http://www.ijwhr.net
doi 10.15296/ijwhr.2023.8007
Open Access Original Article
IJWHR
International Journal of Women's Health and Reproduction Sciences
Vol. 12, No. 3, July 2024, 125–135
ISSN 2330-4456

Antitumor Effect of *Lavandula angustifolia* Extracts on the MDA-MB-231 Breast Cancer Cell Line, With Emphasis on the Inhibition of Histone Deacetylase: In Vitro Evaluation and Molecular Docking Studies

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Abstract

Objectives: Complementary medicine for the treatment of breast cancer has received a considerable research attention over the past years. Studies have also shown the anti-proliferative properties of *Lavandula angustifolia*. This study aimed to investigate the likely anti-proliferative effects of n-hexane, ethyl acetate, and methanol extracted from *L. angustifolia* on a cell line of the breast cancer.

Materials and Methods: The MDA-MB-231 cell line was treated with different concentrations of three extracts from *L. angustifolia*. MTT assay was performed to evaluate cell viability, and flow cytometry was used to examine the cell cycle. The potential of the breast cancer cell line to form the colonies after a treatment with the plant extract was also investigated. The matrix metalloproteinase 2 and 9 (MMP2 and 9) were measured by quantitative real-time polymerase chain reaction (qPCR). Molecular docking was performed using AutoDock Vina in PyRx0.8 to investigate the likely target protein.

Results: The results showed that the methanol extract had considerable cytotoxicity to MDA-MB-231. The G0/G1 phase arrest was significantly increased in MDA-MB-231 after treatment with methanol extract. Furthermore, MMP2 and 9 significantly down-regulated the post treatment with the plant extract. The docking energy (kcal.mol^{-1}) of eight methanolic extract compounds was docked with the histone deacetylase (HDAC), and nine transcription factors were achieved.

Conclusions: In sum, a cytotoxic property was determined for *L. angustifolia* methanol extract on the MDA-MB-231. According to the results from docking studies, the best compound among the investigated compounds was 2-furancarboxaldehyde, which was docked with HDAC with a –7.37 kcal.mol^{-1} docking score. Therefore, it was recommended that future studies should be carried out to further investigate *L. angustifolia* as a possible complementary treatment.

Keywords: Antitumor activity, *Lavandula angustifolia*, Breast cancer cell line, Docking study, MDA-MB-231

Introduction

Breast cancer is the most frequent cancer in women, accounting for 627 000 cancer deaths globally in 2018 (1). In Iran, the age-standardized prevalence rate of the breast cancer is 23.1 per 100 000 (2-4). Chemotherapy, surgical treatment, radiation, hormonotherapy, and targeted therapy are the most commonly used treatment options for breast cancer (5-9). However, therapy approaches have often resulted in immediate and long-term negative effects such as premature menopause/infertility, heart failure, DNA damage, leukemia, and cognitive impairment (5,10).

MMPs, also termed as matrix metallopeptidases or matrixins, are zinc-containing endopeptidases that are calcium-dependent metalloproteinases (11). One of the most important effects of MMPs (matrix metalloproteinase-2) in tumor progression is their role in extracellular matrix (ECM) disintegration, which allows the cancer cells to spread outside of the primary tumor and form metastases. MMP-2 and MMP-9 are particularly capable of destroying type IV collagen, the most common component of the basement membrane. The basement membrane is important for forming tissue structure, cell structural support, as well as cell signaling and polarity. The disintegration of the basement membrane is a necessary stage in the metastatic spread of most malignancies (12). In humans, the MMP2 gene encodes the 72 kDa collagenase (type IV), which is also known as MMP-2 and gelatinase A (13). MMP-9 – also known as type IV collagenase (92 kDa), gelatinase B (GELB) or 92 kDa gelatinase – is a class of zinc-metalloproteinase family implicated in the destruction of extracellular matrix (11). Several transcription factors like nuclear factor kappa B (NF-κB), cAMP response element-binding protein (CREB), C-FOS:C-JUN, CCAAT enhancer-binding protein (CEBP), specificity protein 1 F (SP1F), mitogen-activated protein kinase (MAPK), polyomavirus enhancer

Received 9 August 2023, **Accepted** 30 October 2023, **Available online** 12 January 2024

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Key Messages

- We demonstrated for the first time that the methanol extract of *L. angustifolia* suppresses the growth of a breast cancer cell line.
- Methanol extract of *L. angustifolia* suppresses the growth of a breast cancer cell line. Our findings confirmed that *L. angustifolia* MetOH extract reduced MMP2 and MMP9 expression in the MDA-MB-231 cell line.
- According to the docking studies, among the investigated compounds, the main component of the methanol extract is 2-Furancarboxaldehyde which is docked with HDAC with most docking energy and therefore is the main potent agent for reducing the expression of MMP2 and MMP9.

activator 3 (PEA3), SMAD, signal transducer and activator of transcription 3 (STAT3), and histone deacetylase (HDAC) and others are involved in the regulation of MMP gene expression (14,15).

According to the recent research, supplementary drugs (e.g., natural items) can facilitate treatment of the cancer patients while having few adverse effects (16-18). Controlling cancer cell proliferation, lowering the risk of tumor growth, and enhancing cancer elimination have motivated many researchers to look for new therapy approaches (19). Many of the natural chemical components found in herbs and purified secondary metabolites are utilized as nutraceuticals or dietary supplements. Herbal medicine may offer superior therapeutic techniques due to the improved effectiveness, lesser toxicity, and cheaper treatment costs (19).

Lavandula angustifolia (Lavender) is a member of the mint family and is endemic to the western Mediterranean. The oil extract of *L. angustifolia* flower and leaves has long been used as an herbal treatment in traditional medicine (20). Recently, the cytotoxic characteristics of *L. angustifolia* extracts, as well as the antibacterial, antioxidant, and anticancer activity of these extracts have received a considerable research attention (21-23). Furthermore, *L. angustifolia* extracts have shown therapeutic effects against the rheumatic illnesses (24), neurological disorders (25), and gastrointestinal diseases (26). *L. angustifolia* aqueous extract has also been reported to show anti-tumorigenic activities in prostate cancer (17), MCF-7, and HeLa cell lines (27). The medicinal activities of *L. angustifolia* are derived mostly from linalool (26% to 49%) and linalyl acetate (17.6% to 53%) in aqueous extract (28). The major elements of the ethanol (EtOH) extract are 1-8-cineol, epi-cadinol, and z-phytol, whereas the n-hexane extract mostly includes 1-8-cineol, camphor, and borneol (27). Furthermore, the essential oil of *L. angustifolia* contains a blend of 1-8-cineol and borneol (26). Contrary to earlier observations concerning *L. angustifolia* cytotoxic effects, the recent study results about the given effects are completely contradictory. Several investigations (17,20-22) have shown that aqueous extracts have anti-proliferation properties, although others have failed to record the same findings (27).

The cytotoxicity effects of *L. angustifolia* on breast cancer cells have been suggested in traditional medicine; however, these effects have rarely been reported by scientific sources. The present study, therefore, aimed to investigate the cytotoxic and antiproliferative effects of n-hexane, ethyl acetate, and methanolic extracts of the native *L. angustifolia* collected from Kurdistan province on MDA-MB-231 breast cancer cell line. Aside from the in vitro experiment, a computational examination was carried out to estimate the possible target protein(s). To this end, the 3D structures of identified compounds were docked with targets to determine the likely mechanism of inhibition of MMP2 and MMP9 production.

Materials and Methods

Preparation of *Lavandula angustifolia* Extracts

The aerial parts of *L. angustifolia* were air-dried for a week and then ground into powder. Separate extracts of *L. angustifolia* were prepared by dissolving 50 g of the powdered samples in 500 mL of n-hexane, ethyl acetate, and methanol (MetOH). Afterward, each extract was filtered for preparing the crude extracts, and the achieved solutions were evaporated under decreased pressure at 40 °C. Finally, the extracts were filtered again and allowed to evaporate in order for removing any leftover solvents before weighing the raw extracts.

Gas-Chromatography/Mass Spectrometry Analysis of Components in *Lavandula angustifolia* Extracts

Different extracts of L. angustifolia were analyzed using an Agilent 7890B/5977A GC/MSD instrument (Agilent, USA). A HP-5ms column (30m, 0.25mm, 0.25m) is connected to a quadruple mass detector in the Gas-Chromatography/Mass Spectrometry (GC/MS) equipment. The data was acquired and processed by a machine with the Wiley 7 n.l library installed. The various *L. angustifolia* extracts were evaluated using Agilent 7890B/5977A GC/MSD equipment (Agilent, USA). An HP-5ms column (30m, 0.25mm, 0.25m) interfaced with a quadruple mass detector is part of the GC/MS system. A computer system with the Wiley 7 n.l library was used to collect and process the data. The separation and analysis conditions for chromatography and mass spectrometry were as follows: oven temperatures: 60 °C (1 min), 60 °C–250 °C (5 °C/min), 250 °C (38 min); volume injection: 0.1 µL; injector temperature: 250 °C; ratio of split: 1:50; carrier gas Helium at 1 mL/min; ionization current: 150 µA; ionization potential: 70 eV; mass range: 35–465 mui; ion source temperature: 250 °C. To identify distinct chemicals, the retention indices (RI) and mass spectra of each component were compared with the verified samples and existing literature (27). The corresponding quantities for each chemical were obtained by calculating the area percentage.

Sample Preparation

Extract stock solutions were made by combining 0.1 mg of each crud extract with 1mL of the same solvent. Next, the stock solutions were diluted with Roswell Park Memorial Institute-1640 to make the concentrations of 15–500 g/mL (RPMI-1640). The treatment solutions were freshly prepared prior to initiation of the study.

Cell Culture

An MDA-MB-231 breast cancer cell line was obtained from the ICGEB (International Center for Genetic Engineering and Biotechnology) (Tehran, Iran). MDA-MB-231 cell line was cultured in RPMI-1640 culture media (Invitrogen, Carlsbad, CA) with 10% FBS (Gibco BRL), and incubation was carried out at 5% CO2 and 37 °C (29,30).

Proliferation Assay

To obtain the extract with the maximum cytotoxicity, the cell viability was first tested using a MTT assay kit (3–2, 5-diphenyl tetrazolium bromide) (Sigma, St Louis, MO) adopting the producer's method. In summary, the breast cancer cell line was planted on a 96-well plate at a density of 5000 cells per well and treated with n-hexane, Ethyl acetate, and MetOH extracts (15-500 g/mL) through varied incubation times or kept untreated and given an equivalent quantity of the solvent for three days. As negative controls, the solvent treatment and an experiment group receiving no treatment were employed. The absorbance of each experiment groups was then measured photometrically at 450 nM. Our study procedure was continued using the MetOH extract since it was the only extract capable of reducing the cell growth.

Analysis of MMP 2 and 9 Gene Expression by Quantitative Real-Time PCR

Total RNA was extracted from cells using the RNXTM-Plus kit (Cinnagen, Tehran, Iran) and following the described protocol. The RevertAid First Strand cDNA Synthesis Kit (Ferments – Thermo Scientific) was used to make the cDNA. The MMp-2 and MMP-9 expression levels were determined by specific primers (MMP2-F: GGCGGCGGTCACAGCTACTTC and MMP2-R: CGAAGGCAGTGGAGAGGAAG) by the SYBR Premix Ex Taq II kit (Takara, Japan). As for MMP-9, CGGTTTGGAAACGCAGATGG was used as a forward primer and GGTGTAGAGTCTCTCGCTGG was employed as a reverse one. The qRT-PCR experiment was then conducted using Rotor-Gene 6000 equipment (Corbett Research, Mortlake, NSW, Australia), and the expression data were normalized to the level of HPRT expression and presented as r.u (i.e., relative unit).

Cell Cycle Analysis

Cell line MBA-MD-231 was cultivated in 6-well plates and tested using the treatment medium after they achieved 80%-90% confluent. MetOH extract (500 g/mL) was applied to the cells and allowed to incubate for 72 hours. The same method was followed for control groups. After that, all experiment groups were collected and fixed for 1 hour at 4 °C in 70% ethanol. Propidium iodide (PI) was then applied to the fixed cells for 1 hour at room temperature (Sigma-Aldrich). A BD FACSCalibur flow cytometer was employed to analyze the cell cycle (BD Biosciences, Mountain View, CA). Then 20 000 events were recorded in each evaluation and the FlowJo program (TreeStar, Inc., San Carlos, California, USA) was used to calculate the cell rate in various stages of the cell cycle.

Colony Formation Assay

In a 6-well plate with 2 mL media, the MDA-MB-231 cell line was cultivated with a cell density of 500 per well. The experiment groups were subsequently treated with MetOH extract (500 µg/mL) at 5% CO2 and 37 °C for 14 days. Afterward, PBS was used to wash the cells and a 3:1 Methanol: Acetic Acid solvent was used for fixation for 40 minutes. The 0.04% crystal violet was used to stain the fixed cells (Merck, CI 42555). Ultimately, the stained colonies were rinsed with PBS before enumeration.

Statistical Analysis

The data were analyzed using SPSS 16 (SPSS Inc., Chicago, USA) and reported as the means ± standard deviations of three separate trials, and $P<0.05$ was regarded as statistically significant. The Student's *t* test is performed when there are two independent groups, and one-way ANOVA is used to determine the significance of the difference between two groups. If the one-way ANOVA findings were significant, the Tukey HSD test was then employed.

Molecular Docking Study

The transcription factors and HDAC crystal structures were obtained from the RCSB (https://www.rcsb.org/). The three-dimensional structure of eight active compounds was retrieved in SDF format from the PubChem website. The Open Babel program was used to convert SDF molecules into mol2 format. Then each of the nine transcription factors and HDAC were docked with each one of the eight ligands. In the present study, molecular docking was performed using AutoDock Vina in PyRx0.8, a significant tool for computer-aided drug development and an open-source application (31). The unified atom scoring function, which only examines heavy atoms, is used by AutoDock Vina. A plain text editor was used to erase all water molecules and, then, Gasteiger algorithm charges were used to add missing hydrogen atoms (32). Polar hydrogen atoms were also added to the proteins. Ligands were loaded into the Open Babel program, and energy was minimized. AutoDock Vina was used to convert ligands and proteins into pdbqt format. As cubic angstroms, each protein binding site was identified as a

search box. The docking operation was completed in the next stage.

Results

Chemical composition of *Lavandula angustifolia* Extracts

A GC/MS technique was implemented to investigate the principal ingredients of the n-hexane, Ethyl acetate, and MetOH extracts of *L. angustifolia*. The GC/MS analysis revealed that the extracts of n-hexane, Ethyl acetate, and MetOH contained a total of 61 compounds (Table 1). Coumarin (59.44%), Tricosane (15.22%), 7-methoxy coumarin (12.69%), and 2-furancarboxaldehyde (6.7%) were the main components of the MetOH extract. The main constituents of the n-hexane extract were 1,8 cineol (33.7%), decane (25.1%), borneol (8.56%), L-camphor (6.36%), and dodecane (4.92 %). Finally, the primary components of ethyl acetate extract were 1,8 cineol (52.79%) and α-pinene (27.05%).

Effects of the n-Hexane, Ethyl acetate and MetOH Extracts of *Lavandula angustifolia* on Proliferation of MDA-MB-231

The cell line of breast cancer was treated with varied doses of MetOH, n-Hexane, and ethyl acetate extracts of *L. angustifolia* (15–500 µg/mL) for 72 hours. The morphology of MDA-MB-231 is shown in Figures 1a-d, before and after the treatment with *L. angustifolia* extracts.

The viability of the MDA-MB-231 cell line was dramatically reduced after a treatment with the MetOH extract (Figure 2c), but the n-hexane and ethyl acetate extracts of *L. angustifolia* had no significant cytotoxic effect on the multiplication of the malignant cell line (Figure 2b-c).

According to our study findings, none of the investigated solvents had any harmful effects in the same concentration range. The 50% inhibitory concentration (IC50) was then measured. According to our findings, the IC50 for *L.*

Table 1. Chemical and Volatile Components of n-Hexane, Methanol, and Ethyl Acetate Extracts from Aeri-al Parts of *L. angustifolia*

Compound	Retention Time	n-Hexane	Ethyl acetate	Methanol
α-Phellandrene	4.118		0.01	
α-Pinene	4.385	0.44	27.05	
Camphene	4.501		0.26	
Octane, 4-ethyl	4.662	0.66		
Sabinene	4.907		0.07	
β-Pinene	5.134	0.14	0.52	
Myrcene	5.174		0.45	
Decane	5.575	25.1	0.11	
1-Decene	5.678	0.28		
Delta-3-carene	5.593		6.71	
α-Terpinene	5.705		0.15	
l-Limonene	5.965		3.94	
o-Cymene	6.034	0.50	2.13	
1,8-Cineol	6.257	33.70	52.79	0.54
Lavender lactone	6.367	0.22		
Gamma-terpinene	6.547		1.94	
Linalool oxide	7.031	2.53	0.09	
Terpinolene	7.160		0.86	
Transe-linalool oxide	7.169	2.01		
Linalool	7.383		0.11	
Butanoic acid	7.482		0.13	
Linalool L	7.616	5.24		
Sabinol	8.304		0.16	
L-camphor	8.662	6.36	0.27	
Pinocarvone	9.004	0.12		
Levomenthol	9.026		0.10	
Terpinen-4-ol	9.142		0.37	
Borneol L	9.172	8.69	0.35	1.05
Terpinene-4-ol	9.343	0.30		
Linalyl propanoate	9.435		0.39	
Dodecane	9.786	4.92	0.15	
Transe-(+)-Carveol	10.245	0.15		
(.+-.) 2-exo-Hydroxycineole	10.352	0.30		
Isobornyl acetate	10.462	0.90		
2-Furancarboxaldehyde	10.710			6.80

Table 1. Continued

Compound	Retention Time	n-Hexane	Ethyl acetate	Methanol
Benzaldehyde, 4-(1-methylethyl	10.717	0.40		
Carvone	10.798	0.18	0.05	
Linalyl acetate	10.990	0.82		
Carvacrol	11.661		0.07	
(-)-Bornyl acetate	11.718	0.26		
p-Cymen-7-ol	11.833	0.31		
3-Carene	12.321	0.14		
Thiophene, 2,4-dimethyl	12.515	0.46		
3-Octanol	12.843	0.18		
Tridecane, 3-methyl	13.443	0.19		
Tetradecane	14.110	1.81	0.09	
Valencene 1	14.884		0.11	
Coumarin	15.197			59.44
Gamma-cadinene	16.565	0.14		
Caryophyllene oxide	17.985	0.49		
Hexadecane	18.116	0.51		
β-Cubebene	19.046	0.30		
7-Methoxy Coumarin	20.678			12.70
Octadecane	21.759	0.16		
Palmitic acid	24.520			1.71
Hexadecanoic acid	24.578	0.27		
Heptacosane	34.910	0.15		
Tricosane	35.888			15.22
Vitamin E	36.689			2.55
Squalene	37.210	0.42		

angustifolia MetOH extract was 500 µg/mL for the MDA-MB-231 cell line (Figure 3).

Lavandula angustifolia Methanol Extract Inhibits Cell Cycle in Breast Cancer Cell Lines

The inhibitory effect of the MetOH extract from *L. angustifolia* on the MDA-MB-231 was also investigated, and it was found that G0/G1 phase arrest was considerably enhanced following a treatment with MetOH extract, whereas S phase increased in the negative control groups (Figure 4).

In addition, ANOVA analysis revealed that a 500 mg dosage of the given extract substantially increased the cell cycle arrest in the G0/G1 phase in breast cancer cell line compared to the control group ($P=0.002$).

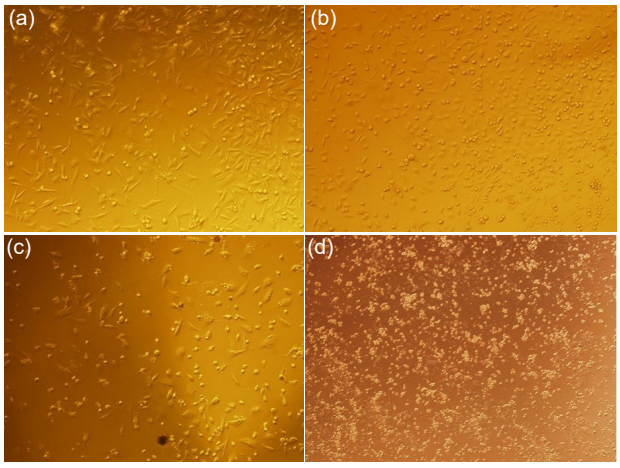


Figure 1. Morphologic Characteristics of MDA-MB-231 Cell Line of Breast Cancer. (a) MDA-MB-231 before treatment; (b) MDA-MB-231 after treatment with n-Hexane extract; (c) MDA-MB-231 after treatment with ethyl acetate extract and finally (d) MDA-MB-231 after treatment with methanol extract (Magnification, ×200).

Colony Formation Ability

The capacity of the breast cancer cell line to form colonies was examined after a treatment with the MetOH extract (500 µg/mL), and it was detected that the growth of colonies in MDA-MB-231 cell line was considerably reduced after a treatment with *L. angustifolia* methanol extract in comparison with the control group (Figure 5).

Down-regulation of MMP-2 and MMP-9 After Treatment With the MetOH of *L. angustifolia*

The breast cancer cell line was treated with MetOH extract of *L. angustifolia* for further evaluation, and MMP-2 and MMP-9 gene expression were evaluated. After a treatment with the MetOH extract, MMP-2 and MMP-9 expression were dramatically reduced in the cell line. However, our findings demonstrated that negative control groups had no effects on MMP-2 and MMP-9 expressions (Figure 6).

Docking Study

Virtual screening studies are presently an undeniable

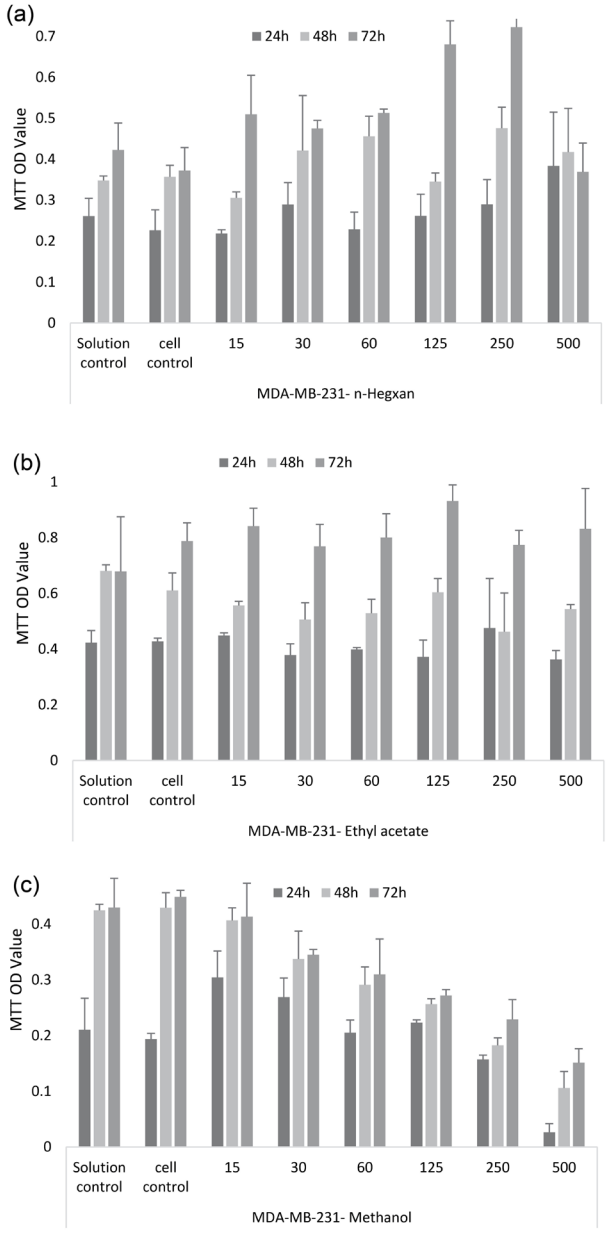


Figure 2. Proliferation Rate of the Breast Cancer Cells Analyzed by MTT Assay. The MTT assay revealed the lack of inhibition in MDA-MB-231 cell line proliferation treatment with n-Hexan (A) and ethyl acetate (B) extracts of *L. angustifolia*. C) the proliferation of MDA-MB-231 cell line was significantly inhibited following a treatment with methanol extract of *L. angustifolia* compared to controls (concentrations are expressed as µg/mL).

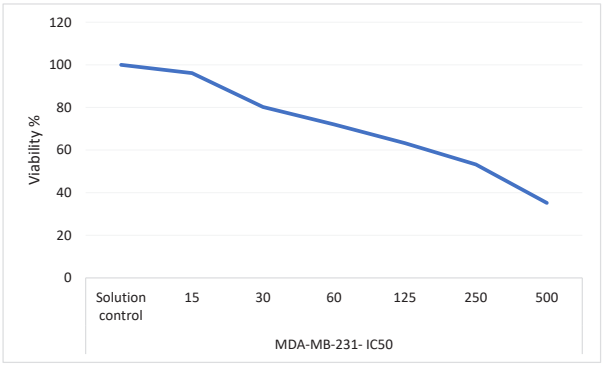


Figure 3. IC50 Values (µg/mL) of Methanol Extract of *L. angustifolia* for MDA-MB-231. The IC50 values of MDA-MB-231 cells for methanol extract of *L. angustifolia* were significantly decreased compared with non-treated control cells.

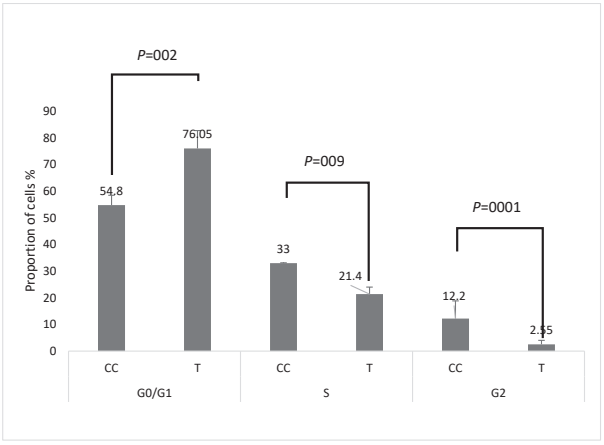


Figure 4. Methanol Extract of *L. angustifolia* Restricted Cell Proliferation in MDA-MB-231 by Inhibiting the G0/G1 Cycle Phase Transition to S Compared to Cell Control (CC).

aspect of the medication discovery procedure worldwide (33). Docking is a method of screening vast databases for possible chemicals that can bind to a target protein (active site) (34). One of the most effective methods for assessing the binding affinity is molecular docking. In this study, AutoDock software was utilized to find a powerful compound among the structures found in the Methanolic extract. In a docking study, a greater negative value is indicative of a stronger protein-ligand binding. The best compounds were docked with HDAC (PDB ID:4A69), NF-κB (PDB ID:1SVC), p38 MAPK (PDB ID:1CM8), SMAD3 (PDB ID:1Mk2), STAT3 (PDB ID:6NUQ), PEA3 (PDB ID:4CO8), CREB (PDB ID:1DH3), C-FOS:C-JUN (PDB ID:1FOS), CEBP (PDB ID:1GTW) and SP1F (PDB ID:1Sp1), were 2-furancarboxaldehyde (-7.37 kcal.mol^{-1}), 7-methoxy Coumarin (-6.51 kcal.mol^{-1}), borneol-L (-6.24 kcal.mol^{-1}), coumarin (-5.74 kcal.mol^{-1}), 1,8-cineol (-5.39 kcal.mol^{-1}), 7-methoxy coumarin (-4.78 kcal.mol^{-1}), 7-methoxy coumarin (-4.77 kcal.mol^{-1}), 7-methoxy coumarin (-4.49 kcal.mol^{-1}), 2-furancarboxaldehyde (-4.29 kcal.mol^{-1}), and 2-furancarboxaldehyde (-3.88 kcal.mol^{-1}), respectively (Table 2).

Table 3 presents the interacting residues of nine transcription factors and HDAC with the best docked ligands and two-dimensional structures of ligands.

The package (LigPlot+) was applied in Figure 7 in order to estimate the interactions between the ligands and protein's active site.

Discussion

The application of herbal medicine, as a complementary medicine to improve the responsiveness of cancer

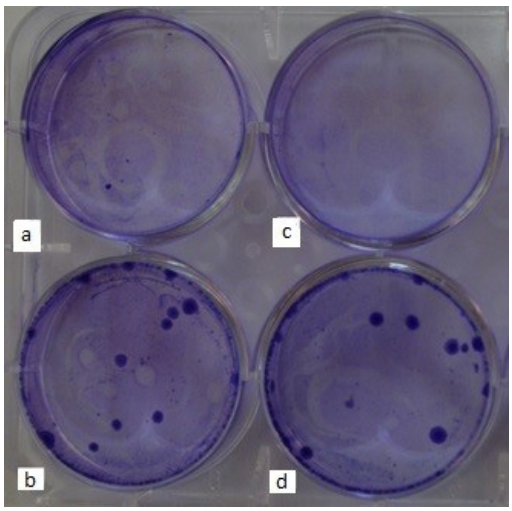


Figure 5. MDA-MB-231 Colonies Formed as Determined by Colony Formation Assay. Treatment with methanol extract of *L. angustifolia* significantly suppressed the number of colonies formed by MDA-MB-231 cells (a) compared to non-treated control (b). In addition, clonogenicity was significantly decreased in MDA-MB-231 cells (c) as compared with the control group (d) after 14 days of a treatment with methanol extract from *L. angustifolia*.

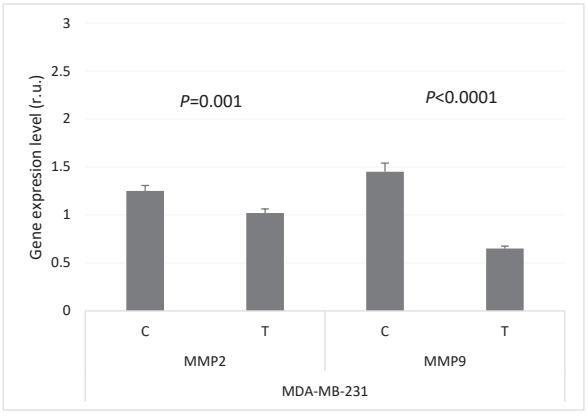


Figure 6. Down regulated Level of MMP2 and MMP9 Genes in MDA-MB-231 Cell Line After Treatment With MetOH Extract of *Lavandula angustifolia* (C: control, T: treatment, r.u: relative unit).

treatment techniques and reduce their negative effects, has been highlighted recently. *L. angustifolia*, for instance, has been reported to exert a strong antiproliferative impact on prostate cancer (35). In this study and for the first time, it was demonstrated that the methanol extract of *L. angustifolia* suppressed the growth of a breast cancer cell line. Our findings confirmed that *L. angustifolia* MetOH extract reduced MMP2 and MMP9 expression in the MDA-MB-231 cell line. Our findings further revealed that the antiproliferative activity of n-hexane and ethyl acetate extracts was much lower than that of Methanol extract, which may have been attributed to the extract composition differences. It has been discovered that *L. angustifolia* from different sources has distinct components. In our study, it was discovered that the MetOH extract was rich in coumarin (59.44%), Tricosane (15.22%), 7-methoxy coumarin (12.69%), and 2-furancarboxaldehyde (6.7%), whereas 1,8-cineol (33.7%) and decane (25.1%) were the main elements of the n-hexane extract. Ethyl acetate extract mainly contained 1,8 cineol (52.79%) and α-pinene (27.05%). Our findings revealed that certain concentrations of MetOH extract from *L. angustifolia* reduced the viability of the breast cancer cells. Our findings, compared to the results from earlier studies, demonstrated the anti-tumorigenic effect of *L. angustifolia* extract on both metastatic and non-metastatic breast cancer cell lines for the first time.

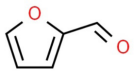
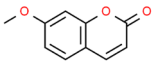
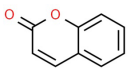
MDA-MB-231 is a triple negative (i.e., negative for estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2) breast cancer subtype (Claudin-low) with aggressive and metastatic behavior and a moderate response to treatment (30). According to our findings, *L. angustifolia* extract may have slowed the growth of triple negative breast cancer, and methanol extract, rather than ethyl acetate or n-hexane extracts,

Table 2. Docking Energy of Eight Compounds Docked with HDAC and 9 Transcription Factors according to kcal.mol^{-1}

Target	Compound							
	Coumarin	Palmitic Acid	Tricosane	Vitamin E	7-Methoxy Coumarin	2-Furan Carboxaldehyde	Borneol-L	1,8-Cineol
HDAC	-5.06	1.59	-1.93	-3.65	-4.78	**-7.37**	-4.23	-4.20
NFkB	-6.17	1.27	-2.20	-3.97	**-6.51**	-6.29	-4.88	-5.97
MAPK-p38	-5.94	-2.06	-1.10	-2.92	-5.45	-4.318	**-6.24**	-4.45
SMAD3	**-5.74**	1.23	-0.94	-2.99	-5.16	-5.45	-5.16	-4.97
STAT3	-4.43	1.05	-1.72	-3.88	-4.51	-4.26	*	**-5.39**
PEA3	-4.36	*	*	-3.43	**-4.78**	-4.02	*	*
CREB	-4.68	-3.18	0.058	-3.033	**-4.77**	-4.53	-4.76	*
C-FOS_C-JUN	-4.20	-1.02	-0.43	-1.82	**-4.49**	-4.05	*	*
CEBP	-4.18	0.90	-2.31	-2.27	-3.78	**-4.29**	-4.26	-3.40
SP1F3	-3.49	-1.72	-098	-2.58	-3.56	**-3.88**	*	*

Note: Bold figures stand for most docking energy.

Table 3. Interactions of 9 Transcription Factors and Histone Deacetylase With the Best Docked Ligands

Receptor	Compound name	Compound Structure	Interacting Residues	Docking energy (kcal.mol^{-1})
HDAC	2-Furancarboxaldehyde		Met24, Gly132, Leu133, His134, HIS135, Gly143, Cys145, Asp170, His172, Asp259, Gly295, Gly296, Tyr298, Zn	-7.37
NF-kB	7-methoxy Coumarin		Ser243, Lys244, Ser249, Asn250, Asp274	-6.51
MAPK-p38	Borneol-L		Leu58, Glu74, Phe67, Asp171, Gly173, Mg	-6.24
SMAD3	Coumarin		Gln315, Pro317, Asn320,Pro327, Ala328, Arg367	-5.74
STAT3	1,8-Cineol		Lys531, Leu532, Lys548, Asn553, Met554, Ala555	-5.39

had the capacity of suppressing the proliferation of MDA-MB-231. Coumarin, Tricosane, 7-methoxy coumarin, and 2-furancarboxaldehyde were the major components of the methanol extract, and their presence was likely associated with anti-tumorigenic effects of the *L. angustifolia* extract against MDA-MB-231 cell line. Flow cytometry studies also showed an increase in cell cycle arrest following the treatment of this breast cancer cell line with MetOH extract of *L. angustifolia*.

Previous research on *L. angustifolia* extract, which has the most anti-cancer impact, has generated widely-disparate findings. A study by Tayarani-Najaran et al investigated the Lavender's anti-proliferative effects, and found that ethanol and n-hexane extracts from *L. angustifolia* were

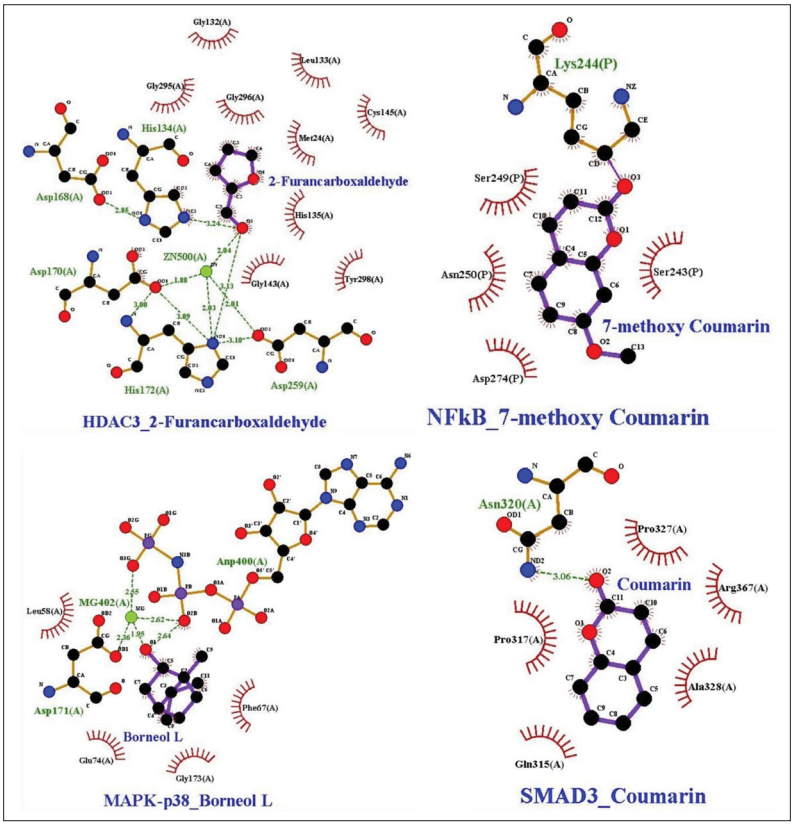


Figure 7. 2D Interaction of Protein Ligands in Active Site.

more cytotoxic than H2O extracts against MCF7 and HeLa cell lines (27). Lavender's effective concentration for reducing cell viability was 60 µg/mL, which was half the therapeutic amount. Their findings further demonstrated that this anti-tumorigenic feature was activated by increasing the cellular apoptosis. Another study by Dalilan et al found that an aqueous extract of *L. angustifolia* inhibited the lymphocyte proliferation (36). The effective concentration of *L. angustifolia* extract used in their investigation was 100µg/mL. Furthermore, their study indicated that the antiproliferative action of *L. angustifolia* extract was achieved by the enhanced apoptosis. A study by Zhao et al examined the anti-tumorigenic effect of *L. angustifolia* essential oil on human prostate cancer, and reported that the essential oil of *L. angustifolia* had the potential to prevent the development of a prostate tumor xenograft in nude mice, with linalool being the major component responsible for this action (35). Their findings clearly illustrated that the essential oil of *L. angustifolia* significantly boosted apoptosis, thereby inhibited cell proliferation.

In summary, our findings indicated that MetOH extracts had more anti-proliferative potentials than ethyl acetate and n-hexane extracts. Daniel et al argued that MMP inhibitors caused cell cycle arrest and apoptosis in glomerular mesangial cells (37). Also, our findings revealed that the MetOH extract from *L. angustifolia* suppressed MDA-MB-231 cell line proliferation by triggering apoptosis, lowering MMP2 and MMP9 expression, and enhancing G0/G1 cell cycle arrest. Various factors (e.g., HDAC, NF-kappa B, MAPK-p38, SMAD3, and STAT3) have been suggested to control the expression of MMP genes and G0/G1 cell cycle arrest (14). Several pathways have been found to modulate the flexibility of chromatin and facilitate the variable expression of the genes. Between these pathways, modifications of chromatin caused by histone tail deacetylation and acetylation have received a lot of research attention (38). Deacetylation of core histones is commonly linked to transcriptional inhibition (15).

Out of all investigated compounds, according to the docking studies, the best one is 2-Furancarboxaldehyde, which is docked with HDAC with a –7.37 kcal.mol^{-1} docking score (Figure 8). By lowering proteasome activity, HDAC inhibition limits NF-κB activation (39). Nuclear factor-B (NF-B) is a main transcription factor associated with breast cancer metastasis since it increases the production of MMP-9 (40). TNF-alpha has been shown to activate numerous transduction pathways, including NF-κB, via its receptors (42). Also, 2-furancarboxaldehyde, coumarin and 7-methoxy coumarin and 1,8-cineol have a good affinity for binding NF-κB. MAPK-p38 is a target that coumarin, 7-methoxy coumarin, and borneol-L almost tightly bind to it. Numerous investigations have revealed that p38 modulates the production of several MMPs, implying a link between p38 activation and

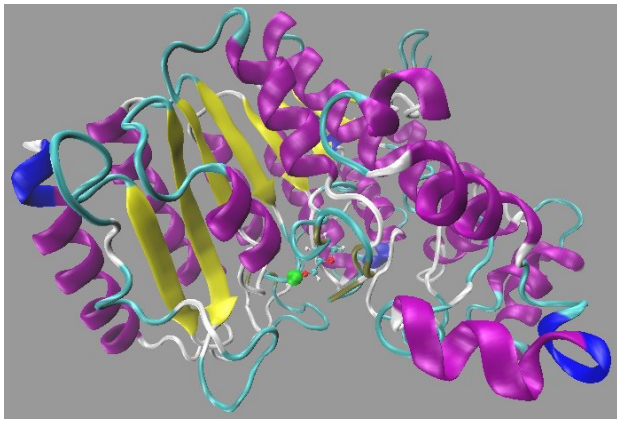


Figure 8. 3D Structure of Histone Deacetylase as Best Docked Enzyme with 2-Furancarboxaldehyde.

metastasis. In fact, inhibiting p38 reduces MMP-2/9 activity and the invasive potential of bladder cancer cells in vitro. Furthermore, inhibiting p38 blocks the TGF-dependent MMP-9 production and lowers the bone metastases from breast cancer in mice (42,43). In addition, Koul et al reported that the p38 MAPK pathway regulates MMP-2 activation and cell invasion by phosphorylating heat shock protein 27 (44). Another transcription factor, SMAD3, has been discovered to function as a transcriptional suppressor of tissue inhibitors of metalloproteinase-2 (TIMP2) and a transcriptional activator of MMP2 by binding to TIMP2's 3′ UTR and MMP2's 5′ UTR, respectively (45). 7-methoxycoumarin, coumarin, 2-furancarboxaldehyde, and 1, 8-cineol are methanolic extract compounds with good affinity for SMAD3. The upregulation of MMP2, MMP9, Twist, Snail, Slug, and vimentin is a well-studied mechanism of STAT3-mediated cell metastasis. Furthermore, smad3 activates state3 which, in turn, activates MMP 2,9 (46). Taking into account the computational score of our study, it was suggested that 1,8-cineol may have bound to STAT3; however, it seemed that 1,8-cineol was unlikely to exert an inhibitory effect on STAT3 because n-hexane and ethyl acetate extract had no effect on the cell growth. In summary, coumarin and 7-methoxy coumarin strongly bind to HDAC, NF-κB, MAPK-p38, and SMAD3. Moreover, 2-Furancarboxaldehyde has the lowest energy or the most tendencies for SMAD3 and HDAC, as well as NF-κB. Borneol-L binds to MAPK-p38 and SMAD3 with great affinity. However, three substances, including Vitamin E, Tricosane, and Palmitic acid, did not show a high affinity for the targets.

Limitations of the Study

As it is the case with almost all studies, the design of the current study was subjected to few limitations. First, the presence of several and complex compounds in the methanol extract made it difficult to determine the effective substance(s) from the extract in laboratory conditions. Therefore, it was recommended that further

docking studies should be carried out in this regard. Second, the polar and heavy compounds of the methanol extract were not determined due to the lack of access to an expert to interpret the LC-MS spectrum of the effective extract. Therefore, only those compounds detected from the GC-MS spectrum were examined.

Conclusions

It was concluded that the anti-proliferative properties of MetOH extracts were higher than those of the n-hexane or Ethyl acetate extracts. It was also found that the MetOH extract of *L. angustifolia* inhibited MDA-MB-231 cell line proliferation, and this cytotoxicity was applied via inducing apoptosis, decreasing MMP2 and MMP9 expression, and increasing the G0/G1 cell cycle arrest. According to our findings from ducking studies, the main component of the methanol extract was 2-Furancarboxaldehyde docked with HDAC with the most docking energy, which may have been an important factor responsible for the anti-cancer effects of methanolic extract of L. angustifolia through reducing the expression of MMP2 and MMP9. Therefore, it was recommended that *L. angustifolia* should be considered as a potential complementary medicine in future in vivo studies.

Authors' Contribution

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Writing–review & editing: Mohammad-Karim Khosropanah.

Conflict of Interests

Authors declare that they have no conflict of interests.

Ethical Issues

This study was approved by the ethical committee of Kurdistan University of Medical Sciences (No. IR.MUK.REC.1397/98).

Financial Support

The authors received no specific funding for this work.

Acknowledgments

The authors would like to heartly thank Dr. Abdulrahman Rahimi for supporting us by preparing and approving the aerial parts of the lavender plant from the greenhouse of the Faculty of Agriculture, Islamic Azad University Sanandaj. The results from flowcytometry were kindly analyzed by Dr. Assadollah Mohammadi. The authors also wish to thank all stuffs of the Cellular and Molecular Research Center of Kurdistan University of Medical Sciences for their kind assistance.

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