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Original Article

The effect of the essential oils from five different *Lippia* species on the viability of tumor cell lines

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ABSTRACT

Several *Lippia* species have been used in folk medicine mainly for gastrointestinal and respiratory diseases. Their biological properties have been partially associated to the terpenoids found in their essential oils. According to the World Health Organization, cancer is the leading cause of death worldwide and is described as a complex group of diseases with several hallmarks. One of its acceptable defining features is the cell proliferation beyond their boundaries forming the tumors. Importantly, some drugs currently available were discovered by the investigation of plant secondary metabolites. Thus, this study aimed to evaluate *in vitro* cytotoxic effect of the essential oils extracted from five *Lippia* species against tumor cell lines. The results indicated that mouse colon carcinoma CT26.WT cell line viability was significantly reduced showing an IC_{50} of 19.05, 30.20 and 36.30 µg/ml when treated with the essential oils of *L. sidoides*, *L. salviifolia* and *L. rotundifolia*, respectively. Human lung carcinoma A549 cell line also had a compromised viability to the action of *L. alba* carvone chemotype essential oil. The tested essential oils did not compromise viability of the normal cell line CHO. These finds suggest that the studied *Lippia* essential oils might be good candidates for further in-depth studies.

Introduction

It has been reported that incidence and mortality rates for most cancers are decreasing in the United States and many other western countries, however they are increasing in several less developed and economically transitioning countries. The proportion of new cancer cases diagnosed in less developed countries is projected to increase from about 56% of the total world in 2008 to more than 60% in 2030 (Jemal et al., 2010). Worldwide, lung cancer is the most common in term of cases and deaths. Colorectal cancer is the third most common cancer in men and the second in women, and breast and prostate cancer are the leading cause of cancer death in women and men worldwide, respectively (Ferlay et al., 2010).

Several strategies, including surgery, radio and chemotherapy have been developed to treat different types

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of cancer. Chemotherapy has proven efficient in many cases, however the development of resistance and toxicity continues to be problems during the treatment. Therefore, it is still essential the identification of new agents demonstrating chemotherapeutic and chemopreventive activities. In this sense the monoterpenes have been suggested as good candidates for the study of antitumor agents. Shoff et al. (1991) demonstrated that the monoterpene geraniol increase the population doubling time of leukemia and melanoma cells. Several authors demonstrated that synthetic geraniol compromise growth of *in vitro* tumor cell lines and the wild variety of *in vivo* tumors types, including hepatoma, pancreatic and colon which is highly resistant to chemotherapy (Yu et al., 1995; Burke et al., 1997; Duncan et al., 2004; Carnesecchi et al., 2001; 2002; Ong et al., 2006; Wiseman et al., 2007). In the same way, the monoterpene limonene exerts therapeutic activity against breast, skin, liver, lung and stomach tumors in rodents (Elegbede et al., 1986; Wattenberg and Coccia, 1991; Crowell and Gould, 1994; Kawamori et al., 1996; Crowell, 1999). Additionally, tumor-suppressive activity has been reported to monoterpenes like carvone, carveol, mentol and perillyl alcohol (He et al., 1997).

Brazil has one of the richest biodiversity worldwide with nearly 19% of the world flora (MMA, 1998). Monoterpenes are found in several plant species, including species of the *Lippia* genus. Furthermore, Brazil has one of the main diversity centers of the genus *Lippia*, which is located at the Espinhaço Mountain Range, in the State of Minas Gerais, Brazil (Salimena-Pires 1991; Viccini et al., 2004). The genus *Lippia* belongs to the Verbenaceae family and comprises approximately 200 species of herbs, shrubs and small trees (Sanders, 2001). Several species of the genus *Lippia* are used for treatment of respiratory disorders, being employed as remedy for colds, grippe, bronchitis, coughs and asthma (Caceres et al., 1991; Forestieri et al., 1996; Pascual et al., 2001; Jean et al., 2012). They are also commonly utilized as gastrointestinal remedies and as seasoning for food preparation (Hennebelle et al., 2008; Oliveira et al., 2007, Pascual et al., 2001). Additionally, some *Lippia* species are used to treat hepatic diseases, burns, wounds, fever, syphilis, gonorrhea, diarrhea, dysentery, malaria, among others. (Pascual et al., 2001). Importantly for the two best studied species of this genus, *L. alba* and *L. sidoides*, previous studies reported antioxidant activity, indicating that these plants might be a potential source of antitumor biomolecules (Ramos et al., 2003; Monteiro et al., 2007). Yet, other specie is which antioxidant activity was reported was *L. salviifolia* (Silva, 2008). The pharmacological properties of the *Lippia* species have been related to the components of their secondary metabolism, specifically to their essential oils (Pascual et al., 2001). The main constituents of *Lippia* essential oil are the monoterpenes, and according to the major monoterpene present in their oil they can be classified in different chemotypes (Pascual et al., 2001). Although several pharmacological studies have been performed for many *Lippia* species, very few studies were performed for the ones native to Brazil, such as *L. lacunosa* and *L. rotundifolia* (Jardim Botânico do Rio de Janeiro, 2013).

The aim of the present study was to chemically characterize the essential oils extracted from *L. alba, L. sidoides, L. salviifolia, L. rotundifolia* and *L. lacunosa* and to evaluate their effect on viability of tumor cell lines.

Materials and methods

Plant material

Fresh leaves were collected for each one of the *Lippia* species, Verbenaceae, *L. alba* (Mill.) N.E. Brown geraniol and carvone chemotypes, *L. sidoides* Cham., *L. salviifollia* Cham., *L. rotundifolia* Cham. and *L. lacunosa* Mart. and Schauer at the Experimental Station located on the campus of the Federal University of Juiz de Fora, Juiz de Fora, Brazil (21°46'48.4"S 43°22'24.4" W). Each one of the *Lippia* species was collected in the early morning or late afternoon, without rain, from October 2009 to February 2010. The voucher specimens of the *Lippia* species evaluated in this study are deposited at the CESJ Herbarium of Federal University of Juiz de Fora and the voucher specimens numbers are: *L. alba* geraniol chemotype: 48374, *L. alba* carvone chemotype: 48463, *L. sidoides*: 49007, *L. salviifolia*: 47444, *L. rotundifolia*: 31376 and *L. lacunosa*: 51920.

Essential oil extraction

The essential oils from fresh leaves of the *Lippia* species were obtained separately by hydrodistillation in a Clevenger-type apparatus for 4 h. The oils were measured and aliquots of 5 mg of each one of them were stored at -80°C in sealed vials covered with aluminum foil until use. For each one of the assays described below one aliquot was defrost and dissolved in 4% dimethyl sulfoxide, DMSO (Sigma, St. Louis, MO, USA) and purified water, making up a stock solution of 1 mg/ml.

Gas chromatography/mass spectrometry analysis

The chemical composition of the essential oil of each *Lippia* specie was determined by gas chromatography coupled to mass spectrometry performed on a Shimadzu QP5050A GC/MS instrument, equipped with a PTE-5 Supelco column (30 m × 0,25 mm × 0,25 µm). The carrier gas was helium (1 ml/ml). The column temperature ranged from 30° to 250°C. The split rate was 1:20 and the injector and interface temperature were 250°C. The total analysis time was 39.17 min with a flux of 19.8 ml/min. Retention indexes (RI) were calculated from retention times generated from the analysis of each oil in comparison with the standard *n*-alkanes solution, C8-C20, analyzed, and used to determine the components of each one of the essential oils, according to Adams (1995). The amount of compounds was determined by peaks area integration of the spectrograms.

Cell line and culture condition

Mouse colon carcinoma CT26.WT cells were provided by Dra. Lucíola Bastos from the Department of Physiology and Biophysics, Biological Sciences Institute, Federal University of Minas Gerais, Brazil; human lung carcinoma A549 cells was obtained by cell bank of Rio de Janeiro, Brazil; human mammary gland adenocarcinoma MDA MB-231 cells was provided by Dr. Ricardo R. Brentani from Ludwig Institute for Cancer Research, São Paulo, Brazil; human colon

adenocarcinoma CACO-2 cells was obtained by cell bank of Rio de Janeiro, Brazil and the hamster normal ovary CHO cells was obtained by Pan American Foot-and-Mouth Disease Center, Rio de Janeiro, Brazil. Each one of the cancer cell lines were cultured in appropriate medium at 37°C with 5% CO_2. CT26.WT, MDA MB-231 and CHO were grown in RPMI 1640 medium, pH 7.4 (Cultilab, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (FBS), 0.1 mg/ml ampicillin, 0.1 mg/ml kanamycin, 0.005 mg/ml amphotericin, 0.2% $NaHCO_3$ and 0.2% HEPES (Sigma, St. Louis, MO, USA). A549 and CACO-2 were grown in DMEM medium (Cultilab, Campinas, SP, Brazil) supplemented with 10% FBS, 0.1 mg/ml kanamycin, 0.005 mg/ml amphotericin and 0.37% $NaHCO_3$ (Sigma, St. Louis, MO, USA).

Cytotoxicity assay

The cytotoxicity of all six essential oils were measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetratozolium bromide] (Sigma, St. Louis, MO, USA) colorimetric assay (Denizot and Lang, 1986). Cells from each cell line were seeded into 96-well plates at a density of 2×10^3 cells/well in 100 µl of appropriated medium supplemented with 10% FBS. After 48 h of incubation at 37°C, cells were treated with medium supplemented with 10% FBS containing different concentrations of each one of the six essential oils, separately. The stock solutions of each essential oil were diluted in four concentrations, 0.1, 1.0, 10 and 100 µg/ml. The negative control samples contained 0.4% DMSO, which is equivalent to the percentage found in the highest concentration evaluated. The positive control samples contained 5-fluorouracil (5-FU), an analogue of the pyrimidine uracil and one of the oldest anticancer agents (Casale et al., 2004), at four different concentrations, 0.1, 1.0, 10 and 100 µM. The blank control contained only RPMI medium supplemented with 10% FBS. After 72 h of incubation at 37°C, medium was discarded and 10 µl tetrazolium dye (MTT) solution (5 mg/ml in PBS) was added to each well. Cells were incubated at 37°C for 4 h, then the solution was discarded and 100 µl of dimethyl sulfoxide (DMSO) was added to dissolve the formed formazan crystals. The microplates were read in a TP-reader (Thermoplate) at 540 nm.

Statistical analysis

At each experimental plate, all the samples were repeated six time and all MTT assays were performed in different independent experiments. The results were analyzed using the software GraphPad Prisma 5.0 (GraphPad Software, Inc.). Each treated group and the control was compared by Bonferroni Test and when $p < 0.05$ the differences were considered significant. Cell viability was calculated using the following equation: A/C × 100, where A is the arithmetic average of the OD of each sample and C is the arithmetic average of the OD observed in the negative control. The cell viabilities values were used to calculate the concentration that corresponds to 50% of cell lethality (IC_{50}), through linear regression using the software Sigma Plot 10.0 (Systat Software, Inc.).

Results

Composition of the essential oils obtained from five Lippia *species*

Table 1 presents the compounds identified by gas-chromatography followed by mass spectrometry in the essential oils of *L. alba* geraniol and carvone chemotypes, *L. sidoides, L. salviifolia, L.rotundifolia and L. lacunosa*. Approximately fourteen main compounds were detected, but to the most of them the contents were very low. For *L. alba* geraniol chemotype the compounds with the highest percentages were geranial and citral and to carvone chemotype were carvone and limonene. In the oil of *L. sidoides* the main compounds were thymol and *o*-cymene. The major constituents of *L. salviifolia* essential oil were nerolidol and germacrene D and of *L. rotundifolia* were β-myrcene and (*E,Z*)-, (*E,E*)-, and (*Z,Z*)-farnesol. Finally, the most abundant components for the essential oil of *L .lacunosa* were myrcenone and β-myrcene.

Effect of the essential oils of Lippia *species on tumor cell lines viability*

Table 2 shows the IC_{50} values (which are the concentrations that corresponds to 50% cell lethality) of cells treated with each one of the essential oils extracted from the *Lippia* species. It was determined that IC_{50} values were obtained only in the presence of essential oils of L. sidoides (19.05 µg/ml), *L. salviifolia* (30.20 µg/ml) and *L. rotundifolia* (36.30 µg/ml) on CT26.WT cell line and *L. alba* carvone chemotype essential oil (47.80 µg/ml) on A549 cell line. In the Fig. 1 we observe the representative curve concentration-response as a measurement of the OD values determined by the MTT assay. It illustrates the reduction of CT26.WT cell viability after treatment with the essential oils of *L. sidoides*, *L. salviifolia* and *L. rotundifolia* and the reduction of A549 cell line viability with the essential oil from *L. alba* carvone chemotype. On the other hand, MDA MB-231 and CACO-2 cell lines did not have their viability significantly compromised by the *Lippia* essential oils evaluated in this study (Table 2). Except for CACO-2 cell line, the positive control 5-FU reached IC_{50} levels were 7.94 µg/ml to CT26.WT, 8.30 µg/ml to A549 and 7.60 µg/ml to MDA MB-231 cell lines. The essential oils of *Lippia* also did not compromise cell viability of normal cell line CHO (Table 2).

Discussion

Historically, numerous drugs have been developed from compounds originally isolated from medicinal plants (Lee, 1999). Since 1961 plant-derived compounds have been approved as anticancer drugs to, such as vinblastine (Velban), vincristine (Oncovin), etoposide (VP-16), teniposide (VM-26), paclitaxel (Taxol), navelbine (Vinorelbine), taxotere (Docetaxel), topotecan (Hycamitin) and irinotecan (Camptosar) (Dholwani et al., 2008). Recently, several studies have suggested that monoterpenes could represent a new class of agents to be used as anticancer drugs, which is especially

important for tumors highly resistant to chemotherapy and to minimize the side effects of the current treatments (Shoff et al., 1991; Yu et al., 1995; Burke et al., 1997; He et al., 1997; Crowell, 1999; Duncan et al., 2004; Wiseman et al., 2007; Chaouki et al., 2009). Several of these monoterpenes are found in *Lippia* species and Brazil is one of the largest centers of diversity of this genus, comprising 70-75% of the known species (Viccini et al., 2006). In this study, it was initially identified the chemical compounds of the essential oils extracted from each one of the five *Lippia* species. GC-MS was performed and the results are shown in Table 1. For *Lippia alba* geraniol chemotype, geranial and citral were the major compounds, comprising approximately 63%. Tavares et al. (2005) obtained the same major compounds. For the chemotype carvone, carvone and limonene were the main constituents, comprising around 70% of the essential oil. Zoghbi et al. (1998) obtained these same components among the majority ones. For *L. sidoides*, the major compound was thymol (63.20%), which is in agreement with Costa et al. (2005). *L. salviifolia* had nerolidol (49.22%) being the major compound of its essential oil, as Singulani et al. (2012). For *L. rotundifolia* and *L. lacunosa* the major compounds were β-myrcene (18.48%) and myrcenone (58.57%) respectively. Leitão et al. (2008) obtained the same ones for *L. lacunosa*. Therefore, these results showed that the major components identified in each one of the five species are in agreement with previous studies, and confirmed the identity of the *Lippia* chemotypes used in this study.

In order to evaluate effect of the essential oil extracted from *Lippia* species on tumor cell viability, the MTT assay was undertaken. According to the National Cancer Institute (USA), vegetables crude extracts are cytotoxic considered when their IC_{50} values are less than 30 µg/*ml* (Hennebelle et al., 2008; Mesa-Arango et al., 2009). This assay showed that 50% of cell inhibition was obtained with concentrations under or near to 30 µg/ml for CT26. WT with the essential oils of *L. sidoides* (19.05 µg/ml) and *L. salviifolia* (30.20 µg/ml) (Fig. 1 and Table 2). The growth suppression effect of thymol, the major compound of *L. sidoides* essential oil, has been reported through IC_{50} value on the B16F10 mouse melanoma cell line (He et al., 1997). Nerolidol, the major compound of *L. salviifolia* essential oil, had been previously also demonstrated antiproliferative action on human leukemia HL-60. (Tatman and Mo, 2002). The IC_{50} values obtained for A549 cells treated with *L. alba* carvone chemotype essential oil (47.80 µg/*ml*) and CT26. WT cell treated with *L. rotundifolia* essential oil (36.30 µg/*ml*) (Fig. 1 and Table 2) did not classify them as cytotoxic crude extracts by the National Cancer Institute. Besides the selectivity on colon tumor cell line, it was not observed

Table 1
Percentage chemical composition of the compounds of the essential oils extracted from leaves of *L. alba* geraniol and carvone chemotypes, *L. sidoides, L. salviifolia, L. rotundifolia and L. lacunosa*, as determined by gas-chromatography followed by mass spectrometry. The high content compounds were highlighted.

Compounds	RI[a]	*L. alba* geraniol	*L. alba* carvone	*L. sidoides*	*L. salviifolia*	*L. rotundifolia*	*L. lacunosa*
sabinense	976	0.72	1.62	-	0.79	0.80	-
β-pinene	980	-	-	-	1.24	1.88	-
β-myrcene	991	0.50	-	2.14	-	**18.48**	**19.47**
α-phellandrene	1005	-	-	-	-	4.44	-
α-terpinene	1018	-	-	0.91	-	-	-
o-cymene	1022	1.95	-	**10.89**	-	0.62	-
β-phellandrene	1031	-	-	-	-	2.21	-
limonene	1031	9.62	**21.34**	-	0.99	-	1.21
(*E*)-β-ocimene	1050	-	-	-	-	0.45	-
γ-terpinene	1062	4.23	-	4.61	-	-	-
linalool	1098	1.07	1.43	3.14	0.83	1.03	0.96
myrtenol	1194	-	-	-	-	1.13	-
myrcenone	1148	-	-	-	-	-	**58.57**
terpinen-4-ol	1177	-	-	0.41	-	-	-
α-terpineol	1189	-	-	-	-	-	0.53
verbenone	1204	-	-	-	-	-	-
nerol	1228	0.62	-	-	-	-	-

Compounds	RI[a]	*L. alba* geraniol	*L. alba* carvona	*L. sidoides*	*L. salviifolia*	*L. rotundifolia*	*L. lacunosa*
(*Z*)-ocimenone	1231	-	-	-	-	-	3.60
thymol methyl ether	1235	-	-	3.82	-	-	-
(*E*)-ocimenone	1239	-	-	-	-	-	4.60
citral	1240	**26.13**	-	-	-	-	-
carvone	1242	-	**48.35**	-	-	-	-
geraniale	1270	**36.53**	-	-	-	-	-
thymol	1290	-	-	**63.20**	-	-	-
carvacrol	1298	-	-	3.46	-	-	-
α-copaene	1376	-	-	-	1.72	-	-
β-bourbonene	1384	-	0.74	-	-	-	-
geranyl acetate	1383	-	-	-	-	-	1.12
β-elemene	1391	0.67	0.90	-	4.03	3.45	-
(*E*)-caryophyllene	1418	-	-	0.53	7.06	3.68	3.89
α-humulene	1454	-	-	5.06	1.48	0.66	0.57
9-*epi*-(*E*)-caryophyllene	1467	-	-	-	0.91	-	-
γ-muurolene	1477	4.72	-	-	-	-	-
germacrene D	1480	-	7.91	-	**18.30**	-	0.71
ar-curcumene	1483	-	-	-	-	2.87	-
bicyclogermacrene	1494	-	-	-	3.11	-	-
α-zingiberene	1495	0.69	-	-	-	1.93	-
α-chamigrene	1500	-	-	-	-	2.65	-
β-curcumene	1512	-	-	-	-	4.13	-
γ-cadinene	1513	0.55	0.56	-	-	-	-
δ-cadinene	1524	-	-	-	1.52	-	-
elemol	1549	8.24	10.66	-	-	3.66	-
nerolidol	1564	0.73	1.03	-	**49.22**	-	-
espatulenol	1576	-	-	-	0.91	2.05	-
caryophyllene oxide	1581	-	-	-	0.88	2.02	1.73
guaiol	1595	0.83	1.10	-	-	-	-
γ-eudesmol	1630	-	0.64	-	-	0.57	-
α-eudesmol	1652	-	-	-	-	0.72	-
α-cadinol	1653	-	-	-	1.35	-	-
7-*epi*-α-eudesmol	1658	-	0.71	-	-	-	-
epi-α-bisabolol	1686	-	-	-	1.73	-	-
(*Z*,*Z*)-farnesol	1713	-	-	-	-	**10.60**	-
(*E*,*E*)-farnesol	1722	-	-	-	-	**10.91**	-
(*E*,*Z*)-farnesol	1742	-	-	-	-	**16.47**	1.67
TOTAL		97.80	96.99	98.17	96.07	97.41	98.63

[a] RI, Retention index.

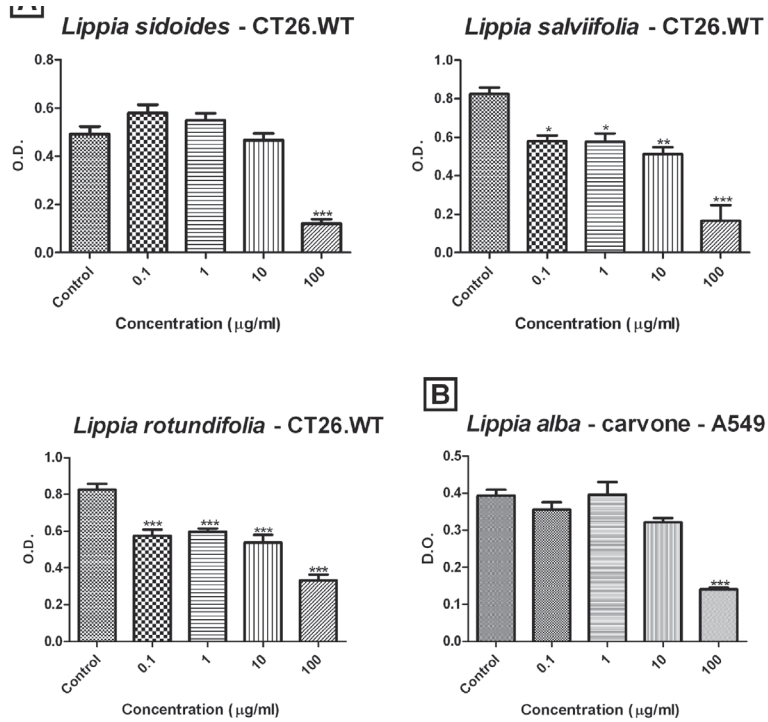

Figure 1 - The cytotoxic effect, as a measurement of the OD values determined by the MTT assay, of the different concentrations (0.1-100 μg/ml) of *L. sidoides*, *L. salviifolia* and *L. rotundifolia* essential oils on CT26.WT cell line (A) and *L. alba* carvone chemotype essential oil on A549 cell line (B). The control samples contained 0.4% DMSO, which is equivalent to the percentage found in the highest concentration evaluated. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$ are significantly different from the control value.

Table 2
IC_{50} values (μg/ml) of cell lines treated for 72 h with the four different concentrations, 0.1, 1, 10 and 100 μg/ml of each one of the essential oils extracted from the *Lippia* species. The negative control samples contained 0.4% DMSO, which is equivalent to the percentage found in the highest concentration evaluated. The positive control samples contained 5-FU at four different concentrations, 0.1, 1, 10 and 100 μM.

Cell lines		*L. alba* geraniol	*L. alba* carvone	*L. sidoides*	*L. salviifolia*	*L. rotundifolia*	*L. lacunosa*	5-FU
Tumor	CT26.WT	-	-	19.05	30.20	36.30	-	7.94
	A549	-	47.80	-	-	-	-	8.30
	MDA MB-231	-	-	-	-	-	-	7.60
Normal	CACO-2	-	-	-	-	-	-	-
	CHO	-	-	-	-	-	-	79.40

toxic effect in normal cell (CHO) exposed to each one of the essential oil tested.

In conclusion the present results showed a significant cytotoxic effect of *L. sidoides* and *L. salviifolia* essential oils on CT26.WT colon tumor cells which might be attributed to their major compounds. Also importantly it showed the absence of this effect on the normal cell line. Therefore, these finds stimulates further studies on their mechanisms of action in order to be considered for *in vivo* studies.

Authors' contributions

MSG (master course student) contributed in collecting plant sample, analyzing and extracting the essential oil, running the laboratory work, analysis of the date and drafted the paper. FOL and MTPL contributed to biological studies. TMAA contributed in GC/MS analysis. LFV contributed with plant collection of the *Lippia* species. CMC designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

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