Citral and carvone chemotypes from the essential oils of Colombian *Lippia alba* (Mill.) N.E. Brown: composition, cytotoxicity and antifungal activity

Ana Cecilia Mesa-Arango[1]/+, Jehidys Montiel-Ramos[1], Bibiana Zapata[1], Camilo Durán[2], Liliana Betancur-Galvis[1], Elena Stashenko[2]

[1]Cancer and Infection Group, School of Medicine, University of Antioquia, 51D # 62-29 Lab 283 B, Medellín, Colombia [2]Research Centre for Biomolecules, Research Centre of Excellence, Industrial University of Santander, Bucaramanga, Colombia

Two essential oils of Lippia alba *(Mill.) N.E. Brown (Verbenacea), the carvone and citral chemotypes and 15 of their compounds were evaluated to determine cytotoxicity and antifungal activity. Cytotoxicity assays for both the citral and carvone chemotypes were carried out with tetrazolium-dye, which showed a dose-dependent cytotoxic effect against HeLa cells. Interestingly, this effect on the evaluated cells (HeLa and the non-tumoural cell line, Vero) was lower than that of commercial citral alone. Commercial citral showed the highest cytotoxic activity on HeLa cells. The antifungal activity was evaluated against* Candida parapsilosis, Candida krusei, Aspergillus flavus *and* Aspergillus fumigatus *strains following the standard protocols, Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing and CLSI M38-A. Results demonstrated that the most active essential oil was the citral chemotype, with geometric means-minimal inhibitory concentration (GM-MIC) values of 78.7 and 270.8 µg/mL for* A. fumigatus *and* C. krusei, *respectively. Commercial citral showed an antifungal activity similar to that of the citral chemotype (GM-MIC values of 62.5 µg/mL for* A. fumigatus *and 39.7 µg/mL for* C. krusei*). Although the citronellal and geraniol were found in lower concentrations in the citral chemotype, they had significant antifungal activity, with GM-MIC values of 49.6 µg/mL for* C. krusei *and 176.8 µg/mL for* A. fumigatus*.*

Key words: *Lippia alba* - essential oil - antifungal activity - cytotoxicity - monoterpenes

Lippia alba (Miller) N.E. Brown (Verbenaceae) is an aromatic plant that grows in Africa and Latin America that is utilised to make the infusion of "pronto alivio" ("soon relief") or "Hill oregano", as it is popularly known in Colombia, which is used for various medicinal purposes (Garcia-Barriga 1974, Pascual et al. 2001, Oliveira et al. 2006). Essential oils from *L. alba* exhibit a great chemical variety, suggesting the existence of a high number of chemotypes (Hennebelle et al. 2008). Monoterpenes such as limonene, carvone, citral, β-caryophyllene, tagetenone, myrcene, γ-terpinene, camphor, 1,8-cineole and estragole are frequently found in the essential oils of this plant (Hennebelle et al. 2006).

Different biological activities, such as cytotoxic, antifungal, antibacterial, antiviral and anti-inflammatory have been identified in essential oils or extracts from *L. alba* (Viana et al. 1998, Holetz et al. 2002, Costa et al. 2004, Andrighetti-Frohner et al. 2005, Oliveira et al. 2006). The cytotoxic and antitumoural effects of *L. alba* extracts and some major components of its essential oils, such as limonene and citral, have been demonstrated in HL-60 human promyelocytic leukaemia cells, K562 human erythroleukemic cells, HepG2 human hepatocellular liver carcinoma cells and HeLa human cervix epithelioid carcinoma cells (Paik et al. 2005, Ji et al. 2006, Wang et al. 2006). However, little effort has been made to study these activities in different chemotypes and main components of the essential oils found in *L. alba*.

Recently, there has been a growing interest in medicinal plants as alternatives to synthetic drugs, particularly against microbial agents, due to increasing antibiotic resistance of pathogens associated with infectious diseases to the common antimicrobial drugs used in clinical practice (Tavares et al. 2008). The antifungal activity of the essential oils of *L. alba* against human pathogenic fungi such as *Candida albicans*, *Candida guilliermondii, Candida parapsilosis*, *Candida neoformans*, *Trichophytum rubrum* and *Fonsecaea pedrosoi* has been previously demonstrated for the citral and myrcene-citral chemotypes (Oliveira et al. 2006).

L. alba occurs in various regions of Colombia (Durán et al. 2007) and although the antifungal activity of some essential oils of this plant have been evaluated in other countries (Duarte et al. 2005, Hennebelle et al. 2006), in Colombia the biological activities of the *L. alba* chemotypes have not yet been fully elucidated. The aim of this study was to evaluate the chemical composition, cytotoxic effect and antifungal activity of essential oils of *L. alba*, as well as some of their components, from two regions of Colombia.

MATERIALS AND METHODS

Plant material and essential oil extraction - Two samples (1 kg each) of *L. alba* stems and leaves were collected from two regions in Colombia as part of a survey

Financial support: COLCIENCIAS, Bogotá, Colombia (RC 432-2004)
+ Corresponding author: amesa@medicina.udea.edu.co
Received 16 February 2009
Accepted 26 May 2009

conducted by CENIVAM, a Research Centre devoted to aromatic plants and essential oil studies in Colombia. The taxonomic identification of the botanical samples was performed by Dr. José Luís Fernández at Herbario Nacional de Colombia, Institute of Natural Sciences, Faculty of Sciences, Universidad Nacional de Colombia (Bogotá), where exsiccata of each plant remain as permanent samples. One *L. alba* sample, voucher specimen 512084, was collected in March 2005 in Saravena, Arauca, at a mean altitude of 220 m; the second *L. alba* sample, voucher specimen 516929, was collected in May 2005 at Turbaco-Bolivar, at a mean altitude of 80 m. The essential oils were extracted from dried *L. alba* stems and leaves (300 g) by microwave-assisted hydrodistillation (30 min, 250 mL water), using a Clevenger-type distillation apparatus and a Dean-Stark distillation trap in a domestic microwave oven (Kendo MO-124, 2.5 GHz, 800 W), as described previously (Stashenko et al. 2004). Sodium sulphate (Merck, Darmstadt, Germany) was added to the decanted essential oil as a drying agent.

Essential oil analysis - Compound identification was based on mass spectra (EI, 70 eV) obtained with a gas chromatograph (GC) (Agilent Technologies 6890 Plus, Palo Alto, CA, USA), equipped with a mass selective detector (Agilent Technologies 5973), split/splitless injector (1:50 split ratio) and a data system (HP ChemStation 1.05) with WILEY 138K, NIST 2002 and QUADLIB 2004 mass spectra libraries. A DB-5MS fused-silica capillary column (J & W Scientific, Folsom, CA, USA) was used. The GC oven temperature was programmed from 45°C (15 min) to 250°C (15 min) at 5°C/min. The temperatures of the ionisation chamber and of the transfer line were set at 230°C and 285°C, respectively. Mass spectra and reconstructed ion currents (chromatograms) were obtained by automatic scanning at 5.19 scans/s, within the mass range *m/z* 30-300. Chromatographic peaks were checked for their homogeneity with the aid of the mass chromatograms for the characteristic fragment ions. A GC (HP 5890 A Series II), equipped with flame ionisation detection (FID), split/splitless injector (1:50 split ratio) and a data system [HP ChemStation HP Rev. A.06.03 (509)], was used for GC/FID analysis of the oils and quantification of their components. The detector and injector temperatures were set at 250°C. The same capillary column used for the GC/mass spectrometry (MS) analysis was used for GC/FID separation and detection. The oven temperature was programmed to go from 40°C (15 min) to 250°C (20 min) at 5°C/min. Helium was used as the carrier gas, with 152 kPa column head pressure and 35.7 cm/s linear velocity. Hydrogen and air, at 30 and 300 mL/min, respectively, were used in the GC/FID, with N_2 (30 mL/min) as a make-up gas. Individual components were identified by comparison of their retention indices (RI) (Stashenko et al. 2004), which were determined using a linear scale on the DB-5MS (60 m x 0.25 mm x 0.25 µL, J & W Scientific, Folsom, CA, USA) column and of the mass spectra of standard substances, including *cis*-3-hexenol, α-pinene, camphene, 1-octen-3-ol, beta-myrcene, alpha-phellandrene, *p*-cymene, limonene, terpinolene, linalool, *cis*-limonene oxide, citronellal, borneol, citronellol, *trans*-carveol, nerol, isogeraniol, neral, geraniol, carvone, geranial, thymol, eugenol, geranyl acetate, alpha-copaene and *trans*-beta-caryophyllene. All standards were bought from Sigma-Aldrich (USA).

Monoterpenes and drugs - (+) limonene oxide, alpha-pinene, 1S(-) beta-pinene, (±) linalool, (+) dihydrocarvone, R(-) carvone, eugenol, S(+) carvone, geraniol, nerol, citral (40:60% ratio of neral: geranial), R(+) citronellal, S(-) limonene, *trans*-beta-caryophyllene and R(+) limonene were purchased from Sigma (Chemical Company St Louis, MO, USA). Stock solutions of both oils and terpenes were prepared in DMSO for cytotoxicity and antifungal assays. Amphotericine B and itraconazole were bought from Sigma (USA).

Cytotoxicity assay - Human cervix epithelioid carcinoma cells (HeLa cell line ATCC, CCL-2) and *Cercopithecus aethiops* African green monkey kidney cells (Vero cell line, ATCC CCL-81) were used. The cells were grown in MEM supplemented with 10% FBS, 100 units/mL of penicillin, 100 mg/mL of streptomycin, 20 mg/mL of l-glutamine, 0.14% $NaHCO_3$, 1% each nonessential amino acid and a vitamin solution. The cytotoxicity of the essential oils and their components was examined in vitro with an tetrazolium-dye MTT (dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma, New Jersey, USA) assay, following a previously described protocol (Betancur-Galvis et al. 2002). Briefly, cells were plated at 15 x 10^3 cells per well in a 96-well flat-bottomed plate and incubated at 37°C in a humidified incubator with 5% CO_2. After a 24 h incubation, each diluted oil or monoterpene was added to the appropriate wells and the plates were incubated for an additional 48 h at 37°C. Supernatants were removed from the wells and 28 µL (2 mg/mL) of an MTT solution in MEM supplemented with 10% FBS were added to each well. Plates were incubated for 1 h at 37°C and then 130 µL of DMSO were added to the wells to dissolve the purple formazan crystals that were produced. The plates were placed on a shaker for 25 min and absorbance was read at 550 nm on a multiwell spectrophotometer (Titertek Uniskan). In order to define the cytotoxic effect, cytotoxic concentration 50 (CC_{50}), defined as the minimal dilution of the compound that induces 50% killing of the cells, was calculated. Pyrogallol (Fluka, USA) was used as a positive control (Han et al. 2008, Kim et al. 2008). The hydrodistillation process ensured an endotoxin-free material, since such a technique is not able to remove high-molecular mass molecules such as endotoxins (10 kDa) from the plant material and the molecular weight of essential oil components does not surpass 0.3 kDa.

Antifungal activity assay - The antifungal activity of the oils and monoterpenes was evaluated following the Clinical and Laboratory Standards Institute M38-A protocol (NCCLSI 2002) for filamentous fungi and the standard method proposed by the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST) (Cuenca-Estrella et al. 2003) for fermentative yeasts.

C. parapsilosis (ATCC 22019), *C. krusei* (ATCC 6258), *Aspergillus flavus* (ATCC 204304) and *A. fumigatus* (ATCC 204305) were used to evaluate antifungal activity. Briefly, 100 µL of five serial dilutions of the essential oils and monoterpenes were dispensed into flat-bottom 96-well microtitration plates (Becton Dickinson, New Jersey, USA) in duplicate, with final concentrations between 31.25-500 µg/mL. The plates were then frozen at -70ºC until required. Amphotericine B and itraconazole (Sigma-Aldrich, Co, MO, USA) were used as positive controls at a range of 0.031-16 µg/mL. Tween 80 was included at a final concentration of 0.001% (v/v) to enhance oil and monoterpene solubility. One hundred microlitres of the fungal inoculum size of 1-5 x 10^5 CFU/mL and 0.8 x 10^4-1x10^5 CFU/mL for yeast and filamentous fungi, respectively, were added. For the AFST- EUCAST method, the minimal inhibitory concentration (MIC) was determined after a 24 h incubation and defined as the lowest concentration that resulted in a 90% reduction of growth. For the CLSI M38-A method, the MICs were determined after a 48 h incubation and defined as the lowest essential oil dilution that resulted in total inhibition of visible growth. Evaluation of activity against *Aspergillus* spp required the use of CLSI M38-A broth macrodilution adaptation to avoid loss of the volatile monoterpene during the 48 h incubation. A similar concentration of all reagents as in the microdilution method was used and the final volume was 2 mL. Susceptibility testing was performed in duplicate in three different assays. Essential oils and terpenes were considered active when they exhibited MIC values below 500 µg/mL.

Data analysis - The CC_{50} values for each compound were obtained by lineal regression analysis of the dose-response curves generated from the absorbance data with the statistical package R (Development Core Team, Vienna, Austria, 2008). CC_{50} values were expressed as the Mean ± SEM of at least four dilutions by quadruplicate. MIC values were expressed as geometric means (GM)-MIC of tests performed in duplicate in three different assays against each of the fungal species.

RESULTS

The GC analyses demonstrated the presence of 58 compounds in the essential oils of *L. alba* (both samples data not shown). Forty-eight were characterised by GC/MS. Table I shows the predominant constituents and their respective RI. In this study, essential oils could be classified, based on their chemical constituents, into two chemotypes: citral and carvone, according to their major compounds. The monoterpenes neral (23.6%), geranial (30.5%), *trans*-beta-caryophyllene (6.2%), geraniol (6.3%), nerol (2.6%), 6-methyl-5-hepten-2-one (6%), limonene (3.7%) and linalool (2.1%) were the main constituents of the citral chemotype. The carvone chemotype was characterised by limonene (22.4%), carvone (25.3%), *trans*-beta-caryophyllene (2.4%), neral (10.4%) and geranial (10.4%) (Table I). The citral chemotype showed the highest concentration of oxygenated monoterpenes (76%), while in the carvone chemotype, the compound distribution was as follows: 25% monoterpene hydrocarbons, 56.6% oxygenated monoterpenes, 17% sesquiterpene hydrocarbons and 1.5% oxygenated sesquiterpenes.

Cytotoxic and antifungal activities of the essential oils of *L. alba* and some of their constituents are presented in Table II. Minor compounds such as (+)-limonene oxide, (-)-limonene oxide, alpha-pinene, 1*S*-(-)-beta-pinene, (±)-linalool, (+)-dihydrocarvone, R(-)-carvone, S(+)-carvone, S(-)-limonene and R(+)-limonene were not active (data not shown). The monoterpenes with cytotoxic activity (Table II) exhibited a dose-dependent inhibition on the growth of the HeLa tumoural cell line and the Vero non-tumoural cell line, with R^2 determination coefficients of linear regression > 0.8. Both, the citral chemotype oil and its major component, citral, showed the highest cytotoxic activity on the HeLa tumour line with CC50 values of 3.5 µg/mL and < 0.1 µg/mL, respectively. This amount of cytotoxic activity on tumoural cells was comparable to pyrogallol activity. The cytotoxic effect of the citral chemotype on Vero non-tumoural cells was lower than that of commercial citral alone. On the other hand, the citral chemotype showed antifungal activity against *A. fumigatus* and *C. krusei,* with GM/MIC values of 78 µg/mL and 270.8 µg/mL, respectively. The carvone chemotype oil did not show any activity against the evaluated fungi. The major components of the carvone chemotype, such as limonene and carvone, were not active at concentrations < 500 µg/mL. Oxygenated monoterpenes present in the citral chemotype, such as geraniol, citral and R(+) citronellal, showed activity against all four strains assayed; however, citral showed the lowest MIC value. Commercial citral showed the highest activity against *A. fumigatus* and *C. krusei*, with GM/MIC values of 62.5 µg/mL and 39.4 µg/mL, respectively. *A. fumigatus* was more susceptible to the citral chemotype oil and the monoterpenes geraniol, nerol, citral and R-(+)-citronellal than *A. flavus*. MIC values for the two reference antifungal drugs that were used as positive controls, amphotericine B and itraconazole, were within the established values for the AFST-EUCAST and CLSI M38-A protocols.

DISCUSSION

From a pharmacological point of view, *L. alba* is probably the most studied species in the *Lippia* genus (Hennebelle et al. 2008). In spite of the diversity and the medicinal use of *L. alba* in different regions of Colombia, no previous reports have evaluated and compared the cytotoxic and antifungal activity of *L. alba* oil chemotypes. The two *L. alba* (Mill.) N.E. Brown essential oils studied here were classified as citral and carvone chemotypes, which correspond to chemotypes I and III, respectively, according to the classification suggested by Hennebelle et al. (2008). The citral chemotype, which contains 54.1% of citral corresponding to 23.6% of neral plus 30.5% of geranial and commercial citral (Sigma, USA), which is made up of 40% neral and 60% geranial, showed the highest cytotoxic activity on the HeLa tumoural line and the lowest activity on the Vero cell line. However, in general, the order of cytotoxic activity on both cell lines was found to follow citral ≥ citral

TABLE I

Essential oil composition (%) of two *Lippia alba* chemotypes

Constituent	RI-5[b]	RI-W[c]	Relative amount (%)[a]	
			L. alba Carvone chemotype	*L. alba* Citral chemotype
cis-3-hexenol[d]	840	1289	0.1	0.0
alpha-pinene[d]	939	921	0.1	0.2
Camphene[d]	954	965	0.3	0.0
1-octen-3-ol[d]	979	1351	0.1	0.0
6-methyl-5-hepten-2-one[d]	986	1241	1.9	6.0
β-myrcene[d]	991	1064	0.7	0.1
6-methyl-5-hepten-2-ol	992	1365	0.0	0.2
alpha-phellandrene[d]	1009	1066	0.5	0.1
isopentyl isobutanoate	1015	994	0.1	0.1
p-cymene[d]	1028	1172	0.0	0.1
Limonene[d]	1034	1105	22.4	3.7
beta-phellandrene	1036	1111	0.0	0.1
trans- beta-ocimene	1047	1153	0.5	0.3
Terpinolene[d]	1089	1186	0.05	0.0
Rosefuran	1092	1303	0.1	0.4
Linalool[d]	1100	1453	0.6	2.1
trans-p-mentha-2,8-diene-1-ol	1126	1580	0.1	0.0
exo-fenchol	1128	1491	0.0	0.0
cis-limonene oxide[d]	1137	1350	0.1	0.0
*cis-p-m*entha-2,8-diene-1-ol	1141	1707	0.1	0.0
Citronellal[d]	1154	1381	0.3	0.8
cis-chrysanthenol	1162	1449	0.1	0.6
rosefuran epoxide	1171	1509	0.1	1.1
Borneol[d]	1181	1613	0.6	0.9
cis-dihydrocarvone	1203	1517	0.4	0.0
trans-dihydrocarvone	1211	1537	0.3	0.0
Citronellol[d]	1224	1673	0.0	0.1
trans-carveol[d]	1228	1745	0.6	0.0
Nerol[d]	1231	1708	0.7	2.6
Isogeraniol[d]	1236	1719	0.1	0.5
Nerald	1248	1589	10.4	23.6
Geraniol[d]	1252	1755	0.0	6.3
Carvone[d]	1258	1653	25.3	0.0
Piperitone	1264	1641	1.1	0.1
Geranial[d]	1275	1643	10.4	30.5
isopiperitone	1278	1754	0.2	0.0
trans-carvone oxide	1284	1745	0.1	0.0
Thymol[d]	1291	2092	0.1	0.0
piperitenone	1349	1842	2.2	0.0
Eugenol[d]	1357	2080	0.2	0.3
geranyl acetate[d]	1379	1662	0.4	0.7
alpha-copaene[d]	1385	1399	0.1	0.0
beta-elemene	1397	1496	1.7	0.9
beta-ylangene	1432	1483	0.2	0.0
trans-beta-caryophyllene[d]	1436	1506	2.4	6.2
beta-gurjunene	1444	1447	0.2	0.0
beta-guaiane	1447	1498	1.3	1.6
trans- beta-farnesene	1456	1570	0.2	0.1

a: relative amounts (percentages) were calculated from relative peak areas in gas chromatograph flame ionisation detection chromatograms; *b*: retention indices on DB-5MS Column; *c*: retention indices on DB-WAX Column; *d*: a standard compound was available for identification purposes.

TABLE II

Cytotoxic concentration 50 (CC_{50}) and geometric means of minimal inhibitory concentration (GM-MIC) (µg/mL) of two chemotypes of essential oil of *Lippia alba* and some of their constituents

	Cytotoxic activity				Antimicotic activity GM-MIC (µg/mL)			
	Vero cell line		HeLa cell line					
Samples	CC_{50}	R^2	CC_{50}	R^2	*Candida parapsilosis*	*Candida krusei*	*Aspergillus flavus*	*Aspergillus fumigatus*
Carv Carvone chemotype	> 200	-	74.5 ± 13.1	0.85	> 500	> 500	> 500	> 500
Citral chemotype	> 100	-	3.5 ± 0.7	0.77	> 500	270.8[a]	> 500	78.7[a]
eugenol	71.3 ± 13.3	0.83	86.6 ± 18.3	0.86	500	500	500	250[a]
geraniol	59.3 ± 6.7	0.94	131 ± 3.7	0.99	314.9[a]	500	250[a]	176.8[a]
nerol	47.8 ± 9.9	0.83	85.6 ± 4.2	0.97	500	500	500	250[a]
citral	124.1 ± 12.2	0.92	< 0.1	0.87	125[a]	39.72[a]	250[a]	62.5[a]
trans-beta-caryophyllene	39.7 ± 6.9	0.75	-		> 500	> 500	> 500	> 500
R(+)citronellal	64.4 ± 11	0.86	160.9 ± 6.1	0.98	314.9[a]	49.6[a]	500	250[a]
Itraconazole	NC	-	NC	-	0.06 - 0.12	0.06-0.25	0.25	0.0625-0.25
Amphotericin B	NC	-	NC	-	1	0.5	1	2
Pyrogallol	120.6 ± 12.6	0.91	4.1 ± 1.2	0.81	-	-	-	-

a: oils and monoterpenes with MICs below 500 µg/mL; CC_{50}: the minimal dilution of the compound that induces 50% killing of the cells; NC: not calculated; R^2: coefficient of determination of linear regression.

chemotype oil > carvone chemotype oil, which suggests a citral-dependent cytotoxicity. The absence of cytotoxicity in the carvone chemotype oil could be explained by either the low content of oxygenated monoterpene citral (20.8%) compared to that in the citral chemotype oil (54.1%), or by the fact that the major compounds (limonene and carvone) in the former oil were antagonistic to the oxygenated monoterpene citral.

According to the National Cancer Institute (USA), the threshold of significance for crude extracts is inhibitory concentration 50 (IC_{50}) < 30 µg/mL (Hennebelle et al. 2008) and therefore the citral chemotype was not cytotoxic on Vero non-tumoural cells. Previous studies have shown the cytotoxic activity of citral on tumoural cells (Dudai et al. 2005). Citral effectively inhibits the growth of a mouse leukaemia cell line (BS-24-1), with a CC_{50} value of 47 µg/mL, but is ineffective on normal spleen cells. Furthermore, 45 µg/mL citral, through caspase 3 activation, induces apoptosis of human (U397, HL60) and mouse (RL-12, BS-24-1) leukaemic cells. In contrast, its derivates, citronellal, citronellol and a product of its metabolism, geraniol, have no effect on the survival of leukaemic cells (Dudai et al. 2005), similar to what was observed in this study in HeLa cells.

Several methods for detecting antifungal activity of natural products are available, but since they are not equally sensitive or not based upon the same principle, results can be profoundly influenced by the method (Cos et al. 2006). Sharma and Tripathi (2008) have shown that the antifungal activity of essential oils can be more effectively evaluated in liquid rather than in solid media, since in the latter, oil diffusion may not be appropriate. In this study, the standard methods of microdilution for the evaluation of antifungal susceptibility to drugs were used. These methods allow for reduction of the amount of oil used and the simultaneous evaluation of several samples, as well as data reproducibility. However, long-term incubation for testing monoterpenes against *Aspergillus* spp required the use of the macrodilution test, since the active and volatile terpenes interfered with the evaluation of activity of other less volatile terpenes when a microdilution method was used. The disadvantage to this method is that macrodilution requires a larger sample size.

Currently, there is no agreement on the acceptable level of activity for plant material when compared to standard drugs; therefore, some authors consider activity only if it is comparable to antibiotics, while others only consider higher values. Aligiannis et al. (2001) suggested a classification for plant materials, based on MIC results, as follows: strong inhibitors, MIC up to 0.5 mg/mL, moderate inhibitors, MIC between 0.6 and 1.5 mg/mL, and weak inhibitors, MIC above 1.6 mg/mL. Based on these criteria, Duarte et al. (2005) indicated a strong activity of *L. alba* oil against *C. albicans*, with an MIC of 0.06 mg/mL, while MIC of the positive control nystatin is 0.05 mg/mL. On the other hand, Borges-Argáez et al. (2007) have suggested that a MIC value of 100-200 µg/mL can be considered acceptable for plant material. According to these criteria, in this study, *C. krusei* was moderately sensitive to the citral chemotype essential oil (GM/MIC 270 µg/mL) and highly sensitive to the commercial monoterpenes, citral (39.72 µg/mL) and R-(+)-citronellal (49.6 µg/mL). This finding is important because *C. krusei* has been recognised as a potential multidrug-resistant pathogen due to its intrinsic fluconazole resistance and recent reports showing a decreased susceptibility of the pathogen to both flucytosine and amphotericine B (Pfaller et al. 2008). *Aspergillus fumi-*

gatus is the main cause of invasive aspergillosis in immunocompromised patients and only a limited number of drugs are available for treatment (Brock et al. 2008). In addition, recent studies have reported emergence of azole resistance in this species (Snelders et al. 2008). Therefore, new antifungals are urgently needed.

In this study, the lowest MIC values with *A. fumigatus* were obtained with the citral chemotype (78.7 µg/mL) and citral (62.5 µg/mL). Hayes and Markovic (2002) showed that *Backhousia citriodora* essential oil, with a 92% composition of citral, was active against *C. albicans* and *Aspergillus niger* with MICs comparable to commercial citral. Recently, Sharma and Tripathi (2008) determined, through an agar diffusion method, a MIC value of 0.3 µg/mL against *A. niger* for a *Citrus sinensis* essential oil, which has a limonene concentration of 87%. Linalool and linalool-rich essential oils from *Croton cajucara* are active against *C. albicans*, with MIC values of 0.7 µg/mL and 13.4 µg/mL, respectively (Alviano et al. 2005). Duarte et al. (2005) showed that a *L. alba* linalool-chemotype essential oil, with 76.3% of linalool, is active against *C. albicans*, with an MIC of 0.6 mg/mL. In this study, which used the AFST-EUCAST and CLSI M38-A protocols and a threshold of antifungal activity of MIC $\leq$ 500 µg/mL, the carvone chemotype essential oil of *L. alba* and the monoterpenoids carvone, linalool and limonene, were not active against *C. parapsilosis*, *C. krusei*, *A. flavus*, or *A. fumigatus*.

The antifungal activity of the citral chemotype of *L. alba* demonstrated in this study could be explained by a higher concentration of oxygenated monoterpenes, such as neral/geranial (54%) and nerol/geraniol (8.9%), which has been previously described by Oliveira et al. (2006). Essential oils can be useful as antifungal agents because they affect several targets simultaneously and there are no reports of particular resistance or adaptation to essential oils by yeast (Bakkali et al. 2008).

Skin sensitisation has been reported during topical application of citral at concentrations higher than 1% (Hayes & Markovic 2002). Dijoux et al. (2006) have shown that lemongrass (*Cymbopogon citrates*) oil with citral (70-90%) and geraniol (1.5-7.5%) is phototoxic and cytotoxic. The present results show that the citral chemotype of *L. alba* was not cytotoxic on non-tumoural cells. The results described for the citral chemotype of *L. alba* should stimulate further studies on the phototoxicity of this compound on primary cells, in order to consider its topical use for clinical applications.

REFERENCES

Aligiannis N, Kalpotzakis E, Mitaku S, Chinou IB 2001. Composition and antimicrobial activity of the essential oils of two *Origanum* species. *J Agric Food Chem 40*: 4168-4170.

Alviano WS, Mendonça-Filho RR, Alviano DS, Bizzo HR, Souto-Padrón T, Rodrigues ML, Bolognese AM, Alviano CS, Souza MM 2005. Antimicrobial activity of *Croton cajucara* Benth linalool-rich essential oil on artificial biofilms and planktonic microorganisms. *Oral Microbiol Immunol 20*: 101-105.

Andrighetti-Frohner CR, Sincero TCM, da Silva AC, Savi LA, Gaido CM, Bettega JMR, Mancini M, de Almeida MTR, Barbosa RA, Farias MR, Barardi CRM, Simoes CMO 2005. Antiviral evaluation of plants from Brazilian atlantic tropical forest. *Fitoterapia 76*: 374-378.

Bakkali F, Averbeck S, Averbeck D, Idaomar M 2008. Biological effects of essential oils - a review. *Food Chem Toxicol 46*: 446-475.

Betancur-Galvis L, Zuluaga C, Arno M, Gonzalez MA, Zaragoza RJ 2002. Cytotoxic effect (on tumor cells) and *in vitro* antiviral activity against herpes simplex virus of synthetic spongiane diterpenes. *J Nat Prod 65*: 189-192.

Borges-Argáez R, Canche-Chay CI, Peña-Rodriguez LM, Said-Fernández S, Molina-Salinas GM 2007. Antimicrobial activity of *Diospyros anisandra*. *Fitoterapia 78*: 370-372.

Brock M, Jouvion G, Droin-Bergère S, Dussurget O, Nicola MA, Ibrahim-Granet O 2008. Bioluminescent *Aspergillus fumigatus*, a new tool for drug efficiency testing and *in vivo* monitoring of invasive aspergillosis. *Appl Environ Microbiol 74*: 7023-7035.

Cos P, Vlietinck AJ, Berghe DV, Maes L 2006. Anti-infective potential of natural products: how to develop a stronger *in vitro* "proof-of-concept". *J Ethnopharmacol 106*: 290-302.

Costa MCCD, Aguilar JS, do Nascimento SC 2004. Atividade citotóxica de extratos brutos de *Lippia alba* (Mill.) N.E. Brown (Verbenaceae). *Acta Farm Bonaerense 23*: 349-352.

Cuenca-Estrella M, Moore CB, Barchiesi F, Bille J, Chryssanthou E, Denning DW, Donnelly JP, Dromer F, Dupont B, Rex JH, Richardson MD, Sancak B, Verweij PE, Rodríguez-Tudela JL, AFST Subcommittee of the European Committee on Antimicrobial Susceptibility Testing 2003. Multicenter evaluation of the reproducibility of the proposed antifungal susceptibility testing method for fermentative yeasts of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (AFST-EUCAST). *Clin Microbiol Infect 9*: 467-474.

Dijoux N, Guingand Y, Bourgeois C, Durand S, Fromageot C, Combe C, Ferret PJ, 2006. Assessment of the phototoxic hazard of some essential oils using modified 3T3 neutral red uptake assay. *Toxicol In Vitro 20*: 480-489.

Duarte MC, Figueira GM, Sartoratto A, Garcia VL, Delarmelina C 2005. Anti-*Candida* activity of Brazilian medicinal plants. *J Ethnopharmacol 97*: 305-311.

Dudai N, Weinstein Y, Krup M, Rabinski T, Ofir R 2005. Citral is a new inducer of caspase-3 in tumor cell lines. *Planta Med 71*: 484-488.

Durán DC, Monsalve LA, Martínez JR, Stashenko EE 2007. Estudio comparativo de la composición química de aceites esenciales de *Lippia alba* provenientes de diferentes regiones de Colombia, y efecto del tiempo de destilación sobre la composición del aceite. *Sci Téc 13*: 435-438.

García-Barriga H 1974. *Plantas medicinales de Colombia*, Universidad Nacional de Colombia, Bogotá, 506 pp.

Han YH, Kim SZ, Kim HS, Park WH 2008. Pyrogallol as a glutathione depletor induces apoptosis in HeLa cells. *Int J Mol Med 21*: 721-730.

Hayes AJ, Markovic B 2002. Toxicity of Australian essential oil *Backhousia citriodora* (Lemon myrtle). Part 1. Antimicrobial activity and *in vitro* cytotoxicity. *Food Chem Toxicol 40*: 535-543.

Hennebelle T, Sahpaz S, Dermont C, Joseph H, Bailleul F 2006. The essential oil of *Lippia alba*: analysis of samples from French overseas departments and review of previous works. *Chem Biodivers 3*: 1116-1125.

Hennebelle T, Sahpaz S, Henry J, Bailleul F 2008. Ethnopharmacology of *Lippia alba*. *J Ethnopharmacol 116*: 211-222.

Holetz FB, Pessini GL, Sanches NR, Cortez DAG, Nakamura CV, Filho BPD 2002. Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases. *Mem Inst Oswaldo Cruz 97*: 1027-1031.

Ji J, Zhang L, Wu YY, Zhu XY, Lv SQ, Sun XZ 2006. Induction of apoptosis by d-limonene is mediated by a caspase-dependent mitochondrial death pathway in human leukemia cells. *Leuk Lymphoma 47*: 2617-2624.

Kim SW, Han YW, Lee ST, Jeong HJ, Kim SH, Kim IH, Lee SO, Kim DG, Kim SH, Kim SZ, Park WH 2008. A superoxide anion generator, pyrogallol, inhibits the growth of HeLa cells via cell cycle arrest and apoptosis. *Mol Carcinog 47*: 114-125.

NCCLSI - National Committee for Clinical Laboratory Standards Institute 2002. *Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard. NCCLSI document M38-A*, Vol 22, National Committee for Clinical Laboratory Standards, Wayne, p. 1-29.

Oliveira DR, Leitao GG, Santos SS, Bizzo DHR, Lopes D, Alviano CS, Alviano DS, Leitão SG 2006. Ethnopharmacological study of two *Lippia* species from Oriximina, Brazil. *J Ethnopharmacol 108*: 103-108.

Paik SY, Koh KH, Beak SM, Paek SH, Kim JA 2005. The essential oils from *Zanthoxylum schinifolium* pericarp induce apoptosis of HepG2 human hepatoma cells through increased production of reactive oxygen species. *Biol Pharm Bull 28*: 802-807.

Pascual ME, Slowing K, Carretero E, Sanchez D, Villar A 2001. *Lippia*: traditional uses, chemistry and pharmacology: a review. *J Ethnopharmacol 76*: 201-214.

Pfaller MA, Diekema DJ, Gibbs DL, Newell VA, Nagy E, Dobiasova S, Rinaldi M, Barton R, Veselov A 2008. Global Antifungal Surveillance Group. *Candida krusei*, a multidrug-resistant opportunistic fungal pathogen: geographic and temporal trends from the ARTEMIS DISK Antifungal Surveillance Program, 2001 to 2005. *J Clin Microbiol 46*: 515-521.

Sharma N, Tripathi A 2008. Effects of *Citrus sinensis* (L.) Osbeck epicarp essential oil on growth and morphogenesis of *Aspergillus niger* (L.) Van Tieghem. *Microbiol Res 163*: 337-344.

Snelders E, van der Lee HA, Kuijpers J, Rijs AJ, Varga J, Samson RA, Mellado E, Donders AR, Melchers WJ, Verweij PE 2008. Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *PLoS Med 5*: e219.

Stashenko EE, Jaramillo BE, Martínez JR 2004. Comparison of different extraction methods for the analysis of volatile secondary metabolites of *Lippia alba* (Mill.) N.E. Brown, grown in Colombia and evaluation of its *in vitro* antioxidant activity. *J Chromatogr A 1025*: 93-103.

Tavares AC, Goncalves MJ, Calaleiro C, Cruz MT, Lopes MC, Canhoto J, Salgueiro LR 2008. Essential oil of *Daucus carota subsp. halophilus*: composition, antifungal activity and cytotoxicity. *J Ethnopharmacol 119*: 129-134.

Viana GSB, do Vale TG, Rao VSN, Matos FJA 1998. Analgesic and antiinflammatory effects of two chemotypes of *Lippia alba*: a comparative study. *Pharm Biol 36*: 347-351.

Wang XS, Yang W, Tao SJ, Li K, Li M, Dong JH, Wang MW 2006. Effect of delta-elemene on Hela cell lines by apoptosis induction. *Yakugaku Zasshi 126*: 979-990.