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Cytotoxic effect of leaf essential oil of *Lippia gracilis* Schauer (Verbenaceae)

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ABSTRACT

Medicinal plants are one of the most important sources of drugs used in the pharmaceutical industry. Among traditional medicinal plants, *Lippia gracilis* Schauer (Verbenaceae) had been used for several medicinal purposes in Brazilian northeastern. In this study, leaf essential oil (EO) of *L. gracilis* was prepared using hydrodistillation. Followed by GC–MS analysis, its composition was characterized by the presence of thymol (55.50%), as major constituent. The effects of EO on cell proliferation and apoptosis induction were investigated in HepG2 cells. Furthermore, mice bearing Sarcoma 180 tumor cells were used to confirm its *in vivo* effectiveness. EO and its constituents (thymol, *p*-cymene, γ-terpinene and myrcene) displayed cytotoxicity to different tumor cell lines. EO treatment caused G_1 arrest in HepG2 cells accompanied by the induction of DNA fragmentation without affecting cell membrane integrity. Cell morphology consistent with apoptosis and a remarkable activation of caspase-3 were also observed, suggesting induction of caspase-dependent apoptotic cell death. *In vivo* antitumor study showed tumor growth inhibition rates of 38.5–41.9%. In conclusion, the tested essential oil of *L. gracilis* leaves, which has thymol as its major constituent, possesses significant *in vitro* and *in vivo* antitumor activity. These data suggest that leaf essential oil of *L. gracilis* is a potential medicinal resource.

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Introduction

Natural products are an interesting source of drugs used in the pharmaceutical industry. Among these, essential oils are complex mixtures of odoriferous substances that usually present multiple pharmacology properties. Each of these constituents contributes to the biological effects of these oils (Bakkali et al. 2008).

Lippia gracilis Schauer (Verbenaceae), popularly known as “alecrim-da-chapada” and “candeia-de-queimar”, had been used for several medicinal purposes in Brazilian northeastern. Among its folk medicinal uses, the treatment of cutaneous diseases, burns, wounds, ulcers, influenza, cough, sinusitis, bronchitis, nasal congestion, headache, jaundice and paralysis have been reported (Pascual et al. 2001; Albuquerque et al. 2007). Usually, its leaves are used to prepare infusion or decoction and used as a tea, as well as a macerate in alcohol for topical application (Lorenzi and Matos 2008). Some studies examining the pharmacological properties of *L. gracilis* have demonstrated that its leaf essential oil presents antibacterial, molluscicidal, larvicidal, antinociceptive and anti-inflammatory actions (Pessoa et al. 2005; Silva et al. 2008; Mendes et al. 2010; Teles et al. 2010). The antinociceptive and anti-inflammatory properties of methanolic extract of leaves of *L. gracilis* have also been reported (Guimarães et al. 2012). Recently, in our cytotoxic drug-screening program, we demonstrated the cytotoxic activity of leaf essential oil of *L. gracilis* to several tumor cell lines (Ribeiro et al. 2012). However, the mechanisms underlying these effects were not explored.

In present work, the chemical composition of leaf essential oil (EO) of *L. gracilis* was characterized by CG–MS. The mechanisms involved in EO cytotoxic activity were investigated in HepG2 cells. *In vivo* effects of EO in mice bearing Sarcoma 180 tumor cells were also evaluated.

Materials and methods

Cells

Cytotoxicity was determined in tumor cells using HepG2 (human hepatocellular carcinoma), K562 (human chronic

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myelocytic leukemia) and B16-F10 (mouse melanoma), all donated by Hospital A.C. Camargo, São Paulo, SP, Brazil. Cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640, Gibco-BRL, Gaithersburg, MD, USA) medium supplemented with 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil), 2 mM L-glutamine (Vetec Química Fina, Duque de Caxias, RJ, Brazil) and 50 μg/ml gentamycin (Novafarma, Anápolis, GO, Brazil). Adherent cells were harvested by treatment with 0.25% trypsin EDTA solution (Gibco-BRL, Gaithersburg, MD, USA). All cell lines were cultured in cell culture flasks at 37 °C in 5% CO_2 and sub-cultured every 3–4 days to maintain exponential growth. Cytotoxicity experiments were conducted with cells in exponential growth phase.

Sarcoma 180 tumor cells, which had been maintained by passages in the peritoneal cavity of Swiss mice, were obtained from the Laboratory of Experimental Oncology at the Federal University of Ceará.

Human lymphocyte cells were obtained by primary culture. Heparinized blood (from healthy, non-smoker donors who had not taken any drug at least 15 days prior to sampling) was collected and peripheral blood mononuclear cells (PBMC) were isolated by a standard protocol using Ficoll density gradient (Ficoll-Paque Plus, GE Healthcare Bio-Sciences AB, Sweden). PBMC were washed and resuspended at a concentration of 0.3×10^6 cells/ml in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 50 μg/ml gentamycin at 37 °C with 5% CO_2. Concanavalin A (ConA, Sigma Chemical Co. St Louis, MO, USA) was used as a mitogen to trigger cell division in T-lymphocytes. ConA (10 μg/ml) was added at the beginning of culture and after 24 h, cells were treated with the test drugs (Brown and Lawce 1997).

For all experiments, cell viability was performed by Trypan blue assay. Over 90% of the cells were viable at the beginning of the culture.

Animals

A total of 36 Swiss mice (males, 25–30 g), obtained from the central animal house of the Federal University of Sergipe, Brazil, were used. Animals were housed in cages with free access to food and water. All animals were kept under a 12:12 h light-dark cycle (lights on at 6:00 a.m.). Animals were treated according to the ethical principles for animal experimentation of SBCAL (Brazilian association of laboratory animal science), Brazil. The Animal Studies Committee from the Federal University of Sergipe approved the experimental protocol (number 60/2010).

Plant material

L. gracilis leaves were collected in the proximity of the “Serra da Guia” [coordinates: 09°58′09″ S, 37°51′52″ W], Poço Redondo, Sergipe State, Brazil in November 2006. Samples were processed and identified according to standard protocol (Mori et al. 1989), being deposited in the herbarium of the Federal University of Sergipe (ASE) under the number 18740. The specie was identified by Dr. Raymond Mervyn Harley, Royal Botanic Gardens, Kew (England).

Hydrodistillation and CG–MS analysis of the essential oil

The essential oil from fresh leaves of *L. gracilis* (50 g) was obtained by hydrodistillation for 2 h using a Clevenger-type apparatus (Amitel, São Paulo, Brazil). The essential oil was dried over anhydrous sodium sulphate, and the percentage content was calculated on the basis of the dry weight of plant material. The essential oil was stored at −4 °C in a sealed amber bottle until chemical analysis. The extractions were performed in triplicate.

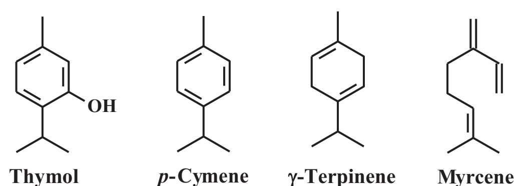

Fig. 1. Chemistry structure of thymol, *p*-cymene, γ-terpinene and myrcene.

Essential oil analysis of *L. gracilis* was performed on a Shimadzu QP5050A GC/MS system equipped with an AOC-20i auto-injector. A J&W Scientific DB-5MS (coated with 5%-phenyl-95%-dimethylpolysiloxane) fused capillary column (30 m × 0.25 mm × 0.25 μm film thickness) was used as the stationary phase. Helium was the carrier gas at 1.2 ml/min flow rate. Column temperatures were programmed from 40 °C for 4 min, raised to 220 °C at 4 °C/min, and then heated to 240 °C at 20 °C/min. The injector and detector temperatures were 250 and 280 °C, respectively. Samples (0.5 μl in CH_2Cl_2) were injected with a 1:20 split ratio. MS were taken at 70 eV with a scan interval of 0.5 s and fragments from 40–350 Da.

The retention indices were obtained by co-injecting the oil sample with a C_8–C_{18} linear hydrocarbon mixture (van Den Dool and Kratz 1963). The volatile components were analyzed by GC/MS, and identification was made by comparing retention indices and mass spectra with those in the literature (Adams 2007), as well as by computerized matching of the acquired mass spectra with those stored in the NIST and Wiley mass spectral library and other published mass spectra. The percentage composition of each component was determined by dividing the area of the component by the total area of all components isolated under these conditions without response factor correction.

Pure compounds

Thymol (purity ≥99.5%), *p*-cymene (purity 99%), γ-terpinene (purity ≥97.0%) and myrcene (purity ≥90%) (Fig. 1) were obtained from Sigma Chemical Co. St Louis, MO, USA.

Cell proliferation assay

Cell growth was quantified by methyl-[^{3}H]-thymidine incorporation assay, as described by Griffiths and Sundaram (2011) with minor modifications. Methyl-[^{3}H]-thymidine is a radiolabelled DNA precursor incorporated into newly synthesized DNA, which the amount of incorporated methyl-[^{3}H]-thymidine is related to the rate of proliferation. For all experiments, 100 μl of a solution of cells (0.7×10^5 cells/ml for adherent cells or 0.3×10^6 cells/ml for suspended cells) were seeded in 96-well plates. After 24 h, the drugs (1.56–50 μg/ml), dissolved in dimethyl sulfoxide (DMSO, LGC Biotechnology, São Paulo, SP, Brazil), was added to each well and incubated for 72 h. Doxorubicin (doxorubicin hydrochloride, Eurofarma, São Paulo, SP) was used as the positive control. Six hours before the end of incubation time, 1 μCi of methyl-[^{3}H]-thymidine (PerkinElmer, USA) was added to each well. After this period, cultures were harvested using a cell harvester (Brandel, Inc. Gaithersburg, MD, USA) to determine the ^{3}H-thymidine incorporation using a liquid scintillation cocktail Hidex Maxilight (PerkinElmer Life Sciences, Groningen, GE, Netherlands) and a plate CHAMELEON V multilabel Counter (Mustionkatu 2, TURKU, Finland) with MikroWin Hidex 2000 v. 4.38 software (Microtek Laborsysteme GmbH, Overath, Germany). The drug effect was quantified as the percentage of control radioactivity.

Analysis of mechanisms involved in the cytotoxic activity

The following experiments were performed to elucidate the mechanisms involved in cytotoxic action of EO. For all experiments, 2 ml of a solution of HepG2 cells (0.7×10^5 cells/ml) were seeded in 24-well plates and incubated by overnight to allow that the cells to adhere to the plate surface. Then, the cells were treated for 24 h with EO at final concentration of 2.5 and 5.0 μg/ml. Negative control was treated with the vehicle (0.1% DMSO) used for diluting the tested drug. Doxorubicin (1.0 μg/ml) was used as the positive control.

Trypan blue dye exclusion test

Cell proliferation was assessed by the Trypan blue dye exclusion test. HepG2 cells were seeded in the absence and presence of EO. After 24 h drug exposure, cell proliferation was assessed. Cells that excluded Trypan blue were counted using a Neubauer chamber.

Cell cycle distribution

Cells were harvested in a lysis solution containing 0.1% Triton X-100 (Sigma Chemical Co. St Louis, MO, USA) and 2 μg/ml propidium iodide (BioSource, USA). Cell fluorescence was determined by flow cytometry in a FACSCalibur cytometer (Bencton Dickinson, San Diego, CA, USA) with CellQuest software (BD Biosciences, San Jose, CA, EUA). Ten thousand events were evaluated per experiment and cellular debris was omitted from the analysis.

Morphological analysis with hematoxylin–eosin staining

Morphological changes were examined by light microscopy (Olympus BX41, Tokyo, Japan) using Image-Pro Express software (Media Cybernetics, Inc. Silver Spring, USA). To evaluate alterations in morphology, cells from cultures were harvested, transferred to cytospin slides, fixed with methanol for 30 s, and stained with hematoxylin–eosin.

Morphological analysis using fluorescence microscope

Morphological changes were examined using fluorescence microscope. Cells were pelleted and resuspended in 25 μl saline. Thereafter, 1 μl of aqueous solution of acridine orange (AO, Sigma Chemical Co. St Louis, MO, USA) and ethidium bromide (EB, Sigma Chemical Co. St Louis, MO, USA) (AO/EB, 100 μg/ml) was added and the cells were observed under a fluorescence microscope (Olympus BX41, Tokyo, Japan). Three hundred cells were counted per sample and classified as viable, apoptotic or necrotic cells.

Cell membrane integrity

The cell membrane integrity was evaluated by the exclusion of propidium iodide. Cell fluorescence was determined by flow cytometry, as described above.

Caspase-3 activation assay

Caspase-3/CPP32 colorimetric assay kit (BioVision Incorporated, CA, USA) was used to investigate caspase-3 activation in treated cells based on the cleavage of Asp-Glu-Val-Asp (DEVD)-pNA. Briefly, cells were lysed by incubation with cell lysis buffer on ice for 10 min and then centrifuged at 10,000 g for 1 min. To each reaction mixture, 50 μl cell lysate (100–200 μg total protein) was added. Enzyme reactions were carried out in a 96-well flat-bottom microplate.

In vivo antitumor assay

The *in vivo* antitumor effect was evaluated using sarcoma 180 ascites tumor cells following protocols previously described (Bezerra et al. 2008; Britto et al. 2012). Ten-day old sarcoma 180 ascites tumor cells (2×10^6 cells per 500 μl) were implanted subcutaneously into the left hind groin of mice. EO was dissolved in 5% DMSO and given to mice intraperitoneally once a day for 7 consecutive days. Negative control was treated with the vehicle (5% DMSO) used for diluting the tested substance. 5-Fluoruoracil (5-FU, Sigma Chemical Co. St Louis, MO, USA) was used as the positive control. At the beginning of the experiment, the mice were divided into four groups, as follows: Group 1: animals treated by i.p. injection of vehicle 5% DMSO ($n = 12$); Group 2: animals treated by i.p. injection of 5-FU (25 mg/kg/day) ($n = 8$); Group 3: animals treated by i.p. injection of EO (40 mg/kg/day) ($n = 8$); Group 4: animals treated by i.p. injection of EO (80 mg/kg/day) ($n = 8$). The treatments were started one day after tumor injection. The dosages were determined based on previous articles. On day 8, the animals were euthanized, by cervical dislocation, and the tumors were excised and weighed. The drug effects were expressed as the percent inhibition of control.

Body weight loss, organ weight alteration and hematological analyses were determined at the end of experiment above, as previously described (Bezerra et al. 2008; Britto et al. 2012). Peripheral blood samples were collected from the retro-orbital plexus under light ether anesthesia and the animals were euthanized by cervical dislocation. After sacrifice, liver, kidney and spleens were removed and weighed. In hematological analysis, total and differential leukocyte counts were determined by standard manual procedures using light microscopy.

Statistical analysis

Data are presented as mean ± SEM (or SD) or IC_{50} values and their 95% confidence intervals (CI 95%) obtained by nonlinear regression. Differences among experimental groups were compared by one-way analysis of variance (ANOVA) followed by Newman–Keuls test ($p < 0.05$). All analyses were carried out using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA, USA).

Results and discussion

The present work investigated the phytochemical and cytotoxic properties of leaf essential oil of *L. gracilis*. It was chemically characterized by CG–MS analysis. The effects of EO on cell proliferation and apoptosis induction were investigated in HepG2 cells. Furthermore, mice bearing Sarcoma 180 tumor cells were used to confirm its *in vivo* effectiveness.

EO was obtained as pale yellowish oil in 4.0% yield (w/v). Previous reports on essential oil composition of *L. gracilis* growing in Brazil, particularly in Ceará, Pernambuco and Sergipe States showed monoterpenes mainly *p*-cymene, γ-terpinene and variable content of carvacrol and/or thymol as its major components (Pessoa et al. 2005; Silva et al. 2008; Neves et al. 2008; Mendes et al. 2010; Teles et al. 2010). In the present study, it was possible to identify 35 compounds in the leaf essential oil of *L. gracilis* that was also constituted predominantly by monoterpenes (Table 1). However, due to the higher percentage of thymol and the presence of other major components identified such as *p*-cymene, thymol methyl ether, γ-terpinene, myrcene and thymol acetate, the chemical composition of this specimen is different from others collected in Sergipe and in others Brazilian localities (Pessoa et al. 2005; Silva et al. 2008; Neves et al. 2008; Mendes et al. 2010; Teles et al. 2010). Moreover,

Table 1
Chemical constituents of leaf essential oil of *L. gracilis*.

	RI[a]	RI[b]	Compounds	% Peak area[c]
1	884	880	3-Methyl-3-buten-1-ol acetate	tr
2	924	924	α-Thujene	1.23 ± 0.12
3	930	932	α-Pinene	0.40 ± 0.00
4	973	974	β-Pinene	tr
5	988	988	Myrcene	4.03 ± 0.29
6	1003	1002	α-Phellandrene	0.10 ± 0.00
7	1005	1008	δ-3-Carene	0.27 ± 0.06
8	1014	1014	α-Terpinene	1.47 ± 0.15
9	1022	1020	*p*-Cymene	10.80 ± 1.35
10	1026	1024	Limonene	0.57 ± 0.06
11	1035	1032	(*Z*)-β-Ocimene	0.20 ± 0.00
12	1045	1044	(*E*)-β-Ocimene	0.20 ± 0.00
13	1056	1054	γ-Terpinene	5.53 ± 0.57
14	1067	1065	*cis*-Sabinene hydrate	tr
15	1082	1086	Terpinolene	0.10 ± 0.00
16	1097	1095	Linalool	0.13 ± 0.06
17	1169	1167	Umbellulone	0.30 ± 0.18
18	1176	1174	Terpinen-4-ol	0.90 ± 0.00
19	1226	1232	Thymol methyl ether	10.53 ± 1.40
20	1290	1289	Thymol	55.50 ± 4.21
21	1294	1298	Carvacrol	0.20 ± 0.00
22	1343	1349	Thymol acetate	3.30 ± 0.44
23	1362	1369	Cyclosativene	0.20 ± 0.00
24	1369	1374	α-Copaene	0.10 ± 0.00
25	1377	1376	(*E*)-Methyl cinnamate	0.30 ± 0.10
26	1416	1417	(*E*)-Caryophyllene	1.43 ± 0.06
27	1435	1439	Aromadendrene	0.20 ± 0.00
28	1452	1452	α-Humulene	0.20 ± 0.00
29	1469	1469[d]	2,6-Dimethoxyacetophenone	0.23 ± 0.06
30	1487	1496	Viridiflorene	0.20 ± 0.00
31	1491	1500	Bicyclogermacrene	0.17 ± 0.06
32	1504	1505	β-Bisabolene	0.27 ± 0.06
33	1511	1511	δ-Amorphene	tr
34	1578	1582	Caryophyllene oxide	0.20 ± 0.00
35	1581	1590	Globulol	0.10 ± 0.01
TOTAL				99.36

tr, trace (mean value < 0.10%).
[a] RI, retention indices on DB-5MS column calculated according to van Den Dool and Kratz (1963).
[b] RI, retention indices according to Adams (2007).
[c] Data are presented as mean ± SD of three analyses.
[d] RI, retention index according to Tret'yakov (2008).

the lowest content of carvacrol and (*E*)-caryophyllene suggests that this may be another chemotype that it is a novel source of thymol.

Three tumor cell lines were treated with increasing concentrations of EO and its constituents (thymol, *p*-cymene, γ-terpinene and myrcene) for 72 h and analyzed by methyl-[^{3}H]-thymidine incorporation assay. Table 2 shows the obtained IC_{50} values. EO showed IC_{50} values ranged from 4.93 to 22.92 μg/ml for HepG2 and K562 cell lines, respectively. Among its constituents, myrcene presented to be the most cytotoxic compound, showing IC_{50} values ranging from 9.23 to 12.27 μg/ml for HepG2 and B16-F10 cell lines, respectively. Thymol, *p*-cymene and γ-terpinene showed cytotoxicity only for B16-F10, showing IC_{50} values of 18.23, 20.06 and 9.28 μg/ml, respectively. Doxorubicin, used as the positive control, showed IC_{50} values from 0.03 to 2.92 μg/ml for B16-F10 and K562 cell lines, respectively. In addition, the cytotoxicity of EO was also evaluated to normal cells (PBMC). The results, presented in Table 2, show that EO was also cytotoxic to normal cells. None of EO constituents showed cytotoxicity to normal cells at the tested concentrations (IC_{50} > 25 μg/ml).

According to our cytotoxic drug-screening program, essential oil that shows IC_{50} values below 30 μg/ml and pure compound that shows IC_{50} values below 1 μg/ml are considered promising (Suffness and Pezzuto 1990; Bezerra et al. 2008). Therefore, EO is considered a potent cytotoxic agent. On the other hand, its constituents thymol, *p*-cymene, γ-terpinene and myrcene are considered weak cytotoxic agents. These compounds were previously

Table 2
Cytotoxic activity of leaf essential oil of *L. gracilis* and its constituents (thymol, *p*-cymene, γ-terpinene and myrcene) on tumor and normal cells.

Cell lines	Histotype	Essential oil	Thymol	*p*-Cymene	γ-Terpinene	Myrcene	Doxorubicin
HepG2	Hepatocellular carcinoma	4.93 (3.09 – 7.85)	>25	>25	>25	9.23 (4.03 – 21.11)	0.62 (0.46 – 0.83)
K562	Chronic myelocytic leukemia	22.92 (19.17 – 27.41)	N.d	N.d	N.d	N.d	2.92 (2.28 – 3.73)
B16-F10	Melanoma	7.01 (2.63 – 18.73)	18.23 (13.87 – 23.95)	20.06 (10.47 – 38.41)	9.28 (7.38 – 11.68)	12.27 (5.13 – 29.37)	0.03 (0.01 – 0.09)
PBMC	Normal lymphocyte	16.64 (7.42 – 37.32)	>25	>25	>25	>25	4.17 (3.19 – 5.46)

Data are presented as IC_{50} values, in μg/ml, and their 95% confidence interval obtained by non-linear regression from two independent experiments performed in duplicate or triplicate by methyl-[^{3}H]-thymidine incorporation assay after 72 h incubation. Doxorubicin was used as the positive control. Nd, not determined.

Table 3
Effect of leaf essential oil of *L. gracilis* on cell cycle distribution of human hepatocellular carcinoma HepG2 cells after 24 h incubation.

Drugs	Concentration (μg/ml)	Cell cycle phases (%)		
		G_1	S	G_2/M
Control	–	62.49 ± 2.12	10.76 ± 1.57	17.63 ± 1.86
Doxorubicin	1.0	39.31 ± 5.06*	9.64 ± 1.73	54.07 ± 7.90*
Essential oil	2.5	72.08 ± 1.10*	9.23 ± 1.32	13.26 ± 0.88
	5.0	74.61 ± 1.31*	8.87 ± 0.85	12.48 ± 0.78

Data are presented as mean values ± S.E.M. from two independent experiments performed in duplicate. Negative control was treated with the vehicle (0.1% DMSO) used for diluting the tested substance. Doxorubicin was used as the positive control. Ten thousand events were analyzed in each experiment.
* $p < 0.05$ compared to control by ANOVA followed by Student–Newman–Keuls test.

assessed against tumor cell lines. Among them, thymol showed IC_{50} value of ∼60 μg/ml to HL-60 cells and γ-terpinene showed IC_{50} value of 156.92 μg/ml to Jurkat cells (Deb et al. 2011; Döll-Boscardin et al. 2012). Probably, the potent cytotoxic activity of tested essential oil might be attributed to mixture of its main and minor constituents.

Since HepG2 cells were especially sensitive to EO cytotoxicity, further studies were performed with this cell line using concentrations corresponding to 2.5 and 5.0 μg/ml. These concentrations were chosen based on its IC_{50} value in this cell line (4.93 μg/ml).

When analyzed by Trypan blue dye exclusion, EO reduced proliferation of HepG2 cells in a concentration-dependent manner after 24 h incubation ($p < 0.05$, Fig. 2).

Cell cycle arrest is a common cause of cell growth inhibition. To determine whether EO cytotoxicity induction involves alterations in cell cycle progression, analysis of cell cycle distribution by flow cytometry were included in this study. All DNA subdiploid in size (sub-G_1) were considered as internucleosomal DNA fragmentation. The results of the effect of EO on cell cycle distribution showed that total number of G_1 cells increased, indicating cell cycle arrest during this phase (Table 3). G1-phase cell cycle arrest creates an opportunity for cells to either undergo repair or enter the apoptotic pathway to maintain tissue homeostasis and eliminate the mutated neoplastic and hyperproliferating neoplastic cells from the system (Pucci et al. 2000). Besides the increasing of cells in G_1, it was also

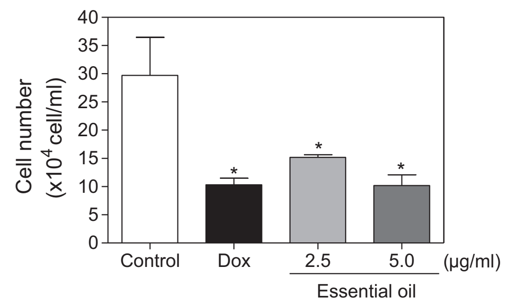

Fig. 2. Effect of leaf essential oil of *L. gracilis* on the proliferation of human hepatocellular carcinoma HepG2 cells measured by Trypan blue dye exclusion method after 24 h incubation. Negative control was treated with the vehicle (0.1% DMSO) used for diluting the tested substance. Doxorubicin (Dox, 1.0 μg/ml) was used as the positive control. Data are presented as mean values ± S.E.M. from two or three independent experiments performed in duplicate. *$p < 0.05$ compared to negative control by ANOVA followed by Student–Newman–Keuls test.

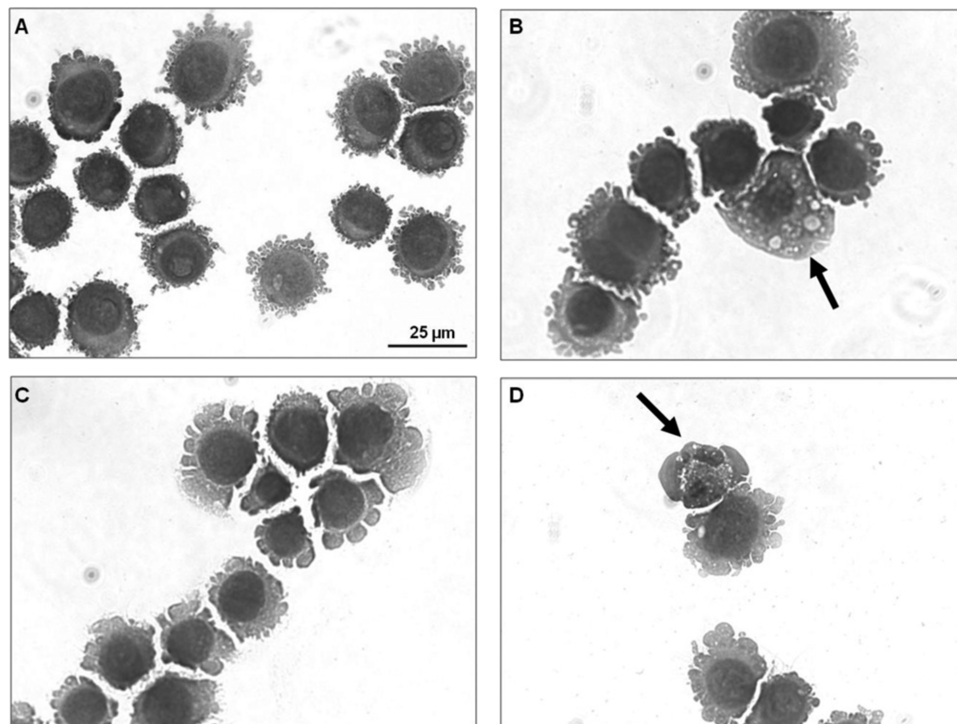

Fig. 3. Effect of leaf essential oil of *L. gracilis* on cell morphology of human hepatocellular carcinoma HepG2 cells. The cells were stained with hematoxylin-eosin and analyzed by optical microscopy after 24 h incubation with the essential oil at concentrations 2.5 (C) and 5.0 (D) μg/ml. Negative control (A) was treated with the vehicle (0.1% DMSO) used for diluting the tested substance. Doxorubicin (1.0 μg/ml) was used as the positive control (B). Black arrows show chromatin condensation or nuclear DNA fragmentation.

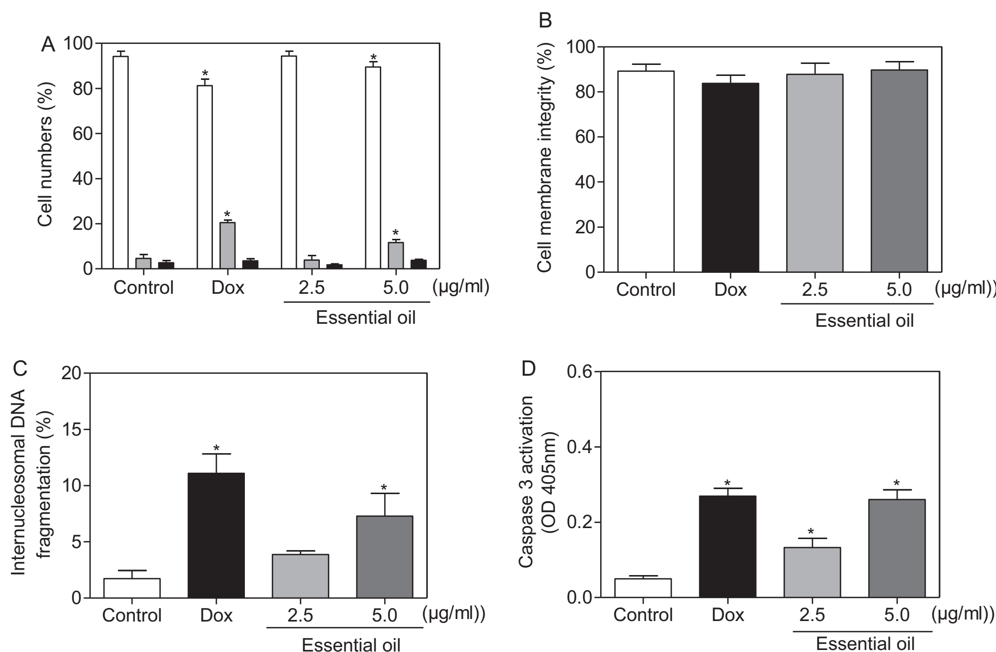

Fig. 4. Effect of leaf essential oil of *L. gracilis* on viability of human hepatocellular carcinoma HepG2 cells after 24 h incubation. (A) Cell viability measured by fluorescence microscope using acridine orange/ethidium bromide – viable cells (white bar), apoptotic cell (gray bar), necrotic cell (black bar). (B) Cell membrane integrity measured by flow cytometry using propidium iodide. (C) Internucleosomal DNA fragmentation determined by flow cytometric using propidium iodide and triton X-100. (D) Caspase 3 activation measured by colorimetric assay. Negative control was treated with the vehicle (0.1% DMSO) used for diluting the tested substance. Doxorubicin (Dox, 1.0 μg/ml) was used as the positive control. Data are presented as mean values ± S.E.M. from two or three independent experiments performed in duplicate. For flow cytometry analysis ten thousand events were analyzed in each experiment. *$p < 0.05$ compared to negative control by ANOVA followed by Student-Newman–Keuls test.

observed an increasing in the internucleosomal DNA fragmentation ($p < 0.05$, Fig. 4C).

Morphological changes were investigated using hematoxylin–eosin staining (Fig. 3). In presence of 5.0 μg/ml of EO, cells presented morphology consistent with apoptosis, including cell volume reduction, chromatin condensation and fragmentation of the nuclei condensation. Morphological changes were also investigated using AO/EB staining and fluorescence microscopy, where the percentages of viable, apoptotic and necrotic cells were calculated. After 24 h of exposure, EO-treated cells presented an increased number of apoptotic cells at concentration of 5 μg/ml ($p < 0.05$, Fig. 4A).

EO did not disrupt membrane at any tested concentration ($p > 0.05$, Fig. 4B). In addition, as cited above, DNA fragmentation increased in EO-treated cells ($p < 0.05$, Fig. 4C). These both modifications were compatible with apoptotic cells. In addition, a remarkable activation of caspase-3 was recorded in lysates from HepG2 cells treated with EO (Fig. 4D), suggesting caspase-dependent apoptotic cell death.

Apoptosis is a regulated cell death process that eliminates damaged or malfunctioning cells. It is characterized by phosphatidylserine exposure, loss of mitochondrial membrane potential, caspase activation, chromatin condensation, nuclear fragmentation, resulting in the phagocytosis of membrane-bound apoptotic bodies (Walsh and Edinger 2010). Herein, we demonstrated that EO is able to induce cell death through caspase-dependent apoptosis pathway in HepG2 cells. Interestingly, Deb et al. (2011) reported that thymol, the main constituent of EO, is able to induce HL-60 cell death by apoptosis pathway associated with the reactive oxygen species production, disruption of mitochondrial membrane potential, increase in mitochondrial H_2O_2 production, a decrease in Bcl-2 protein, an increase in Bax protein levels and caspase-9, -8 and -3 activation. Moreover, thymol was also able to induce caspase-independent apoptosis.

To investigate whether OE has *in vivo* antitumor activity, mice were subcutaneously transplanted with sarcoma 180 cells and treated by intraperitoneal route once a day for 7 consecutive days with EO. The effects of EO on mice transplanted with sarcoma 180 tumor cells are presented in Fig. 5. Tumor growth inhibition rates were 38.5–41.9%. The inhibition was significant at both doses in relation to the control group ($p < 0.05$).

Systemic toxicological parameters were also examined in EO-treated mice. For these, body weight loss, organ weight alteration and leukogram were determined. No statistically significant changes in EO-treated mice were seen in any toxicological parameters analyzed ($p > 0.05$, data not shown). In contrast, 5-FU, used as the positive control, reduced the body weights and spleen organ weights and induced a decrease in total leukocytes ($p < 0.05$, data not shown).

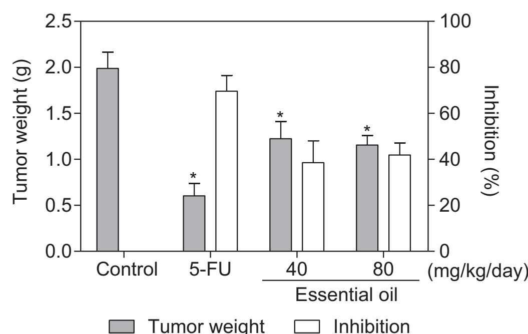

Fig. 5. Effect of leaf essential oil of *L. gracilis* on mice inoculated with sarcoma 180 tumor. The graph shows tumor weight (g) and tumor growth-inhibition levels. Negative control was treated with the vehicle used for diluting the test substance (5% DMSO). 5-Fluorouracil (5-FU) was used as the positive control at dose of 25 mg/kg/day. Data are presented as mean ± S.E.M. of 8–12 animals. *$p < 0.05$ compared with negative control group by ANOVA followed by the Student-Newman–Keuls test.

In conclusion, these data presented that the tested leaf essential oil of *L. gracilis* is chemically characterized by the presence of thymol, as major constituent, and possesses *in vitro* and *in vivo* anticancer activities. In cell-based assay, it was able to induce G_1 arrest and caspase-dependent apoptosis in HepG2 cells. In animal model, it was found to be associated with a decrease in tumor growth.

Conflict of interest

The authors have declared that there is no conflict of interest.

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