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Chemical Characterization of *Lavandula angustifolia* Mill. as a Phytocosmetic Species and Investigation of its Antimicrobial Effect in Cosmetic Products

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Abstract: The content of the extracts obtained from *Lavandula angustifolia,* which were grown in Burdur Örtülü locality, was determined via HPLC and GC-MS analysis and the anti-microbial effect of the essential oil *L. angustifolia* was also investigated. The dried flowers of *L. angustifolia* were extracted and the essential oil was distilled from the remaining part. Various phenolic compounds in the extract were quantitatively determined by HPLC. Quantitatively caffeic acid, rosmarinic acid, and 4-hydroxybenzoic acids were the most abundant phenolic acids in the content in decreasing order. 31 different compounds were determined by GC-MS analysis: Linalool and linalyl acetate have the highest concentration. Anti-microbial effects of the essential oil of *L. angustifolia* were determined against the most frequently encountered microorganisms in the cosmetics: *Candida albicans, Staphylococcus aureus, Pseudomonas aeruginosa, and Aspergillus brasiliensis.* It was observed that the essential oil *L. angustifolia* could completely remove the contamination caused by the microorganisms as of the 14th day. According to the results it is concluded that the essential oil of *L. angustifolia*, can be used either directly or incorporated into the cosmetics without the necessity for any other extra preservative against the above mentioned microrganisms.

Keywords: *Lavandula angustifolia* Mill.*;* cosmetic; phenolic compounds; essential oil; antimicrobial activity**.**

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INTRODUCTION

In the last years, usage of aromatic plants in the perfumery, food, and cosmetics industries as the principal raw material as well as its usage in many other fields have increased the demand for medicinal and aromatic plants. Especially the methods, which are named as return to nature, have increased the interest in these plants both in Turkey and in other countries in the world [1].

Lavender (*Lavandula* sp.) is a very valuable essential oil plant from the *Lamiaceae* family. There are 39 lavender species (*Lavandula* sp.) most of which have Mediterranean origin and among them, three have high commercial value. While the essential oil quality of the lavender species (British lavender) is high the lavandin species (hybrid lavender) have high essential oil yield [2, 3]. It plays an important role in the pharmacology and perfumery industries since it contains essential and aromatic oils [4]. 1.8-2 billion dollar-worth of essential oil is exported each year in the world and lavender oil constitutes 50 million dollars of this export [5, 6, 7]. The three commercial lavender species are Lavender (*Lavandula angustifolia* Mill. = *L. officinalis* L. = *L. vera* DC), Lavandin (*Lavandula intermedia* Emeric ex Loisel. = *L. hybrida* L.) and Spike Lavender (*Lavandula spica* = *L. latifolia* Medik.). While the essential oil quality of the Lavender types, which are named as British lavender, is high the essential oil quantity of lavandin, which is named as hybrid lavender [8].

The most important essential oil components of lavender oil are linalyl acetate, linalool, and cineol. Among them, linalyl acetate is the most important component which determines the quality of the lavender oil [9]. There are many studies which indicate that *L. angustifolia* mostly contains linalool and linalyl acetate as essential oil component in essential oil obtained via water vapor distillation of the flowers of *L. angustifolia* [10, 11, 12]. Due to linalool and linalyl acetate, both of which are present in the plant, lavender is used in production of perfume, skin cleaning lotion, bath soap with odor and bath foams in the cosmetics industry [13, 14, 15]. Lavender and its ethanolic extract have high antioxidant activity and its content is rich in phenolic compounds. It is thought that this effect arises from the protective effect that phenolic compounds show against oxidative damage which is caused by the free radicals. Moreover, it is indicated that lavender inhibits bacterial growth [9, 16].

Cosmetic products are not sterile and are open to the growth of microorganism contamination any time. When these products are studied from the microbiological perspective, since they are not produced under aseptic conditions, microorganisms which are not pathogens are observed. Cosmetic products containing pathogenic

microorganisms would put the health of people who use them in danger rather than beautifying them and would either degrade the products or cause diseases. The dangers, which cosmetic products cause, have attracted the attention of those working in this field and intensive studies on this subject [17]. According to the American Pharmacopoeia, *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Salmonella typhimurium, Candida albicans* and *Aspergillus niger are* microorganisms *which should not be found in c*osmetic products [18]. *Staphylococcus aureus is one of the most common bacterial skin pathogen found in* creams. Presence of these bacteria in creams intensively may cause skin and mucosal infections such as inflammatory skin disease (impetigo), follicular inflammation, and abscess. Infection starts when *Staphylococcus* degrades in the dermal mucosal barrier and enters into the tissues or into the bloodstream [19, 20]. It is reported that the active of the 70% of the skin infections seen in children is *S. aureus* [21]. It is reported that ocular infections are commonly seen in patients who use eye drops. Nosocomial infections and epidemics arising from usage of products contaminated with *P. aeruginosa* are reported [22]. Glycerin used in creams and lotions can be metabolized by microorganisms (especially by *Bacillus, Staphyloccocus* and *Micrococcus)* [23]. Cosmetic products contaminated by *C. albicans* can cause dermatitis [24]. The creams produced by the Ukranian Company Effect which are determined to contain *C. albicans* were collected by the end of 2006 upon notification of Estonia [25].

In the present study, our aim was to examine the content of the extracts obtained from *Lavandula angustifolia,* which were grown in Burdur Örtülü locality and also investigate the anti-microbial effect of the essential oil of *L. angustifolia.* For this purpose, various phenolic compounds were detected in the extracts of Lavender (*Lavandula angustifolia* Mill.) and essential compounds were also detected in its essential oil.

MATERIALS AND METHODS

Chemical Materials

Caffeic acid, p-coumaric acid, and rosmarinic acid were purchased from Sigma, 3,4-dihydroxybenzoic acid, chlorogenic acid, and ferulic acid were purchased from Aldrich, 4-hydroxybenzoic acid, cinnamic acid, and ethanol (absolute for analysis) were purchased from Merck, hexane (for HPLC ≥97.00) and methanol (HPLC grade ≥ 99.9%) were purchased from Sigma-Aldrich, vanilic acid and apigenin were purchased from Fluka, formic acid (analytical reagent grade) was purchased from Fisher Chemical and gallic acid was purchased from Pancreac.

Plant Material (Sampling)

Lavenders (*Lavandula angustifolia* Mill.) collected from Burdur Örtülü locality (37.7167°N-30.2833°E and 959 m above sea level) were used. 2000 g of lavender sample has been dried in the shade after the harvest and the dry flowers were separated from their stems.

Solvent Extraction

Dry flowers separated from their stems were extracted for analysis of their phenolic content. 2-g-sample was dried, powdered and extracted with 10 mL of 96% ethanol for 24 h in water bath at 45 °C. This mixture is centrifuged at 4000 rpm for 5 minutes. Supernatant was concentrated with the rotary evaporator at 45 ^{o}C until complete dryness and dissolved in mobile phase [26].

Clevenger extraction

2000 g stemless dry lavender flowers were weighed. Then the flowers were submitted to hydrodistillation with a Clevenger-type apparatus. As a result of distillation, essential oil yield was found 4.2% .

Instrumentation

Chromatographic analyses were performed using a Shimadzu reversed-phase high-performance liquid chromatography (RP-HPLC) equipped with diode-array detector (DAD). RP-HPLC analysis were done using a 20ACBM system controller, SPD-M20A diode array detector, CTO-10ASVp column oven, SIL 20ACHT autosampler, and a LC20 AT pump. The data were performed using the LC Solution software. The chromatographic separations were achieved on Agilent Eclipse C18 column (250*4,6 mm i.d., 5 µm) the eluates were detected at 280 nm and 320 nm. Analysis of phenolics in Lavandula was achieved by RP-HPLC with gradient elution. The mobile phase used was 3% formic acid in (A) water *vs* (B) methanol. The elution gradient applied at a flow rate of 0.8 mL min^{-1} was: 93%A/7% B for 3 min, 72%A/28%B in 28 min, 67%A/33%B in 60 min, 58%A/42%B in 62 min, 50%A/50%B in 70 min, 30%A/70%B in 75 min, 93%A/7% B in 80 min. Samples were dissolved in mobile phase, and 20 µL of this solution was injected into the column. The column temperature was set at 30 °C. In the HPLC analysis the method of Gomes *et al*. (1999) was modified and used [27]. The volatile compounds were analyzed by using a gas chromatograph 7890 A coupled to a mass spectrometer series MSD 5975 C, (Agilent Technologies) and volatiles were resolved on a CP WAX 52 CB capillary column (50 m * 0.32 mm ID, df :1.2 µm) purchased from Agilent. The carrier gas was helium, at a flow rate of 1.2 mL/min. The temperature program for the GC was as follows; 60 °C initial temperature, after waiting for 2 minutes at 60 °C to

increase 220 °C with 2 °C /min, after reaching this temperature the temperature was kept constant for 20 minutes. The injection was performed in the split mode (20:1). The injection volume was 1 µL. Injector temperature was 240 °C The GC–MS interface was heated at 240°C. MS ion source temperature was 230°C and MS-quadrupole was 150 °C. The electron impact energy was set at 70 eV, and data were collected in the range of 30–500 atomic mass units (amu). Compounds' identification was based on mass spectra by comparison with MS spectral database from Wiley. The integrations were performed with MSDCHEM software [28].

Microorganisms and growth culture

Antimicrobial effects of Lavender essential oil on bacteria-yeast and mold strains were investigated. When choosing the microorganisms, the frequently encountered species in the contaminated creams such as *Candida albicans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Aspergillus brasiliensis* were preferred. The methods used in preparation of reference cultures are given in Table 1 and the working cultures, can be seen in Table 2, prior to inoculation, are given below. The protective activity of Lavender oil is determined via challenge test and according to TS EN ISO 11930:2012 standard which is an international method [53]. According to this method the sample was weighed (g/mL) and the bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa* and the fungi *Candida albicans* and *Aspergillus brasiliensis* were inoculated in the sample at a known level. Following this the samples were incubated in the acclimatization cabin at 25 °C for 28 days. On the 7^{th} and 14^{th} days of incubation and at the end of incubation, samples were analyzed and the number of microorganisms was determined.

Table 1. Preparation of the Reference Stock Cultures.

Reference Strains	Method	Broth Medium Used
Staphylococcus aureus ATCC 6538/ Lot 3221505	Plate	Tryptic Soy Agar
Pseudomonas aeruginosa ATCC 9027/ Lot 3270513	Plate	Tryptic Soy Agar
Candida albicans ATCC 10231/ Lot 8067507	Plate	Sabouraud 4% Dextrose Agar + Supplement
Aspergillus brasiliensis ATCC 16404/ Lot 3175110	Plate	Potato Dextrose Agar

Table 2. Preparation of Working Cultures.

Analysis	Method	Broth Medium Used
Staphylococcus aureus ATCC 6538	Enrichment and Line inoculation in solid broth	Baird-Parker Agar Staphylococcus Selective Agar
Pseudomonas aeruginosa ATCC 9027	Enrichment and Line inoculation in solid broth	Cetrimide Agar Pseudomonas Selective Agar
Candida albicans ATCC 10231	Enrichment and Line inoculation in solid broth	Sabouraud 4% Dextrose Agar + Supplement
Mould- Yeast	Diffusion Plate	Sabouraud 4% Dextrose Agar
Total aerobic mesophilic microorganisms	Diffusion Plate	Tryptic Soy Agar With Polysorbate 80 And Lecithin

RESULTS AND DISCUSSION

HPLC Results

Eleven different phenolic compounds were determined *via* HPLC analysis as it can be seen in Table 3. The antimicrobial phytochemicals are collected in five groups as phenolics, terpenoids-essential oils, alkaloids, lectins-polypeptides and polyacetylenes [29]. Phenolics constitute the largest group among the vegetative antimicrobial agents [30]. The phenolic compounds determined in the lavender extract are gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, p-coumaric acid, ferulic acid, apigenin, rosmarinic acid, and cinnamic acid can be seen in Table 4. Its standard chromatogram is given in Figure 1 and the chromatogram of its extract is presented in Figure 2. It is well known that the polyphenols show goof antibacterial effect [31]. High concentrations of phenolic compounds are toxic both for the plants and the microorganisms [32, 33].

Table 3. Concentration of 11 phenolic acids in Lavender sample.

Sample	Gallic Acid	3,4-dihydroxy benzoic Acid	4-hydroxybenzoic Acid	Chlorogenic Acid	Vanillic Acid	Caffeic Acid	p-Coumaric Acid	Ferulic Acid	Apigenin	Rosmarinic Acid	Cinnamic Acid
Lavender	*0.14	3.37	10.70	7.41	0.67	15.20	5.03	0.18	7.80	10.13	0.98

*All the values in the same line are given as µg/g.

Table 4. LOD values, wavelengths, retention time (RT), and linear regression coefficients (R^2) for phenolic compounds.

	Gallic Acid (1)	3,4-dihydroxy benzoic Acid (2)	4-hydroxybenzoic Acid (3)	Chlorogenic Acid (4)	Vanillic Acid (5)	Caffeic Acid (6)	p-Coumaric Acid (7)	Ferulic Acid (8)	Apigenin (9)	Rosmarinic Acid (10)	Cinnamic Acid (11)
LOD (mg/L)	0.19	0.027	0.036	0.017	0.019	0.019	0.022	0.021	0.010	0.016	0.016
Detection wavelength (nm)	280	280	280	320	320	280	320	320	320	320	280
RT	7.8	12.2	16.9	19.4	21.7	24	29.3	34.7	64.1	66.7	70.7
R^2	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999

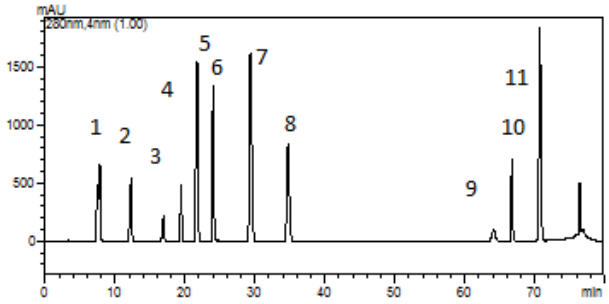

Figure 1. HPLC chromatogram of standard mixtures. 1. Gallic acid, 2. 3,4-dihydroxybenzoic acid, 3. 4-hydroxybenzoic acid, 4. chlorogenic acid, 5. vanillic acid, 6. caffeic acid, 7. p-coumaric acid, 8. ferulic acid, 9. apigenin, 10. rosmarinic acid, 11. cinnamic acid. (Agilent Eclipse C18 column (250*4.6 mm i.d., 5µm), flow rate: 0.8 mL min^{-1}, wavelength: 280 nm)

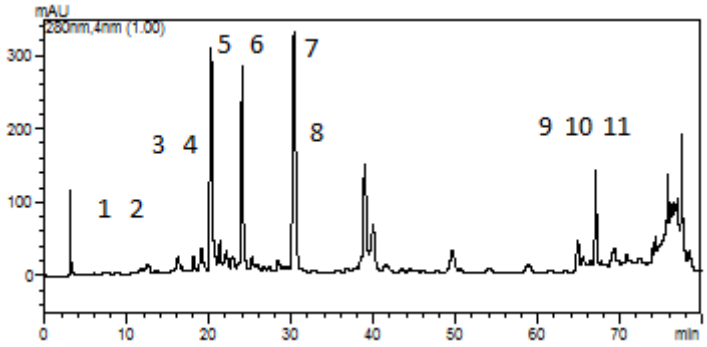

Figure 2. HPLC chromatogram of the sample. 1. Gallic acid, 2. 3,4-dihydroxybenzoic acid, 3. 4-hydroxybenzoic acid, 4. chlorogenic acid, 5. vanillic acid, 6. caffeic acid, 7. p-coumaric acid, 8. ferulic acid, 9. apigenin, 10. rosmarinic acid, 11. cinnamic acid. (Agilent Eclipse C18 column (250*4.6 mm i.d., 5 µm), flow rate: 0.8 mL min^{-1}, wavelength: 280 nm).

GC-MS Results

The Lavender essential oil is reported to be rich in linalyl acetate and linalool in the studies [34, 35]. The results of the analysis that we made also support these findings. Our results are given in Table 5.

Linalool, linalyl acetate, and α-terpineol have the highest ratio with 42.215%, 23.116% and 4.912%, respectively. The results can also be seen in Figure 3 as the chromatogram of the samples.

Lavender oil inhibited the growth of *C. Albicans*. Many researchers have also reported that especially essential oils rich in phenolics, aldehydes, and alcohols are effective in inhibiting pathogenic microorganisms and in preventing degradation [37]. Therefore, phenolic compounds are natural alternative to the synthetic antimicrobials used in the cosmetics, food and pharmaceutical industries [38] and they can be active even at low concentrations [37]. It is reported that linalool and α-terpineol have strong antimicrobial

activity against periodontopathic and cariogenic bacteria [36]. Furthermore, the antibacterial activity of lavender oil and its main components; *i.e.* 1,8-cineol, linalool, linalyl acetate, limonene, α-pinene, and β-pinene have been assayed against the human pathogenic bacteria and they have been successful [46]. These results are consistent with our findings.

Table 5. Essential oil composition of Lavender.

No	Component	RT*	Ratio(%)
1.	α-pinene	4.2	0.028
2.	Camphene	4.9	0.046
3.	β-pinene	5.5	0.019
4.	β-myrcene	6.2	0.341
5.	α-terpinene	6.5	0.007
6.	Limonene	6.7	0.234
7.	1,8 cineol	6.9	0.677
8.	Cymene	7.1	0.955
9.	3-octanone	7.4	0.163
10.	Acetic acid hexyl ester	7.5	0.257
11.	α –terpinolene	7.7	0.156
12.	3-octayl acetate	8.2	0.089
13.	n-hexyl isobutyrate	8.28	0.228
14.	1-hexene	8.3	0.033
15.	1-octen 3 yl acetate	8.5	0.193
16.	3-octanol	8.6	0.071
17.	Butanoic acid hexyl ester	8.9	1.323
18.	Hexyl -2-methyl butanoate	9	0.231
19.	Linalool oxide	9.1	0.592
20.	Linalool	9.8	42.215
21.	Linalyl acetate	10	23.116
22.	Cyclohexanone	10.1	2.053
23.	Neryl acetate	10.3	0.211
24.	Trans caryphyllene	10.4	0.935
25.	Farnesene	10.7	0.28
26.	α-terpineol	11	4.912
27.	Borneol	11.1	3.394
28.	Geranyl acetate	11.2	0.935
29.	Nerol	11.4	4.229
30.	Geraniol	12.2	2.975
31.	Caryophyllene oxide	14.5	4.5
	TOTAL		**95.398**
	Unknown		**4.602**

* RT: Retention time

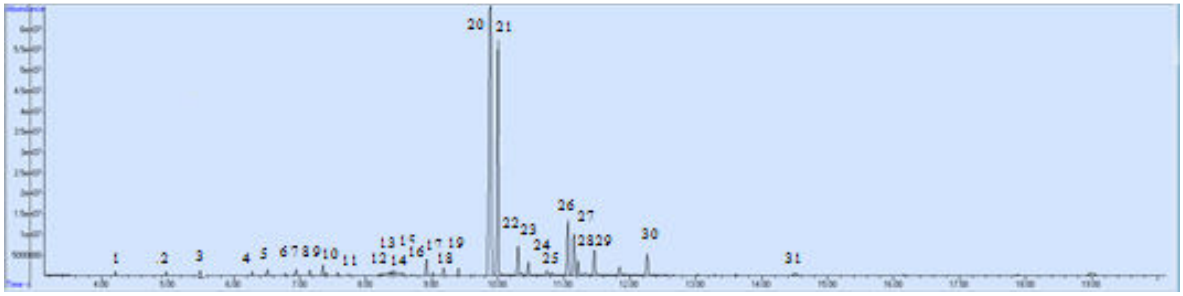

Figure 3. GC-MS Chromatogram of the sample.

Microbiological Results

According to the ISO 11930 standard challenge test was applied on lavender essential oil for 28 days and their logarithmic decreases were evaluated can be seen in Table 6-8.

Table 6. Microbial activity test performed according to the ISO standard.

Parameter	Unit	Results of the Analysis	Standard No	Limit Value
Total aerobic mesophilic microorganism	cfu/g	<1000 cfu/g	ISO 21149	<1000 cfu/g
Candida albicans	cfu/g	Negative	ISO 18416	Negative
Staphylococcus aureus	cfu/g	Negative	ISO 22718	Negative
Pseudomonas aeruginosa	cfu/g	Negative	ISO 22717	Negative
Mold-Yeast	cfu/g	<1000 cfu/g	ISO 16212	<1000 cfu/g

The antimicrobial activity of the essential oil studied on the microorganism strains was found to be pretty high. The results of the total aerobic mesophilic microorganism and Total Mold-Yeast counting, which determine the microbial quality of the oil were found to be coherent with the limit values. Moreover, *Candida albicans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and pathogenic microrganisms were not encountered in the analysis. The microbiologic quality of the product is sufficient for its usage in production of cosmetics can be seen in Table 6-8. In the analysis the ISO standards were applied in the microbiology of cosmetics were used and the analysis were made in an accredited laboratory.

Table 7. Logarithmic Evaluation Table.

Microorganism	1st Hour		7 th Day			14 th Day	28 th Day
	CFU/g	log CFU/g	CFU/g	log CFU/g	log reduction	CFU/g	CFU/g
Staphylococcus aureus ATCC 6538/ Lot 3221505	1.0E+06	6.00	9.8E+01	2.0	4.01	<10	<10
Pseudomonas aeruginosa ATCC 9027/ Lot 3270513	1.2E+06	6.09	1.5E+02	2.2	3.93	<10	<10
Candida albicans ATCC 10231/ Lot 8067507	2.3E+05	5.36	6.5E+01	1.8	3.55	<10	<10
Aspergillus brasiliensis ATCC 16404/ Lot 3175110	1.5E+04	4.18	1.5E+01	1.2	3.00	<10	<10

Many antimicrobial studies were made regarding *L. angustifolia* on various microorganisms [34, 35, 39-48] and our findings supported these results.

Table 8. Result Control Table.

Log decrease values (*Rx*= lg*N*0 - lg*Nx*) required [a]								
Microorganisms	**Bacteria**			***C. albicans***			***A.brasiliensis***	
Sampling Time	**T7**	**T14**	**T28**	**T7**	**T14**	**T28**	**T14**	**T28**
Criteria A	≥3	≥ 3 and NI[b]	≥ 3 and NI	≥ 1	≥ 1 and NI	≥ 1 and NI	≥ 0[c]	≥ 1
Criteria B	Not applied	≥ 3	≥ 3 and NI	Not applied	≥ 1	≥ 1 and NI	≥ 0	≥ 0 and NI

a: In this test, 0,5 log deviation are evaluated as acceptable.
b:NI: No increase in the time since the previous count
c :*Rx*= 0 lg*N*0 = When lgnx (no increase after the first count)

Lavender (*Lavandula angustifolia*) essential oil has been pretty effective on the mentioned microorganisms (Table7-8) and as of the 14th day, they could eliminate contamination completely.

CONCLUSION

The content of the essential oils extracted from *Lavandula angustifolia,* which were grown in Burdur Örtülü locality, was determined via HPLC and GC-MS analysis. Furthermore, the anti-microbial effect of the essential oil *L. angustifolia* was also investigated. To obtain lavandula oil, Clevenger and solvent extraction methods were used. Various phenolic compounds in the extracts were quantitatively determined by HPLC.

Eleven different phenolic compounds, which are gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, p-coumaric acid, ferulic acid, apigenin, rosmarinic acid, and cinnamic acid were determined via HPLC analysis. In a quantitative sense, caffeic acid, rosmarinic acid, and 4-hydroxybenzoic acids were the most abundant phenolic acids in the content in decreasing order. It is known that the phenolic compounds in lavender extract show antimicrobial effect. Thirty-one different volatile compounds were determined by GC-MS analysis. It was found that linalool and linalyl acetate have the highest concentration. Anti-microbial effects of the essential oil of *L. angustifolia* were determined against the most frequently encountered microorganisms in the cosmetics such as *Candida albicans, Staphylococcus aureus, Pseudomonas aeruginosa, Aspergillus brasiliensis.* According to our results it is revealed that the essential oil, *L. angustifolia*, can be used either directly or incorporated into the cosmetics without the necessity for any other extra preservative against the

microrganisms mentioned above. Also, it was found that the essential oil of *L. angustifolia* completely removed the contamination caused by some microorganisms.

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Türkçe Öz ve Anahtar Kelimeler

Fitokozmetik Tür olarak *Lavandula angustifolia* Mill.'in Kimyasal Karakterizasyonu ve Kozmetik Ürünlerde Antimikrobiyal Etkisinin İncelenmesi

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Öz: Burdur-Örtülü'de yetişen *Lavandula angustifolia* türünden elde edilen ekstraktların bileşimi HPLC ve GC-MS analizleriyle belirlenmiştir ve *L. angustifolia*'nın esansiyel yağındaki antimikrobiyal etki araştırılmıştır. *L. angustifolia*'nın kurutulmuş çiçekleri ekstrakte edilmiş ve esansiyel yağı geri kalan kısmından damıtılmıştır. Ekstrakttaki çeşitli fenolik bileşikler HPLC ile kantitatif olarak belirlenmiştir. Kantitatif olarak kafeik asit, rosmarinik asit ve 4-hidroksibenzoik asitler, bu sırada azalacak şekilde, en bol bulunan fenolik asitler olarak bulunmuştur. GC-MS analizinde 31 farklı bileşik belirlenmiştir: Linalool ve linalil asetat en yüksek derişimlere sahiptir. *L. angustifolia*'nin esansiyel yağının antimikrobiyal aktivitesi, kozmetik ürünlerde en çok rastlanan mikroorganizmalara karşı tespit edilmiştir (*Candida albicans, Staphylococcs aureus, Pseudomonas aeruginosa* ve *Aspergillus brasiliensis*). *L. angustifolia*'nın esansiyel yağının 14.günden itibaren mikroorganizmalar tarafından oluşturulan kirliliği tamamen giderdiği görülmüştür. Sonuçlara göre, *L. angustifolia*'nın esansiyel yağının yukarıda bahsedilen mikroorganizmalara karşı başka bir koruyucu madde gereksinimi olmaksızın, kozmetik ürünlerde doğrudan veya dolaylı olarak kullanılabileceği anlaşılmaktadır.

Anahtar kelimeler: *Lavandula angustifolia* Mill.*;* kozmetik ürünler; fenolik bileşikler; esansiyle yağ; antimikrobiyal aktivite**.**

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