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Synergistic antibacterial combination of *Lavandula latifolia* Medik. essential oil with camphor

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Abstract: Combination of various compounds and essential oils for pharmaceutical formulations withdraw attention. In this present study, it was aimed to evaluate the *in vitro* potential synergistic antibacterial effect of *Lavandula latifolia* (spike lavender) essential oil with camphor by using the checkerboard method against the human pathogens; *Staphylococcus aureus* and *Listeria monocytogenes*. Pharmacopoeia quality *L. latifolia* essential oil and racemic camphor were analyzed and verified by GC-FID and GC/MS, simultaneously. *In vitro* antibacterial activity of essential oil and camphor (MIC range: 0.16–20 mg/mL) and standard antimicrobial clarithromycin (MIC range: 0.125–16 µg/mL) were carried out by broth microdilution against *S. aureus* and *L. monocytogenes* standard strains, respectively. Resulting antibacterial effects were evaluated for their fractional inhibitory concentrations (FICs) as antagonistic, additive and synergistic effects. The analytical results showed that the major component of essential oil was linalool (45.2%) and 1,8-cineole (25.6%). Antibacterial effects of essential oil were determined as MIC 1.25–5 mg/mL. As a result of the experiments, *L. latifolia* essential oil–camphor combinations were identified as “synergistic (FIC ≤ 0.5), and additive (0.5 < FIC ≤ 1)” in the respective combinations, suggesting further evaluation for formulations for potential antimicrobial applications in food and pharmaceuticals.

This work is dedicated to Prof. K. Hüsni Can Başer for his 70th Birthday.

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Keywords: antibacterial activity; camphor; checkerboard method; *Lavandula latifolia*; synergistic.

1 Introduction

Lavandula sp. (Lamiaceae) is a valuable essential oil plant crop, which is cultivated around the world mainly for cosmetics and pharmaceutical utilization since centuries [1, 2]. *Lavandula* essential oils are especially used for nervous system stimulation, sedation, tranquilization and stress relieve. In addition, it has useful dermatological uses in the treatment of sunburn and skin rashes, as well as strong antiseptic, and antimicrobial effects [3]. There are 39 *Lavandula* species, which are mostly of Mediterranean origin. However, there are three commercially important species within the genus among other cultivars, producing lavender (*Lavandula angustifolia* Mill. = *L. officinalis* L. = *L. vera* DC), lavandin (*L. intermedia* Emeric ex Loisel. = *L. hybrida* L.) and Spike lavender (*L. spica* = *Lavandula latifolia* Medik.) [4, 5].

L. latifolia Medik. essential oil is one of the medicinal herbal products, which contains as main components 1,8-cineole and linalool, having utilization in cosmetics and pharmaceuticals as well. Linalool has sedative [6, 7] and local anesthetic effects [8]; antimicrobial [9, 10], and insecticidal effects [11, 12]; whereas the main component 1,8-cineole has antispasmodic [13] and antimicrobial [10] properties. 1,8-Cineole is also present in *L. angustifolia* and *L. hybrida*, although in much lower concentrations [14–16].

The study material camphor is a white crystalline monoterpene, generally obtained from the tropical *Cinnamomum camphora* tree, if not from synthetic origin from turpentine. In addition, camphor is found in many essential oils including *Lavandula* sp., *Artemisia* sp., *Rosmarinus officinalis* etc. Camphor is traditionally used to treat colds, pain and inflammation. *In vitro* antiviral, anticancer, anti-inflammatory, analgesic, antispasmodic, nasal decongestant and rubefacient effects are also reported. The use of 3–11% as a topical pain reliever and anesthetic is approved by the FDA. Also, it is well known that the enantiomeric structure of essential oil components such as camphor affects the bioactivity results, each enantiomer

may have different activity and effects. However, overdose may cause toxic effects, especially in children, which urges careful use [17, 18].

The selected microorganisms of the present study; *Staphylococcus aureus* is among the human pathogens causing a wide spectrum of diseases such as respiratory tract infections, endocarditis, arthritis among others [19], whereas *Listeria monocytogenes* is a foodborne pathogen, and its infection may cause brain invasion, listeriosis resulting fewer, diarrhea and muscle aches etc. [20, 21].

Side effects of drugs and antimicrobial resistance caused using excessive and unnecessary antibiotics require the development of new antimicrobials [22, 23]. Recently, efforts to increase antimicrobial activity have gained importance with the combination of essential oils with natural ingredients and standard antibiotics [24, 25].

The aim of this work was to compare the antimicrobial efficacy by using binary combinations of *L. latifolia* essential oil with racemic camphor, alone and in combination using the checkerboard method, against human pathogenic *S. aureus* and *L. monocytogenes* standard strains.

2 Materials and methods

2.1 Materials

Pharma-grade [26] *L. latifolia* essential oil was purchased at Apoth. Bauer & Co., Germany. (±)-Camphor (Ph. Eur., ≥95%) and other chemicals were acquired from Sigma-Aldrich, Merck, Fluka if not otherwise indicated.

2.2 Analytical

2.2.1 Gas chromatography/mass spectrometry (GC/MS): The analyses were carried out using the Agilent 5975 GC-MSD system (Sem Ltd., Istanbul, Turkey). Innnowax FSC column (60 m × 0.25 mm, 0.25 µm film thickness) was used with helium as carrier gas (0.8 mL/min). GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min and kept constant at 220 °C for 10 min and then programmed to 240 °C at a rate of 1 °C/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250 °C. Mass spectra were recorded at 70 eV, where the mass range was from m/z 35 to 450.

2.2.2 Gas chromatography-flame ionization detector (GC-FID): The GC analyses were carried out using an Agilent 6890 N GC system (Sem Ltd., Istanbul, Turkey). Flame ionization detector (FID) temperature was set to 300 °C. To obtain the same elution order with the GC/MS system, simultaneous auto-injection was done on a duplicate of the same column applying the same operational conditions. Relative percentages (%) of the separated compounds were calculated from FID chromatograms.

Table 1: *Lavandula latifolia* essential oil analysis, relative percentages (%).

RRI	Compounds	%	IM
1032	α -pinene	2.4	RRI, MS
1076	Camphene	0.5	RRI, MS
1118	β -pinene	2.0	RRI, MS
1174	Myrcene	0.4	RRI, MS
1203	Limonene	1.3	RRI, MS
1213	1,8-cineole	25.6	RRI, MS
1246	(Z)- β -ocimene	0.1	MS
1255	γ -terpinene	tr	RRI, MS
1266	(E)- β -ocimene	0.1	MS
1280	<i>p</i> -cymene	0.6	RRI, MS
1532	Camphor	12.2	RRI, MS
1553	Linalool	45.2	RRI, MS
1565	linalyl acetate	2.3	RRI, MS
1612	β -caryophyllene	1.0	RRI, MS
1611	terpinen-4-ol	0.2	RRI, MS
1617	lavandulyl acetate	0.1	RRI, MS
1684	Isoborneol	0.5	MS
1706	α -terpineol	1.5	RRI, MS
1719	Borneol	0.9	RRI, MS
1733	neryl acetate	0.3	RRI, MS
1795	geranyl acetate	tr	RRI, MS
1808	Nerol	0.2	RRI, MS
1857	Geraniol	0.4	RRI, MS
2008	caryophyllene oxide	0.2	RRI, MS
Total		97.9	

RRI: Relative retention indices calculated against *n*-alkanestr. tr: Trace (< 0.1 %). IM: Identification method based on the relative retention indices (RRI) of authentic compounds on the HP Innnowax column; MS, identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data.

2.2.3 Identification of the components: Identification of the essential oil components was carried out by comparison of their relative retention times (RT) with those of authentic samples or by comparison of their relative retention index (RRI) to that of a series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS Library, MassFinder 4 Library) and in-house “Başer Library of Essential Oil Constituents” libraries built up by genuine compounds and components of known oils as well as MS literature data was used [25, 27]. Results are given in Table 1.

2.3 Antibacterial activity

2.3.1 Bacterial strains: *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 19111 were obtained from the American Type Culture Collection (ATCC). All microorganisms were stored at –85 °C in 15% glycerol prior to the experiments. Bacterial strains were refreshed on Mueller Hinton Agar (MHA, Merck) plates at 37 °C. Strains were inoculated and standardized versus McFarland No: 0.5 turbidimetrically in sterile saline (0.85%) to 5×10^3 CFU/ per well in Mueller Hinton Broth (Sigma-Aldrich) [29].

2.3.2 *In vitro* antibacterial activity by microdilution method: The lavender essential oil and camphor were evaluated for their potential antibacterial activity by using the microdilution broth susceptibility assay [27, 28, 30]. Essential oil and camphor dilution series were prepared in 96-well microtiter plates at a concentration range of 0.16–20 mg/mL. About 100 µL of 1:100 diluted bacterial suspensions were then added to each well. The last row containing medium with microorganism was used as negative control and the well containing only medium served as a positive growth control. Clarithromycin (0.125–16 µg/mL) was used for standard antimicrobial. The microplates were covered with airtight films just before they were placed in the incubator. After incubation at 37 °C for 24 h, for staining of viable microorganisms, 20 µL of resazurin of 0.01% was added to all the plates. The first blue well was determined as the minimal inhibitory concentration (MIC, mg/mL). All experiments were repeated in triplicate, and average MICs were reported in Table 2.

2.3.3 *In vitro* synergistic antibacterial activity by checkerboard method: Serial dilutions of the samples were prepared. About 50 µL dilutions of the first test substance (*L. latifolia* essential oil) were added to the microtiter plate from A to H, while 50 µL dilutions of the other test substance (camphor) were added vertically from 1 to 8. About 100 µL 1:100 diluted bacterial suspensions were then added to each well. After 24 h of incubation at 37 °C, 20 µL of 0.01% resazurin solution was added onto the Petri dish for visualization. The microplates were covered with airtight films just before they were placed in the incubator. The incubation was continued for 3 h at 37 °C. The determined MIC values were used for fractional inhibition concentration (FIC) calculation following the formula:

$$FIC(A + B) = (MIC_{AB}/MIC_A) + (MIC_{AB}/MIC_B).$$

The results were interpreted as synergistic (FIC < 0.5), additive (0.5 ≤ FIC ≤ 1), indifference (1 < FIC ≤ 4) or antagonism (FIC > 4) [25, 31], as shown in Table 2.

Table 2: Minimum inhibitory concentrations (MIC, mg/mL) and fractional inhibitory concentrations (FIC) of essential oil and camphor combinations determined by checkerboard microdilution method.

Standard strains						FIC	Effect
	Clarithromycin (µg/mL)	Essential oil	Camphor	Combination of essential oil	Combination of camphor		
<i>L. monocytogenes</i>	0.125	1.25	2.5	0.62	0.31	0.6	additive
				0.62	0.62	0.7	additive
				0.31	0.62	0.5	additive
				0.16	0.62	0.4	synergistic
				0.08	0.62	0.3	synergistic
<i>S. aureus</i>	0.25	2.5	5	1.25	0.31	0.5	additive
				1.25	0.62	0.6	additive
				1.25	1.25	0.7	additive
				1.25	2.5	1.0	additive
				0.62	1.25	0.5	additive
				0.62	2.5	0.7	additive
				0.31	1.25	0.4	synergistic
				0.31	2.5	0.6	additive
				0.16	2.5	0.6	additive
				0.08	2.5	0.5	additive

3 Results and discussion

3.1 Chemical components of *L. latifolia* essential oil

For the verification of the pharmacopoeia quality, the essential oil was analyzed by both GC/FID and GC-MS, simultaneously. A total of 24 of the main components were detected in *L. latifolia* essential oil, with a ratio of 97.9%. In this present study, the major compounds of the spike lavender essential oil were identified as linalool (45.2%), 1,8-cineole (25.6%) and camphor (12.2%) confirming the quality, which confirms the Ph. Eur. 9.0 [26] and are listed in Table 1.

According to previous literature, major compounds of *L. latifolia* essential oils are reported as linalool 41.0–43.2%, camphor 12.5–13.0%, 1,8-cineole 11–27% [32, 33]. In a recent study, chemical analyses of spike lavender essential oil from Spain were reported, where linalool (32.3%), 1,8-cineole (11.7%) and camphor (12.4%) were determined as main components [34]. In the study reported by Carrasco et al. 2016, linalool (35–51%), 1,8-cineole (26–32%) and camphor (10–18%), respectively, were identified as the main components for *L. latifolia* essential oil [35].

3.2 Antimicrobial activity

Initially, antibacterial activity of the essential oil, camphor (MIC range: 0.16–20 mg/mL) and standard antimicrobial

clarithromycin (MIC range: 0.125–16 µg/mL) were determined by *in vitro* microdilution method against *S. aureus* and *L. monocytogenes*, respectively. The results of the antibacterial activity as MIC values were listed in Table 2. Accordingly, in this method, essential oil and camphor were effective at 2.5 and 5 mg/mL against *S. aureus* and 1.25 and 2.5 mg/mL against *L. monocytogenes* pathogen, respectively.

The antimicrobial effect of the lavender essential oil was previously reported, where the agar dilution results showed MIC values 0.2 and >0.8 % (vol/vol) against *S. aureus* and *L. monocytogenes* strains, respectively [36]. The inhibitory activity of *L. latifolia* essential oil against *S. aureus* and *L. monocytogenes* strains was reported in the concentration range of 0.5–2 µL/mL [37]. In another publication, the effect of *L. latifolia* essential oil vapor against *S. aureus* showed no effect at a test concentration of 500 µL/L [38].

Overall, 64 different essential oil + camphor combinations were evaluated against *S. aureus*, and *L. monocytogenes* using the checkerboard microdilution method. Results of combination tests with the checkerboard assay are shown in Table 2. Synergistic effects were observed from 0.16 + 0.62 and 0.08 + 0.62 (mg/mL) essential oil + camphor combinations against *L. monocytogenes* and 0.31 + 1.25 (mg/mL) against *S. aureus*. In addition to the synergistic effects, additive effects were observed against the particular studied microorganisms.

De Rapper and coworkers (2013) analyzed the antimicrobial activity of *L. angustifolia* essential oil in combination with other 45 essential oils against pathogenic microorganisms like *S. aureus*, *Pseudomonas aeruginosa* and *Candida albicans*, and reported that the combinations showed 26.7% synergistic, and 48.9% additive effects [39]. Magi et al. 2015 evaluated the *in vitro* antibacterial activity of *L. angustifolia*, *Origanum vulgare*, *Thymus vulgaris*, *Mentha piperita*, and *Melaleuca alternifolia* essential oils; as well as carvacrol, and synergy of carvacrol and erythromycin, against 32 clinical, erythromycin-resistant groups of *Streptococci* [40]. Stevic et al. 2014 determined antifungal synergistic effects, with lavender–rose oil and lavender–oregano oil against *Fusarium subglutinans* and *F. semitectum* [41].

Camphor was present in the tested *L. latifolia* essential oil, in relatively high amounts. The combination of *L. latifolia* essential oil and racemic camphor indeed increases the relative amount, which may have an impact to the antimicrobial effects. To confirm the combination effects of camphor on an essential oil, more detailed work is needed such as using an essential oil without camphor content.

As a result, additive and synergistic effects of *L. latifolia* essential oil and the monoterpene camphor were

demonstrated for the first time, with an overall success rate of 23.4 + 4.7%, respectively. The results also suggested that approx. 72% of the random mixtures lead to “indifferent activity” outcomes. Essential oil + camphor combinations were identified as “synergistic (FIC≤0.5) and additive (0.5 < FIC≤1)” in the respective combinations, suggesting a positive impact on antimicrobial activity holding promise for future in-depth formulation studies.

4 Conclusion

One of the current approaches toward synergic antimicrobial combination evaluation is the *in vitro* checkerboard method used in this present study. As observed in the present study, *L. latifolia* and camphor combinations were relatively more inhibitory against the pathogens *L. monocytogenes* and *S. aureus*, respectively. For safe future utilization in formulations, more detailed *in vitro* and *in vivo* toxicological evaluations are suggested and needed.

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