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Comparative Studies of Cytotoxic and Apoptotic Properties of Different Extracts and the Essential Oil of Lavandula angustifolia on Malignant and Normal Cells

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Comparative Studies of Cytotoxic and Apoptotic Properties of Different Extracts and the Essential Oil of *Lavandula angustifolia* on Malignant and Normal Cells

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Lavender (*Lavandula angustifolia* Mill.) is a bush-like shrub from Lamiaceae. The herb has been used in alternative medicine for several centuries. In this study, the cytotoxicity and the mechanisms of cell death induced by 3 different extracts of aerial parts and the essential oil of *L. angustifolia* were compared in normal and cancerous human cells. Malignant (HeLa and MCF-7 cell lines) and nonmalignant (human fibroblasts) cells were incubated with different concentrations of the plant extracts. Cell viability was quantified by MTS assay. Apoptotic cells were determined using propidium iodide staining of DNA fragmentation by flow cytometry (sub-G1 peak). The molecules as apoptotic signal translation, including Bax and cleaved PARP, were identified by Western blot. Ethanol and *n*-hexane extracts and essential oil exhibited significant cytotoxicity to malignant cells but marginal cytotoxicity to human fibroblasts in vitro and induced a sub-G1 peak in flow cytometry histogram of treated cells compared to the control. Western blot analysis demonstrated that EtOH and *n*-hexane extracts upregulated Bax expression, also it induced cleavage of PARP in HeLa cells compared to the control. In conclusion, *L. angustifolia* has cytotoxic and apoptotic effects in HeLa and MCF-7 cell lines, and apoptosis is proposed as the possible mechanism of action.

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INTRODUCTION

Cancer as a growing health difficulty causes problems mostly in developed countries and its incidence is increasing rapidly worldwide. Some problems such as population growth, drug side effects, high cost of treatment, increasing drug resistance and inadequate supplies of drugs have caused natural products to be used in treatment of many diseases like cancer (1).

A vast majority of aromatic medicinal plants contain chemical compounds exhibiting different biological properties (2). *Lavandula angustifolia* (Lamiaceae) is a popular medicinal herb in aromatherapy and it has long been used as a relaxant. The plant has antimutagenic (3), antiflatulence, anticolic, spasmolytic, analgesic, and antiinflammatory activities and can reduce skeletal muscle tone in rats (4). *Lavandula angustifolia* is used for the therapy of several gastrointestinal, nervous, and rheumatic disorders (5).

Lavandula angustifolia essential oil has antiseptic, antifungal, and antibacterial properties (6). Lavender oil has been shown to be cytotoxic to human skin cells in vitro at a concentration of 0.25% (v/v) (7).

Some constituents of *L. angustifolia* essential oil increases sedation and relaxation and decreases anxiety and agitation both in animals and humans (8–10). Furthermore, some studies on mice and rats have indicated antinociception and antiinflammation activity of *L. angustifolia* (11,12). Several constituents of

L. angustifolia essential oil possess anticancer and antimutagenic properties (13–15).

Lavender is comprised of numerous constituents including essential oil, tannins, triterpenes, coumarins, and flavonoids. Some components of *L. angustifolia* essential oil include monoterpenoids such as linalool; linalyl acetate; perillyl alcohol, 1, 8-cineole; β-ocimene; limonene, terpinen-4-ol, and camphor (6). Perillyl alcohol has been shown to reduce cell proliferation in pancreatic tumor cells in a concentration-dependent manner in human and hamster cell lines (16). Perillyl alcohol inhibits cell proliferation in murine B16 melanoma cells. It has also cytotoxic activity against rodent mammary and pancreatic tumors and rat liver cancer (17).

In addition, 1, 8-cineole can suppress growth in leukemia cell lines (18) and terpinen-4-ol is able to inhibit the growth of human M14 melanoma cells (19).

Commercially, lavender is cultivated mainly for preparation of monofloral honey and essential oil production; the plant is also used for culinary application, perfumes, cosmetics, and perfumed soap production.

The composition of the essential oil extracted from *L. angustifolia* and their cytotoxic effects has been investigated previously (20,21). Nevertheless, the cytotoxic effects of different extracts obtained from *L. angustifolia* and a through comparative investigation on different extracts of aerial parts and its essential oil has not been properly addressed.

The aim of this study was to compare the cytotoxic and proapoptotic properties of 3 different extracts (EtOH, *n*-hexane, and H_2O) obtained from aerial parts of *L. angustifolia* and its essential oil on 2 cancer cell lines including human cervix carcinoma cell line (HeLa) and human breast cancer cell line (MCF-7) and human fibroblasts as nonmalignant cells. Paclitaxel (Taxol®) is a chemotherapy drug given to treat ovarian, breast, and nonsmall cell lung cancer. In this study, paclitaxel was used as a positive control.

MATERIALS AND METHODS

Chemicals

MTS [3-(4, 5-Dimethylthiazol-2-yl) -5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] from Promega (Madison, WI, USA); RPMI-1640 and FCS from Gibco; Lympholyte®-H from Cedarlane (Canada); β-actin and PARP antibodies, antirabbit IgG and HRP linked antibody from Cell Signaling Technology (Boston, MA); ECL Western blotting detection reagent from Bio-RaD (USA); the fluorescent probe propidium iodide (PI), protease inhibitor cocktail, phosphatase inhibitor cocktail, sodium citrate, Triton X-100, phenylmethylsulfonyl fluoride and QuantiPro BCA Assay Kit were purchased from Sigma (Steinheim, Germany).

Plant Materials

Aerial parts of *L. angustifolia* were collected in August 2010 from the Ferdowsi University Campus and identified in the Herbarium of School of Pharmacy, Mashhad University of Medical Sciences. Voucher specimen (No: 254-1804-01) was deposited in the herbarium of School of Pharmacy, Mashhad University of Medical Sciences. The plant extracts were prepared as follows: Dried aerial parts of *L. angustifolia* (100 g × 3) was ground into powder and then was percolated with 100 ml of each solvent (EtOH 70%, *n*-hexane and H_2O) for 4 h separately. The extracts were filtered and the solvents were evaporated under reduced pressure at 45°C to afford crude extracts (Fig. 1).

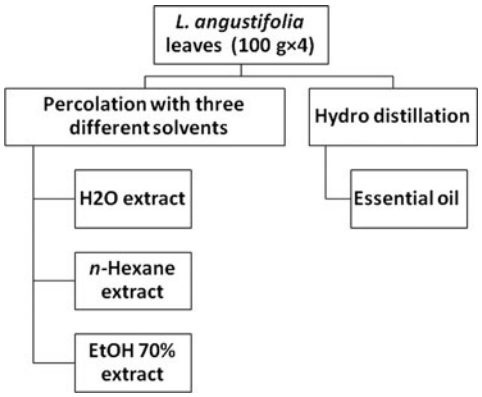

FIG. 1. Extraction scheme of *L. angustifolia*.

For preparing of the essential oil, the aerial parts (100 g) of *L. angustifolia* and the dried extracts (3 g) were subjected to hydrodistillation using a Cleavenger-type apparatus for 3 h. After decanting and drying over anhydrous sodium sulfate, the slightly yellow colored oil was recovered. The essential oil obtained from aerial parts of the plant then subjected to cytotoxic assay and the oils prepared from the plant and extracts were analyzed with GC to compare the volatile contents of each oil.

Gas-Chromatography and Gas-Chromatography–Mass Spectrometry

The GC analysis was performed using a Varian CP-3800 equipped with a FID detector, fused-silica column (CP-Sil 8CB, 50 m × 0.25 mm, film thickness 0.12 μm). The operating conditions were: oven temperature 50°C (5 min), 50°C–250°C (3°C/min), 250°C (10 min); injector temperature 260°C, split ratio 1:5, with the carrier gas, N2 (2 ml/min); detector temperature 280°C.

The GC–MS analyses were performed using a Agilent 5975 apparatus with a HP-5ms column (30 m × 0.25 mm i.d., 0.25 μm film thickness) interfaced with a quadruple mass detector and a computer equipped with Wiley 7n.l library; oven temperature 50°C (5 min), 50°C–250°C (3°C/min), 250°C (10 min); injector temperature 250°C; volume injection, 0.1 μL; split ration, 1:50; carrier gas Helium at 1.1 ml min; ionization potential, 70 eV; ionization current, 150 μA; ion source temperature, 250°C; mass range, 35–465 mui.

The constituents of the oils were identified by calculation of their retention indices under temperature programmed conditions for *n*-alkanes (C8–C20) and the oil on a CP-Sil 8CB column. Identification of individual compounds was made by comparison of their mass spectra and retention indices (RI) with those authentic samples and those given in the literature (22). Quantification of the relative amount of the individual components was performed according to the area percentage method without consideration of calibration factor.

Sample Preparation

To prepare the stock solutions (100 mg/ml), all extracts and the essential oil were dissolved in DMSO. The concentrations of 15–500 μg/ml were then obtained by diluting these solutions with Roswell Park Memorial Institute-1640 (RPMI-1640) so that the final concentrations of DMSO did not exceed 0.05%. All dilutions were prepared fresh before addition to the cells.

Cell Culture

HeLa and MCF-7 cells were obtained from the Pasteur Institute (Tehran, Iran) and maintained at 37°C in a humidified atmosphere (90%) containing 5% CO_2. Cells were cultured in RPMI-1640 with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For each concentration and time course study, there was a control sample that remained untreated and received an equal volume of the solvent.

Isolation and Culture of Human Fibroblasts

Periodontal fibroblast cells obtained from a healthy volunteer who was undergoing oral surgery (third molar extraction) only for dentistry reasons in the Clinic of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran. The tissue specimens were cut into small pieces and digested in phosphate-buffered saline containing collagenase (2 mg/ml) under shaking (60 cycles/min) at 37°C (23,24). After centrifugation, the pelleted cells were suspended in DMEM medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and seeded tissue culture flasks. All the assays were performed using fibroblasts between passages 4 and 10.

Cell Viability

The effects on inhibition of cell growth were measured by the MTS (3-(4, 5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium).
The MTS growth inhibition assay (25,26) was performed according to the instructions provided by the manufacturer (Promega, Madison, WI, USA). Briefly, the cells were seeded (10,000 cell/well) onto flat-bottomed 96-well culture plates on Day 1. On Day 2, the cells were treated with EtOH, *n*-hexane, and H_2O extracts and essential oil (15–500 μg/ml) over different incubation periods (24, 48, and 72 h) or remained as untreated controls and received equal amount of the solvent. At the end of each time point, fresh complete medium containing 10 μl of MTS solution was added and further incubated for 3 h. Optical density of each culture was then recorded at 490 nm using an ELISA reader. Each experiment was performed in triplicate and all the extracts were compared with Paclitaxel (0.7 μM) as a positive control. Results are expressed as the percentage growth inhibition with respect to the untreated cells.

Apoptosis: PI Staining

PI, a florescent dye capable of binding to DNA, is used for detection of apoptotic cells. Because of DNA fragmentation, apoptotic cells lose DNA fragments after the cells become permeable with triton-X 100 and therefore absorb less PI than the control unaffected cells. The low florescent intensity PI stained DNA fragment peak appears before G1 peak in the flowcytometry histogram (27). Briefly, HeLa cells were cultured overnight in a 24-well plate and treated with the extracts (EtOH, *n*-hexane, and essential oil) for 48 h. Floating and adherent cells were collected and incubated at 4°C overnight in the dark with 500 μl of a hypotonic buffer (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100). Flow cytometric analysis was done by using a FACScan flow cytometer (Becton Dickinson, California, USA). About 10,000 events were counted with FACS (27,28).

Western Blotting Analysis

HeLa cells were treated with 70 and 100 μg/ml of the EtOH and *n*-hexane extracts of *L. angustifolia* for 48 h. The cells were harvested and rinsed with ice-cold PBS. The cell pellet was resuspended in a lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% triton X-100, 1 mM EDTA, 0.2% SDS, 1% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride and then left on ice for 30 min. After centrifugation at 10,000 rpm for 20 min at 4°C, the cell lysate was collected and protein concentration was determined according to the BCA detection kit (29). Equal amounts of proteins were subjected to 12.5% SDS–PAGE (w/v). The proteins were transferred to a polyvinylidene fluoride membrane and subjected to immunoblotting using β-actin, PARP, and Bax antibodies as the primary antibodies and antirabbit IgG, HRP linked antibody, as secondary antibodies. PARP cleavage and Bax protein bands in HeLa cells were detected by enhanced chemiluminescence using the ECL Western blotting detection reagent. Images were quantified using the Gel-Pro Analyzer v. 6. 0 Gel Analysis Software (29).

Statistical Analysis

One-way analysis of variance and Bonferroni's posthoc were used for data analysis. All results were expressed as mean ± SEM, and P values below 0.05 were considered statistically significant.

RESULTS

Essential Oil Composition

The oil constituents of EtOH and *n*-hexane extracts obtained from aerial parts of *L. angustifolia* and its essential oil were

TABLE 1
Chemical composition and volatile components of essential oil, *n*-hexane, and ethanol extracts obtained by hydro-distillation from aerial parts of *L. angustifolia*

			%		
No.	Compound	RI*	Essential oil	*n*-Hexane extract	Ethanol extract
1	tricyclene	923	0.1	0.1	—
2	α-thujene	930	0.3	0.1	—
3	α-pinene	938	1.9	1.3	—
4	camphene	951	0.7	0.7	—
5	sabinene	974	1.3	0.4	—
6	β-pinene	978	2.5	0.8	—
7	1-octen-3-ol	984	0.1	0.7	—
8	2-3 dehydro-1-8,cineol	991	0.1	0.1	—
9	myrcene	995	1.7	0.1	—
10	n-decane	1000	—	6.0	—
11	α-phellandrene	1005	0.4	—	—
12	δ-3-carene	1012	2.9	0.2	—
13	α-terpinene	1019	0.1	0.2	—
14	*o*-cymene	1025	*t* **	1.8	*t*
15	β-phellandrene	1028	7.7	—	*t*
16	1-8-cineol	1031	18.8	23.8	*t*
17	*cis*-ocimene	1047	0.6	—	—
18	*trans*-ocimene	1056	0.1	—	—
19	γ-terpinene	1064	0.2	0.3	0.1
20	*cis*-sabinene hydrate	1071	0.8	0.3	—
21	*cis*-linalool oxide	1077	*t*	0.2	—
22	mentha-2-4(8)-diene	1088	0.2		—
23	terpinolene	1090	0.4		—
24	6-camphonene	1094	—	0.1	—
25	trans-sabinene hydrate	1098	0.2	*t*	—
26	undecane	1100	—	0.3	—
27	perillen	1102	0.1	—	—
28	linalool	1104	0.1	—	—
29	β-thujone	1117	0.1	0.1	—
30	*cis*-ρ-menth-2-en-1ol	1126	0.4	0.3	*t*
31	α-campholenal	1128	0.4	0.5	0.2
32	*cis*-limonene oxid	1136	0.2	—	0.1
33	nopinone	1138	—	0.4	—
34	trans-pinocarveol	1141	0.1	0.6	—
35	camphor	1150	6.5	11.2	0.1
36	pinocarvone	1164	0.2	0.5	—
37	borneol	1169	19.6	17.8	2.3
38	terpinen-4-ol	1175	*t*	0.8	—
39	cryptone	1193	3.7	5.3	0.6
40	α-terpineol	1198	1.9	1.2	0.3
41	myrtenol	1201	0.2	0.9	0.1
42	*trans*-piperitol	1213	0.5	—	*t*
43	*trans*-carveol	1225	0.6	0.9	*t*

(Continued on the next page)

TABLE 1
Chemical composition and volatile components of essential oil, *n*-hexane, and ethanol extracts obtained by hydro-distillation from aerial parts of *L. angustifolia (Continued)*

			%		
No.	Compound	RI*	Essential oil	*n*-Hexane extract	Ethanol extract
44	isobornyl formate	1231	0.6	1.6	—
45	*m*-cumenol	1235	—	0.3	0.2
46	cumine aldehyde	1245	1.6	2.2	0.4
47	carvone	1249	0.5	0.9	0.1
48	car-3-en-2-one	1255	0.1	0.2	—
49	unknown	1256	—	0.4	—
50	piperitone	1258	0.2	0.1	*t*
51	geraniol	1261	0.1	—	—
52	ρ-menth-1-en-7-al	1279	—	—	0.4
53	bornyl acetate	1287	0.2	0.4	—
54	ρ-cymene-7-ol	1294	0.9	1.2	0.5
55	tridecane	1300	—	0.1	—
56	carvacrol	1306	*t*	—	—
57	unknown	1336	—	0.7	—
58	eugenol	1360	*t*	—	—
59	neryl acetate	1369	*t*	_	—
60	geranyl acetate	1388	0.6	1.0	0.2
61	β-elemene	1392	*t*	—	—
62	*n*-tetradecane	1400	—	0.2	—
63	α-gurjunene	1408	0.3	—	—
64	α-cedrene	1411	—	0.1	—
65	β-caryophyllene	1418	1.0	0.2	0.2
66	α-santalene	1420	—	0.1	—
67	coumarine	1434	*t*	0.1	—
68	α-humulene	1452	0.1	*t*	—
69	allo-aromadenderene	1460	0.1	—	—
70	cis-cadina-1-(6)-diene	1462	0.1	—	—
71	α-acoradiene	1466	*t*	—	—
72	linalool isovalerate	1469	0.1	—	—
73	β-acoradiene	1473	*t*	—	—
74	germacrene-D	1480	0.1	—	—
75	β-E-ionone	1486	0.1	0.1	0.1
76	α-murrolene	1501	—	—	0.1
77	germacrene A	1508	—	—	0.1
78	δ-amorphene	1512	0.7	—	1.5
79	γ-cadinene	1515	1.2	2.5	1.1
80	trans-calamenene	1522	0.3	0.2	—
81	α-calacorene	1569	—	—	0.3
82	cis-muurol-5-e-4-β-ol	1556	0.1	—	—
83	palustrol	1566	0.2	0.2	0.4
84	germacrene-D-4-ol	1578	0.4	0.2	0.5
85	caryophyllene oxide	1584	2.2	3.4	4.2
86	viridiflorol	1601	0.3	0.4	1.1
87	1,10-di-epi-cubenol	1614	0.7	—	2.3

(Continued on the next page)

TABLE 1
Chemical composition and volatile components of essential oil, *n*-hexane, and ethanol extracts obtained by hydro-distillation from aerial parts of *L. angustifolia (Continued)*

No.	Compound	RI*	% Essential oil	% *n*-Hexane extract	% Ethanol extract
88	1-epi-cubenol	1631	—	—	0.3
89	epi-α-cadinol	1644	7.7	4.5	31.8
90	α-muurolol	1649	—	—	*t*
91	α-cadinol	1657	0.3	0.2	4.5
92	cadalene	1676	—	0.1	0.6
93	α-bisabolol	1685	—	—	4.2
94	cis-14-nor-muurol-5-en-4-one	1688	0.8	0.9	2.2
95	unknown	1694	2.5	—	—
96	nootkatol	1699	—	—	1.7
97	10-nor-calamenen-10-one	1710	—	—	0.7
98	cyclocolorenone	1745	—	—	1.9
99	tri-methyl 2-pentadecanone †	1846	—	—	1.2
100	unknown	1893	—	—	0.5
101	hexadecanoic acid ‡	1985	—	—	1.7
102	*z*-phytol #	2115	—	—	31.1
	Major grouped compounds				
	Monoterpene hydrocarbons		21.2	6.0	0.3
	Oxygenated monoterpenes		59.6	72.5	5.3
	Sespuiterpene hydrocarbons		3.9	3.2	3.9
	Oxygented sesquiterpenes		12.5	9.8	55.8
	Diterpenoides		—	—	31.1
	Miscellaneous compounds		0.2	7.3	2.9
	Total identified		97.4	98.9	99.3

*RI: The Kovats retention indices relative to C8-C20 *n*-alkanes were determined on CP-Sil 8CB capillary column. **t: trace <0.05%. †Trimethyl 2-pentadecanone was identified by comparison of its RI with that given in the literature: Magiatisa P, Skaltsounisa AL, Chinoua I, and Haroutounian SA: Chemical composition and in-vitro antimicrobial activity of the essential oils of three Greek *Achillea* species. *Z Naturforsch C* 57, 287–290, 2002. ‡Hexadecanoic acid was identified by comparison of its RI with that given in the literature: Priestap HA, Van Baren CM, Di Leo Lira P, Coussio JD, Bandoni AL: Volatile constituents of *Aristolochia argentina*. *Phytoche* 63 221–225, 2003. #z-phytol was identified by comparison of its RI with that given in the literature: Skaltsa HD, Demetzos C, Lazari D, Sokovic M: Essential oil analysis and antimicrobial activity of eight *Stachys* species from Greece. *Phytochem* 64, 43–52, 2003.

attained by hydro-distillation and analyzed by GC and GC-MS. Totally, 102 components were identified as volatiles of the EtOH and *n*-hexane extracts and essential oil, representing 99.3%, 98.9%, and 97.4% of the EtOH and *n*-hexane extracts and essential oil composition respectively (Table 1). The grouped contents of the EtOH and *n*-hexane extracts and essential oil were determined as monoterpene hydrocarbons (0.3%, 6.0%, and 21.2%), oxygenated monoterpenes (5.3%, 72.5%, and 59.6%), sesquiterpene hydrocarbons (3.9%, 3.2%, and 3.9%), oxygenated sesquiterpens (55.8%, 9.8%, and 12.5%), and the miscellaneous (31.1%, 0.0%, and 0.0%), respectively. The major oil components of the EtOH and *n*-hexane extracts and essential oil were β-phellandrene (t, 0.0% and 7.7%); 1-8-cineol (t, 23.8% and 18.8%); camphor (0.1%, 11.2%, and 6.5%); borneol (2.3%, 17.8%, and 19.6%); cryptone (0.6%, 5.3%, and 3.7%); epi-α-cadinol (31.8%, 4.5%, and 7.7%); z-phytol (31.1%, 0.0%, and 0.0%), respectively.

Cytotoxicity of Various Extracts of *L. angustifolia*

Malignant cells and human fibroblasts (nonmalignant control cells) were incubated with various concentrations of EtOH, *n*-hexane, and H_2O extracts and the essential oil of *L. angustifolia* (15–500 μg/ml) for 24, 48, and 72 h. The essential oil and EtOH and n-hexane but not H_2O extracts decreased cell

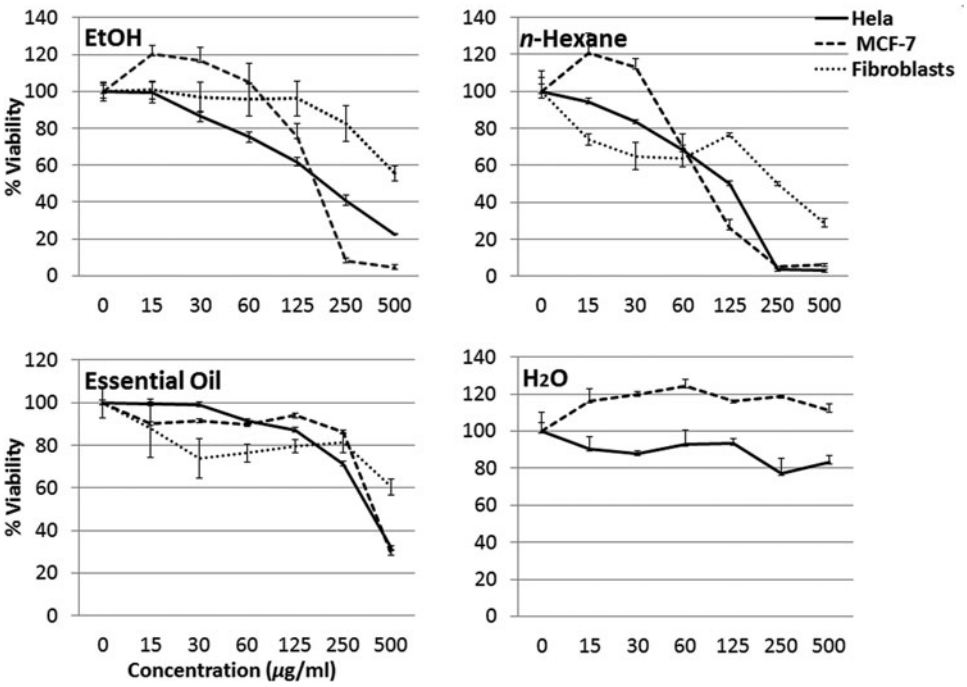

FIG. 2. Dose-dependent growth inhibition of malignant (MCF-7 and HeLa cells, respectively) and nonmalignant control cells (human fibroblasts) by the EtOH, *n*-hexane, and H_2O extracts and essential oil (15–500 μg/ml) after 48 h. Viability was quantitated by MTS assay. Control cells remained untreated and received an equal volume of the solvent.

viability in malignant cells, as a concentration and time-dependent manner (Fig. 2). This toxicity was consistent with morphologic changes including reduction in cell volume and rounding (Fig. 3). According to the MTS assay, incubation of EtOH and *n*-hexane extracts and the essential oil in the same range of concentrations produced less toxicity to human fibroblasts than tumor cells (Fig. 2). Together, these data point to the selective activity of EtOH and *n*-hexane extracts and the essential oil against tumor cells. Doses inducing 50% cell growth inhibition (IC_{50}) against HeLa and MCF-7 cells are presented in Table 2.

Paclitaxel (700 nM) was used as a positive control. Paclitaxel at this concentration decreased the viability of HeLa and MCF-7 cells to $14.4\% \pm 0.5$ and 4.4 ± 0.4 in comparison with untreated control received an equal volume of the solvent respectively (data not shown).

Role of Apoptosis: PI Staining

It has been reported that DNA fragmentation creates small fragments of DNA that can be eluted following incubation in a hypotonic phosphatecitrate buffer. When stained with a quantitative DNA-binding dye such as PI, cells that have lost DNA will take up less stain and will appear to the left of the G1 peak (28,30). Apoptosis following treatment with EtOH and *n*-hexane extracts and the essential oil of *L. angustifolia* was measured with PI staining to detect the sub-G1 peak resulting from DNA fragmentation. Sub-G1 peak as an indicative of apoptotic cells was induced in EtOH and *n*-hexane extracts and essential oil of *L. angustifolia* treated but not in control cells. EtOH and *n*-hexane extracts and the essential oil of *L. angustifolia*-treated cells exhibited a sub-G1 peak in HeLa cells in a concentration-dependent manner that indicates the involvement of an apoptotic process in EtOH and *n*-hexane extracts and the essential oil-induced cell death. (Fig. 4).

Effect of *L. angustifolia* on Poly (ADP Ribose) Polymerase Cleavage and Expression of Bax Protein

The cleavage of the 116 kDa PARP-1 to 89 kDa fragment was used as the sign of apoptosis. In HeLa cells, PARP-1 was cleaved to the 89 kDa fragment in treated cells with *n*-hexane and EtOH extract after 48 h (Fig. 5). The difference between treatments and control were statistically significant (P value $=$ 0.03).

Bax protein has an important role in induction of apoptosis via the mitochondrial pathway (31). The *n*-hexane and EtOH extract of *L. angustifolia* enhanced the expression of Bax protein in HeLa cells (Fig. 5). The difference between treatments and control were statistically significant (P value $=$ 0.008).

DISCUSSION

Given the growing incidence of cancer, natural compounds are considered more over the chemical counterparts to prevent and suppress tumor growth because of their beneficial effects on human health. Apoptosis is a very important mechanism of

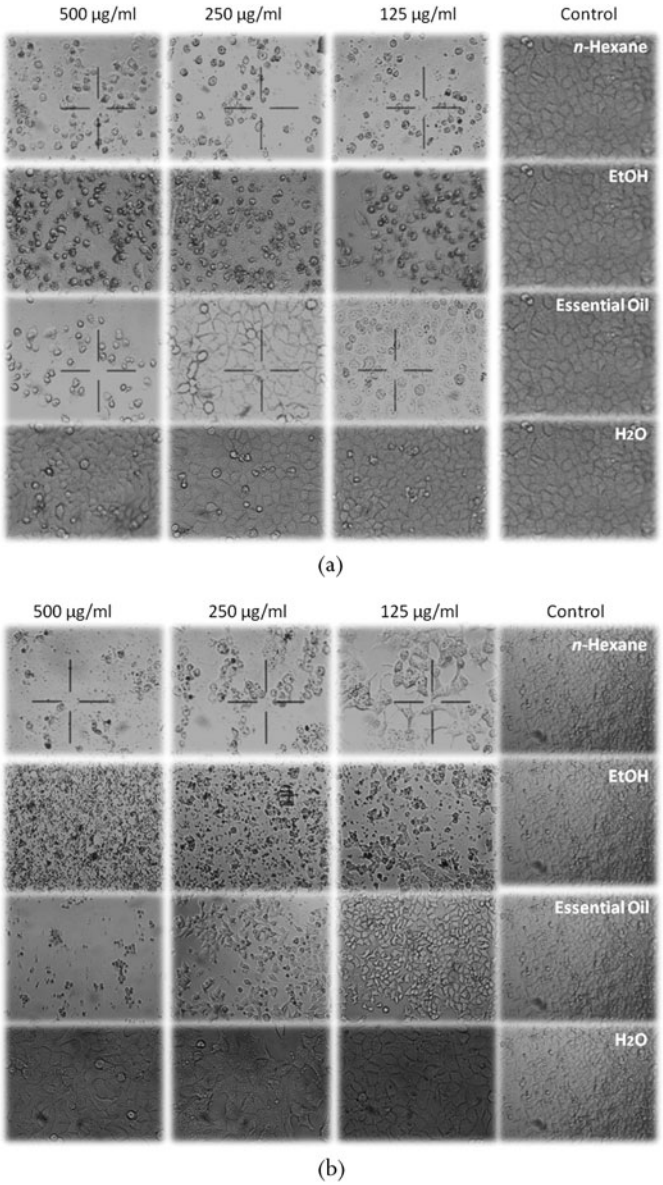

FIG. 3. a: Morphological changes of HeLa cells after treatment with EtOH, *n*-hexane, and H_2O extracts and essential oil of *L. angustifolia* for 48 h. Control cells remained untreated and received an equal volume of the solvent. b: Morphological changes of MCF-7 cells after treatment with EtOH, *n*-hexane, and H_2O extracts and essential oil of *L. angustifolia* for 48 h. Control cells remained untreated and received an equal volume of the solvent.

cell death in cancer treatment. Therefore, natural products that can activate apoptosis signaling pathways attract a lot of interest (32).

In this study for the first time a thorough comparison of the cytotoxic and proapoptotic properties of 3 different extracts (EtOH, *n*-hexane, and H_2O) obtained from *L. angustifolia* and its essential oil was done on 2 cancer cell lines including human cervix carcinoma cell line (HeLa) and human breast cancer cell line (MCF-7) and human fibroblasts as nonmalignant cells. Our data verified that *L. angustifolia* extracts and the essential oil have cytotoxic activity against HeLa and MCF-7 cell lines. We showed that the *n*-hexane and EtOH extracts of *L. angustifolia* had strong antiproliferative effects against both MCF-7 and HeLa cell lines while the activity of H_2O extract and leaf oil were considerably less, which can be attributed to the different composition of extracts and essential oil. The constituents of the essential oil present in EtOH and *n*-hexane extracts obtained from *L. angustifolia* and its essential oil and their relative amount are listed in Table 1. The oil components identified in the EtOH and *n*-hexane extracts and essential oils obtained from *L. angustifolia* were different from each other (33). In comparison, the EtOH extract oil was rich in epi-α-cadinol (31.8%) and *z*-phytol (31.1%), whereas the major oil components of the *n*-hexane extracts were 1-8-cineol (23.8%), camphor (11.2%), borneol (17.8%), and cryptone (5.3%), and essential oil mainly contained β-phellandrene (7.7%), 1-8-cineol (18.8%), camphor (6.5%), borneol (19.6%), cryptone (3.7%), and epi-α-cadinol (7.7%).

It should be noted that the low concentrations (less than 60 μg/ml) of 3 different extracts (EtOH, *n*-hexane, and H_2O) obtained from *L. angustifolia* increased the viability of MCF-7 cells. Interestingly this finding is in accordance with published review by Henley et al. in 2010. It has been shown that

TABLE 2
Doses of solvent extracts and essential oil of *L. angustifolia* inducing 50% cell growth inhibition (IC_{50}) against malignant (MCF-7 and HeLa cells) and nonmalignant control cells (human fibroblasts)

		IC_{50} μg/ml		
Time	Plant materials	HeLa	MCF-7	Human fibroblasts
24 h	Essential oil	290	451	>500
	n-Hexane extract	102	85.68	>500
	EtOH extract	301	179.6	>500
	H_2O extract	>500	>500	>500
48 h	Essential oil	359.7	397	>500
	n-Hexane extract	96.73	86.61	>500
	EtOH extract	177.8	155.2	>500
	H_2O extract	>500	>500	>500
72 h	Essential oil	31.92	185	>500
	n-Hexane extract	30.34	81.34	>500
	EtOH extract	29.47	82.86	>500
	H_2O extract	>500	>500	>500

Malignant and nonmalignant cells were incubated with various concentrations of EtOH, *n*-hexane, and H_2O extracts and the essential oil of *L. angustifolia* (15-500 μg/ml) for 24, 48, and 72 h. For each concentration and time course study, there was a control sample that remained untreated and received an equal volume of the solvent. Data are representative of 3 independent experiments. *P* values given as compared to untreated control cells.

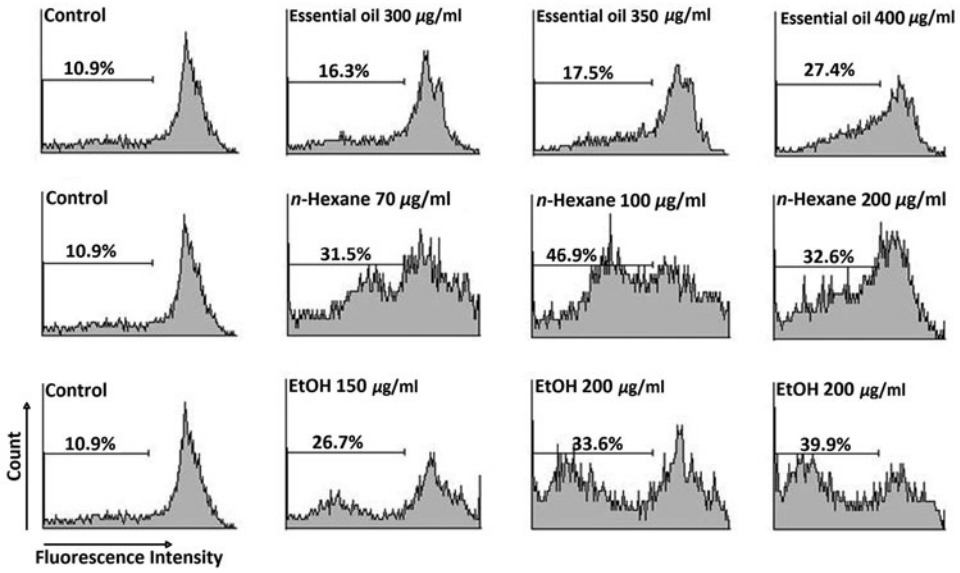

FIG. 4. Flow cytometry histograms of apoptosis assays by propidium iodide (PI) method in HeLa cell. Cells were treated with different concentration of EtOH and *n*-hexane extracts and the essential oil of *L. angustifolia* for 48 h. Sub-G1 peak as an indicative of apoptotic cells was induced in EtOH and *n*-hexane extracts and essential oil of *L. angustifolia* treated but not in control cells. Data are representative of 3 independent experiments.

lavender oil modulates the endogenous expression of the estrogen-responsive genes in MCF-7 cells and possesses estrogenic and antiandrogenic properties (34).

Aromatic plants have a long history of medicinal uses from skin treatments to remedies for cancer. Volatile oil fractions (essential oils) have been postulated as the active components of the aromatic plants (35).

Because of the great number of constituents present in essential oils, they affect multiple cellular targets. Apoptosis induction and necrosis have been proposed as the main causes of essential oil cytotoxicity in mammalian cells. Thyme, clove, cinnamon, carvacrol, thymol, and eugenol were found to possess cytotoxic effects in Caco-2 cells (36,37).

In this study strong antiproliferative effects against both MCF-7 and HeLa cell lines at least partly may related to the presence of epi-α-cadinol, z-phytol, 1-8-cineol, camphor, borneol, and cryptone as the major components of the extracts (EtOH and *n*-hexane) obtained from *L. angustifolia* and its essential oil.

The apoptosis was involved in this cytotoxicity in the HeLa cells. Apoptosis or programmed cell death can be disrupted by the occurrence of a gene mutation and the defect in the apoptosis pathway, playing a role in the incidence of some human diseases such as malignancies (38). The reduction of mitochondrial membrane potential, externalization of phosphatidyle-serine to the outer membranes layer of the mitochondria, producing reactive oxygen species, decreasing intracellular pH, and selective degradation of proteins and DNA damage are the biochemical hallmarks of apoptosis (38). The sub-G1 peak in the flow cytometry histogram of treated cells verified the apoptosis induction by EtOH and *n*-hexane extracts of *L. angustifolia*. These data suggest that the cytotoxicy of *L. angustifolia* is at least partially due to apoptosis induction.

Balance between pro- and antiapoptotic Bcl-2 proteins determines the sensitivity of cells to apoptosis; this balance is disturbed by stress and cell damage (39). Therefore, increasing levels of proapoptotic proteins such as Bid, Bax, and Bak and decreasing levels of antiapoptotic proteins like Bcl-2 and Bcl-xL play an important role (40). Activation of caspase-9 is a sign of activation of the intrinsic mitochondrion-dependent pathway in response to increased levels of Bax (24). EtOH and *n*-hexane extracts increased levels of Bax and the cleaved form of PARP (89 kDa) as shown in Fig. 5. PARP has an important role in repairing single-stranded DNA and is activated in response to the damage caused by radiation, chemicals, and so on. On the other hand, in the apoptotic process PARP is inactivated by caspase cleavage.

In vitro studies on the anticancer effects of *L. angustifolia* essential oils indicate that the essential oil of the plant contains compounds in trace amounts that have significant anticancer activity (6). But in this study, obtained data confirmed that cytotoxic and proapoptotic activities of EtOH and *n*-hexane extracts are much stronger than the essential oil. It can be concluded that in these extracts, especially *n*-hexane extract, cytotoxic compounds have higher levels or are more potent than those in the essential oil or both.

The cytotoxic and apoptotic activity of EtOAc and *n*-hexane extracts were higher than that of the H_2O extract. The extraction of *L. angustifolia* with *n*-hexane and EtOAc extract generated fractions that were cytotoxic for tumor cells. These findings suggest that EtOAc and *n*-hexane extracts are more efficacious than H_2O extraction and cells are more resistant to H_2O extract. This

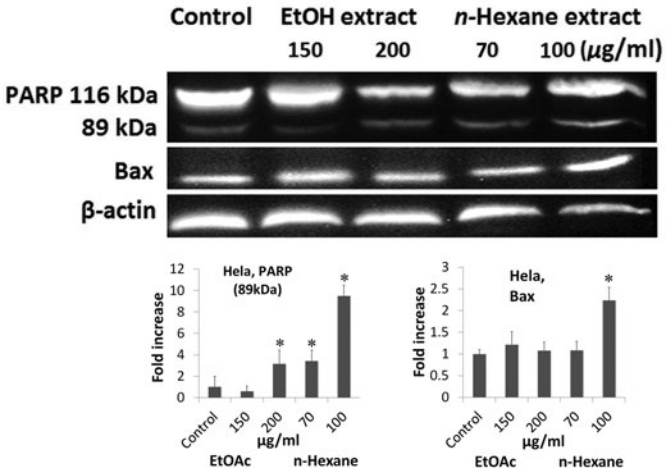

FIG. 5. Western blotting analysis of Bax, and poly (ADP ribose) polymerase (PARP) of the HeLa cells treated with 150 and 200 μg/ml of EtOH extract (150, 200) and 70 and 100 μg/ml of *n*-hexane extract (70, 100) of *L. angustifolia* for 48 h. Results are mean ± SEM ($n = 3$). $^*P < 0.05$ compared to control.

can be concluded that the low polar compounds of the extracts in general and volatile oils in particular are cytotoxic/apoptotic to cells.

Our study has shown that EtOH and *n*-hexane extracts and the essential oil of *L. angustifolia* inhibited the cell proliferation of HeLa and MCF-7 cells in vitro. In addition, our results have clearly shown that EtOH and *n*-hexane extracts inhibit HeLa cell growth via inducing apoptosis. More studies are needed in the future to fully identify the active phytochemicals and the mechanisms involved in cell death induced by *L. angustifolia*.

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