarity, the β-carotene bleaching test is affected by the so-called "polar-paradox" and, as a consequence of the presence of an emulsion, polar

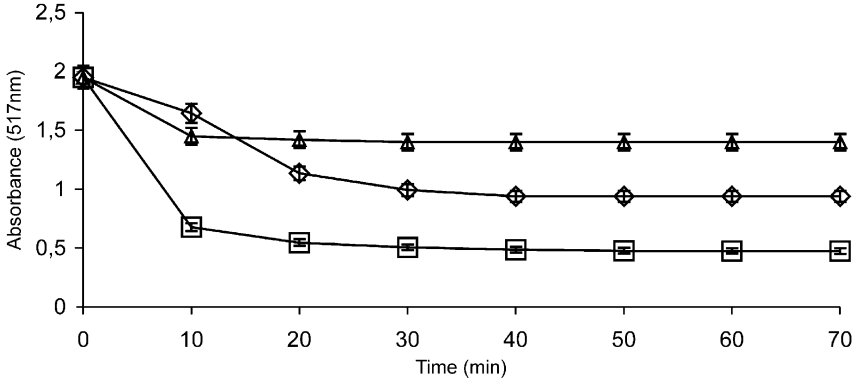

Fig. 2. Antioxidant activity of *O. quixos* (Ishpingo) essential oil by the DPPH assay over time and comparison with commercial *T. vulgaris* (Thyme) essential oil and reference compound (Trolox). ◇ *Ocotea quixos* (Ishpingo) essential oil; □ *Thymus vulgaris* (Thyme) essential oil; △ Trolox. Anova repeated measures showed that all the absorbance values varied significantly until 30 min ($P < 0.001$; $F = 197.19$). *t*-Test dependent variables showed that all the absorbance data were significantly different among essential oils and Trolox ($P < 0.001$; df = 23).

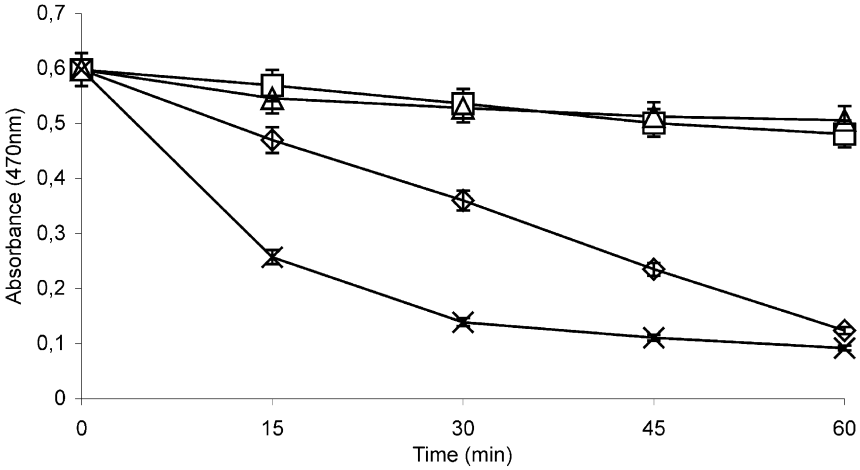

Fig. 3. Antioxidant activity of *Ocotea quixos* (Ishpingo) essential oil by the β-carotene bleaching test and comparison with commercial thyme essential oil and reference compound (BHA). ◇ *O. quixos* (Ishpingo) essential oil; □ *T. vulgaris* (Thyme) essential oil; △ BHA (butylated hydroxy anisole); X Negative control. Anova repeated measures showed that all the absorbance values detected varied significantly with respect to time ($P < 0.001$; $F = 38.06$). *t*-Test dependent variables showed that the absorbance values detected for Ishpingo essential oil were significantly different from those detected for Thyme essential oil, BHA and negative control ($P < 0.001$; df = 14).

molecules may be less effective in preventing linoleic acid oxidation. This leads to the suggestion that the polar components of the essential oil could be responsible for the activity revealed by the DPPH test (Frankel, Huang, Kanner, & German, 1994; Koleva, van Beek, Linssen, de Groot, & Evstatieva, 2002; Porter, 1993).

3.3. Antibacterial and antifungal activity

Table 2 shows in vitro inhibitory effects of pure essential oil of *O. quixos* calices, compared with *T. vulgaris* essential oil, against four bacterial and two yeast strains. The antibacterial activity showed higher MIC values, never lower than 0.12 mg/ml. *P. aeruginosa* was an exception, showing an evident sensitivity towards Ishpingo essential oil (0.049 mg/ml) and resistance towards thyme oil (0.24 mg/ml). Whereas the activity of Ishpingo essential oil against both the yeasts *S. cerevisiae* and *C. albicans* was homogeneous (0.24 mg/ml) and slightly better than that of thyme. Those results can be linked to the specificity of *trans*-cinnamadehyde-rich oils against fungal strains rather than bacteria (Ferhout, Bohatier, Guillot, & Chalchat, 1999; Mastura, Azah, Khozirah, Mawardi, & Manaf, 1999).

As shown in Fig. 4, the antifungal activity against the dermatophyte and phytopathogen strains of Ishpingo essential oil was relatively good. In particular, the growth inhibition percentage against the dermatophyte *T. mentagrophytes* var. *mentagrophytes* was 60% lower than that shown by commercial thyme essential oil at the highest concentration tested. However, Ishpingo essential oil performed better against the phytopathogen *P. ultimum,* with an inhibitory action (85%) slightly lower than that of thyme (100%) at 500 μg/ml.

Table 2
Antibacterial activity expressed as minimum inhibitory concentration (MIC[a]) of *O. quixos* (Ishpingo) essential oil compared with that of commercial thyme essential oil taken as positive control

	Ishpingo essential oil (*Ocotea quixos*)	Thyme essential oil (*Thymus vulgaris*)
	MIC (mg/ml)	
Gram positive bacteria		
Staphylococcus aureus subsp. *aureus* ATCC 29213	0.12	0.12
Enterococcus foecalis ATCC 29212	0.24	0.12
Gram negative bacteria		
P. aeruginosa ATCC 17934	0.049	0.24
Escherichia coli ATCC 4350	0.12	0.12
Yeasts		
Saccharomyces cerevisiae ATCC 2365	0.024	0.049
Candida albicans ATCC 48274	0.024	0.049

[a] The MIC was considered as the lowest concentration of each essential oil showing a clear zone of inhibition.

4. Conclusions

The essential oil of *O. quixos* calices proved to possess interesting properties, emerging from both its chemical composition and from the evaluation of its in vitro biological activities. In fact, the flavouring of food products with *O. quixos* essential oil could be an interesting possibility for the future, as a consequence of the high value of cinnamon-like aromas in the food industry (soft drinks, flavoured teas and milk, chewing gums and baked products). Moreover, the antiradical activity exerted by the oil and its specific action against pathogenic strains such as *C. albicans* and against a gram negative bacteria, known for their resistance (Paz et al., 1995), may suggest its use as a functional fragrance. The activity of the oil against the phytopathogen tested may also open the possibility of its use as a constituent of phytocomplexes for agrobiological cultures. Commercially the main drawback of this spice resides in its low and localized production. The ecological sustainability of its harvesting, however, suggests its exploitation as a valuable non-timber product from the Eastern areas of the Amazon.

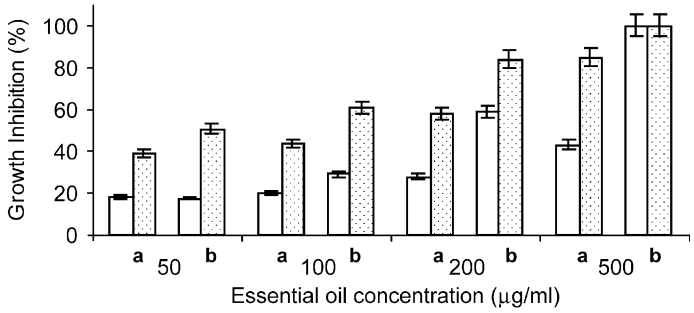

Fig. 4. Antifungal activity expressed as growth inhibition percentage induced in *Trichophyton mentagrophytes* var. *mentagrophytes* (blank histograms) and *Pythium ultimum* (dotted histograms). **a**: *Ocotea quixos* (Ishpingo) essential oil; **b**: *Thymus vulgaris* (Thyme) essential oil. ANOVA factorial tests showed that all the data of growth inhibition percentage varied significantly with respect to all the independent variables ($P < 0.001$).

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