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COMPOSITION AND ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS FROM AROMATIC PLANTS USED IN BRAZIL

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ABSTRACT

Essential oils from aerial parts of *Mentha piperita*, *M. spicata*, *Thymus vulgaris*, *Origanum vulgare*, *O. applii*, *Aloysia triphylla*, *Ocimum gratissimum*, *O. basilicum* were obtained by steam destillation using a Clevenger-type system. These oils were screened for antibacterial and anti-*Candida albicans* activity using bioautographic method. Subsequently, minimal inhibitory concentration from oils was determined by microdilution method. Most essential oil studied were effective against *Enterococcus faecium* and *Salmonella cholerasuis*. *Aloysia triphylla* and *O. basilicum* presented moderate inhibition against *Staphylococcus aureus* while only *A. tryphila* and *M. piperita* were able to control the yeast *Candida albicans*. The oils were analyzed by GC and GC-MS techniques in order to determine the majoritary compounds.

Key words: essential oil, medicinal plants, antimicrobial activity, minimal inhibitory concentration

INTRODUCTION

Higher and aromatics plants have traditionally been used in folk medicine as well as to extend the shelf life of foods, showing inhibition against bacteria, fungi and yeasts (17). Most of their properties are due to essential oils produced by their secondary metabolism (1). Essential oils and extracts from several plant species are able to control microorganisms related to skin (1), dental caries (7), and food spoilage, including Gram-negative and Gram-positive bacteria (14).

Many countries have maintained research programs to screen traditional medicines for antimicrobial activity, as is the case of India (2), Palestin (4), Africa (6), Honduras (19), Jordan (20), Cuba (21) and Italy (23). Plants from Brazilian biomes have also been used as natural medicines by local populations in the treatment of several tropical diseases, including schistosomiasis, leishmaniasis, malaria and fungal and bacterial infections (5). However, despite the rich flora, only data from a few plants is available, including both native and exotic species.

Medicinal plants from CPQBA/UNICAMP germoplasm collection have been studied against bacteria and the yeast *Candida albicans* (Robin) Berkhout ATCC 10231. Extracts, fractions and compounds isolated from *Mikania laevigata* Sch. Bip ex Baker, *M. glomerata* Sprengel, *Artemisia annua* L., *Phyllanthus niruri* L., *Phyllanthus amarus* Schumach. & Thonn and *Achyrocline satureioides* (Lam.) DC were able to control one or more microorganisms (11,12,13,24).

Aromatic plants and spices have great importance for food, cosmetics and pharmaceutical industries. Their use have taken place since ancient times, and despite many of them were substituted by synthetic ones, the demand for natural products is increasing (15). Leafs from *O. vulgare* L., *O. applii* L., *O. basilicum* L., *O. gratissimum* L., *M. spicata* L. e *M. piperita* L. var. *citrata* have been used as spices and teas after drying, while the essential oil is utilized in cosmetics and pharmaceuticals. The essential oils contents in different species is influenced by genetic material, culture conditions and environment, (8) and finally, by crop and post-crop processing (22).

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In the present study, the *in vitro* antimicrobial activity of the essential oils from eight aromatic plants used in Brazil was investigated. The levels and oil composition were characterized by gas-chromatography/mass spectrophotometrical analyses.

MATERIALS AND METHODS

Aromatic Plants

Mentha piperita L. (UEC 127.110), *M. spicata* L. (UEC 121.401), *Thymus vulgaris* L. (UEC 121.405), *Origanum vulgare* L. (UEC 121.409), *O. applii* (Domin) Borus (UEC 121.410), *Aloysia triphylla* (L´Hér.) Britton (UEC 121.412), *Ocimum gratissimum* L. (UEC 121.407) and *O. basilicum* L. (UEC 121.408) were chosen to the present study. The aromatic plants were collected from CPQBA/UNICAMP experimental field, between 9:00 and 10:00 am, in the first week on March, in full flowering, except to *O. vulgare* L. in vegetative stage.

Essential oil extraction

The oil extraction was obtained from 40g fresh plants by steam destillation using Clevenger system, during 3 h. The aqueous phase was extracted with dichloromethane (3 x 50 mL). The organic phase was dried with sodium sulphate, filtered and the solvent evaporated until dryness. The oil was solubilized in ethyl acetate for gas chromatography and mass spectrometry analysis.

Chromatography conditions

The identification of volatile constituents was conducted by gas-chromatography in Hewlett-Packard 5890 Series II (Palo Alto, CA, USA) equipment, with selective mass detector HP-5971 in the electron impact (EI) ionization mode (70 eV), injector *split/splitless*, capillary column HP-5 (25 m x 0.2 mm x 0.33 µm). Temperature: injector = 220ºC, column = 60ºC, 3ºC.min^{-1}, 240ºC (7 min). Carrier gas (He) = 1.0 mL.min^{-1}. Retention indices (RI) have been obtained according to the method of Van den Dool (26).

Microorganisms

Antimicrobial activity tests were carried out against the bacteria *Pseudomonas aeruginosa* (Schroeter) ATCC13388, *Salmonella choleraesuis* (Smith) CCT4296, *Rhodococcus equi* (Magnussom) CCT0541, *Micrococcus luteus* (Schroeter) CCT2692, *Staphylococcus aureus* (Rosenbach) CCT2740, *S. epidermides* (Winslow & Winslow) ATCC12228, *Escherichia coli* CCT0547, *Bacillus subtilis* (Ehrenberg) Cohn CCT2576, *Enterococcus faecium* ATCC10541 (Orla-Jensen) Schleifer and Kilpper-Balz (registered at ATCC as *Streptococcus faecium*), *Enterococcus faecium* (Orla Jensen) CCT5079 and against the yeast *Candida albicans* (Robin) Berkhout ATCC 10231.

Culture Media

Bacteria were assayed on Nutrient Agar (Merck, g/L): peptone from meat, 5.0; meat extract, 3.0 and agar-agar, 12.0, and *C. albicans* on Sabouraud Dextrose Agar (Merck, g/L): peptone, 10.0; glucose, 40.0; agar-agar, 15.0.

Inocula

Inocula for the assays were prepared by diluting scraped cell mass in 0.85% NaCl solution, adjusted to McFarland scale 0.5 and confirmed by spectrophotometrical reading at 580 nm. Cell suspensions were finally diluted to 10^4 UFC.mL^{-1} for being used in the activity assays.

Bioautography assays

Antibacterial activity tests were carried out prior by bioautography method on thin layer chromatography (TLC) plates (25). After dilution in ethyl acetate, the essential oils (3 µL at 10 mg/mL) were applied on duplicate TLC plates, and hexane:acetate (85:15, v/v) was used as eluent. Subsequently, the first plate was developed with anisaldehyde and the other one was submitted to microbiological assays. Chloramphenicol was used as positive control.

The bacterial inocula suspensions prepared as described were inoculated by pour-plate in the respective medium (1:100) around 42ºC. A 0.5 mL aliquot of 1 mg/mL trifenil tetrazolium chloride solution (TTC) was added as growth indicator. The media were transferred to the Petri dishes where the TLC plates were previously deposited. After homogenization, the cultures were incubated at 37ºC during 24h.

The oils activity against *C. albicans* (Robin) Berkhout was evaluated only by MIC (Minimal Inhibition Concentration) test.

Minimal Inhibitory Concentration (MIC) Tests

MIC tests were carried out according to Eloff (10), using a tissue culture testplate (96 wells). The stock solutions of the oils were diluted and transferred into the first well, and serial dilutions were performed so that concentrations in the range of 2-0.03 mg.mL^{-1} were obtained. Chloramphenicol or nistatin (Merck) was used as the reference antibiotic control. The inoculum was added to all wells and the plates were incubated at 37ºC during 24 h (bacteria) or at 30ºC for 48 h (yeast). Antimicrobial activity was detected by adding 20 µL of 0.5% TTC (triphenyl tetrazolium chloride, Merck) aqueous solution. MIC was defined as the lowest concentration of oil that inhibited visible growth, as indicated by the TCC staining.

RESULTS AND DISCUSSION

Oil Yield and Chemical Constituents

Oil yields expressed in relation to dry weight plant material are shown in Table 1. The highest (0.74% w/w) and lowest (0.10% w/w) yields were obtained from *O. gratissimum* L. and *O. basilicum* L., respectively.

The chemical composition of the essential oils obtained was analyzed by GC and GC-MS, which allowed identification

Table 1. Essential oil concentration (% w/w) from aromatic plants studied, including the botanical name, family and traditional use.

Botanical name	Family	Traditional Use	Essential oil (% w/w)
Aloysia tryphila (L´Hér.) Britton	Verbenaceae	spice, digestive, sedative	0.22
Thymus vulgaris L.	Lamiaceae	antiseptic, antispasmodic	0.56
Mentha piperita L.	Lamiaceae	antiseptic, vermifug	0.42
M. spicata L.	Lamiaceae	antispasmodic, diuretic	0.32
Ocimum basilicum L.	Lamiaceae	digestive, vermifug	0.10
O. gratissimum L.	Lamiaceae	anticold, diuretic	0.74
Origanum vulgare L.	Lamiaceae	analgesic, expectorant	0.13
O. applii L.	Lamiaceae	analgesic, expectorant	0.20

of about 80% of oil constituents (Table 2). The main compounds from *Aloysia triphylla* (L´Hér.) Britton oil were geranial (21.83%), neral (17.45%) and limonene (11.03%). *Thymus vulgaris* L. main components were thymol (79.15%), carvacrol (4.63%) and p-cimene (3.27%). The *Mentha* species showed different oil composition. Linalool (51.0%), carvone (23.42%) and 3-octanol (10.1%) were identified from *M. piperita* L. and piperitenone oxide (94.8%) was present in *M. spicata* L. Thymol was the main constituent of *O. applii* (Domin) Borus (64.5%) and *O. vulgare* L. (38.0%) while eugenol was the component obtained from *O. gratissimum* (93.9%) and *O. basilicum* L. (28.1%).

Antimicrobial activity

Many microorganisms, which cause damage to human health, exhibit drug resistance due to inadequate use of antibiotics. Thus, there is a need for the discovery of new substances from natural sources, including plants. In this work, the antimicrobial activity of essential oils from aromatic species used in Brazil was previously evaluated by bioautographic assay that allows identification of oils active fractions. The trifenil tetrazolium chloride indicates cellular growth, once alive cells turn red. Thus, white spots indicate regions where the oil fraction was active. According to results the oils presented one or more active fractions against the microorganisms studied (Table 3).

Table 2. Identified compounds from aromatic plants: **AT** = *A. triphyla*; **TV** = *T. vulgaris*; **MS** = *M. spicata*; **MP** = *M. piperita*; **OB** = *O. basilicum*; **OG** = *O. gratissimum*; **OV** = *O. vulgare* and **OA** = *O. applii*. **(a) RI** = retention index; **(b)** Results expressed as % Area.

Analyte	(a) RI	(b) Sample							
		AT	TV	MP	MS	OV	OA	OB	OG
1-octen-3-ol	978	0.38	0.78			0.68			
3-octanol	998			10.1					
p-cimene	1024	1.13	3.27			1.50			
limonene	1028	11.03							
1,8-cineol	1031							1.05	
trans-β-ocimene	1035	0.41				0.39			
γ-terpinene	1057	0.47	2.57			1.99			
isopentyl n-butirate	1068			0.67					
α-terpinolene	1083					0.31			
fenchone	1088							0.53	
linalool	1093	1.05	1.62	51.0			4.92	32.6	
cis-p-menth-2-en-1-ol	1122			0.52		1.47			
trans-p-menth-2-en-1-ol	1140					0.86			
camphor	1150							10.1	
borneol	1169		2.29			2.52			
Terpin-4-ol	1176		1.20	8.00		33.3	3.08	0.99	0.26
cimen-8-ol	1182						0.64		
α-terpineol	1188	0.45		1.31		4.25		3.90	

trans-piperitol	1208					0.16			
thymol methyl ether	1231		0.76			0.24	1.47		
neral	1238	17.45							
carvacrol methyl ether	1241					1.33	5.91		
carvone	1241			23.42					
geranial	1270	21.83							
thymol	1294		79.15			38.0	64.5		
carvacrol	1298		4.63						
eugenol	1358							28.1	93.9
piperitenone oxide	1373				94.8				
Geranyl acetate	1379	3.29				0.33			
β-bourbonene	1385			0.18			1.35	0.23	
β-elemene	1393							1.92	
Cedrene <1,7-di-epi-alpha>	1397	0.28							
trans-caryophillene	1422	6.65	2.62	2.31		2.66		2.00	1.08
β-gurjunene	1430						0.36		
β-bergamotene	1435							1.66	
α-guaiene	1438							0.29	
β-farnesene	1454				0.76				
α-humulene	1455							0.77	
allo-aromadendrene	1461						0.28		
Germacrene D	1482			0.44		1.47	4.79	5.49	4.23
Curcumene <AR>	1483	5.14							
γ-muurolene	1484				1.06				
zingiberene	1495	0.51							
δ-guaiene	1506							0.68	
β-bisabolene	1508					1.05	1.98		
γ-cadinene	1516		0.38				0.89	1.27	
δ-cadinene	1524	0.57						0.29	0.19
espatulenol	1580	4.31				1.44	1.62		
caryophillene oxide	1586	6.96				1.07			
epi-α-muurolol	1643						0.72	5.81	
α-eudesmol	1655							0.24	
α-cadinol	1656					0.29	1.53	0.38	0.16
SUM OF IDENTIFIED PEAKS		**81.9**	**94.6**	**98.0**	**96.6**	**95.3**	**94.1**	**98.3**	**99.9**

The MIC was determined only for oils that presented positive results on bioautographic assays. Comparing with literature results (3) strong activity is for MIC values between 0.05 – 0.50 mg/mL, moderate activity MIC values between 0.6 – 1.50 mg/mL and weak activity above 1.50 mg/mL.

The results show a variable effect of the oils on the microorganisms (Table 3). Essential oil from *Aloysia tryphila* (L´Hér.) Britton was active against six of the microorganisms tested, showing the lowest MIC values (0.05 mg/mL and 0.50 mg/mL) against *E. faecium* ATCC 10541 (Orla-Jensen) Schleifer & Kilpper-Balz and *B. subtilis* (Ehrenberg) Cohn, respectively. *T. vulgaris* L., *M. piperita* L., *O. gratissimum* L., *O. vulgare* L. e *O. applii* L. showed strong activity against *E. faecium* ATCC 10541 (Orla-Jensen) Schleifer & Kilpper-Balz (0.05 – 0.40 mg/mL) and moderate activity against *S. choleraesuis* (Smith) (0.60 mg/mL) and *S. aureus* (Rosenbach) (1.00 mg/mL). All oils studied presented moderate activity against *S. aureus* (Rosenbach) and *B. subtilis* (Ehrenberg) Cohn, except *M. spicata* L. which was inactive. The fact of *M. spicata* presents piperitone oxide (94.8%) as main component and not shows any activity allows concluding that it is not a antimicrobial compound. Finally, *Aloysia triphylla* (L´Hér.) Britton and *M. piperita* L. exhibited moderate activity against *C. albicans* (Robin) Berkhout (0.80 and 0.74 mg/mL, respectively).

Among the aromatic plants studied, the major constituents found were the monoterpenes linalool, eugenol and thymol.

Table 3. Bioautography and Minimal Inhibitory Concentration (MIC – mg.mL^{-1}) of the essential oils from aromatic plants.

Microorganism	Controls (Antibiotic MIC) mg/mL	*A. tryphila* B	*A. tryphila* MIC mg/mL	*T. vulgaris* B	*T. vulgaris* MIC mg/mL	*M. spicata* B	*M. spicata* MIC mg/mL	*M. piperita* B	*M. piperita* MIC mg/mL	*O. basilicum* B	*O. basilicum* MIC mg/mL	*O. gratissimum* B	*O. gratissimum* MIC mg/mL	*O. vulgare* B	*O. vulgare* MIC mg/mL	*O. applii* B	*O. applii* MIC mg/mL
S. aureus	0.02	++	0.80	+	1.00	-	> 2.00	++	1.00	+	0.70	+	1.00	++	1.00	++	1.00
E. faecium[1]	0.12	+++	0.05	++	0.15	+	> 2.00	+++	0.15	+	1.00	++	0.30	++	0.40	++	0.20
S. choleraesuis	0.06	+++	0.60	+	0.60	+	> 2.00	++	0.60	+	> 2.00	+	0.60	++	0.60	++	0.60
P. aeruginosa	0.85	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. subtilis	0.02	+	0.50	+	0.49	+	> 2.00	++	1.00	+	0.80	+	1.10	++	1.00	++	1.20
E. faecium[2]	0.07	++	> 2.00	+	> 2.00	-	-	-	-	-	-	-	-	-	-	-	-
S. epidermidis	0.04	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
R. equi	0.04	++	> 2.00	+	> 2.00	-	-	-	-	+	> 2.00	+++	> 2.00	-	-	-	-
M. luteus	0.05	++	1.60	+	2.00	-	-	+	> 2.00	+	2.00	+	> 2.00	+	> 2.00	+	> 2.00
E. coli	0.04	-	-	-	-	-	-	-	-	+	> 2.00	++	> 2.00	-	-	-	-
C. albicans	0.05	-	0.80	-	2.00	-	> 2.00	-	0.74	-	> 2.00	-	> 2.00	-	2.00	-	> 2.00

+ one active fraction; ++ two active fractions; +++ three active fractions on Bioautography Test (B);
[1]*E. faecium* ATCC 10541; [2]*E. faecium* CCT 5079.

These compounds are previously known for its antimicrobial activity (9,16,18). Helander *et al.* (16) attributed the thymol antimicrobial action to its phenolic character, which can cause membrane-disturbing activities.

Finally, regarding to effects of the antibiotics used as positive control, *A. tryphilla* (L´Hér.) Britton presented MIC value lower than chloramphenicol, showing the antimicrobial potential of the essential oil.

Subsequently, bioguided fractionation will be conducted to the potential plants for identification of the active compounds. Evaluations of the oils from other medicinal plants are also being conducted.

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RESUMO

Composição e atividade antimicrobiana de óleos essenciais de plantas aromáticas usadas no Brasil

Óleos essenciais foram obtidos a partir das partes aéreas de *Mentha piperita*, *M. spicata*, *Thymus vulgaris*, *Origanum vulgare*, *O. applii*, *Aloysia triphylla*, *Ocimum gratissimum* e *O. basilicum* através de arraste de vapor em sistema tipo Clevenger. Os óleos foram avaliados quanto à atividade antimicrobiana contra bactérias e contra a levedura *Candida albicans* pelo método de bioautografia. A concentração mínima inibitória dos óleos com atividade positiva foi em seguida determinada pelo método da microdiluição. De acordo com os resultados, a maioria dos óleos essenciais estudados foram efetivos contra *Enterococcus faecium* e *Salmonella cholerasuis*. *A.triphylla* e *O. basilicum* apresentaram inibição moderada contra *Staphylococcus aureus* enquanto apenas *A. tryphila* e *M. piperita* foram capazes de inibir a levedura *Candida albicans*. Os óleos foram analisados quimicamente por técnicas de CG e CG-EM de modo a determinar os compostos majoritários presentes.

Palavras-chave: óleos essenciais, plantas medicinais, atividade antimicrobiana, concentração mínima inibitória

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