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Antimicrobial activity of European Pharmacopoeia quality essential oil combinations against oral pathogens

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ABSTRACT

This study evaluates the antimicrobial activity of European Pharmacopeia quality essential oils from *Foeniculum vulgare* Mill., *Lavandula angustifolia* Mill., *Carum carvi* L., and *Pinus mugo* Turra in mouthwash formulations with various combinations. These essential oils are ethnobotanically recognized for treating throat infections. Antimicrobial testing was conducted using microdilution and disc diffusion methods against oral pathogens. The mouthwash formulations were created as binary and quadruple combinations of the four oils. Formulations contains essential oils in the range of 0.5-0.55 mg/mL. Among the seven combinations tested, the most potent were the *F. vulgare* - *P. mugo* (25 mm zone diameter) and *L. angustifolia* - *C. carvi* (30 mm zone diameter) blends against *Streptococcus mitis*. While none of the formulations were effective against *Moraxella catarrhalis*, all were generally effective against *S. mutans*. Consequently, it is suggested that the mouthwash formulations developed with these essential oils may be particularly useful in preventing dental caries.

Keywords: mouthwash, disc diffusion, essential oil, European Pharmacopeia

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INTRODUCTION

The oral cavity is considered one of the most extensively studied ecosystems in the human body. The oral cavity is highly susceptible to infections and various pathologies, with common issues including dental diseases, periodontitis, oral mucosa infections, and oral cancer[1].

Mouthwashes play a key role in reducing microbial plaque, which helps in maintaining oral hygiene[2]. Mouthwashes are oral solutions designed to rinse the mouth, eliminate bacteria, act as astringents, deodorize the oral cavity, and provide therapeutic benefits by alleviating infections. The efficacy of mouthwashes in controlling microbial plaque is important, but they should also be designed to offer a pleasant mouthfeel and flavor to encourage consistent use and consumer preference. They often contain antiseptics used for treating such infections[3]. Mouthwashes have the potential to function as targeted therapeutic agents by addressing either the host's inflammatory responses or specific pathogens. By focusing on pro-inflammatory pathways or specific bacterial targets, mouthwashes could effectively reduce inflammation and alleviate oral disease symptoms[4]. Many strategies focus on the biological activities of alternative natural products due to the increased microbial resistance of common antibiotics[5].

Essential oils in mouthwashes are effective against oral pathogens[6]. *In vitro* studies showed that an essential oil blend can more effectively eliminate *Staphylococcus aureus* and *Streptococcus* biofilms on hydroxyapatite discs than chlorhexidine[7]. Additionally, essential oils were reported to decrease plaque and bleeding scores when used by tooth brushing[8]. Additionally, it is well-established that essential oils possess antimicrobial effects on the gingiva while exhibiting low toxicity. It is highly significant in terms of enabling the development of new pharmaceutical formulations for oral hygiene[2].

There are several reports on antimicrobial activity of *Foeniculum vulgare* Mill. (fennel, bitter fennel oil) essential oil that belongs to Lamiaceae family[9,10]. Essential oil contains major 55.0 to 75.0% anethole according to European Pharmacopeia. Fennel essential oil known, along with active compounds within it, has shown strong inhibitory effects *in vitro* against a diverse range of bacterial and fungal pathogens. *Lavandula angustifolia* L. of the Lamiaceae family, is uses against insomnia and mental stress in the EMA monographs[11,12]. Also, previous studies demonstrated that lavender essential oil a wide range of bioactivities, including antibacterial, antifungal, antioxidant, and anti-inflammatory[10,13,14]. Lavender essential oil includes linalool (20-50%) and linaly acetate

(25-46%) as major components according to EMA monographs[12]. Caraway (*Carum carvi* L.), which are members of the Apiaceae family, widely used to flavor foods, enhance fragrances, and in medical preparations. Specifically, *C. carvi* essential oil is commonly found in liqueurs, mouthwashes, toothpastes, soaps, and perfumes due to its strong antimicrobial effects[15,16]. The main components of essential oil were reported limonene and carvone[17]. *Pine mugo* Turra, also named mountain pine, studies were examined the chemical composition of dwarf pine essential oil. The major components of the mountain pine essential oil were found as obtained δ-3-carene, α-pinene, β-phellandrene previously[18].

In this study, the potential antimicrobial effects of 4 different (*F. vulgare, L. angustifolia, C. carvi, P. mugo*) European Pharmacopoeia quality essential oils were evaluated. The *in vitro* antimicrobial activity of essential oils mouthwash formulations consisting of a blend in various proportions were studied. To the best of our knowledge, this is the first study on mouthwashes composed of seven different combinations of *Foeniculum vulgare, Lavandula angustifolia, Carum carvi*, and *Pinus mugo* essential oils.

METHODOLOGY

Materials

All essential oils were obtained from Caesar & Loretz GmbH (Germany). Antimicrobial test materials and excipients of the mouthwash formulations (sodium chloride, sodium bicarbonate, sodium saccharin and ethanol) were purchased from Sigma Aldrich (Germany).

Antimicrobial activity

The *in vitro* antibacterial activity was determined using the broth microdilution assay following the methods according to the Clinical and Laboratory Standards Institute (CLSI) to determine the minimum inhibitory concentrations (MIC)[19]. *Staphylococcus aureus* ATCC 6538, *Streptococcus mitis* NCIMB 13770, *Moraxella catarrhalis* ATCC 23245 and *Streptococcus mutans* ATCC 25125 strains were grown in Mueller Hinton Broth (MHB, Merck, Germany) in aerobic conditions at 37°C for 24 h. All microorganisms were adjusted to 1×10^8 CFU/mL using McFarland No: 0.5 in sterile saline (0.85%) solution. Stock solutions and serial dilutions of the test samples were prepared in dimethyl sulfoxide (DMSO). The minimum non-reproductive concentration was reported as minimum inhibitory concentration (MIC, as μg/mL). The MIC was calculated and reported as the mean of three repetitions compared to positive standards.

In addition, the *in vitro* antimicrobial activity of formulations was evaluated using the disc diffusion method following the methodology described by the Clinical and Laboratory Standards Institute (CLSI). The same human pathogenic strains were used here as well. The inoculation of the pathogens was performed using Mueller Hinton Broth (MHB, Merck, Germany) at 37°C under aerobic conditions for 24 h and standardized to 1×10^8 CFU/mL using McFarland No: 0.5 in sterile saline (0.85%). The mouthwash sample's stock solution was prepared in dimethyl sulfoxide (DMSO) at 10 mg/mL concentration, and the antibacterial evaluation was performed in triplicates[20], where the results were reported as average values.

Mouthwash formulations

The mouthwashes were prepared using combinations of the essential oils. Initially, the mouthwash solutions were formulated using 5.0-5.5% of essential oil. As sweetener saccharine sodium was applied. Furthermore, the essential oils were weighed and dissolved in ethanol while sodium chloride and sodium bicarbonate were added gradually using a mechanical stirrer (500 rpm, 30 minutes), respectively. The combination blend was filtered, and the volume of the filtrate was completed to 10 mL by using distilled water. No preservative was added since the mouthwashes included high content of ethanol (>15%), as well as essential oils (Table 1)[20].

Table 1. The mouthwash formulations containing the essential oils (EO)

Formulation code	*F. vulgare* EO (%)	*L. angustifolia* EO (%)	*C. carvi* EO (%)	*P. mugo* EO (%)	Sodium Chloride (%)	Sodium Bicarbonate (%)	Sodium Saccharine (%)	EtOH (%)	Distilled water
MF1	2.5	2.5	-	-	0.1	0.05	0.001	60	q.s. 10 mL
MF2	2.5	-	2.5	-	0.1	0.05	0.001	60	q.s. 10 mL
MF3	2.5	-	-	2.5	0.1	0.05	0.001	60	q.s. 10 mL
MF4	-	2.5	2.5	-	0.1	0.05	0.001	60	q.s. 10 mL
MF5	-	2.5	-	2.5	0.1	0.05	0.001	60	q.s. 10 mL
MF6	-	-	2.5	2.5	0.1	0.05	0.001	60	q.s. 10 mL
MF7	2.5	1	1	1	0.1	0.05	0.001	60	q.s. 10 mL

RESULTS and DISCUSSION

In this study, we report on the antibacterial activity of individual essential oils and combined mouthwash formulations. Seven different formulations exhibited the same amount of the excipients, however, in different concentrations of essential oils.

The essential oil of *F. vulgare* used in the study should contains at more than 55.0% anethole, 12.0% fenchone, and less than 6.0% estragole, according to Ph. Eur. monograph. The analysis study of the composition of essential oil of *F. vulgare*, cultivated from different places, reported predominant compounds estragole (60.01%-35.33%), anethole (22.15%-52.27%), and fenchone (6.50%-4.32%)[21]. The main components of the *L. angustifolia* essential oil obtained through steam distillation are monoterpenes such as linalool (20-50%) and linalyl acetate (25-46%)[22]. In the analysis of volatile components of *Lavandula aetheroleum* of main components was identified linalool (20.0-45.0%) and linalyl acetate (20.79-39.91%). According to the Ph. Eur., *C. carvi* essential oil should contain 50-65% carvone, 30-45% limonene, and a maximum of 2.5% of trans-dihydrocarvone and trans-carveol, respectively. The identification of the major components of *C. carvi* essential oil studies[23,24] was found to be consistent with the Ph. Eur. Essential oil of *C. carvi* obtained from the needles and twigs is used. This essential oil consists of monoterpene hydrocarbons such as α-pinene, δ-3-carene, myrcene, limonene, according to Ph. Eur. The phytochemical analysis studies showed that the major components were consistent with in the pharmacopeia[25,26].

In the present study, the antimicrobial activity of 4 different essential oils of pharmacopoeia quality at 1 mg/mL concentration was evaluated individually using the broth two-serial dilution method against oral pathogens, as shown Table 2. According to the result, essential oils at >1000 μg/mL concentration were comparatively ineffective against several oral pathogens as compared to the control group.

Table 2. Antimicrobial activity of Pharmacopeia quality essential oils

Essential oils	Minimum inhibitory concentration (mg/mL)			
	S. aureus	*S. mitis*	*M. catarrhalis*	*S. mutans*
F. vulgare	>1000	>1000	>1000	>1000
L. angustifolia	>1000	>1000	>1000	>1000
C. carvi	>1000	>1000	>1000	>1000
P. mugo	>1000	>1000	>1000	>1000
Tetracycline	8	0.5	8	0.5

Anethole was reported as the major component of *F. vulgare* essential oil, and its antimicrobial activity was found to be of low efficacy[27]. In contrast, the antimicrobial activity of *F. vulgare* essential oil was tested against various microorganisms, and the MIC value against *S. aureus* was found at concentration 250 mg/mL. Additionally, this study reported that the main components of essential oil were α-pinene, estragole, and β-pinene[28]. The results of this study differ when compared to our findings. The differences in the MIC values observed for the same microorganism could be attributed to the varying percentages of the compounds in the essential oils.

The antimicrobial effect of *L. angustifolia* essential oil was reported against human pathogens. Several studies have shown that *Lavandula* essential oil possesses significant antibacterial properties against a wide range of microorganisms. It was showed that formulations containing lavender essential oil demonstrate a stronger antimicrobial activity against *S. aureus*[29]. Other studies showed that *C. carvi* seems to have notable antibacterial activity[23,30,31]. Moreover, *P. mugo* essential oil was identified with outcomes varying based on the specific microbial pathogens targeted[26,32,33].

The oral cavity provides space for the colonization by various microorganisms that predominant components of microflora. The majority of the oral bacterial infections are polymicrobial so the combination of antimicrobial molecules should be combined. Currently, there is an increasing demand for mouthwashes formulated with combinations of essential oils. Studies demonstrated the effectiveness of Listerine® mouthwash, which is available in a combination of essential oils[34].

The antimicrobial effects of the formulations against four oral pathogens were determined using the disc diffusion test. As a result of, it was determined that the combination of *L. angustifolia-C. carvi* provided a selective antimicrobial effect against *S. mutans* than other combinations (Table 3). Additionally, the combination of *F. vulgare-P. mugo* was effective against *S. mutans* bacteria. According to the experimental results, mouthwash combination of *F. vulgare-P. mugo, L. angustifolia-C. carvi,* and quadruple combination (*F. vulgare-L. angustifolia-C. carvi-P. mugo*) showed that the combination has a significantly antimicrobial effect (Figure 1). In the evaluation of the three combinations effective against *S. mutans*, the percentage concentrations of essential oils in the formulations are of particular interest. Both the *F. vulgare-P. mugo* and *L. angustifolia-C. carvi* formulations contain 0.25 mg/mL each of essential oil. The quadruple combination contains 0.25 mg/mL of *F. vulgare*, while the other essential oils are present at 0.1 mg/mL each. The results show that the effectiveness of the *L. angustifolia*, *C. carvi*, and *P. mugo* essential oils decreases at lower doses.

Table 3. Growth of inhibition zones of mouthwash formulations (in mm)

	S. mitis	***M. catarrhalis***	***S. mutans***	***S. aureus***
F. vulgare- L. angustifolia	18	-	17	8
F. vulgare-C. carvi	14	-	12	10
F. vulgare-P. mugo	12	-	25	7
L. angustifolia-C. carvi	12	-	30	8
L. angustifolia-P. mugo	14	-	17	8
C. carvi-P. mugo	10	-	18	8
F. vulgare-L. angustifolia C. carvi- P. mugo	19	-	22	9
Tetracycline	24	19	22	20

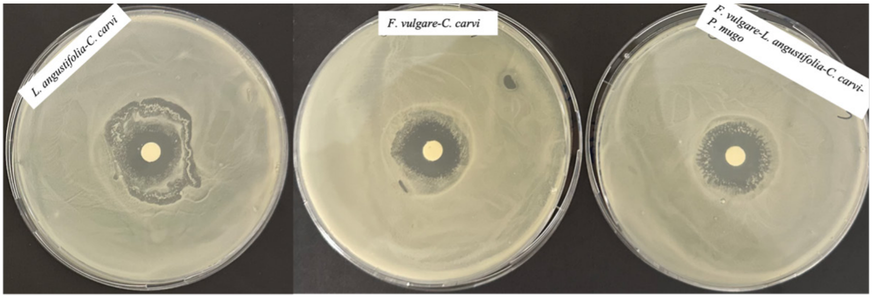

Figure 1. Disk diffusion test results against *S. mutans*

In the combination study of lavender essential oil with *Artemisia herba alba* and *Rosmarinus officinalis* essential oils, a synergistic effect was observed against the tested microorganisms[35]. A combination study of lavender and fennel essential oils also revealed an additive effect against *S. aureus*[10]. In our study, the combination of lavender and fennel essential oils showed greater effectiveness against *S. mitis* than the positive control in antimicrobial activity. Based on these findings, it can be inferred that the formulations exhibited a synergistic effect resulting from the various combinations. Essential oils used in the combinations found to have antibacterial activity exhibited a synergistic effect against *S. mutans*. Furthermore, it can be concluded that the combinations exhibiting a synergistic effect could be considered for use as mouthwashes. The first report on the antimicrobial activity of mouthwashes prepared from different combination of *F. vulgare*, *L. angustifolia*, *C. carvi*, and *P. mugo* essential oils. As a result, it is thought that mouthwash formulations developed with these pharmacopeia quality essential oils can be used especially in preventing dental caries.

STATEMENT OF ETHICS

Ethics approval is not required in this study, as no human and experimental animal samples is involved.

CONFLICT OF INTEREST STATEMENT

Declared none.

AUTHOR CONTRIBUTIONS

Data collection: A. E. Karadağ, D. Kırcı, S. E. Kahya; design of the study: A. E. Karadağ, D. Kırcı, F. Demirci, S. E. Kahya; analysis and interpretation of the data: A. E. Karadağ, S. E. Kahya; drafting the manuscript: A. E. Karadağ, S. E. Kahya.

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