Research Article

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Chemical composition and *in vitro* mutagenic, antioxidant and anti-inflammatory activities of *Lavandula angustifolia* Mill. essential oil from Turkey

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Received: 17 February 2022 / Revised: 21 April 2022 / Accepted: 21 April 2022

ABSTRACT: The essential oil obtained from *Lavandula angustifolia* Mill. (Lavander) has many pharmacological effects, including antioxidant, antifungal, antibacterial and carminative effects. *Lavandula angustifolia* essential oil (LEO) contains monoterpene compounds, mainly linalool and linalyl acetate and various phenolic acids such as caffeic acid, vanillic acid, ferulic acid and chlorogenic acid. The hydrodistillation of the aerial parts of *L. angustifolia* produced a pale yellow oil. Gas chromatography-Gas chromatography/Massspectrometry (GC-GC/MS) results showed that LEO contains monoterpene alcohols that primarily include linalool (35.91%), 4-Terpineol (6.10%), α-Terpineol (4.49%) and lavandulol (2.49%). As a result of the cytotoxicity analysis of LEO, the IC_{50} value was found to be 0.372 mg/mL. LEO was found to be non-mutagenic against the bacterial test strain *Salmonella typhimurium* TA 98 with S9 fraction while the highest concentration (9.58 mg/mL) was found to be either mutagenic or cytotoxic against TA 98 without S9 fraction. The essential oil's antioxidant capacity was found 6.522±0.069 mg/mL while the total phenolic content was 1.22±0.04 mg GAE/g extract (Gallic acid equivalent/g extract). Anti-inflammatory activity of LEO was 1.238± 0.026 mg/mL while that of indomethacine was 0.022±0.003 mg/mL. In conclusion, when the results are evaluated in terms of *in vitro* biological activities, LEO can be considered as a potential herbal product with active compounds that should be examined in future studies.

KEYWORDS: *Lavandula angustifolia*; essential oil; mutagenity; antioxidant activity; cytotoxicity.

1. INTRODUCTION

Lavandula species belongs to the family Lamiaceae (Labiatae) [1]. The genus *Lavandula* is a well-known medicinal plant that has been shown valuable for the treatment of depression, stress, headache, and migraine in traditional medicine [2]. Essential oil or dried samples of genus *Lavandula* have been used in traditional medicine [1]. Essential oil obtained from *Lavandula angustifolia* Mill. (Lavander) has been reported to have many pharmacological effects such as antioxidant, antifungal, antibacterial and carminative effects [1,3].

Essential oils are complex mixtures formed as a result of the protection mechanisms of plants [4]. Essential oil products that are obtained from aromatic plants have rich chemical content [5]. Conditions such as the place where the plant is collected, climatic conditions, and seasonal differences affect the chemical composition of essential oils [6]. *L. angustifolia* essential oil (LEO) has been documented to contain monoterpene compounds, mainly linalool and linalyl acetate and various phenolic acids such as caffeic acid, vanillic acid, ferulic acid, and chlorogenic acid [7,8]. Natural antioxidants have been investigated and shown to protect our body aganist free radicals. Essential oils are natural mixtures with antioxidant effects.

How to cite this article: Omurtag Özgen PS, Biltekin SN, İduğ T, Macit C, Ayran İ, Çelik SA, Kültür Ş, Kan Y, Omurtag GZ. Chemical composition and in vitro mutagenic, antioxidant and anti-inflammatory activities of Lavandula angustifolia Mill. essential oil from Turkey. J Res Pharm. 2022; 26(4): 781-789.

© 2022 Marmara University Press
ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.175

Antioxidant and anti-inflammatory effects of linalool and linalyl acetate compounds, which are abundant in lavender essential oil, were also reported [6,8,9].

The Ames test measures the mutagenic potential of chemicals [10,11]. Most of the mutagenity tests were performed on bacteria. The Ames microplate fluctuation (MPF) test has essentially the same principles as the traditional Ames test, but new standards have been established for this type of test [10,11,12]. It has many advantages over the traditional Ames test and complies with OECD (Organization for Economic Co-operation and Development) and FDA (Food and Drug Administration) guidelines. The Ames MPF test is a short-term mutagenicity test that is frequently used for the detection of antimutagens/anticarcinogens or reverse mutagens/carcinogens. It has also been used to measure the mutagenic potential of medicinal plants in recent years [11,13,14].

The current study scrutinized chemical composition, total phenolic content, antioxidant, anti-inflammatory activities, cytotoxicity and the mutagenic effect of *L. angustifolia* essential oil.

2. RESULTS

2.1. Essential oil yields and composition

The aerial parts of *L. angustifolia* were subjected to hydrodistillation for 3 h using Clevenger type apparatus to resulted in 1.5% v/w of the pale-yellow oil. Table 1 shows the chemical composition of the LEO in percent. 15 compounds (containing more than 1%) of LEO were accounted for 96.25% of the total oil.

LEO contains monoterpene alcohols primarily including linalool (35.91%), 4-terpineol (6.10%), α-terpineol (4.49%) and lavandulol (2.49%) (Table 1).

2.2. Cytotoxicity, antioxidant and anti-inflammatory activity analysis

As a result of the cytotoxicity analysis of LEO, IC_{50} value was found to be 0.372 mg/ml. *In vitro* antioxidant activity of LEO was examined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. Antioxidant capacity of LEO was detected as 6.522±0.069 mg/mL while total phenolic content was determined as 1.22±0.04 mg GAE/g extract (Gallic acid equivalent/g extract) at stated concentrations. Moreover, anti-inflammatory activity was found 1.238± 0.026 mg/mL (Table 2).

2.3. Results of mutagenity assay

Results showed that in the Ames MPF assay, all tested concentrations of LEO were calculated to have a baseline of 2.00 and 1.91 (revertants), and did not show two- and three- fold increases over the baseline or in any statistical significance in the tested doses against TA 98 with/without S9 enzyme, respectively. Table 3, and Figure 1 showed that in the presence or absence of metabolic activation (S9) with TA 98 strain, the number of revertant colonies were not statistically significant compared to the number of spontaneous revertant colonies at the study concentrations, except for the 6th concentration (9.58 mg/mL) in the absence of S9 ($p \leq 0.05$). LEO was found to be non-mutagenic against TA 98 with S9 ($p > 0.05$), while the highest concentration (9.58 mg/mL) was found to be either mutagenic or cytotoxic against TA 98 without S9.

3. DISCUSSION

The cytotoxicity (IC_{50}) of lavender oil was reported to be 0.28 µg/mL on HEL 12469 human embryo lung cells by Puškárová et al. [5]. In another study, Miastkowska et al. compared commercial and harvested Lavender essential oils and reported 0.20 v/v and 0.36 v/v for the HaCaT cell line, respectively [15]. In addition, Cardia et al. reported that lavender essential oil showed low cytotoxicity on mice leukocytes obtained from the peritoneal cavity, with cell viability greater than 75% up to a concentration of 10 µg/mL [16]. Our result is compatible with the findings of previous literature. Different cytotoxicity results may vary depending on the profile of the essential oil as well as the use of different cell lines.

In a study conducted with natural flavor complexes (NFCs), lavender oil was detected as non-genotoxic with its linalool and linalyl acetate contents [17]. In a different study, lavender oil was reported not to induce mutagenicity in the *S. typhimurium* strains TA98 and TA100 both with and without liver S9 from Aroclor 1254-treated rats [8]. Studies examined a variety of tertiary alcohol constituents found in different plants for their genotoxicity and summarized that linalool and linalyl acetate did not have mutagenic effects [18,19,20]. These results are in accordance with our results, since they have found lavender oil to be non-mutagenic. On the other hand, our results did not confirm the study in that a reverse mutation assay conducted with lavender oil at concentrations of 4.4 and 8.8 ng/plate, it was found to be mutagenic in *S. typhimurium* strain TA98

(concentration of 8.8 ng/plate) [21]. In our study evaluation of results was done according to the criteria described in the literature [22] and the results are classified as negative.

Table 1 The chemical composition of LEO in GC-GC/MS analysis

Compound	Relative Percent (%)	Retention Index (RI)
Thujene	0.16	3.51
α-Pinen	0.23	3.63
3-Octanone	0.94	4.20
Myrcene	1.38	4.29
Acetic acid, hexyl ester	0.45	4.57
Cis-Ocimene	5.61	5.01
α-Ocimene	3.03	5.20
Linalool	35.91	6.17
Octen-1-ol, acetate	1.61	6.29
Alloocimene	1.33	6.70
Camphor	0.33	7.14
Lavandulol	2.49	7.45
1-Borneol	1.57	7.54
4-Terpineol	6.10	7.76
Hexyl butanoate	1.08	7.91
α-Terpineol	4.49	8.01
Nerol	0.63	8.71
Hexyl 2-methyl butyrate	0.25	8.85
Linalyl acetate	16.75	9.28
Lavandulyl acetate	4.94	9.99
Neryl acetate	0.82	11.54
Geranyl acetate	1.64	11.94
α-Santalane	0.60	12.85
Trans-Caryophyllene	2.12	12.92
Trans-Farnesene	0.12	13.49
Cis-Farnesol	0.14	13.61
Germacrene-D	1.07	14.16
(-)-Caryophyllene oxide	0.46	16.20
Total	96.25	

Medicinal plants and compounds derived from plants are potential drug candidates. For this reason, it is important to evaluate plants in toxicological tests as well as their pharmacological activities [23]. Both toxicological and antioxidant activity studies on lavender essential oil are important in terms of contributing to the literature. Altogether, the evidence provided by the results of all of the Ames and *in vitro* micronucleus studies reported here, in addition to the negative genotoxicity reported for linalool and linalyl acetate, the major constituents of lavender oil, supports the conclusion that lavender oil is not genotoxic.

Table 2 Cytotoxic, antioxidant and anti-inflammatory activities of LEO

Cytotoxicity	**Antioxidant activity**		**Anti-inflammatory activity**
MTT (IC_{50})	**DPPH (IC_{50})**	**TPC (IC_{50})**	**5-LOX (IC_{50})**
372.6 ± 5.03 µg/mL	6.522 ± 0.069 mg/mL	1.22 ± 0.04 mg GAE /g extract	1.238 ± 0.026 mg/mL
Standard	2.46±0.005 mg/mL		0.022±0.003 mg/mL

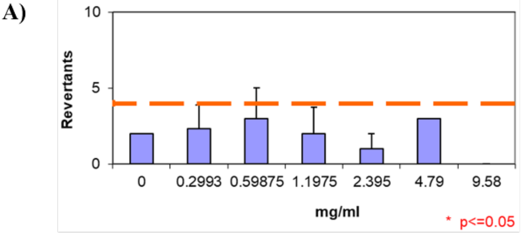
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, DPPH: 2,2-diphenyl-1-picrylhydrazyl, TPC: Total phenolic compound, 5-LOX: 5-Lipoxygenase, IC_{50}: Inhibitory concentration 50

Table 3 Mutagenic effect of LEO to *Salmonella typhimurium* (TA98) with and without metabolic activation (S9)

Compound	**Concentrations of Mutagens (µL and µg/mL) and LEO (mg/mL)**	**Number of Revertants/Plate**			
		TA98			
		S9+		**S9-**	
		MEAN	**±SD**	**MEAN**	**±SD**
DMSO	300 µL	2.00	0.00		
2-NF	2.0 µg/mL			33.00	5.20
4-NQO	0.1 µg/mL				
2-AA	5.0 µg/mL	48.00	0.00		
	0.2993	2.33	1.53	1.53	1.73
	0.5987	3.00	2.00	2.00	1.15
	1.1975	2.00	1.73	1.73	1.00
LEO	2.3950	1.00	1.00	1.00	0.58
	4.7900	3.00	0.00	0.00	0.58
	9.5800	0.00	0.00	0.00*	0.00*

Vehicle: DMSO; Mutagens: 2-NF: 2-nitrofluorene and 4-NQO: 4-nitroquinoline-*N*-oxide; LEO: Essential oil of *Lavandula angustifolia*. Result is given as Mean ± SD and * indicates significance when $p<0.05$.

In previous studies, DPPH radical scavenging activity was found 0.421 mg/mL by Niksic et al. (2017), while Rasooli et al. (2007) stated that 18.86 µg/ml. In another study, Zhao et al. (2015) obtained 51.05 mg/ml [6,24,25]. In a study in which the effect of plant harvesting time on the content and antioxidant capacity was measured, the activity was found to be 1.0558 mg/mL in a sample collected in July, while it was found to be 1.2995 mg/mL the sample collected in September; in same study total phenolic contents were found 2.8980 g/100 g and 2.0870 g/100 g respectively [26]. In a Turkish study, the antioxidant capacities of different *L. angustifola* cultivars were found to vary from 91.5 to 101.27 µg/ml [27]. In a study by Lin et al. (2009), the total phenolic content of the essential oil was found to be 6.76 µg/5 mg [28]. Total phenolic content result of current study is compatible with the literature.

A)


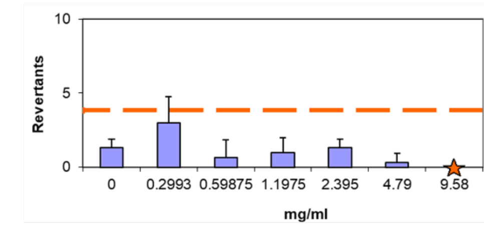
B)


Figure 1 Ames MPF result graphs of LEO against TA 98 with/without S9 **A)** TA 98 with metabolic activation and **B)** TA 98 without metabolic activation.

In vivo and *in vitro* studies have shown that *L. angustifolia* extracts and essential oils have anti-inflammatory activity [29,30]. Moreover, in a different *in vitro* study, the anti-inflammatory property of *L. angustifolia* essential oil extracted from plants grown in a natural environment was performed with primary cultures of human macrophages infected with *S. aureus.* Results showed that LEO had anti-inflammatory effects [31]. All studies stated that the anti-inflammatory activity of *L. angustifolia* was dependent upon the main constituents, namely linalool and linalyl acetate. In our study anti-inflammatory activity was found to be 1.238± 0.026 mg/mL while indomethacine was found to be 0.022±0.003 mg/ml. As stated, the reason for the relatively low anti-inflammatory activity may be related to the amount of linalool and linalyl acetate.

4. CONCLUSION

In conclusion, LEO was found to be non-genotoxic against TA98 with or without metabolic activation according to the Ames results, indicating that it is a potential candidate for further studies.

In this study, the mutagenicity effect of six different concentrations of LEO was performed by using the Ames MPF™ assay with a TA98 strain in the presence and absence of metabolic activation (S9). The results of these experiments are shown in Figure 1. The plot of concentration (mg/mL) versus the number of revertants shows a baseline of 2.00 and 1.91 for the TA98 strain in the presence and absence of S9, respectively. All concentrations do not show 2 or 3-fold increases over the baseline. Neither a 2 or 3-fold increase over the baseline nor statistical significance are obtained from the results.

When our biological activity results are compared with previous studies, the results are compatible with the literature and the differences can be attributed to climate, temperature, harvesting time, cultivar conditions and the region where the plant is obtained. All these conditions affect the composition of the oil. Changes in chemical composition also affect biological activities.

5. MATERIALS AND METHODS

5.1. Plant material

Lavandula angustifolia Mill. was obtained from Selcuk University, Faculty of Agriculture, Medicinal and Endemic Plants Education and Research Farm, Konya/Turkey. The plant material was identified by Şükran Kültür from Istanbul University, Department of Pharmaceutical Botany, Faculty of Pharmacy. A voucher specimen was deposited to the herbarium of the Faculty of Pharmacy at Istanbul University. (Herbarium no: ISTE116009).

5.2. Isolation of the essential oil

Dried aerial parts of *L. angustifolia* (100 g) was subjected to hydrodistillation via Clevenger apparatus system for 3 h according to the procedure described in European Pharmacopeia [32]. The oil was filtered over anhydrous sodium sulphate and stored at +4°C in the dark for further investigation.

5.3. Gas chromatography- Gas chromatography/Mass spectrometry (GC-GC/MS) analysis

Mass spectrophotometric analyses were performed on an Agilent 7890B GC System equipped with FID. The separation was achieved using an Agilent HP-Innowax column (60 m x 0.25 mm i.d., 0.25 µm film thickness). Column temperature: 60 °C (10 min.), 4 °C /min 220 °C (10 min) 240 °C, Total 80 min.; injection mode (split (40:1), 1 µL of a 10% v/v n-hexane solution). Injector and detector temperatures were 250 °C. Helium was used as carrier gas (0.7 mL/min).

After that, the second phase of separation occurred in GC/MS. These analyses were performed on an Agilent 7890B Gc5977B Mass Selective Dedector System. The separation was carried out with an Agilent HP-

Innowax column (60 m x 0.25 mm i.d.; 0.25 µm film thickness); ionization energy voltage 70 eV. Mass spectra were scanned in the 35–450 amu range. Gas chromatographic conditions were as reported in the previous paragraph.

5.4. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity was performed as described previously, along with minor modifications [33]. Briefly, stock extracts of LEO were prepared in MeOH as 10 mg/ml starting from 2.5 mg/ml of the stock solution; serial dilutions were performed as follows: 2.5 mg/ml, 1.25 mg/mL, 0.625 mg/ml and 0.312 mg/ml. 10 µl from each dilution was mixed with 190 µl of 0.1 mM DPPH radical solution (Sigma-Aldrich) prepared in MeOH. The mixture was than loaded into 96 well-plate, in triplicates. The plate was shaken gently and left in room temperature in the dark for 40 minutes until measured. Absorbance was measured at 517 nm (Synergy HTX Biotech multimode ELISA reader). The half inhibitory concentration (IC_{50}) values, which represent the concentration that caused a 50% inhibition of radical formation, were used to interpret the results. Gallic acid was used as the positive control. All tests were carried out in triplicate unless otherwise noted.

5.5. Total phenolic content

We performed a total phenolic content (TPC) analysis which was developed previously [34]. Briefly, 5 µL of the test samples (2.5, 1.25, 0.625 and 0.312 mg/mL in MeOH), 25 µl of 0.2 N Folin-Ciocalteu reagent and 145 µL distilled water were mixed at 96-well plates and the mixture was shaken gently. Following 6 min incubation in the dark, 75 µl of Na_2CO_3 (7%) solution was added to the mixture and shaken again. The plates were incubated for 2 hours in darkness and at room temperature. Absorbances at 765 nm was measured. Gallic acid was used as the standard. All tests were carried out in triplicate, unless otherwise noted.

5.6. Anti-inflammatory activity

5-lipoxygenase (5-LOX) inhibition activity was observed with the method of Phosrithong and Nuchtavorn with the slight modifications described by Yıldırım et al. [35,36]. 10 µl of the extract or the standard indomethacin were added to 20 µl ethanol, 20 µl distilled water, 25 µl of sodium borate buffer solution (0.1 M, pH 9) and 25 µl of type V soybean lipoxygenase solution in the buffer (pH 9, 20.000 U/mL). The reaction mixture was pre-incubated at 25 °C for 5 min. Then, 100 µl of 0.6 mM linoleic acid solution was added to the reaction mixture, mixed well and the change in absorbance at 234 nm was followed for 6 min. Each reaction was run in triplicate.

5.7. Mutagenity assay

Ames MPF™ assay was applied with *Salmonella typhimurium* TA98 strain in the presence and absence of S9 to evaluate the mutagenic effect of LEO. The mutagenity assay was performed following the instructions of the manufacturer (Xenometrix, Allschwil-Switzerland) according to given references [22,37]. Samples were tested in triplicate and the results were expressed as mean ± standard deviation (SD).

5.8. Preparation of *L. angustifolia* essential oil solution for mutagenity assay

To investigate the mutagenic effect, six different stock solutions of LEO at 25 times concentrated (25x)-concentration (0.2993, 0.5987, 1.1975, 2.3950, 4.7900, 9.5800 mg/mL) were prepared in dimethylsulfoxide (DMSO). The preparation of 25x stock concentration is necessary to achieve the desired 1x assay concentration in each well. The highest concentration of essential oil was prepared by diluting 46 mg LEO/300 µL DMSO stock solution with 16-fold DMSO.

5.9. Cell culture conditions

The cytotoxicity of LEO was evaluated on NIH/3T3 mouse embryonic fibroblasts cell lines which were obtained from the American Type Culture Collection (ATCC, USA). The NIH/3T3 cell line was grown in Dulbecco's Modified Eagle's Medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin (10.000 units/mL) and streptomycin (10.000 µg/mL) at 37°C in a humidified atmosphere of 5% CO_2. The cells were passaged every 3 days [38].

5.10. Cytotoxicity assay

Cytotoxic activities were determined by cell proliferation analysis using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [38,39]. NIH/3T3 cells were seeded at a density of 5×103 cells per well into a 96 well culture plate and left to incubated for 24 hours. Then the cells were treated with different concentrations (10, 50, 100, 250, 500, 1000, 1500 µg/mL) of LEO. After the 24 hours incubation period, the cell culture medium was removed. Then, 30 µL MTT reagent (5 mg/mL) was added to all wells and cells were incubated for an additional 4 h at 37°C. After the incubation period, cell culture medium was removed and 150 µL of DMSO was applied to wells to dissolve formazan crystals. The absorbance was measured at 570 nm using ELISA microplate reader. The percentage of cell viability was calculated using the following equation [40]:

$$\text{Viability\%} = (\text{Absorbance}_T) / (\text{Absorbance}_C) \times 100\%$$

5.11. Statistical analysis

Data are presented as mean ± standard deviation. All experiments were performed at least in triplicates. Statistical comparisons for mutagenity assay were performed through the Student's t-test. Statistical analyses were performed using Excel. Statistical comparisons for cytotoxicity assay were performed through one-way ANOVA followed by Tukey test. Statistical analyses were performed using Graph Pad Prism 7.0d (Graph Pad Software, La Jolla, USA). Statistical significance was set at $p < 0.05$.

Acknowledgements: This work was supported by two Istanbul Medipol University Grants (BAP-2020/15, BAP-2019/03).

Author contributions: Concept – G.Z.O., T.I., P.S.O.O.; Design – G.Z.O., T.I., P.S.O.O.; Supervision – G.Z.O.; Resources – I.A., S.A.C., Y.K.; Materials – T.I., I.A., S.A.C., Y.K.; Data Collection and/or Processing –C.M., P.S.O.O., S.N.B., T.I., S.K.; Analysis and/or Interpretation – C.M., T.I., S.N.B., P.S.O.O.; Literature Search – G.Z.O., T.I., P.S.O.O., S.N.B., C.M.; Writing – P.S.O.O., T.I., C.M., S.N.B.; Critical Reviews – G.Z.O., C.M., T.I., S.N.B., P.S.O.O.

Conflict of interest statement: The authors declared no conflict of interestin the manuscript.

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