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Biotechnological potential of essential oils from different chemotypes of *Lippia alba* (Mill.) N.E.Br. ex Britton & P. Wilson

[Potencial biotecnológico de los aceites esenciales de diferentes quimiotipos de *Lippia alba* (Mill.) N.E.Br. ex Britton y P. Wilson]

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Abstract: This study reports the biotechnological importance of seven *Lippia alba* specimens collected in different places in Brazil, and evaluation of some activities as larvicidal against *Aedes* spp., antifungal against dermatophytes; cytotoxicity against SNB-19 (astrocytoma), HCT-116 (human colon) and PC-3 (human prostate) cancer cell lines, and inhibition of the enzyme acetylcholinesterase (AChE). The essential oils, whose extraction yield was about 1.24 w/w ± 0.9%, showed differences in their chemical composition and considered as chemotypes. The essential oils containing neral and geranial as main constituents showed better action against HCT-116 cell lines (IC50 value was 9.22 µg/mL), larvicidal activity against arbovirus vectors (LC50 value against *A. aegypti* was 1.59 µg/mL) and inhibition of AChE (halo inhibition zone was 1 cm). The essential oils containing mainly monoterpenoids showed better antifungal action with MIC values range from 0.15 to 1.25 mg/mL. This chemical and biological characterization may be useful for biotechnological applications.

Keywords: Vector control; Antitumor effect; Natural products; *Lippia alba*; Chemotypes.

Resumen: Este estudio reporta la importancia biotecnológica de siete especímenes de *Lippia alba* recolectados en diferentes lugares de Brasil, y evaluación de algunas actividades como larvicida contra *Aedes* spp., Antifúngico contra dermatofitos; citotoxicidad contra líneas celulares de cáncer SNB-19 (astrocitoma), HCT-116 (colon humano) y PC-3 (próstata humana), e inhibición de la enzima acetilcolinesterasa (AChE). Los aceites esenciales, cuyo rendimiento de extracción fue de aproximadamente 1,24 p/p ± 0,9%, mostraron diferencias en su composición química y se consideraron quimiotipos. Los aceites esenciales que contienen neral y geranial como constituyentes principales mostraron una mejor acción contra las líneas celulares HCT-116 (el valor de CI50 fue de 9.22 µg/mL), actividad larvicida contra los vectores de arbovirus (el valor de LC50 contra *A. aegypti* fue de 1.59 µg/mL) e inhibición de AChE (la zona de inhibición del halo era de 1 cm). Los aceites esenciales que contienen principalmente monoterpenoides mostraron una mejor acción antifúngica con valores de CMI en el rango de 0.15 a 1.25 mg/mL. Esta caracterización química y biológica puede ser útil para aplicaciones biotecnológicas.

Palabras clave: Control de vectores; Efecto antitumoral; Productos naturales; *Lippia alba*; Quimiotipos

INTRODUCTION

Lippia alba (Mill.) N.E.Br. ex Britton & P. Wilson (Verbenaceae family) is a highly branched aromatic shrub with height up to 2 m (Hennebelle *et al*., 2008). Popularly known as bushy matgrass, bushy Lippia or lemon balm in English language countries, in Brazil it is variously called *erva cidreira*, *falsa melissa* and *salvia*. It is widely distributed in tropical and subtropical regions, with considerable rates of endemism (Salimena *et al*., 2016).

Previous studies indicate a large use the *L. alba* by the population including indigenous and rural communities. Infusions made from leaves and aerial parts are used against hypertension, states of excitement, nausea, flu, digestive troubles, colds and pain, as well as topically to heal wounds, and as a syrup against cough and bronchitis (Albuquerque *et al*., 2007; Suárez, 2019).

Different biological activities, such as antibacterial (Porfírio *et al*., 2017), antifungal (Mesa-Arango *et al*., 2009), antiviral (Ocazionez *et al*., 2010), antigenotoxic (López *et al*., 2011), antiprotozoal (Escobar *et al*., 2010), antioxidant (Stashenko *et al*., 2004) and vasorelaxant (Silva *et al*., 2018), have been identified in *L. alba* essential oils.

The essential oil of *L. alba* presents varied chemical composition, with great chemical diversity, prompting classification into different chemotypes, such as linalool, carvone, citral, myrcene-camphor, myrcene and limonene-carvone (Matos, 1996; Yamamoto *et al*., 2008).

Natural products have attracted increasing interest by the pharmaceutical industry due to their medicinal and pharmacological properties, as alternatives to synthetic drugs (Harvey *et al*., 2015). The larvicidal action of natural products has been useful in the development of bioproducts for the control of arbovirus vectors, such as dengue fever, zika fever, chikungunya fever, yellow fever and Japanese encephalitis, which are serious public health problems in tropical and subtropical regions (Raj *et al*., 2015; Londoño-Renteria *et al*., 2016).

The acetylcholinesterase (AChE) inhibitory enzyme assay is useful to prospect for molecules with pharmacological action for the treatment of Alzheimer's disease (Ali-Shtayeh *et al*., 2018). AChE is the major mode of action of most insecticides, such as carbamates and organophosphates, and this effect may be responsible for the larvicidal activity against *Aedes aegypti* and *Aedes albopictus* (Gade *et al*., 2017; Mesquita *et al*., 2018).

This article compares the chemical composition of the volatile oils extracted from seven *L. alba* chemotypes, and characterizes some biological properties, such as larvicidal against *Aedes aegypti* and *A, albopictus*, antifungal against dermatophytes, and cytotoxicity against SNB-19 (astrocytoma), HCT-116 (human colon) and PC-3 (human prostate) cancer cell lines, as well as evaluating these essential oils for the inhibition of acetylcholinesterase, to provide useful information on possible medicinal and other biotechnological applications.

MATERIALS AND METHODS

Plant material

Aerial parts of *L. alba* chemotypes (leaf, inflorescence and stem) were collected in the flowering period in the medicinal plants garden of Embrapa Agroindústria Tropical (Agroindustry Research Unit), Fortaleza, Ceará, Brazil, in August 2016 (latitude 3º 45', 47", longitude 38º 31' 23" W, altitude 21 m). A voucher specimen (No. EAC59269) was deposited in the Prisco Bezerra Herbarium (EAC) and authenticated by botanist Dr. Maria Iracema Bezerra Loiola of the Department of Biology, Federal University of Ceará. The chemotypes were coded according to their region of origin and culture in Brazil. Embrapa also uses this standard of identification. This study involved the use of seven *L. alba* chemotypes from different regions of Brazil: LA1, LA2, LA3, LA4, LA5, LA6, LA7, all plants grown at Embrapa Tropical Agroindustry (Table No. 1).

Extraction and analysis of essential oils

Fresh aerial parts of *L. alba* (500 g of each chemotype) were submitted to hydrodistillation for 4 h in a modified Clevenger-type apparatus (Craveiro *et al*., 1976). The oil was dried over anhydrous Na_2SO_4 (~1 g), filtered and preserved in a sealed vial at 4ºC prior to further analysis, with different yields of 0.2-2% (w/w). The chemical analysis of the essential oil constituents was performed with a Shimadzu QP-2010 Ultra instrument employing the following conditions: Column: Rtx-5MS (Crossbond, 5% diphenyl/95% dimethyl polysiloxane) measuring 30 m x 0.25 mm x 0.25 µm df; carrier gas: He (24.2 mL/min, in constant linear velocity mode); injector

temperature of 250ºC, in split mode (1:100); and detector temperature of 250ºC. The column temperature was programmed for 35–180ºC at 4ºC/min, then 180–280ºC at 17ºC/min, and 280ºC for 10 min. Mass spectra were obtained at electron impact of 70 eV. The volume of sample injected was 1 µL. The components were identified from their GC retention times, calculated by linear interpolation relative to retention times of main compounds and by comparison of their mass spectra with those present in the computer data bank and published literature (Adams, 2012).

Table No. 1
Variety/chemotype of *Lippia alba* obtained from the experimental garden of Brazilian Agricultural Research Corporation - Embrapa Tropical Agroindustry in Ceará State, Brazil

Variety/chemotype	Municipality/State/Country	Yield (%)
LA1	Atibaia/ São Paulo /Brazil	0.99
LA2	São Gonçalo do Rio Abaixo/ Minas Gerais /Brazil	1.44
LA3	Botucatu/ São Paulo/ Brazil	0.72
LA4	Brasília/ Federal District/ Brazil	0.61
LA5	Cruzeiro Grande/ Federal District/ Brazil	3.31
LA6	Embrapa/ Cenargem/ Federal District/ Brazil	1.00
LA7	Estrutural/ Federal District/ Brazil	0.63

Acetylcholinesterase inhibition assay

The acetylcholinesterase inhibition (AChE inhibition) was qualitatively assessed using the method described by Ellman *et al*. (1961), as adapted for thin-layer chromatography (TLC) by Rhee *et al*. (2001), and was quantified using a BioTek Elisa microplate reader (model ELX 800 with Gen5 V2.04.11 software) at 405 nm, based on the method described by Ellman *et al*. (1961) and modified by Trevisan *et al*. (2003). This in a very sensitive method based on measuring thiocholine production from hydrolysis of acetylthiocholine. This is accomplished by the continuous reaction of thiol with 5, 50-dithiobis (2-nitrobenzoic acid). The following solutions were used: A. Tris/HCl 50 mM, pH 8; B. Tris/HCl 50 mM, pH 8, with 0.1% bovine albumin fraction V; and C. Tris/HCl 50 mM, pH 8, with NaCl (0.1 M) and $MgCl_2 \cdot 6H_2O$ (0.02 M). To each well of a 96-well microplate, 25 µL of acetylthiocholine iodide (15 µM), 125 µL of 5,50-dithiobis in Solution C (3 µM DTNB or Ellman's reagent), 50 µL of Solution B, and 25 µL of compound dissolved in MeOH and diluted in Solution A at concentrations ranged from 1.56 to 400 µg/mL.

Larvicidal assay

The larvicidal assay against *Aedes aeypti* was performed by the following method: *L. alba* essential oils were placed in beakers and dissolved in 20 mL of H_2O/DMSO 1.5% (v/v) at concentrations of 50-500 mg/mL, followed by the addition of 50 *A. aegypti* and *A. albopictus* larvae of the third instar. Mortality was recorded after 24 h of exposure, during which no nutritional supplement was added. The experiments were carried out at 28 ± 2°C. Each test was performed in triplicate. Data were evaluated through regression analysis. From the regression line, the LC_{50} values were read, representing the concentration causing 50% larval mortality. The insecticide Temephos™ was used as positive control and a solution of water and 3% DMSO was used as negative control (Tabanca *et al*., 2013; Pereira *et al*., 2018). The *A. aegypti* and *A. albopictus* larvae of the third instar were obtained at the Etymology Laboratory of the Vector Group of the Ceará State Health Secretariat.

Antifungal activity

The antifungal activity was determined in accordance with guidelines of the Clinical and Laboratory

Standards Institute (CLSI, 2008) by the broth microdilution method. Two strains of *Trichophyton rubrum* were used. Dermatophytes strains were obtained from the fungal collection of the Microbiology Laboratory State University of Vale do Acaraú. Each strain was recovered and identified based on the macromorphology and micromorphology of the colonies (De Hoog *et al*., 2008). Aliquots of suspensions were prepared in potato dextrose agar (Difco, Detroit, MI, USA), and then incubated at 28ºC for 5 –10 days (*T. rubrum* strains). The suspensions were diluted to 1:500 for *T. rubrum*, with RPMI 1640 medium supplemented with l-glutamine without sodium bicarbonate, and then buffered to pH 7.0 with 0.165 M MOPS. *L. alba* chemotypes were tested in concentrations ranging from 0.002 to 2.5 mg/mL. The microdilution test was performed in 96-well microdilution plates incubated at 37ºC, and the results were expressed as minimum inhibitory concentration (MIC), while the antifungal effect was analyzed visually after 5 days (*T. rubrum*). The minimum fungicidal concentration (MFC) was determined by subculturing 100 µL of solution from wells without turbidity on potato dextrose, at 28°C, and was determined as the lowest concentration resulting in no growth of the subculture (Fontenelle *et al*., 2008).

Cytotoxicity assay

The cytotoxicity of the essential oils was determined by the MTT assay, following the protocol described in previous studies (Victor *et al*., 2017; Guedes *et al*., 2018). The cytotoxicity was tested against SNB-19 (astrocytoma), HCT-116 (human colon) and PC-3 (human prostate) cancer cell lines, obtained from the National Cancer Institute, Bethesda (MD, USA). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin, and incubated at 37ºC under a 5% CO_2 atmosphere. Cells were plated in 96-well plates at concentrations of 1 x 10^5 cell mL^{-1}. After 24 h, all fractions (50 µg/mL) dissolved in 1% DMSO were added to each well using a high-throughput screening system (Biomek 3000 – Beckman Coulter, Inc. Fullerton, CA, USA), and the cultures were incubated for 72 h. The fractions were centrifuged at 3000 × g for 10 min at 25ºC. and the supernatant removed. Then 200 µL of a solution of MTT (tetrazolium salt) was added and the plates were incubated for 3 h at 37ºC. The absorbance was measured at 595 nm in a spectrophotometer, after dissolution of the precipitate with 150 µL of DMSO. The control groups received the same amount of DMSO. The positive control used was doxorubicin at concentration of 0.3 mg/L.

In the statistical analysis**,** all experiments were performed in triplicate. One-way ANOVA with the Tukey test was performed followed by multiple comparison testing where appropriate. IC_{50} values were calculated using the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA). Significance of difference was accepted at $p<0.05$.

RESULTS AND DISCUSSION

The volatile oils extracted from *L. alba* chemotypes ranged from yellow to red, whose extraction yield were: 0.99% (LA1), 1.44% (LA2), 0.72% (LA3), 0.61% (LA4), 3.31% (LA5). 1.0% (LA6), 0.63% (LA7). The chemical composition of the essential oil chemotypes, including the retention index and the relative percentage of each constituent, are shown in Table No. 2. For all analyses, more than 90% of the total essential oil composition was obtained.

The chemotypes were characterized by their major constituents: camphor (31.76%) and 1,8-cineole (18.67%) for essential oil LA1; camphor (17.36%) and β-caryophyllene (15.75%) for LA2; geranial (35.60%) and neral (23.55%) for LA3; linalool (96.66%) for LA4; β-caryophyllene (26.08%) for LA5; carvone (46.68%) and geranial (15.65%) for LA6; and geranial (22.52%) and β-caryophyllene (16.50%) for LA7.

Genotypes of *L. alba* have been classified as phenotypes with the different chemotypes due to variation in the concentration of their constituents and the aromatic differences, such as citral aroma. Matos *et al*. (1996) described six types *L. alba* in Brazil, with the prevalence between the clones of two chemotypes, carvone in three samples and citral (geranial + neral) and three other clones.

Environmental factors are probably the main determinants for the formation of chemotypes. Also, cytogenetic differences in the number of chromosomes in two chemotypes were found, and may be related to the speciation processes (Tavares *et al*., 2005). The chemotype LA4 basically presented one constituent, linalool, a monoterpene of great commercial importance in the perfume and cosmetics industry and in pharmacology due to its medicinal properties (Julião *et al*., 2003; Camargo &

Vasconcelos, 2015).

Table No. 2
Comparative chemical composition (%) of the *Lippia alba* essential oils obtained from different places

Constituents	KI[a]	Peak area (%)						
		LA1	LA2	LA3	LA4	LA5	LA6	LA7
α-Pinene	940	14.41	17.33	-	-	-	-	-
Camphene	954	3.07	2.11	-	-	-	-	-
β-Pinene	979	-	0.96	-	-	-	-	-
6-Methyl-hepten-2-one	984	-	-	4.38	-	-	2.48	4.72
Myrcene	988	0.66	1.02	6.07	-	-	-	2.73
α-Terpinene	1016	0.64	0.81	-	-	-	-	-
p-Cymene	1024	6.48	13.63	-	-	-	-	-
o-Cymene	1022	-	-	-	-	-	2.28	-
Limonene	1026	3.74	3.92	7.83	-	-	11.94	-
1,8-Cineole (eucalyptol)	1031	18.67	15.07	-	-	-	-	
γ-Terpinene	1057	1.73	0.64	-	-	-	-	-
Linalool	1099	-	-	3.62	96.66	-	1.58	2.86
Camphor	1146	31.76	17.36	-	-	-	-	-
Terpinen-4-ol	1177	0.95	0.65	-	-	-	-	-
Myrtenol	1201	-	-	-	-	-	-	1.14
Nerol	1235	-	-	1.75	-	-	-	3.61
Neral	1249	-	-	23.55	-	-	11.60	14.20
Carvone	1252	-	-	3.20	-	-	46.68	-
Geraniol	1263	-	-	1.74	-	-	-	4.10
Geranial	1280	-	-	35.60	-	-	15.65	22.52
α-Copaene	1377					1.43		
β-Elemene	1381	-	-	-	-	10.96	-	-
β-Caryophyllene	1427	7.70	15.75	2.47	-	26.08	-	16.50
Allo-aromadendrene	1428	3.02	2.72	-	-	-	-	-
α-Humulene	1486	-	-	-	-	7.20	-	-
D-Germacrene	1500	-	-	-	-	9.60	1.80	1.84
γ-Cadinene	1514					4.38		
δ-Cadinene	1523					9.52		
α-Cadinene	1538					3.46		
Elemol	1550						4.89	
β-Germacrene	1562	-	-	-	-	6.92	-	-
Nerolidol	1570	-	-	1.12	-	-	-	1.93
Spathulenol	1678	-	-	-	-	2.92	-	-
Caryophyllene oxide	1583	0.93	2.71	1.04	0.84	3.35	-	2.04
Globulol	1585	-	-	4.73	-	-	-	8.67
Viridiflorol	1591	0.64	-	-	-	-	-	-
Epi-α-cadinol	1640	0.93	-	-	-	4.28	-	-
α- Muurolol	1646	-	-	-	-	0.76	-	-
α-Cadinol	1654	-	-	-	-	4.22	-	1.35
2Z, 6E-Farnesol	1694	-	-	-	-	0.37	-	-
Totarene	1885	-	-	-	-	-	-	3.93
Total identified		97.41	94.68	97.10	100.00	95.45	98.90	92.14

[a]KI refers to the retention index of the column Rtx-5MS and were estimated by linear regression of retention times of main compounds in the chromatograms and respective Kovats index from the literature

Table No. 3 shows the results of the AChE inhibition by qualitative (inhibition zone – cm) and quantitative (inhibition – µg/mL) assays. The formation of a white halo around the TLC spots is indicative of AChE inhibition. All samples presented results close to or greater than those found for physostigmine, used as a positive control. However, the LA7 chemotype showed a stronger positive result (1.0 cm) than that of physostigmine in the qualitative assay.

Table No. 3
Acetylcholinesterase (AChE) inhibition assays (by TLC and ELISA) of the *Lippia alba* essential oils obtained from different chemotypes

L. alba chemotypes	AChE Inhibition Zone (cm)	AChE Inhibition (µg/mL)
LA1	0.8^{b}±0.01	13.20^{c}±0.88
LA2	0.7^{a}±0.04	35.26^{e}±0.71
LA3	0.9^{c}±0.02	11.49^{b}±0.04
LA4	0.8^{b}±0.04	40.50^{f}±0.04
LA5	0.7^{a}±0.02	48.64^{g}±0.35
LA6	0.8^{b}± 0.04	49.06^{g}±0.13
LA7	1.0^{d}±0.02	32.00^{d}±0.10
Physostigmine	0.9^{c}±0.04	1.15^{a}±0,05
Potassium dichromate ($K_2Cr_2O_7$)	-	-

Different letters mean significant differences between samples. ANOVA and Tukey's Multiple Comparison tests were used at $p<0.05$. (-) test not performed

For quantitative evaluation, this inhibition was determined by the enzyme-linked immunosorbent assay (ELISA), whose the order of AChE inhibition showed that the highest action occurred in LA3 (11.49 ± 0.04 µg/mL), followed by LA1 (13.20 ± 0.88 µg/mL), which contained geranial and neral, camphor and 1,8-cineole as major constituents, respectively. Bioactive molecules that inhibit AChE have therapeutic benefits due to the ability to penetrate the blood-brain barrier, increasing the levels of endogenous acetylcholine, a useful property in therapy for Alzheimer's disease (Morais *et al*., 2017).

According to Jukic *et al*. (2007), monoterpenes like thymol, carvacrol and linalool are weak inhibitors of AChE. This may explain the weak activity found for the LA4 chemotype, which is rich in linalool. However, 1,8-cineole and limonene are monoterpenes with potent AChE inhibitory activity (Abdelgaleil *et al*., 2009), which may explain the activity in the LA1 chemotype. Essential oils are complex mixtures of aromatic compounds whose production depends on abiotic factors, and synergism between their constituents can modulate the inhibition of AChE.

The larvicidal assay was carried out to determine the median lethal concentration (LC_{50}) against instar III larvae of *Aedes aegypti* and *A. albopictus*. The results are shown in Table No. 4. As positive control, Temephos™ (O,O'-(thiodi-4,1-phenylene)bis(O,O-dimethyl phosphorothioate) was used, whose LC_{50} value was 1.5 ± 0.2 µg/mL.

Compounds with LC_{50} values of up to 100 µg/mL are considered good larvicidal agents (Cheng

et al., 2003). The strongest larvicidal activities expressed by LC_{50} and LC_{90} were for the LA7 chemotype against the larval stages of *A. aegypti*. This oil is rich in neral and geranial and showed an LC_{50} value of 1.59 ± 0.66 µg/mL. Among essential oils with insecticidal activity against larvae of *A. aegypti*, the oil from *Cymbopogon flexuosus* (Nees ex Steud.) W. Watson, with citral (geranial + neral) as main component, showed the highest larvicidal activity (LC_{50} = 17.1 µg/mL) (Vera *et al*., 2014). The carvone chemotype LA6 also showed good action, with LC_{50} of 62.98 ± 1.08. A previous study indicated a LC_{50} value of 43.8 µg/mL for carvone alone (Simas *et al*., 2004).

Table No. 4
Larvicidal activity of essential oils of *Lippia alba* obtained from different chemotypes against third-instar larvae of *Aedes aegypti* and *Aedes albopictus* after 24 h of exposure

***L. alba* chemotypes**	***Aedes aegypti***		***Aedes albopictus***	
	LC_{50} (µg/mL)	LC_{90} (µg/mL)	LC_{50} (µg/mL)	LC_{90} (µg/mL)
LA1	92.13^{c}±1.18	220.35^{d}± 1.09	164.2^{c}± 1.65	446.7^{d}± 3.74
LA2	190.5^{f}±3.79	457.32^{f}± 3.36	205.6^{d}± 9.55	538.3^{e}± 39.31
LA3	96.49^{c}±3.82	237.27^{e}± 4.84	-	-
LA4	148.56^{e}±3.32	503.50^{g}± 5.66	450.6^{f}± 2,0	3043.6^{g}± 6.09
LA5	113.99^{d}± 1.31	210.92^{d}± 6.05	275.1^{e}± 6.95	1100.0^{f}± 5.14
LA6	62.98^{b}± 1.08	158.18^{c}± 2.63	113.4^{b}± 0.92	286.8^{b}± 0.19
LA7	1.59^{a}± 0.66	74.73^{b}± 1.23	169.2^{c}± 1.0	396.2^{c}± 3.64
Temephos™	1.5^{a}± 0.2	3.27^{a}± 0.17	0.01^{a}± 0.20	1.78^{a}± 0.10

Different letters mean significant differences between samples. ANOVA and Tukey's Multiple Comparison tests were used at $p<0.05$. (-) test not performed

LA7 showed the best larvicidal action against *A. aegypti* of all investigated *L. alba* chemotypes, with LC_{50} value of 1.59 ± 0.66 µg/mL, a result very close to that of Temephos™ (positive control). This action is due to the synergism of its major constituents geranial, β-caryophyllene and neral. A previous study of *Cymbopogon citratus* (DC.) Stapf essential oil, whose main chemical constituents were geranial (60.3%) and neral (39.7%), showed larvicidal action with LC_{50} value of 69 µg/mL against *A. aegypti* (Cavalcanti *et al*., 2004).

The larvicidal action described for LA7 can be related to the AChE inhibitory activity, due to the presence of many larvicidal substances that inhibit the action of acetylcholinesterase, by inhibiting the hydrolysis of the neurotransmitter acetylcholine into acetate and choline (Colovic *et al*., 2013).

Regarding the antifungal activity against pathogenic fungal strains, shown in Table No. 5, the chemotypes LA1, LA2, LA3 and LA6 showed the highest antifungal activity against *T. rubrum* strains compared to the other chemotypes. These antifungal essential oils contain mainly monoterpenoids, such as α-pinene, camphor, 1,8-cineole, neral and geranial. A

previous study identified antifungal activity against yeasts and filamentous fungi of the citral and carvone chemotypes of *L. alba* (Mesa-Arango *et al*., 2009). The chemical composition of a commercial sample of essential oil from *Eucalyptus smithii* R.T. containing 1,8-cineole as the main constituent was reported to be active against several strains of *Microsporum* and *Trichophyton* (Baptista *et al*., 2015). Our data corroborate the previous findings of the better antifungal action of oils containing these constituents.

Table No. 5
Minimum inhibitory concentration of the *Lippia alba* essential oils obtained from different chemotypes against *T. rubrum*

Strains	MIC/MFC (mg/mL)							
	LA1	LA2	LA3	LA4	LA5	LA6	LA7	KTC
***T. rubrum* LABMIC 0208**	0.62/1.25	1.25/2.5	0.62/1.25	1.25/2.5	NI	0.62/1.25	1.25/2.5	0.25
***T. rubrum* LABMIC 0209**	0.62/1.25	0.15/0.31	0.62/1.25	1.25/2.5	NI	0.62/1.25	1.25/2.5	0.25
Geometric mean	0.62/1.25	0.43/0.88	0.62/1.25	1.25/2.5	NI	0.62/1.25	1.25/2.5	0.25

NI: no inhibition; KTC: ketoconazole; MIC: minimum inhibitory concentration; MFC: minimum fungicidal concentration

The cytotoxicity activity was determined by the MTT assay, which is a well-established colorimetric assay based on the enzymatic reduction of the tetrazolium salt MTT in living, metabolically active cells, but not in dead cells (Macêdo *et al*., 2018). The inhibition percentage of cell growth was high (75–100%) to moderate (51–74%) (ISO 10993-5: 2009). All the chemotypes showed high cytotoxic activity against the cancer cell lines used in this study, except LA5, whose inhibition percentage ranged from 31.70-73.94 (Table No. 6).

Table No. 6
Cell proliferation inhibition (%) of the *Lippia alba* essential oils obtained from different chemotypes determined by MTT assay at the concentration of 50 mg/mL

Chemotype	Cell proliferation inhibition (%)			IC_{50} (µg/mL)
	HCT-116	PC-3	SNB-19	HCT-116
LA1	89.69^{c}± 1.01	82.41^{c}± 1.37	79.50^{c}± 1.11	24.89
LA2	91.02^{c}± 0.32	84.20^{c}± 1.26	64.90^{d}± 1.20	33.81
LA3	95.28^{b}± 0.64	97.37^{a}± 0.97	89.83^{b}± 0.72	5.49
LA4	88.41^{c}± 1.08	82.27^{c}± 1.49	49.44^{e}± 1.59	42.76
LA5	73.94^{d}± 1.18	47.83^{d}± 1.99	31.70^{f}± 1.11	> 100
LA6	93.15^{b}± 0.93	87.80^{b}± 1.21	87.41^{b}± 0.11	> 100
LA7	93.07^{b}± 1.80	91.02^{b}± 1.43	89.23^{b}± 1.48	9.22
Doxorubicin	99.99^{a}± 0.01	99.99^{a}± 0.01	99.99^{a}± 0.01	0.21

Different letters mean significant differences between samples. ANOVA and Tukey's Multiple Comparison tests were used at $p < 0.05$. (-) test not performed

Previous studies have determined the cytotoxic potential of *L. alba* essential oil, rich in geraniol (18.9%) and citral (15.9%), against CHO-Chinese hamster ovary cells (Tofiño-Rivera *et al*., 2016) and K562-leukemia cells (García *et al*., 2017). These results are in line withthose of present study, according to which *L. alba* demonstrated good antiproliferative effect (Figure No. 1), due to the content of oxygenated monoterpenoids in the oils. There also may be a synergistic effect, by modifying the response to the various investigated chemotypes.

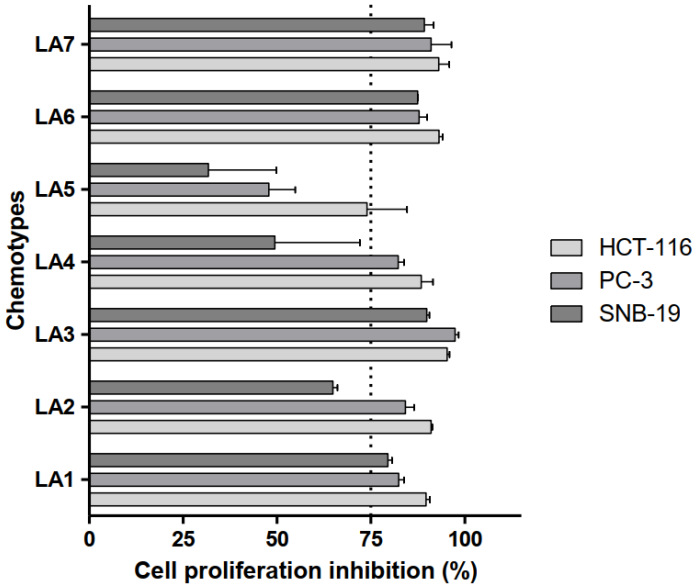

Figure No. 1
Cell proliferation inhibition (%) of the *Lippia alba* essential oils obtained from different chemotypes

Among all the cancer cell lines tested, HCT-116 presented the highest cell proliferation inhibitory effect at the concentration of 50 µg/mL in all the tested chemotypes. Thus, the IC_{50} was determined, whose values ranged from 5.49-42.76 µg/mL (Table No. 7). The LA5 and LA6 chemotypes only had weak antiproliferative action, with IC_{50} of more than 100 µg/mL. However, the LA3 and LA7 chemotypes were able to reduce cell viability of HCT-116 in low concentrations, approaching the results obtained for the standard drug, doxorubicin.

β-caryophyllene and *β*-caryophyllene oxide are present in a large number of plants worldwide. Both compounds possess significant anticancer activities, affecting growth and proliferation of numerous cancer cell lines (Fidyt *et al*., 2016). The aldehydes geranial and neral demonstrated cytotoxic and antitumor effect against the HeLa cell line (Mesa-Arango *et al*., 2009). Citral (geranial + neral) and *β*-caryophyllene was major constituents found in some chemotypes, like LA3 and LA7, which could explain their better anticancer activity.

CONCLUSIONS

The chemical diversity allowed the identification of different chemotypes, whose medicinal properties were investigated, revealing a biotechnological potential of this plant. The results presented here indicate the main constituents of each chemotype, as well as the biological activities, which include antioxidant, antiacetylcholinesterase, antifungal and larvicidal activities, along with cytotoxicity against cancer cells. The chemical composition of the essential oils undergoes changes due to environmental factors and the circadian cycle. In addition, major constituents may influence on the biological action, then there is a need to study the constituents separately through *in silico* and *in vitro* assays.

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REFERENCES

Abdelgaleil SAM, Mohamed MIE, Badawy MEI, El-arami SAA. 2009. Fumigant and contact toxicities of monoterpenes to *Sitophilus oryzae* (L.) and *Tribolium castaneum* (Herbst) and their inhibitory effects on acetylcholinesterase activity. **J Chem Ecol** 35: 518 - 525. **https://doi.org/10.1007/s10886-009-9635-3**

Adams RP. 2012. **Identification of essential oil components by gas chromatography/mass spectrometry.** Allured Publishing Corporation, Illinois, USA.

Albuquerque UP, Medeiros PM, Almeida ALS, Monteiro JM, Neto EMFL, Melo JG, Santos JP. 2007. Medicinal plants of the caatinga (semi-arid) vegetation of NE Brazil: a quantitative approach. **J Ethnopharmacol** 114: 325 - 354. **https://doi.org/10.1016/j.jep.2007.08.017**

Ali-Shtayeh MS, Jamous RM, Abu-Zaitoun SY, Akkawi RJ, Kalbouneh SR, Dudai N, Bernstein N. 2018. Secondary treated effluent irrigation did not impact chemical composition, and enzyme inhibition activities of essential oils from *Origanum syriacum* var. *syriacum*. **Ind Crop Prod** 111: 775 - 786. **https://doi.org/10.1016/j.indcrop.2017.11.055**

Baptista EB, Zimmermann-Franco DC, Lataliza AAB, Raposo NRB. 2015. Chemical composition and antifungal activity of essential oil from *Eucalyptus smithii* against dermatophytes. **Rev Soc Bras Med Trop** 48: 746 - 752. **https://doi.org/10.1590/0037-8682-0188-2015**

Camargo SB, Vasconcelos DFSA. 2015. Atividades biológicas de Linalol: conceitos atuais e possibilidades futuras deste monoterpeno. **Rev Ciênc Méd Biol** 13: 381 - 387.

Cardoso JLMS, Soares MJS, Leite JRSA, Malaquias LCC, Coelho LFL. 2013. Antiviral activity of dermaseptin 01 against Dengue virus type 2, Herpes simplex virus type 1 and Vaccinia virus. **Sci Med** 23: 18 - 21.

Cavalcanti ESB, Morais SM, Lima MAA, Santana EWP. 2004. Larvicidal activity of essential oils from Brazilian plants against *Aedes aegypti* L. **Mem Inst Oswaldo Cruz** 99: 541 - 544. **https://doi.org/10.1590/s0074-02762004000500015**

Cheng SS, Chang HT, Chang ST, Tsai KH, Chen WJ. 2003. Bioactivity of selected plant essential oils against the yellow fever mosquito *Aedes aegypti* larvae. **Bioresour Technol** 89: 99 - 102. **https://doi.org/10.1016/s0960-8524(03)00008-7**

Colovic MB, Krstic DZ, Lazarevic-Pasti TD, Bondzic AM, Vasic VM. 2013. Acetylcholinesterase inhibitors: pharmacology and toxicology. **Curr Neuropharmacol** 11: 315 - 335.

Craveiro AA, Matos FJA, Alencar JW. 1976. A simple and inexpensive steam generator for essential oils extraction. **J Chem Educ** 3: 652. **https://doi.org/10.1021/ed053p652**

De Hoog GS, Guarro J, Gené J, Figueras MJ. 2008. **Atlas of Clinical Fungi.** Centralalbureau voor Schimmelcultures/Universitat Rovira I Virgili Reus. Utrecht, The Netherlands:

Ellman GL, Courtney KD, Andres Jr V, Featherstone RM. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. **Biochem Pharmacol** 7: 88 - 95. **https://doi.org/10.1016/0006-2952(61)90145-9**

Escobar P, Leal SM, Herrera LV, Martinez JR, Stashenko E. 2010. Chemical composition and antiprotozoal activities of Colombian *Lippia* spp essential oils and their major components. **Mem Inst Oswaldo Cruz** 105: 184 - 190. **https://doi.org/10.1590/s0074-02762010000200013**

Fidyt K, Fiedorowicz A, Strządała L, Szumny A. 2016. β-caryophyllene and β-caryophyllene oxide-natural compounds of anticancer and analgesic properties. **Cancer Med** 5: 3007 - 3017. **https://doi.org/10.1002/cam4.816**

Fontenelle ROS, Morais SM, Brito EHS, Brilhante RSN, Cordeiro RA, Nascimento NRF, Kerntopf MR, Sidrim JJC, Rocha MFG. 2008. Antifungal activity of essential oils of *Croton* species from the Brazilian Caatinga biome. **J Appl Microbiol** 104: 1383 - 1390. **https://doi.org/10.1111/j.1365-2672.2007.03707.x**

Gade S, Rajamanikyam M, Vadlapudi V, Nukala KM, Aluvala R, Giddigari C, Karanam NJ, Barua NC, Pandey R, Upadhyayula VSV, Sripadi P, Amanchy R, Upadhyayula SM. 2017. Acetylcholinesterase inhibitory

activity of stigmasterol & hexacosanol is responsible for larvicidal and repellent properties of *Chromolaena odorata*. **Biochim Biophys Acta Gen Subj** 1861: 541 - 550. **https://doi.org/10.1016/j.bbagen.2016.11.044**

García LT, Leal AF, Moreno ÉM, Stashenko EE, Arteaga HJ. 2017. Differential anti-proliferative effect on K562 leukemia cells of *Lippia alba* (Verbenaceae) essential oils produced under diverse growing, collection and extraction conditions. **Ind Crop Prod** 96: 140 - 148. **https://doi.org/10.1016/j.indcrop.2016.11.057**

Guedes JAC, Filho EGA, Rodrigues THS, Silva MFS, Souza FVD, Silva LMA, Alves RE, Canuto KM, Brito ES, Pessoa CÓ, Nascimento RF, Zocolo GJ. 2018. Metabolic profile and cytotoxicity of non-polar extracts of pineapple leaves and chemometric analysis of different pineapple cultivars. **Ind Crop Prod** 124: 466 - 474. **https://doi.org/10.1016/j.indcrop.2018.08.026**

Harvey AL, Edrada-Ebel R, Quinn RJ. 2015. The re-emergence of natural products for drug discovery in the genomics era. **Nat Rev Drug Discov** 14: 111. **https://doi.org/10.1038/nrd4510**

Hennebelle T, Sahpaz S, Joseph H, Bailleul F. 2008. Ethnopharmacology of *Lippia alba.* **J Ethnopharmacol** 116: 211 - 222. **https://doi.org/10.1016/j.jep.2007.11.044**

Jukic M, Politeo O, Maksimovic M, Milos M, Milos M. 2007. *In vitro* acetylcholinesterase inhibitory properties of thymol, carvacrol and their derivatives thymoquinone and thymohydroquinone. **Phytother Res** 21: 259 - 261. **https://doi.org/10.1002/ptr.2063**

Julião LS, Tavares ES, Lage CLS, Leitão SG. 2003. Cromatografia em camada fina de extratos de três quimiotipos de *Lippia alba* (Mill) NE Br. (erva-cidreira). **Rev Bras Farmacogn** 13: 36 - 38. **https://doi.org/10.1590/s0102-695x2003000300014**

Londoño-Renteria B, Troupin A, Colpitts TM. 2016. Arbovirosis and potential transmission blocking vaccines. **Parasit Vectors** 9: 516 - 527. **https://doi.org/10.1186/s13071-016-1802-0**

López MA, Stashenko EE, Fuentes JL. 2011. Chemical composition and antigenotoxic properties of *Lippia alba* essential oils. **Genet Mol Biol** 34: 479 - 488. **https://doi.org/10.1590/s1415-47572011005000030**

Macedo AL, Silva DP, Moreira DL, Queiroz LN, Vasconcelos TR, Araujo GF, Kaplan MAC, Pereira SSC, Almeida ECP, Valverde AL, Robbs BK. 2019. Cytotoxicity and selectiveness of Brazilian Piper species towards oral carcinoma cells. **Biomed Pharmacother** 110: 342 - 352. **https://doi.org/10.1016/j.biopha.2018.11.129**

Matos FJA. 1996. As ervas cidreiras do Nordeste do Brasil. Estudo de três quimiotipos de *Lippia alba* (Mill.) N. E. Brown (Verbenaceae) Parte II – Farmacoquímica. **Rev Bras Farm** 77: 137 - 141.

Mesa-Arango AC, Montiel-Ramos J, Zapata B, Durán C, Betancur-Galvis L, Stashenko E. 2009. Citral and carvone chemotypes from the essential oils of Colombian *Lippia alba* (Mill.) NE Brown: composition, cytotoxicity and antifungal activity. **Mem Inst Oswaldo Cruz** 104: 878 - 884. **https://doi.org/10.1590/S0074-02762009000600010**

Mesquita BMD, Nascimento PG, Souza LG, Farias IFD, Silva RA, Lemos TL, Monte FJQ, Oliveira IR, Trevisan MTS, Silva HC, Santiago GMP. 2018. Synthesis, larvicidal and acetylcholinesterase inhibitory activities of carvacrol/thymol and derivatives. **Quim Nova** 41: 412 - 416. **https://doi.org/10.21577/0100-4042.20170189**

Morais SM, Silva KA, Araujo H, Vieira IG, Alves DR, Fontenelle ROS, Silva AMS. 2017. Anacardic acid constituents from cashew nut shell liquid: NMR characterization and the effect of unsaturation on its biological activities. **Pharmaceuticals** 10: 31 - 41. **https://doi.org/10.3390/ph10010031**

Ocazionez RE, Meneses R, Torres FÁ, Stashenko E. 2010. Virucidal activity of Colombian Lippia essential oils on dengue virus replication *in vitro*. **Mem Inst Oswaldo Cruz** 105: 304 - 309. **https://doi.org/10.1590/S0074-02762010000300010**

Porfírio EM, Melo HM, Pereira AMG, Cavalcante TTA, Gomes GA, Carvalho MGD, Costa RA, Júnior FEAC. 2017. *In vitro* antibacterial and antibiofilm activity of *Lippia alba* essential oil, citral, and carvone against *Staphylococcus aureus*. **Sci World J** 1 - 8. **https://doi.org/10.1155/2017/4962707**

Pereira EJP, Silva HC, Holanda CL, Menezes JESA, Siqueira SMC, Rodrigues THS, Fontenelle ROS, Vale JPC, Silva PT, Santiago GMP, Santos HS. 2018. Chemical composition, cytotoxicity and larvicidal activity against *Aedes aegypti* of essential oils from *Vitex gardineriana* Schauer. **Bol Latinoam Caribe Plant Med Aromat** 17: 302 - 308.

Raj GA, Chandrasekaran M, Krishnamoorthy S, Jayaraman M, Venkatesalu V. 2015. Phytochemical profile and

larvicidal properties of seed essential oil from *Nigella sativa* L. (Ranunculaceae), against *Aedes aegypti*, *Anopheles stephensi*, and *Culex quinquefasciatus* (Diptera: Culicidae). **Parasitol Res** 114: 3385 - 3391. **https://doi.org/10.1007/s00436-015-4563-3**

Rhee IK, van de Meent M, Ingkaninan K, Verpoorte R. 2001. Screening for acetylcholinesterase inhibitors from Amaryllidaceae using silica gel thin-layer chromatography in combination with bioactivity staining. **J Chromatogr A** 915: 217 - 223. **https://doi.org/10.1016/S0021-9673(01)00624-0**

Salimena FRG, Ferreira SC, Cardoso PH, Valério VIR. 2016. **Verbenaceae**. In: Rizzo JA (ed.) Flora dos estados de Goiás e Tocantins. Coleção Rizzo. Vol. 47. Universidade Federal de Goiás, Goiânia, Brazil.

Silva RER, Morais LP, Silva AA, Bastos CMS, Pereira-Gonçalves Á, Kerntopf MR, Menezes IRA, Leal-Cardoso JH, Barbosa R. 2018. Vasorelaxant effect of the *Lippia alba* essential oil and its major constituent, citral, on the contractility of isolated rat aorta. **Biomed Pharmacother** 108: 792 - 798. **https://doi.org/10.1016/j.biopha.2018.09.073**

Simas NK, Lima EDC, Conceicao SDR, Kuster RM, Oliveira Filho AD, Lage CLS. 2004. Produtos naturais para o controle da transmissão da dengue-atividade larvicida de *Myroxylon balsamum* (óleo vermelho) e de terpenóides e fenilpropanóides. **Quim Nova** 27: 46 - 49. **https://doi.org/10.1590/S0100-40422004000100009**

Stashenko EE, Jaramillo BE, Martínez JR. 2004. Comparison of different extraction methods for the analysis of volatile secondary metabolites of *Lippia alba* (Mill.) NE Brown, grown in Colombia, and evaluation of its in vitro antioxidant activity. **J Chromatogr A** 1025: 93 - 103. **https://doi.org/10.1016/j.chroma.2003.10.058**

Suárez ME. 2019. Medicines in the forest: Ethnobotany of wild medicinal plants in the pharmacopeia of the Wichí people of Salta province (Argentina). **J Ethnopharmacol** 231: 525 - 544. **https://doi.org/10.1016/j.jep.2018.10.026**

Tabanca N, Avonto C, Wang M, Parcher JF, Ali A, Demirci B, Raman V Khan IA. 2013. Comparative investigation of *Umbellularia californica* and *Laurus nobilis* leaf essential oils and identification of constituents active against *Aedes aegypti*. **J Agric Food Chem** 61: 12283 - 12291. **https://doi.org/10.1021/jf4052682**

Tavares ES, Lopes D, Bizzo HR, Lage CL, Leitão SG. 2004. Kinetin enhanced linalool production by *in vitro* plantlets of *Lippia alba*. **J Essent Oil Res** 16: 405 - 408. **https://doi.org/10.1080/10412905.2004.9698756**

Tavares ES, Julião LS, Lopes D, Bizzo HR, Lage CLS, Leitão SG. 2005. Analysis of the essential oil from leaves of three *Lippia alba* (Mill.) NE Br. (Verbenaceae) chemotypes cultivated on the same conditions. **Rev Bras Farmacogn** 15: 1 - 5. **https://doi.org/10.1590/S0102-695X2005000100002**

Tofiño-Rivera A, Ortega-Cuadros M, Galvis-Pareja D, Jiménez-Rios H, Merini L.J, Martínez-Pabón MC. 2016. Effect of *Lippia alba* and *Cymbopogon citratus* essential oils on biofilms of *Streptococcus mutans* and cytotoxicity in CHO cells. **J Ethnopharmacol** 194: 749 - 754. **https://doi.org/10.1016/j.jep.2016.10.044**

Trevisan MTS, Macedo FVV, Van De Meent M, Rhee IK, Verpoorte R. 2003. Seleção de plantas com atividade anticolinasterase para tratamento da doença de Alzheimer. **Quim Nova** 26: 301 - 304. **https://doi.org/10.1590/S0100-40422003000300002**

Vera SS, Zambrano DF, Méndez-Sanchez SC, Rodríguez-Sanabria F, Stashenko EE, Luna JED. 2014. Essential oils with insecticidal activity against larvae of *Aedes aegypti* (Diptera: Culicidae). **Parasitol Res** 113: 2647 - 2654. **https://doi.org/10.1007/s00436-014-3917-6**

Victor MM, David JM, Santos MA, Barreiros AL, Barreiros ML, Andrade FS, Carvalho AA, Luciano MCS, Moraes MO, Baros-Nepomuceno FWA, Pessoa C. 2017. Synthesis and evaluation of cytotoxic effects of amino-ester derivatives of natural α, β-Amyrin mixture. **J Braz Chem Soc** 28: 2155 - 2162. **https://doi.org/10.21577/0103-5053.20170064**

Yamamoto PY, Colombo CA, Azevedo Filho JA, Lourenção AL, Marques MOM, Morais GDDS, Chiorato AF, Martins ALM, Siqueira WJ. 2008. Performance of ginger grass (*Lippia alba*) for traits related to the production of essential oil. **Sci Agric** 65: 481 - 489. **https://doi.org/10.1590/S0103-90162008000500006**