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Comparative *in vitro* evaluation of the antimicrobial properties of essential oils from *Lamiaceae*, *Cistaceae*, and *Asteraceae* families against *Enterococcus faecalis*

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Abstract

The urgent need for effective antimicrobial agents to combat bacterial infections and the growing problem of antibiotic resistance has driven research into natural alternatives. This study investigates the antimicrobial properties of thirteen essential oils (EOs) from the *Lamiaceae, Cistaceae,* and *Asteraceae* families against the pathogen *Enterococcus faecalis*. The chemical composition of these EOs was analysed using gas chromatography-mass spectrometry (GC-MS), while their antimicrobial activities were assessed through the disk diffusion method. Additionally, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined for each EO. Our results revealed that oregano, thyme, and dittany EOs exhibited strong antimicrobial effects, with inhibition zones ranging from 19.5 to 50 mm and MIC values as low as 0.15 mg/mL, alongside MBC values of 0.30 mg/mL. In contrast, peppermint, rosemary, rockrose, and immortelle EOs showed considerably weaker antimicrobial efficacy, with larger MIC and MBC values and smaller inhibition zones. These findings underscore the potential of oregano, thyme, and dittany EOs as promising antimicrobial agents.

Keywords: Essential oils; *Enterococcus faecalis*; Minimum inhibitory concentration (MIC); Antibiotic; Antimicrobial activity

Abbreviations: CDC: Centers for Disease Control and Prevention; EO: essential oil; PBS: phosphate-buffered saline; OD: optical density; DMSO: dimethyl sulfoxide; MSD: mass selective detector; EI: electron impact; TSB: Tryptone Soya Broth; TSA: Tryptone Soya Agar; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration;

Introduction

The EOs industry has experienced significant growth in recent years, reflecting a sustained trend that is expected to continue. For centuries, EOs derived from plants have been integral to human culture, used as spices, medicinal products, and aromatic agents due to their unique organoleptic properties and therapeutic benefits. These oils are complex mixtures of secondary metabolites, primarily composed of low molecular weight terpenes, such as monoterpenes and sesquiterpenes [1]. The chemical composition of EOs varies widely, influenced by factors such as plant genotype, environmental conditions, the specific plant part used, harvest timing, and production methods. This variation directly impacts their antimicrobial properties, which are well-documented but closely tied to the specific components of each oil [2].

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Among the many EOs studied, oregano and thyme have consistently demonstrated potent antibacterial activity against a wide variety of pathogenic bacteria. This effectiveness is largely due to the presence of key monoterpenes, such as carvacrol and thymol, which are known for their strong antimicrobial effects [3,4]. These findings have expanded the applications of EOs beyond traditional uses in cosmetology and aromatherapy, positioning them as potential antimicrobial agents and natural preservatives in the pharmaceutical and food industries.

Over the past two decades, *enterococci* have garnered substantial attention within the scientific community. They are prevalent in diverse environmental and clinical contexts, serving as indicators for gastrointestinal and dermatological infections [5]. *Enterococci* have become particularly significant in hospital environments, where the widespread use of antibiotics has led to the selection of highly resistant strains. These bacteria are notably resistant to common antibiotics, including ampicillin and vancomycin, which complicates treatment options and increases the risk of severe health outcomes, particularly in immunocompromised patients [6]. *Enterococcus faecalis*, one of the most studied species within this genus, is predominantly found in the gastrointestinal tract, where it forms part of the normal flora. However, it is also present in various foods, particularly in meats and fermented products, as well as in municipal sewage and water [5]. Nevertheless, the identification of this pathogen extends beyond those boundaries, with a growing body of reports emphasizing its presence in the oral cavity. Recent research has highlighted the presence of *E. faecalis* in the oral cavity, where it has been associated with conditions such as periodontitis and dental caries. Despite not being a native resident of the oral microbiome, *E. faecalis* has been frequently identified in these infections due to its ability to survive within biofilms and establish synergistic relationships with other oral bacteria [7].

This pathogen's presence in both food and the oral cavity underscores the interconnectedness between foodborne pathogens and dental diseases, highlighting the dual role that EOs could play in both food preservation and oral care [8]. The Centers for Disease Control and Prevention (CDC) have identified vancomycin-resistant *E. faecalis* as a serious pathogen, with reports of 54500 cases and 5400 associated deaths [9]. The increasing prevalence of antibiotic-resistant *E. faecalis* presents significant challenges in clinical settings, making it more difficult to manage infections and resulting in higher healthcare costs.

In this study, we aim to provide a comprehensive evaluation of the antimicrobial potential of thirteen essential oils against *Enterococcus faecalis*, a bacterium of considerable clinical importance. Our research is particularly innovative in its detailed assessment of the antimicrobial efficacy of lesser-studied EOs, such as those from rosemary, sage, and helichrysum, against *E. faecalis*. By addressing this critical gap in the literature, our study contributes valuable insights into the potential use of EOs as alternative antimicrobial agents. These findings have broad implications, extending beyond the medical and dental fields to potentially benefit the food industry. This preliminary investigation serves as a strategic foundation for future research on the application of EOs in combating antibiotic-resistant strains.

Materials and Methods

Essential Oils

The essential EOs utilized in this study encompass a diverse range of botanical sources (Table 1). All EOs were produced with steam distillation from Greek organic crops. The oils were meticulously stored at 4 °C.

Table 1: Essential oil catalogue with Plant Family, the Systematic Name, the Plant Part, and the Manufacturers (Vessel Essential Oils or Myrtis, Thessaloniki, Greece)

Essential Oil	Plant Family	Latin Name	Plant Part	Manufacturers
P.A	*Lamiaceae*	*Mentha × piperita*	Aerial parts	Vessel EOs
P.B	*Lamiaceae*	*Mentha × piperita*	Aerial parts	Myrtis
O.A	*Lamiaceae*	*Origanum vulgare ssp. hirtum*	Aerial parts of flowering plant	Myrtis
O.B	*Lamiaceae*	*Origanum vulgare*	Aerial parts of flowering plant	Vessel EOs
O.C	*Lamiaceae*	*Origanum vulgare (liquid fire/91.3% carvacrol)*	Aerial parts of flowering plant	Vessel EOs
DIT.	*Lamiaceae*	*Origanum dictamnus*	Aerial parts of flowering plant	Vessel EOs
R.A	*Lamiaceae*	*Rosmarinus officinalis*	Branches and Flowering tops	Vessel EOs
R.B	*Lamiaceae*	*Rosmarinus officinalis (ct-1,8-cineole)*	Branches and Flowering tops	Vessel EOs
TH.	*Lamiaceae*	*Thymus vulgare*	Flowering tops	Vessel EOs
SG.	*Lamiaceae*	*Salvia fruticosa Mill.*	Flowering tops and leaves	Myrtis
LVN.	*Lamiaceae*	*Lavandula angustifolia Mill.*	Flowering tops	Myrtis
RCS.	*Cistaceae*	*Cistus ladanifer*	Leaves	Vessel EOs
HLC.	*Asteraceae*	*Helichrysum italicum*	Flowering tops	Vessel EOs

[1]Data are the mean diameter of the inhibitory zones (mm) ± standard deviation of nine repeats, in six different concentrations (%) of EOs. The diameter of the paper disk (mm) is included. Note: OMZ: Over-Maximum Zone (>50 mm); Plant abbreviations: P.A: Peppermint A; P.B: Peppermint B; O.A: Oregano A; O.B: Oregano B; O.C: Oregano C; DIT.: Dittany; R.A: Rosemary A; R.B: Rosemary B; TH.: Thyme; SG.: Sage; LVN.: Lavender; RCS.: Rockrose; HLC.: Helichrysum

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Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

For the EOs whose chemical profile was not provided by the company, GC–MS analysis was performed on a 6890 Network GC System equipped with a 5975B mass selective detector (MSD) from Agilent Technologies (Santa Clara, CA, USA) in the electron impact (EI) mode of 70eV, located at the central Laboratory of Instrumental Analysis of the University of Patras. The capillary column was an HP-5MS (30 m × 0.25 mm, 0.25 μm) with helium as carrier gas with flow 1 mL/min, in a spitless mode. Samples were diluted 1:40 v/v with ethyl acetate and then 1 μL of each sample was injected via a microsyringe. Four different temperature programs were developed for each EO sample. In particular, the oven temperature program for the peppermint B EO was: 60 ˚C - 120 ˚C (6 ˚C/min), then 120 ˚C - 180 ˚C (6 ˚C /min) and finally, 180 ˚C - 280 ˚C (12 ˚C/min), 280 ˚C for 3 minutes. For the lavender EO, the temperature sequence was: 40 °C - 80°C (3 ˚C/min), 80 ˚C for 2 minutes, 80 ˚C - 110°C (1.5 °C /min), 110 ˚C for 1 minute, 110 ˚C - 130 °C (4 °C/min), 130 °C for 1 minute, 130 ˚C - 150 °C (4 °C/min), 150°C - 260 °C (8°C/min), 260 °C for 2 minutes. For the oregano A EO, the temperature was kept at 50 °C for 2 minutes and then, 50 ˚C - 80 °C (2 °C/min), 80 ˚C for 2 minutes, 80 ˚C - 200 °C (5 °C/ min), 200 ˚C for 2 minutes, 200 ˚C - 280 °C (15 °C/min) and 280 ˚C for 3 minutes. Lastly, for the sage EO analysis, oven temperature started from 50 ˚C and raised to 60 ˚C, at 2 ˚C / min and held for 10 minutes, then from 60 ˚C - 64 ˚C (1˚C / min), 64 ˚C – 121 ˚C (2˚C /min), 121 ˚C for 2 minutes, 121 ˚C - 181 ˚C (3˚C/min) and in the end 181 ˚C - 300°C (20 ˚C / min) with a 5 minute hold at 300 ˚C.

The identification of the components was conducted by comparing the retention times, retention indices (RI_{exp}), and mass spectra of volatiles to Wiley/NIST05 and other libraries (created by assembling relevant bibliography). The RI_{exp} values were calculated via the equation of van den Dool and Kratz, using a series of saturated alkane standards C_8–C_{20} (Sigma-Aldrich, St. Louis, MO, USA), run under the same chromatographic conditions as the EO samples. The quantitative determination of the compounds was expressed as the relative percentage of the ratio of each compound peak area, using the program WSEARCH32 (Ver. 16/2005).

Bacterial Strain

The microorganism used in this study was *Enterococcus faecalis* ATCC 19433 (Merck KGaA, Supelco, Darmstadt, Germany). The *E. faecalis* culture was reconstituted in phosphate-buffered water (PBW) to facilitate colony isolation. Following reconstitution, 100 μL of the sample was inoculated onto Slanetz & Bartley agar plates and incubated at 37 °C under aerobic conditions for 48 hours. After incubation, the resulting colonies were collected and transferred into 30 mL of tryptone soy broth (TSB) in a 50 mL tube. The suspension was then agitated for 16-18 hours, followed by centrifugation at 3500 rpm for 5 minutes. The supernatant was discarded, and the sediment was retained. The precipitate was resuspended in physiological saline until it reached an optical density (OD) of 0.5 at 546 nm, measured using a Model U-2001 UV/Vis Spectrophotometer (Hitachi, USA). Finally, the resulting suspension was diluted at a 1:100 ratio by combining 990 μL of phosphate-buffered saline (PBS) with 10 μL of the prepared solution.

Disk Diffusion Method

The antibacterial activity of the essential oils (EOs) was evaluated using the disk diffusion method, as described by EUCAST [10], with slight modifications. A suspension of *Enterococcus faecalis* containing 10^8 colony-forming units per milliliter (cfu/mL) was prepared, and 100 μL of this suspension was evenly spread onto Slanetz & Bartley Agar plates. Each essential oil was diluted with dimethyl sulfoxide (DMSO) to create five concentration groups: 100%, 50%, 25%, 12.5%, 6.25%, and 3.125%. Sterile disks, 6 mm in diameter, were impregnated with 30 μL of each EO concentration and placed on the inoculated agar surfaces.

DMSO served as the negative control, while three antibiotics-ampicillin (10 μg), erythromycin (15 μg), and oxytetracycline (30 μg)- were used as positive controls. The plates were incubated at 37 °C for 48 to 72 hours to allow for bacterial growth and to observe the effects of the EOs on *E. faecalis*. The experiment was conducted in nine replicates (3 per antibiotic) to ensure the reliability and reproducibility of the results.

Evaluating antimicrobial activity

The evaluation of EO antibacterial activity involved the measurement of inhibition zone diameters in millimeters. To determine the extent of the inhibitory activity for each essential oil, the overall diameter of the inhibition zone was assessed without deducting the disk diameter.

Determination of MIC and MBC: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the essential oils (EOs) were determined using a microdilution method with slight modifications. Two TSB mixtures were prepared: one with 5% dimethyl sulfoxide (DMSO) (Solution 1) and another with 5% DMSO plus 4% EO (Solution 2). A 0.5 McFarland standard of *Enterococcus faecalis* was prepared (Solution 3). In a 96-well microplate, 150 μL of Solution 1 was added to the first 14 wells. Serial dilutions were performed by mixing 150 μL of Solution 2 with Solution 1 across the first 12 wells. Each well then received 10 μL of Solution 3. The positive control contained only Solution 3, and the negative control contained only Solution 2. MIC was defined as the lowest EO concentration inhibiting visible growth after incubation at 37 °C for 18-24 hours. For MBC determination, samples

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were taken from wells around the MIC, plated on TSA, and incubated for 48 hours. All procedures were conducted in triplicate.

Statistical analysis

Normality of the data was first assessed using the Shapiro-Wilk test, a reliable method for small sample sizes. Given that the data did not follow a normal distribution, non-parametric statistical methods were deemed appropriate for the analysis. The mean and standard deviation of the inhibition zone values of each essential oil against *E. faecalis* were calculated using Microsoft Excel. Due to the non-normality and unequal variances in the data, the Kruskal-Wallis test was applied to compare inhibition zones across different concentration levels, a choice more suitable than parametric alternatives like ANOVA. Spearman's rank correlation was then utilized to analyse the relationship between essential oil concentrations (25%-3.125%) and inhibition zones, as well as their dominant components. In the Spearman correlation analysis, the inhibition zones for oils exhibiting Over Maximum Zones (OMZ) were standardized by assigning a value of 50 mm. This adjustment ensured consistency in comparing the relative antibacterial efficacy of the essential oils, facilitating a robust assessment of the correlation between essential oil concentrations and inhibition zones. All other statistical analyses were conducted using SPSS.

Results

Antimicrobial activity of EOs

According to table 2, the results highlight the inhibitory effects of essential oils at different concentrations on *Enterococcus faecalis* (100%, 50%, 25%, 12.5%, 6.25%, 3.125%). At concentrations of 100% and 50%, all the EOs displayed inhibitory zones (IZs) above the maximum border, which was set at 50 mm. The lowest tested concentration of 3.125% revealed distinctive patterns among the oils. The EOs of oregano and dittany showed large zones of inhibition (OMZ) at concentration of 12.5% or higher, surpassing the potency of the antibiotics. The columns serve to indicate the efficacy of the oils, suggesting that oregano C emerges as the most potent among the EOs, displaying the largest IZ, followed by the remaining oregano oils, thyme, and dittany.

In table 3, according to the data supplied by the bacterial strain manufacturers, the specified strain was resistant to the antibiotics aztreonam, colistin, pipepimidic acid, polymyxin B and nystatin.

GC–MS analysis of EOs

The EO of the *Lamiaceae s*pecies exhibited a significant number of similar volatile components, with differences in their percentage quantity in each EO. As shown in table 4, 1,8-cineole was one of the main constituents of the EOs from sage (45.91%), rosemary A (13.41%) and B (46.3%), lavender (18.39%), peppermint A (6.7%) and peppermint B (6.58%) and thyme (6.25%). p-Cymene (33.44%), γ-terpinene (20.71%), and carvacrol (12.24%) were the most significant ingredients in oregano A. Oregano B was characterized by an exceptional concentration of carvacrol (82.1%). Oregano C has an even higher concentration of carvacrol (91.3%). Dittany indicated a significant proportion of carvacrol (54.1%). Peppermint A and B displayed a prominent presence of menthone (24.6% and 37.11% respectively) and menthol (46% and 31.01%). Rosemary A showcased a complex profile with notable constituents, such as 1,8-cineole, α-pinene (14.6%) and

Table 2: Inhibition zones of essential oils against *E.faecalis* in the presence of the 3 antibiotics.

EOs	Mean Inhibition Zones (mm)/ Concentration (%)[1]					
	100%	50%	25%	12.50%	6.25%	3.125%
P.A	OMZ	OMZ	24.8 ± 1.4	19.9 ± 1.9	16 ± 0.9	13.9 ± 0.8
P.B	OMZ	OMZ	21.3 ± 4.5	17.6 ± 3.1	14.7 ± 2.6	11.9 ± 2.4
O.A	OMZ	OMZ	OMZ	OMZ	24.8 ± 6.9	21.6 ± 6.1
O.B	OMZ	OMZ	OMZ	OMZ	22.9 ± 4.6	19.1 ± 4.5
O.C	OMZ	OMZ	OMZ	OMZ	33 ± 12.8	29.6 ± 15.4
DIT.	OMZ	OMZ	OMZ	OMZ	22 ± 2.6	16.2 ± 1.3
R.A	OMZ	OMZ	19 ± 0.9	14.7 ± 1.4	12.2 ± 1.5	10.8 ± 0.7
R.B	OMZ	OMZ	18.9 ±4.2	14.1 ± 3.1	12.3 ± 2.2	10.2 ± 1.7
TH.	OMZ	OMZ	OMZ	35.3 ± 0.6	24.4 ± 3.5	19.1 ± 3.6
SG.	OMZ	OMZ	25.1 ± 3.3	20.4 ± 3.6	16.6 ± 2.5	13.9 ± 3.0
LVN.	OMZ	OMZ	21.4 ± 2.1	17.4 ± 2.4	14.4 ± 1.7	11.7 ± 2.3
RCS.	OMZ	OMZ	20.7 ± 5.6	17.2 ± 3.2	15.4 ± 2.8	12.8 ± 1.8
HLC.	OMZ	OMZ	21.9 ± 5.4	18.3 ± 3.6	15.1 ± 2.9	12.9 ± 2.1

[1]Data are the mean diameter of the inhibitory zones (mm) ± standard deviation of nine repeats, in six different concentrations (%) of EOs. The diameter of the paper disk (mm) is included. Note: OMZ: Over-Maximum Zone (>50 mm); Plant abbreviations: P.A: Peppermint A; P.B: Peppermint B; O.A: Oregano A; O.B: Oregano B; O.C: Oregano C; DIT.: Dittany; R.A: Rosemary A; R.B: Rosemary B; TH.: Thyme; SG.: Sage; LVN.: Lavender; RCS.: Rockrose; HLC.: Helichrysum

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Table 3: Inhibition zones of antibiotics against *E. faecalis*

Antibiotics	Mean IZ (mm)
Penicillin G (6 µg)	22 ± 9
Oxacillin (5 µg)	10 ± 4
Ampicillin (10 µg)	30 ± 11
Ticarcillin (75 µg)	26 ± 10
Mezlocillin (30 µg)	31 ± 12
Cefalotin (30 µg)	16 ± 6
Cefazolin (30 µg)	17 ± 7
Cefotaxime (30 µg)	24 ± 10
Aztreonam (30 µg)	0
Imipenem (10 µg)	29 ± 11
Tetracycline (30 µg)	30 ± 12
Chloramphenicol (30 µg)	26 ± 10
Gentamycin (10 µg)	14 ± 6
Amikacin (30 µg)	11 ± 5
Vancomycin (30 µg)	20 ± 8
Erythromycin (15 µg)	25 ± 10
Lincomycin (15 µg)	10 ± 4
Ofloxacin (5 µg)	18 ± 7
Norfloxacin (10 µg)	17 ± 7
Colistin (10 µg)	0
Pipemidic acid (20 µg)	0
Nitrofurantoin (100 µg)	23 ± 9
Bacitracin (10 µg)	20 ± 8
Polymyxin B (300 unit)	0
Kanamycin (30 µg)	14 ± 5
Neomycin (30 µg)	12 ± 5
Doxycycline (30 µg)	30 ± 12
Ceftriaxone (30 µg)	20 ± 8
Clindamycin (10 µg)	12 ± 5
Fosfomycin (50 µg)	26 ± 10
Moxifloxacin (5 µg)	23 ± 9
Linezolid (10 µg)	30 ± 11
Nystatin (100 unit)	0
Quinupristin/Dalfopristin (15 µg)	22 ± 8
Teicoplanin (30 µg)	20 ± 7
Piperacillin/Tazobactam (30/10 µg)	28 ± 10

[1]The information supplied by the manufacturers regarding the bacterial strain *E. faecalis* NCTC 19433 pertains to the IZ values for each antibiotic. These IZs were obtained using Mueller–Hinton agar as the medium and a 0.5 McFarland inoculum.

camphor (26.91%). Additionally, rosemary B had a high concentration of 1,8-cineole. The sage EO analysis confirmed the EO's high content in 1,8-cineole, camphor (16.22%) and β-thujone (14.85%). For lavender, twenty-nine compounds were identified in total. Linalyl acetate (23.07%) and camphor (22%) were the major components, followed by 1,8-cineole and linalool (15.16%). Among the *Cistaceae* and *Asteraceae* families, rockrose and helichrysum featured a diverse volatile profile, while α-pinene was the dominant component in both EOs: 38.94% for rockrose and 14.94% for helichrysum, alongside γ-curcumene (14.94%). It is worth mentioning the helichrysum EO was rich in sesquiterpenes (37.3% of total EO content), in contrast to the other investigated EOs.

MICs and MBCs of EOs

The investigation of the antimicrobial activity of various EOs against *E. faecalis* revealed intriguing findings. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined for each EO (Table 5).

Statistical results

Shapiro-Wilk Normality Test

The normality of the data was assessed using the Shapiro-Wilk test across four different concentrations (3.125%, 6.250%, 12.5%, and 25%) of EOs and their IZs against *E.faecalis*. The Shapiro-Wilk test results indicated significant deviations from normality for most of the concentrations. Specifically, for the concentration of 3.125%, the p-value was 0.026, indicating a statistically significant deviation from normal distribution. The concentration of 6.250% showed a p-value of 0.055, which is slightly above the 0.05 threshold, suggesting a borderline non-normal distribution. For higher concentrations, such as 12.5% and 25%, the p-values were 0.002 and <0.001, respectively, strongly indicating that the data does not follow a normal distribution. These findings were further supported by visual inspection through Q-Q plots, where data points noticeably deviated from the expected diagonal line, particularly at higher concentrations.

Kruskal-Wallis Test

The results of the Kruskal-Wallis test, which was used to compare the inhibition zones across different concentration levels, indicated a statistically significant difference among the groups ($H = 17.779$, $df = 3$, $p < 0.001$). The mean rank of inhibition zones increased progressively with the concentration, with the lowest mean rank observed at the 3.125% concentration (mean rank = 14.54) and the highest at the 25% concentration (mean rank = 38.04). This suggests that higher concentrations of the essential oils are associated with larger inhibition zones, reflecting greater antibacterial activity as the concentration increases.

Spearman's Correlation Analysis

The Spearman's rank correlation analysis revealed several significant relationships between the concentration of essential oils, their chemical components, and the inhibition zones measured. Specifically, the concentration of essential oils was significantly positively correlated with the inhibition zone ($\rho = 0.639$, $p < 0.01$), indicating that higher concentrations lead to greater antibacterial activity. Both Thymol ($\rho = 0.457$, $p < 0.01$) and Carvacrol ($\rho = 0.357$, $p <$

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Table 4: List of volatile metabolites in the commercial essential oil preparations, presented in descending order according to their % relative content in each EO.

EOs	Volatile Metabolites (%)	Total (%)	Density (g/mL) 25 °C
P.A*	**Menthol (46)**, **Menthone (24.7)**, 1.8-Cineole (6.7), Menthyl Acetate (5.4), Isomenthone (3.9), Limonene (2.5), Menthofuran (1.2), Pulegone (0.5), Carvone (0.1), Isopulegol (0.1).	91	0.896
	M.H. 2.50%, O.M. 88.60%		
P.B	**Menthone (37.11)**, **Menthol (31.01)**, iso-Menthyl acetate (9.03), Isomenthone (7.73), 1,8-Cineole (6.58), Neomenthyl acetate (1.64), Piperitone (1.33), Limonene (1.18), Menthyl Acetate (0.7), Neomenthol (0.60), β-Bourbonene (0.49), Carvone (0.35), Caryophyllene oxide (0.35), Viridiflorol (0.34), γ-Terpinene (0.32), β-Pinene (0.31), α-Pinene (0.28), Spathulenol (0.21), p-Cymene (0.14), Sabinene (0.11), *cis*-p-Menth-2-en-1-ol (0.1), *cis*-Sabinene hydrate (0.09), Camphene (tr), α-Thujene (tr).	100	0.965
	M.H. 2.34%, O.M. 96.27%, S.H. 0.49%, O.S. 0.90%		
O.A	**p-Cymene (33.44)**, γ-**Terpinene (20.71)**, **Carvacrol (12.24)**, β-Myrcene (5.35), α-Terpinene (5.26), α-Thujene (3.87), α-Pinene (3.39), d-Limonene (2.72), Borneol (1.38), *trans*-Sabinene hydrate (1.38), α-Phellandrene (1.08), Camphene (0.92), α-Terpinolene (0.83), 1,8-Cineole (0.83), Caryophyllene oxide (0.81), α-Cadinene (0.78), γ-Muurolene (0.76), β-Pinene (0.68), α-Humulene (0.56), α-Terpineol (0.45), 3-Carene (0.43), *cis*-α-Bergamotene (0.41), *cis*-Dihydro carvone (0.39), β-Bourbonene (0.37), Thymol acetate (0.29), Menthol (0.26), *cis*-β-Ocimene (0.24), Thymol (0.18), Tricyclene (tr).	99.64	0.942
	M.H. 78.92%, O.M. 17.40%, O.S. 3.87 %, S.H. 2.10%		
O.B*	**Carvacrol (82.10)**, p-Cymene (4.89), γ-Terpinene (3.38), Thymol (1.88), *trans*-β-Caryophyllene/ Terpinen-4-ol (1.65), β-Myrcene (0.98), α-Terpinene (0.75), Linalool (0.63), α-Pinene (0.55), Limonene (0.05).	96.86	0.95
	M.H. 10.60%, O.M. 84.61%, S.H. 1.65%		
O.C*	**Carvacrol (91.30)**, p-Cymene (1.26), Thymol (1.10), *trans*-β-Caryophyllene/ Terpinen-4-ol (0.77), γ-Terpinene (0.45), Carvacrol methyl ether (0.44), β-Bisabolene (0.38), Caryophyllene oxide (0.38), Borneol (0.16), β-Myrcene (0.15), α-Pinene (0.15), α-Terpinene (0.15), α-p-Dimethystyrene (0.13), Carvacrol isomer (0.09), p-Cymen-8-ol (0.09), Thymol isomer (0.09), α-Humulene (0.08), α-Terpineol (0.08), α-Thujene (0.08), *trans*-Carveol (0.07), Limonene (0.07), Carvone (0.06), Globulol (0.06), 1-Octen-3-ol (0.06), Humulene epoxide II (0.05), Linalool (0.03).	97.29	0.95
	M.H. 2.38%, O.M. 93.44%, S.H. 0.96%, O.S. 0.44%		
DIT.*	**Carvacrol (54.81)**, **p-Cymene (13.99)**, γ-Terpinene (8.33), α-Thujene (3.03), *trans*-β-Caryophyllene/ Terpinen-4-ol (2.91), β-Myrcene (2.85), Thymol (2.11), α-Terpinene (1.93), α-Pinene (1.21), β-Bisabolene (0.79), Borneol (0.64), 1-Octen-3-ol (0.59), Camphene (0.46), Limonene (0.41), α-Phellandrene (0.36), Linalool (0.20).	95.06	0.95
	M.H. 32.57%, O.M. 57.76%, S.H. 3.70%		
R.A*	**Camphor (26.91)**, **α-Pinene (14.76)**, **1,8-Cineole (13.41)**, Camphene (7.31), β-Myrcene (5.15), Limonene (4.62), Borneol (3.91), β-Pinene (2.20), Verbenone (2.20), β-Phellandrene (1.85), 3-Octanone (1.50), α-Terpineol (1.41), α-Phellandrene (1.30), Linalool (1.29), β-Caryophyllene (1.09), Bornyl acetate (1.08), γ-Terpinene (0.90), p-Cymene (0.85), Terpinen-4-ol (0.77), α-Terpinene (0.71).	93.22	0.879
	M.H. 39.65%, O.M. 50.98%, S.H. 1.09%		
R.B*	**1.8-Cineole (46.3)**, **α-Pinene (11.9)**, **Camphor (10.26)**, β-Pinene (6.8), Camphene (4.5), Borneol (3.2), Limonene (2.5), β-Myrcene (1.7), α-Terpineol (1.6), p-Cymene (1.2), Borneol acetate (0.9), Verbenone (0.2).	91.06	0.879
	M.H. 28.60%, O.M. 62.46%		
TH.*	**p-Cymene (33.53)**, **Thymol (26.59)**, 1,8-Cineole (6.25), Limonene (5.32), Carvacrol (2.77), β-Caryophyllene (2.28), α-Thujene (1.81), Borneol (1.75), Linalool (1.70), α-Pinene (1.70), Camphene (1.68), β-Pinene (1.45), α-Terpinene (1.12), γ-Terpinene (0.81), β-Myrcene (0.19).	90.59	0.9189
	M.H. 47.61%, O.M. 39.06%, S.H. 2.28%		
SG.	**1.8-Cineole (45.91)**, **Camphor (16.22)**, **β-Thujone (14.85)**, Caryophyllene oxide (4.65), p-Cymene (4.65), Camphene (4.11), Terpinyl acetate (3.01), β-Pinene (1.22), α-Pinene (1.09), Bornyl acetate (0.88), *cis*-Sabinene hydrate (0.62), Borneol (0.48), α-Thujene (0.44), Linalyl acetate (0.43), δ-Terpineol (0.41), Viridiflorol (0.34), Myrtenol (0.25), α-Terpineol (0.24), Alloaromadendrene (0.1), Tricyclene (0.1), Thujene (tr).	100	0.9
	M.H. 11.61%, O.M. 83.30%, S.H. 0.10%, O.S. 4.99%		
LVN.	**Linalyl acetate (23.07)**, **Camphor (22.0)**, **1.8-Cineole (18.39)**, **Linalool (15.16)**, Borneol (3.82), *cis*-Linalool oxide (3.71), Terpinen-4-ol (3.33), *trans*-Linalool oxide (2.6), Camphene (0.86), Caryophyllene oxide (0.86), α-Terpineol (0.86), p-Cymene (0.77), Lavandulyl acetate (0.56), α-Pinene (0.56), Cryptone (0.50), β-Pinene (0.48), Hexyl butanoate (0.44), Limonene (0.38), α-Santalene (0.31), Bornyl formate (0.24), Myrcene (0.22), Caryophyllene (0.19), Lavandulol (0.16), Sabinene (0.1), Bergamotene (tr), Bornyl acetate (tr), Neryl acetate (tr), Verbenone (tr).	99.57	0.941
	M.H. 3.37%, O.M. 94.40%, S.H. 0.50%, O.S. 0.86%		

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RCS.	**α-Pinene (38.94)**, Camphene (4.14), Bornyl acetate (3.21), *trans*-Pinocarveol (3.15), p-Cymene (3.13), Globulol (2.29), Trimethylcyclohexanone (2.24), Dehydroparacymene (1.71), Limonene (1.55), γ-Terpinene (1.41), Isopinocamphone (1.38), 1,8-Cineole (1.24), Verbenene (1.20), Terpinen-4-ol (1.16), Borneol (1.04), Picocarvone (0.68), Acetophenone (0.49), β-Pinene (0.47), Ledene (0.45), Viridiflorol (0.38), Myrcene (0.33), α-Terpineol (0.31), Linalool (0.27), β-Caryophyllene (0.19), Geraniol (0.16), Citronellol (0.09), Carvacrol (0.08), Eugenol (0.05).	71.74	0.962
	M.H. 52.88%, O.M. 12.82%, S.H. 0.64%, O.S. 2.67%		
HLC.*	**γ-Curcumene (14.94)**, **α-Pinene (14.94)**, **Neryl acetate (14.14)**, β-Selinene (5.09), Italidione (I+II+III) (3.93), *trans*-β-Caryophyllene (3.67), α-Selinene (3.49), α-Curcumene (3.30), Limonene (3.29), Italicene (3.25), Linalool (1.79), Nerol (1.41), α-Copaene (1.13), *cis*-α-Bergamotene (1.01), *trans*-α-Bergamotene (0.72), Isoitalicene (0.70).	79.17	0.9
	M.H. 18.23%, O.M. 19.71%, S.H. 37.30%		

*Based on the GC-MS data provided by Vessel essential oils' manufacturers. Abbreviations: tr: trace, M.H.: monoterpene hydrocarbons, O.M.: oxygenated monoterpenes, S.H.: sesquiterpene hydrocarbons, O.S.: oxygenated sesquiterpenes. Plant abbreviations: P.A: Peppermint A; P.B: Peppermint B; O.A: Oregano A; O.B: Oregano B; O.C: Oregano C; DIT.: Dittany; R.A: Rosemary A; R.B: Rosemary B; TH.: Thyme; SG.: Sage; LVN.: Lavender; RCS.: Rockrose; HLC.: Helichrysum

Table 5: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the tested EOs against *E. faecalis*

EOs	*Enterococcus faecalis*	
	MIC	MBC
P.A	8.96	17.92
P.B	4.83	9.65
O.A	1.18	1.18
O.B	1.19	1.19
O.C	0.15	0.3
DIT.	1.19	2.38
R.A	8.79	17.58
R.B	8.79	17.58
TH.	2.29	4.59
SG.	4.5	9
LVN.	4.7	9.41
RCS.	9.61	19.24
HLC.	9	18

The MIC and MBC are in mg/mL. Plant abbreviations: P.A: Peppermint A; P.B: Peppermint B; O.A: Oregano A; O.B: Oregano B; O.C: Oregano C; DIT.: Dittany; R.A: Rosemary A; R.B: Rosemary B; TH.: Thyme; SG.: Sage; LVN.: Lavender; RCS.: Rockrose; HLC.: Helichrysum

0.01) were also positively correlated with the inhibition zone, highlighting their contributions to the oils' effectiveness. Additionally, strong positive correlations were found between Menthol and Menthone ($\rho = 0.844$, $p< 0.01$), p-Cymene and Carvacrol ($\rho=0.567$, $p<0.01$), and Carvacrol and Thymol ($\rho = 0.805$, $p < 0.01$), suggesting that these components work synergistically to enhance antibacterial activity.

Discussion

The escalating issue of antibiotic overuse and the emergence of antibiotic-resistant bacteria have propelled the exploration of alternative strategies for combating pathogenic microbes. Essential oils have emerged as promising candidates due to their potent antibacterial properties. The multifaceted composition of essential oils, encompassing a spectrum of bioactive constituents, contributes to their pronounced efficacy against microbial pathogens. Numerous investigations in the scientific literature have elucidated the broad-spectrum antimicrobial potential of essential oils. The ability of these extracts to inhibit the growth and survival of bacteria spanning diverse strains and species underscores their versatility as formidable antimicrobial agents [7,11]. The mode of action involves the perturbation of microbial membranes, interference with cellular integrity, and the initiation of processes leading to bacterial death. Many studies substantiate the efficacy of essential oils, particularly in combating *Enterococcus*, with a specific focus on *E. faecalis* [12,13].

As documented in a published article, peppermint oil has been reported to inhibit *E. faecalis* within the range of 0.125%-1%, with a specified MIC of 0.125% [14]. Our study corroborates these findings, as both peppermint oils A and B demonstrated an inhibitory effect, with MIC values ranging from 0.5-1% and MBC of 1-2%.

The potency of oregano oil in exerting robust antimicrobial effects has been substantiated in the literature. In a study utilizing an inoculum of 10^6, an IZ of 21 mm was detected [15]. Another investigation employing the same microbial load reported an IZ of 13±1 mm and an MIC of 8 mg/mL [16]. Additionally, a separate article, using a microbial count of 10^9, reported an MIC and MBC of 0.60 µL/mL (0.57 mg/mL) [17]. In accordance with our research, another investigation described the antimicrobial efficacy of oregano oil, the MIC ranging from 0.25-0.50 µL/mL (0.23 to 0.47 mg/mL), and the MBC ranging from 0.50-1.00 µL/mL (0.47 to 0.95 mg/mL) [18]. These findings align coherently with the outcomes of our study, revealing a spectrum of IZ (19.5-50 mm), MIC (0.15-1.19 mg/mL) and MBC (0.30-1.19 mg/mL). Dittany oil's antimicrobial effectiveness against *E. faecalis* is relatively underexplored in the available literature, with limited sources providing insights into its potential actions.

Rosemary EOs, which are rarely discussed in the

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literature, demonstrate weak antimicrobial activity. In a particular study, the MIC was reported to be 72 mg/mL, the MBC was 144 mg/mL, and the IZ concentration was 7.00±0.2 mm [19]. Additionally, other articles reported similar MIC and MBC values, recorded at 114.87 and 229.74 mg/mL, respectively [20]. In the context of diversity, sage EO is likewise documented in the literature. A study underscored the presence of 10^8 microorganisms, with IZs of 10.63±0.63 and 12.5±0.58 mm, MIC values of 12.5 or >25 mg/mL and MBC exceeding 25 mg/mL. Although the values of the IZs do not exhibit considerable disparity, it is plausible that the MIC and MBC differ significantly [21].

Studies on thyme essential oil have been conducted, confirming the potent efficacy of this essential oil against *E. faecalis* [22]. A research article using ~10^6 microorganism emphasized the 26.7±4.2 mm oil IZ [23], mirroring the range of values (24.5-50 mm), indicating consistency in the observed antimicrobial effects.

Concerning lavender, a publication noted its considerable promise as an oil against *E. faecalis*, citing a distinctive zone of inhibition measuring 21.78±0.71 mm, aligning well with our experimental framework [24]. Rockrose oil, as reported in the literature [25], exhibited an IZ of 11.7±1.7 mm along with MIC and MBC values of 7.51 and 15.0 mg/mL, respectively, which closely mirrored our obtained results. In conclusion, despite limited references, the oil of helichrysum has divergent values from those observed in our study [26].

Proceeding to the chemical composition, the two peppermint EOs are characterized by substantial quantities of menthone and menthol. Peppermint A manifested notable proportions of menthol (46%) and menthone (24.6%), while peppermint B exhibited concentrations of menthol (31.01%) and menthone (37.11%). Various studies have shown the significant antibacterial activity of both menthone [27,28] and menthol [29]. In our statistical analysis, menthol showed limited correlation with the inhibition zones, indicating that its role may be supportive rather than primary in antimicrobial activity. Menthone, while also correlated, demonstrated a similar modest impact. Both compounds, while important, are less potent compared to other active compounds like carvacrol and thymol, yet they contribute to the overall efficacy of peppermint oil in combating *E. faecalis*. Additionally, peppermint B contains p-cymene (33.44%) and γ-terpinene (20.71%). p-cymene appears to be found in many EOs that exhibit antimicrobial activity and is one of the most active components of thyme EOs [30]. Its moderate antimicrobial activity is well-documented, particularly when it works synergistically with other compounds like carvacrol and thymol. In our analysis, p-cymene exhibited a negative correlation with some other active compounds, suggesting that its role might be complex and context-dependent, potentially enhancing the effects of other more potent components rather than acting as a strong antimicrobial agent on its own.

As previously highlighted, thyme contains p-cymene (33.53%) and the compound thymol (26.59%) as dominant components. Thymol is an important antibacterial agent against both gram-positive and gram-negative bacteria. The correlation analysis indicated that thymol, similar to carvacrol, contributes substantially to the antimicrobial efficacy of the essential oils. Research has shown that thymol may act complementarily or synergistically with conventional antibiotics [31].

In rosemary A, the concentration of 1,8-cineole was 13.41%, α-pinene was 14.76%, and camphor was 26.91%. Conversely, rosemary B had a composition of 1,8-cineole at 46.3%, α-pinene at 11.9% and camphor at 10.26%. Scientific studies confirm the considerable antibacterial activity of 1.8 cineole [32], positioning it as an active component across diverse EOs and demonstrating the potential to enhance the bioavailability of antibiotics [33]. The compound α-pinene is shared among the EOs of rosemary, rockrose (38.94%), and helichrysum and is acknowledged for its antibacterial activity against both gram-positive and gram-negative bacteria [34]. While in vitro experiments suggest a degree of weakness, bibliographic evidence indicates a robust potentiating effect on select antibiotics [35]. Camphor has emerged as a pivotal ingredient in various EOs, such as sage (16.22%), and is a key ingredient of rosemary oil with appropriate antibacterial activity [36], especially when using liposomes [37].

Carvacrol, a focal point in discussions surrounding essential oils, assumes a prominent role in the chromatography of all oregano and dittany EOs. Oregano C's specific composition of 91.3% carvacrol correlates this ingredient with the oil's remarkable inhibition zone, surpassing others under the influence of various antibiotics [38]. The significant positive correlations identified in our study between carvacrol concentration and inhibition zones further confirm its potent antimicrobial and antibiofilm properties. These findings align with previous studies emphasizing carvacrol's effectiveness against *E. faecalis* [39,40].

The difference in IZ among EOs from the same plant is likely attributed to the difference in the proportions of their constituent compounds. Rosemary EOs A and B had relatively feeble weak overall inhibition zones compared to those of the other oils. Oregano A, B and C, along with dittany and thyme, presented potent IZs accompanied by consistently low MICs and MBCs values. By categorizing the essential oils based on their MIC and MBC results, we identified three distinct efficacy groups. Strong efficacy was observed in oregano A, B, and C, thyme, and dittany, with MIC values ranging from 0.15 to 2.29 mg/mL. Moderate efficacy included peppermint B, sage, and lavender, with MIC values between 4.50 and 4.83 mg/mL. Low efficacy was noted in peppermint A,

rosemary A and B, and helichrysum, which exhibited higher MIC values, indicating less potency.

Conclusions

The present study demonstrated that essential oils, particularly oregano, thyme, and dittany, exhibited significant antibacterial effects against *E. faecalis,* with carvacrol and thymol identified as key active components contributing to this efficacy. These findings highlight the potential of essential oils as alternative antimicrobial agents, particularly in the context of increasing bacterial resistance to conventional antibiotics. While oils with limited existing literature, such as rosemary, peppermint, and helichrysum, did not show strong activity, their evaluation against antibiotic-resistant strains is essential for a more comprehensive understanding. Despite the need for further research, this study offers valuable insights into the potential integration of essential oils into dental and food industry practices, providing a novel approach to addressing antimicrobial resistance.

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