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Article

Chemical Composition and Cytotoxicity Evaluation of *Lippia origanoides* Kunth (Verbenaceae) Leaves Essential Oil on Human Gingival Fibroblasts

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Abstract: The present study analyzed the chemical composition and evaluated the *in vitro* cytotoxicity of essential oil extracted from *Lippia origanoides* on human gingival fibroblasts. The essential oil was obtained through hydrodistillation, and the volatile compounds were analyzed by gas chromatography mass spectrometry (GC/MS). The cytotoxicity was evaluated using an MTT assay. The cells were exposed to the essential oil for 1h at concentrations ranging from 600 µg/mL to 650 µg/mL. The major essential oil components were *p*-cymene (27 %), γ-terpinene (11 %), carvacrol (11.8 %), (*E*)-methyl cinnamate (8.3 %), α-pinene (4.6 %), thymol methyl ether (5.2 %), carvacrol methyl ether (5.2 %), (*E*)-nerolidol (3.9 %), and 1,8-cineole (3.5 %). The cell viabilities compared to the control group were 90.33 % (600 µg/mL), 81.17 % (610 µg/mL), and 76.04 % (620 µg/mL). The viability of cells exposed to 630 µg/mL, 640 µg/mL, and 650 µg/mL was 63.01 %, 40.88 %, and 36.15 %, respectively, which indicated an increase in cytotoxic effects as the concentration increased. Lower concentrations of *L. origanoides* essential oil did not result in toxic effects against gingival fibroblasts.

Keywords: Natural products, bioactive compounds, applications, biological activities.

Introduction

The therapeutic potential of medicinal plants has stimulated scientific interest, giving rise to a promising field of study and facilitating the discovery of new medicines [1]. The resistance of microorganisms to conventional medicine in the case of recurring opportunistic infections has stimulated the search for alternative phyto-therapeutic treatments [2]. Chemical components accord plant-derived compounds their biological

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activity [3–7]. However, the same components may cause undesirable cytotoxic effects during therapeutic usage [8]. Knowledge of the biocompatibility of essential oils and plant extracts is of prime importance when establishing treatments for diseases, including oral medications [9].

Species of the *Lippia* genus have antifungal, larvicidal, insecticidal, antibiotic, and antioxidant properties conferred by their extracts and essential oils. Therefore, they are believed to show promise as medicinal plants for pharmacological uses [10-12]. *Lippia* is the second largest genus of the Verbenaceae family and is composed of herbs, shrubs, and small trees [13].

L. origanoides is an aromatic shrub native to Central America and northern South America, including Brazil, especially in the Amazon rainforest, and the Caatinga and Cerrado regions [12]. It is used in popular medicine as a treatment for gastrointestinal and respiratory diseases, and as an oral antiseptic [10].

The major components of *L. origanoides* can vary [5-7,14,15]. The chemotypes thymol and carvacrol are the most frequently found in stalk and leaf samples from Brazil and Colombia [5,8,11-13].

L. origanoides essential oil exhibits potential antimicrobial activity [8]. This characteristic can be attributed to the presence of oxygenated monoterpenes, sesquiterpenic hydrocarbons, and oxygenated sesquiterpenes [8,12,16]. Of particular note is its significant activity against the fungus *Candida albicans*, the main species involved in oral candidiasis and of higher pathogenicity [11,12,16]. Studies on the activity of essential oils against cells and tissues can provide knowledge regarding the chemical composition and bioactivity of such compounds and are fundamental to their use in safe concentrations [8,17,18]. To this end, cell cultures have been extensively used for *in vitro* cytotoxicity experiments with essential oils and natural extracts [19,20].

In this context, gingival fibroblasts, which are the cells that form the connective tissues of the oral mucosa, are commonly used to evaluate the effects of oral products through cell viability tests [21,22].

Considering the importance of the biocompatibility of medicinal plants for use in alternative and complementary medicine, elucidation of the chemical composition and analysis of the toxic potential are fundamental steps for amassing knowledge about *L. origanoides*. There are limited reports on the cytotoxicity of essential oils from this species, and an evaluation using gingival fibroblasts is yet to be performed. Therefore, the present study aimed to analyze the chemical composition and evaluate the *in vitro* cytotoxicity of essential oil from *L. origanoides* Kunth on human gingival fibroblasts.

Materials and methods

Ethics

To carry out this research, the Universidade Federal do Pará required that the project be submitted to the ethics committee. This research project was approved by the Ethics Committee of Research on Human Beings of the Institute of Health Sciences of the Federal University of Pará with registration number 3,898,975.

Plant material and essential oil isolation

2 kg of leaves were collected of *L. origanoides* were obtained from the Carajás National Forest, Pará, Brazil (the geographical coordinates of 05°52’ E 06°33’ S; 49°53 and 50°45’ W). Specimens were collected and botanical techniques were used to make records and transport the material. Mounted exsiccates were deposited at the Herbário João Murça Pires of the Museu Paraense Emílio Goeldi (MPEG), in Belém, and registered as MG 201029.

The samples were dried in a hot-air oven at 30°C and then ground in a blade grinder (Tecnal, model TE-631/3, Piracicaba, São Paolo, Brazil) at a speed of 2,251 rotations/s for 10 min. The particle size of the samples selected for experimentation was in the range of 20-35 mesh.

Hydrodistillation

After drying, 40g of leaves of *L. origanoides* were subjected to hydrodistillation using a Clevenger-type extractor. For the extraction was extracted for 3 hours s at 100°C. After this procedure, anhydrous sodium sulfate was added, and the essential oil was centrifuged to be moisture free [23–25].

Essential oil analysis

The chemical composition of the essential oil was elucidated by gas chromatography-mass spectrometry using a Thermo DSQ-II system equipped with a DB-5MS silica capillary column (30 m × 0.25 mm; 0.25 µm film thickness) under the following operating conditions: temperature program: 60-250°C; gradient: 3°C/min; injector temperature: 240°C; carrier gas: helium (linear velocity of 32 cm/s, measured at 100°C); injection without division of flow (1 µL of solution 2:3000 n-hexane); ion source temperature: 200°C. A quadrupole filter was used to scan every second in the range 39 to 500 Da. Ionization was achieved using the electronic impact technique at 70 eV. Quantitative data regarding the volatile constituents were obtained by normalizing the peak area using a FOCUS GC/FID operated under conditions similar to GC/MS, except with nitrogen as the carrier gas.

The identification of volatile components was carried out using both the linear retention index (IR), which was calculated with reference to the retention times of a homologous series of n-alkanes C_8-C_{20} (Sigma-Aldrich), as well as fragmentation patterns observed in the mass spectra, which were compared with authentic samples in the data system and literature libraries, results from a study by Adams [26], and the NIST database [27].

Cell culture

The gingival fibroblast cell line used in this study was established and characterized at the Cell Culture Laboratory of the Dentistry Faculty of the Federal University of Pará (UFPA) [28]. The samples were cultured in DMEM-F12 culture medium in 25 cm² flasks (Sigma, St. Louis, MO, USA) and supplemented with 10 % fetal bovine serum (Gibco, Carlsbad, CA, USA). The cells were stored in an incubator at 37°C in a humid atmosphere with 5 % CO_2. Cell proliferation was observed daily using an inverted phase-contrast microscope (Axiovert 40 C from Zeiss, Jena, TH, DEU, Göttingen, Germany), with an attached AxioCam MRc camera (Zeiss) until the required confluence of cells was obtained.

***In vitro* cytotoxicity assay**

For the cytotoxicity assay, the gingival fibroblasts were transferred to a 24-well culture plate at a concentration of 10^4 cells/well and incubated at 37°C in a humid atmosphere with 5 % CO_2 for 48h for adhesion and proliferation. DMEM-F12 (Sigma) was used to dilute the essential oil to concentrations of 600 µg/mL, 610 µg/mL, 620 µg/mL, 630 µg/mL, 640 µg/mL, and 650 µg/mL. The cells were exposed to the essential oil for 1h, according to the method described by De Oliveira *et al.*[28]. For the control group, the only culture medium was used.

Cell viability assay

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay was used to analyze cell viability. This technique is based on the conversion of the MTT salt into formazan crystals (which are insoluble in water and purple colored) by the mitochondrial activity of viable cells. After the incubation period, the solutions were removed from the wells, and the MTT solution (5 mg/mL) was added to the culture medium. The cells were incubated with MTT for 4h. Subsequently, the formazan crystals were solubilized with dimethyl sulfoxide (DMSO). Photometric quantification was performed using an ELISA microplate reader (Bio-Rad iMarkTM, UK) with a 595 nm optical filter and Microplate Manager software (Bio-Rad). All experiments were performed in triplicate. The cell viability for each group was calculated as follows [29]:

%viability = 100(mean absorbance of treated wells/mean absorbance of cells without treatment)

Statistical analysis

Statistical analysis was performed using BioEstat version 5.3 (Bioestat, Belém, PA, BR). A Kruskal-Wallis test was performed for variance analysis, followed by a *posthoc* Dunn test. Differences were considered statistically significant at $p<0.05$.

Results

Chemical composition

The moisture content was 12 %, the mass yield was 0.739g of essential oil which represents a yield of 2.1 %. GC/MS analysis resulted in the identification of 65 compounds, including monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesqui-

terpenes, and phenylpropanoids (cinnamates). The major compounds were *p*-cymene (27 %), γ-terpinene (11 %), carvacrol (11.8 %), (*E*)-methyl cinnamate (8.3 %), α-pinene (4.6 %), thymol methyl ether (5.2 %), carvacrol methyl ether (5.2 %), (*E*)-nerolidol (3.9 %), 1,8-cineole (3.5 %), α-phellandrene (2.8 %), α-terpinene (1.6 %), (*E*)-caryophyllene (1.6 %), and caryophyllene oxide (1.2 %) (Table 1).

Cell viability

The values obtained by ELISA were used to determine cell viability. Cell behavior at different concentrations is shown in Figure 1. The 640 µg/mL and 650 µg/mL groups were considered significantly different from the control group. The 630 µg/mL, 640 µg/mL, and 650 µg/mL groups reduced viability by 36.9 %, 59.1 %, and 63.8 %, respectively, demonstrating that these concentrations have potential cytotoxic effects on human gingival fibroblasts, according to ISO 10993-5:2009 [30] (compounds that reduce cell viability by more than 30 % are considered cytotoxic). The results indicate that as the essential oil concentration increased, there was a decrease in the viability of the tested cells (Figure 1).

Discussion

In vitro testing has been used frequently to evaluate the biological behavior of natural products

Table 1. Chemical composition of the *Lippia origanoides* leaves essential oils

RT	RIC	RTL	Constituintes	(%)
5.82	933	934	α-Pinene	4.6
7.03	979	978	β-Pinene	0.2
7.31	992	990	Myrcene	1.1
7.93	1003	1002	α-Phellandrene	2.8
8.35	1016	1014	α-Terpinene	1.6
8.56	1028	1025	*p*-Cymene	27.0
8.72	1029	1026	limonene	0.5
9.79	1057	1056	γ-Terpinene	11.0
Total hydrocarbons monoterpene				**48.8**
8.76	1034	1032	1,8-Cineole	3.5
10.30	1067	1065	*cis*-Linalool oxide (furanoid)	0.2
10.91	1084	1082	*trans*-Linalool oxide (furanoid)	0.4
10.97	1096	1095	Linalool	0.6
12.32	1123	1122	(*Z*)-p-Menth-2-en-1-ol	0.1
12.46	1125	1124	α-Campholenal	0.1
13.00	1137	1135	(*E*)-Pinocarveol	0.3
13.30	1147	1146	Camphor	0.2
14.08	1166	1164	Pinocarvone	0.1
14.32	1169	1167	Borneol	0.2
14.92	1181	1179	*p*-Cymen-8-ol	0.2
15.23	1187	1186	α-Terpineol	0.8
15.25	1188	1187	*trans*-p-Mentha-1(7).8-dien-2-ol	0.2
16.00	1206	1204	Verbenone	0.4
16.45	1217	1215	(*E*)-Carveol	0.1
17.20	1233	1232	Thymol, methyl ether	5.2
17.55	1241	1239	Carvone	0.1
17.63	1243	1241	Carvacrol, methyl ether	5.2
17.73	1245	1244	Carvotanacetone	0.1
17.96	1251	1249	Piperitone	0.2

table 1. (continued).

RT	RIC	RTL	Constituintes	(%)
19.74	1290	1289	Thymol	0.6
20.15	1298	1298	Carvacrol	11.8
20.18	1301	1299	(*Z*)-Methyl cinnamate	0.6
Total oxygenated monoterpenes				**31.2**
23.43	1373	1373	α-Ylangene	0.1
23.51	1376	1374	α-Copaene	0.8
23.58	1378	1376	(*E*)-Methyl cinnamate	8.3
25.38	1418	1417	(*E*)-Caryophyllene	1.6
26.09	1435	1434	γ-Elemene	0.1
26.84	1455	1452	α-Humulene	0.4
27.08	1462	1458	allo-Aromadendrene	0.1
27.93	1479	1478	γ-Muurolene	0.1
27.98	1480	1479	ar-curcumene	0.1
28.38	1489	1489	β-Selinene	0.2
28.85	1500	1500	Bicyclogermacrene	0.1
28.86	1502	1500	α-Muurolene	0.2
29.04	1506	1505	β-Bisabolene	0.1
29.22	1510	1509	α-Bulnesene	0.1
29.7	1522	1521	(*E*)-Calamenene	0.1
30.64	1546	1544	α-Calacorene	0.1
Total hydrocarbons sesquiterpene				**12.5**
31.34	1563	1561	(*E*)-Nerolidol	3.9
32.00	1577	1577	Spathulenol	0.2
32.52	1591	1590	Globulol	0.2
33.21	1609	1608	Humulene epoxide II	0.2
34.08	1632	1630	Muurola-4,10(14)-dien-1-β-ol	0.4
34.45	1640	1639	*allo*-Aromadendrene epoxide	0.2
34.42	1641	1639	Caryophylla-4(12),8(13)-dien-5-α-ol	0.1
34.43	1642	1639	Caryophylla-4(12),8(13)-dien-5-β-ol	0.4
Total oxygenated sesquiterpenes				**5.6**
28.26	1486	1486	Phenyl ethyl 2-methylbutanoate	0.1
28.44	1491	1490	Phenyl ethyl 3-methylbutanoate	0.1
32.27	1585	1584	2-Phenylethyl tiglate	0.5
33.68	1621	1620	dillapiole	0.2
34.63	1645	1644	α-Muurolol	0.1
38.24	1740	1740	(*E*)-Isoamyl cinnamate	0.1
42.07	1850	1846	Phenyl ethyl octanoate	0.1
Total phenylpropanoids				**1.2**
32.74	1596	1596	Fokienol	0.1
56.55	2303	2300	Tricosane	0.1
Others				**0.2**

RT: Retention time

RI^C: Retention Index (on DB-5MS column)

RI_L: literature retention index (Adams[25])

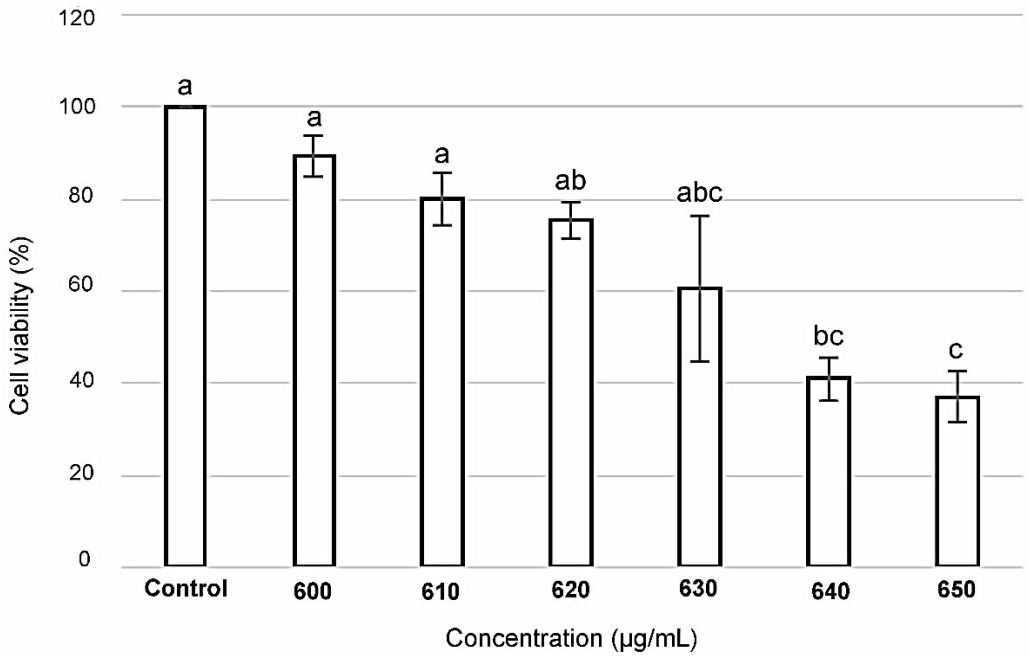

Figure 1. Cell viability of human gingival fibroblasts exposed to different concentrations of *Lippia origanoides* essential oil, evaluated using MTT. The data show the percentage of live cells in three independent experiments. Different letters represent significant difference ($p<0.05$)

[2,11,12,19]. The concentration of essential oil has a causal relationship with cytotoxicity during therapeutic use [31]. Therefore, research into the effects of *L. origanoides* essential oil on gingival fibroblasts is fundamental for its use at safe concentrations. Furthermore, as most Brazilian flora is known to contain medicinal properties [32], it is important to take advantage of this opportunity to develop new treatments for diseases.

In this context, chemical analysis of the components is an important step toward assessing the effects of medicinal plant essential oils [12,16]. Previous studies have demonstrated that common components found in the *Lippia* species are α-pinene, 1,8-cineole, limonene, *p*-cymene, thymol, γ-terpinene, carvacrol, and (*E*)-caryophyllene [8,10, 12,16]. Several chemical profiles have been reported in various regions of Brazil and Colombia, characterized by the following main components: p-cymene/β-caryophyllene/α-phellandrene and β-phellandrene/limonene (chemotype A), carvacrol (chemotype B), thymol (chemotype C), 1,8-cineole, which was found on the leaves of *Lippia schomburgkiana* Schauer and is considered synonymous with *L. origanoides* Kunth (chemotype D) and methyl (*E*)-cinnamate/(*E*)-nerolidol (chemotype E) [11,12,16,33,34].

Many of these components were found in the present study (Table 1). Similar results were reported by Ribeiro *et al.*[33], who claimed that oxygenated sesquiterpenes were present in higher concentrations in samples collected from the Amazon region. Other studies have also demonstrated that *L. origanoides* essential oil from different locations in north and northeast Brazil contains high concentrations of 1,8-cineole [34], (*E*)-nerolidol, and β-caryophyllene [14], supporting the existence of several chemotypes. These chemical profile variations are said to be caused by environmental and climatic differences [5,33].

Many studies have reported the considerable bioactivity of *L. origanoides* essential oil, including its antifungal activity against *C. albicans, C. parapsilosis,* and *C. krusei* [11,12,16]. In addition, the sesquiterpene (*E*)-nerolidol has been shown to work synergistically against *C. albicans*, *C. tropicalis*, and *C. krusei* [35,36]. Similarly, *p*-cymene, 1,8-cineole, and caryophyllene have demonstrated significant antimicrobial activity [5,12,35,37,38], antioxidant [14,34], and antiproliferative [39] activity of different chemotypes of *L. origanoides* have been reported.

However, few studies have evaluated the cytocompatibility of this essential oil. This should be the first step when analyzing materials for medical use, as it allows the evaluation of the cytotoxic potential of the host cells [30]. Therefore, gingival fibroblasts are often used to verify the biocompatibility of essential oils for therapeutic use in the oral cavity [22,31]. These cells are found in the connective tissue of the oral mucosa where many infections occur, including oral candidiasis [40]. Therefore, gingival fibroblasts were selected for this study.

Our results demonstrated that, as the essential oil concentration increased, the viability of the gingival fibroblasts decreased (Figure 1), whereas concentrations of 600 µg/mL, 610 µg/mL, and 620 µg/mL were considered non-cytotoxic. This is probably related to the dosage-dependent toxic potential of terpenes [14,41], which are major components of *L. origanoides* essential oil. This dosage-dependent relationship was previously reported in a study by Mendanha *et al.*[42], who claimed that monoterpene 1,8-cineole was less cytotoxic than sesquiterpene (*E*)-nerolidol on Balb/c3T3-A31 fibroblasts. This also suggests that the cytotoxic effects of such compounds occur through an increase in the fluidity of the cell membrane without rupture.

In addition, Silva *et al.*[43] revealed that p-cymene, the main component of the sample in our study, and carvacrol exhibited no cytotoxicity and low cytotoxicity in L292 fibroblasts, respectively. A previous study demonstrated that carvacrol has a cytotoxic potential similar to 0.2 % chlorhexidine, the main component in oral antiseptics, at 5 min, 1 h, and 24 h [44]. Sperotto *et al.*[41] suggested that *(E)*-nerolidol cytotoxicity is dependent on its concentration and is due to the intracellular increase of reactive oxygen species (ROS). Moreover, caryophyllene, another major component found in our samples, demonstrated low cell toxicity in fibroblast 3T3 cells with 80 % cell viability at 500 µg/mL concentrations [37]. Considering this, the synergistic effects of essential oil constituents must be taken into account when analyzing their effects on cells.

Cytotoxicity studies on *L. origanoides* essential oil are rare in the literature. However, a few studies have indicated that the oil confers low-level toxicity. Betancur-galvis *et al.*[16] and Tangarife-Castaño *et al.*[11] assayed the toxicity of the essential oil on non-tumorous kidney cells from the African green monkey (Vero ATCC CCL-81). However, neither study reported toxicity when using the methodology for IC_{50} determination.

Another study indicated that this essential oil, which has high concentrations of carvacrol and p-cymene, did not produce toxic effects in mice with oral administration of concentrations up to 120 mg/kg, in chronic and acute tests [8]. Likewise, *L. origanoides* leaf extract, which is rich in 1,8-cineole, displayed low acute toxicity during tests in mice. Despite other studies using different methodologies from ours, it should be noted that all studies concluded that *L. origanoides* essential oil has good biocompatibility, which is consistent with the results of this study.

Conclusions

The essential oil extracted from *L. origanoides* leaves contains high concentrations of hydrocarbons monoterpene, oxygenated monoterpenes and hydrocarbons sesquiterpene, with *p*-cymene being the main component. Cytotoxicity on gingival fibroblasts was concentration-dependent. These findings have the potential to guide those using *L. origanoides* essential oil as a therapeutic agent to select concentrations that are non-damaging to tissues.

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Conflict of interest

The authors declare no conflict of interest

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