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In vitro antimicrobial activity of chlorhexidine in combination with essential oils of *Mentha x piperita* L. & *Thymus serpyllum* L. & *Pelargonium graveolens* L'hér

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ABSTRACT

In this study, the antimicrobial potential of *Mentha piperita* & *Thymus serpyllum* &*Pelargonium graveolens* essential oil combinations was evaluated with Chlorhexidine. These species used ethnobotanically against throat infections. The commercial oils were analysed by GC-MS to confirm phytochemistry. Antimicrobial evaluation of the essential oils against *Staphylococcus aureus* and *Streptococcus mutans* were performed using *in vitro* microdilution assay. Binary combinations of Chlorhexidine-essential oils were evaluated using the checkerboard method. *M. piperita* essential oil contained 47% menthol as the major component, whereas the main component of *T. serpyllum* essential oil was 19% geraniol, and the main component of *P. graveolens* was 25% citronellol, respectively. The calculated fractional inhibition concentration index (FICI) of chlorhexidine-*T. serpyllum* combinations against *S. mutans* resulted as antagonistic (FICI 3.3643), while the FICI of chlorhexidine-*P. graveolens* essential oil combination resulted in synergistic (FICI 0.4326). Also, the FICI value = 0.252 of chlorhexidine-*P. graveolens* essential oil combinations against *S. aureus* were synergistic. To the best of our knowledge, combinations of these oils with Chlorhexidine were observed for the first time in this study with the potential application as mouthwash formulation.

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KEYWORDS
Oral care; combination of oils; antimicrobial

1. Introduction

Essential oils are used in oral care preparations due to their multiple actions, such as antimicrobial activities among others. There are numerous reports on antiseptic, antibacterial, antiviral, antioxidant, antiparasitic, antifungal, and insecticidal essential oils (1,2). Additionally, recent studies showed that binary combinations of essential oils showed enhanced antimicrobial potential compared to individual oils (3,4).

As it is well known, Mentha x piperita L. of the Lamiaceae is a cultivated hybrid of Mentha aquatica and Mentha spicata. Although peppermint originates from Mediterranean regions, it is sparsely distributed in North America, Europe, Australia, and Asia (3). This peppermint is used popularly as a flavor, fragrance, medicinal, and for its pharmaceutical application (3,4). Essential oil of M. x piperita showed various biological activities such as antioxidant, antimicrobial, anticancer, antiviral, antiallergic, and anti-inflammatory, as previously reported (5–7). It is also used to treat bronchitis, nausea, flatulence, anorexia, ulcerative colitis, and liver complaints (3). Significant constituents of peppermint essential oils are reported as menthol, menthone, menthofuran, 1,8-cineole, and menthyl acetate, and others (5,6,8).

Thymus serpyllum L., also known as wild thyme of the Lamiaceae family, is a good source of the characteristic essential oil (9). It is native to the Mediterranean, Europe, and North Africa, generally at the higher altitudes (9). In previous studies, T. serpyllum preparations showed antiseptic, antimicrobial, analgesic, antioxidant, carminative, expectorant, and diuretic activities (10), with an essential oil characteristic of geraniol, terpineol, carvacrol, thymol, α-pinene, and limonene content in varying amounts (11,12).

Pelargonium graveolens L'Hér. (Geraniaceae) is an aromatic herb which is used as a flavoring, fragrance, and insecticide, as well as against throat infections, bleeding, and other uses (13). The essential oil composition of P. graveolens is known to contain geraniol, citronellol, trans-geraniol, 10-epi-γ-eudesmol, isomenthone, linalool, geranyl acetate, γ-cadinene, geranyl butyrate, geranyl tiglate, and germacrene D (14,15). The antimicrobial activity of the essential oil and constituents against various bacterial and fungal pathogens was reported (15).

In this present study, it was aimed to evaluate the application as well as the antimicrobial potential of European Pharmacopoeia grade Mentha x piperita and ISO standard grade commercial Thymus serpyllum and

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Pelargonium graveolens essential oil combinations with Chlorhexidine against human pathogens targeting the oral pathogenic microbial flora.

2. Material and methods

The commercial essential oils were acquired by Art De Huile Company (İstanbul, Türkiye).

2.1. GC-FID and GC/MS analysis

The GC-FID analysis was performed using the FID detector at 300°C (Agilent 6890N GC system, CA, USA). Simultaneous automatic injection was carried out using the same conditions in two identical columns in the GC/MS system (Agilent 5975 GC-MSD). Innowax FSC column (60 m x 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*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. Mass range was from *m/z* 35 to 450. Relative percentages of the volatile components were calculated using the FID chromatograms. This process was performed by the GC/MS MassFinder4 Library and the 'in-house Baser Library of Essential Oil Constituents', consisting of authentic sample analyses data or the relative retention index (RRI) of *n*-alkanes (16,17).

2.2. Antimicrobial activity and combination

The *in vitro* antimicrobial activity was determined using the broth microdilution assay following the methods according to the Clinical and Laboratory Standards Institute to determine the minimum inhibitory concentrations (MIC). MRSA *Staphylococcus aureus* and *Streptococcus mutans* ATCC 25,175 strains were inoculated 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 with minimum inhibitory concentration (MIC, as µg/mL). The MIC was calculated and reported as the mean of three repetitions compared to positive standards.

Checkerboard combination testing was performed in triplicate with a 96-wellplate using a 12-by-8 well configuration. Growth control was performed in wells without antibiotic. The checkerboard is prepared in a microtiter plate for multiple combinations of two antimicrobial agents in concentrations equal to, above, and below their minimal inhibitory concentrations for the microorganism. The sample combination in which the growth is completely inhibited, was here also accepted as inhibitory combination concentration (18). The fractional inhibitory concentration (FIC) index range of 0.5 to 4 is commonly used to define additivity; we reassessed this *in vitro* FIC index range based on the experimental variation of the checkerboard technique using multiple replicates. For all the wells of the microtitration plates that corresponded to a MIC, the sum of the FICs (ΣFIC) was calculated for each well with the equation ΣFIC= FICA + FICB. For two antibacterial agents, A and B, acting individually or in combination:

$$FIC(A) = \frac{MIC(A\ in\ the\ presence\ of\ B)}{MIC(A\ alone)}$$

$$FIC(B) = \frac{MIC(B\ in\ the\ presence\ of\ A)}{MIC(B\ alone)}$$

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 standardised 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 (18), where the results were reported as average values, as shown in Table 3.

2.3. Mouthwash formulations

The mouthwashes were prepared using synergistic combinations of the essential oils with chlorhexidine. The lowest synergistic ratios were added to the formulation directly (*P.graveolens* 250 µg/mL+ Chlorhexidine 2 µg/mL; *M. x piperita* 1 mg/mL+ Chlorhexidine 2 µg/mL; *T. serpyllum* 0.5 mg/mL+ Chlorhexidine 1 µg/mL, respectively). Saccharine was also used, for the sweet taste. Furthermore, the essential oils were dissolved in ethanol, while sodium chloride and sodium bicarbonate were added gradually using a stirrer (500 rpm, 30 min),

respectively. The combination blend was filtered, and the filtrate end volume was completed to 10 mL using distilled water. No preservative was added, since the mouthwashes included a relatively high content of ethanol (>15 %) and essential oils (19).

3. Result and discussion

Prior the formulations and biological evaluations, the phytochemical conformity of the commercial oils were verified by GC/MS results; the essential oil data were listed in Table 1., where 98.5% of *M. x piperita*, 97.5% of *T. serpyllum*, and 91.7% of *P. graveolens* essential oil compositions were identified (20).

The major components of *M. x piperita* essential oil were identified as 47.8% menthol, 23.4% menthone, and 5.2% 1,8-cineole, respectively. The *T. serpyllum* essential oil composition was characterised according to the analytics by 19.5% geraniol, 13.7% *p*-cymene, and 5.8% α-thujone, respectively (21). The major components of *P. graveolens* essential oil were identified as 25.7% citronellol, 13.6% citronellyl formate, and 9.1% geraniol, respectively (see Figures 1–3).

The major component of *M. x piperita* essential oil, menthol, was reported as an active compound responsible for the biological activities in previous studies (22). The major constituents of the *P. graveolens* essential oil, such as citronellol and geraniol, were associated with biological activities (16). In the present study, the chemical composition of the analyzed essential oils was confirmed in accordance with previous studies and European Pharmacopoeia and ISO standards.

In this present study, according to the experimental results, the *M. x piperita, T. serpyllum*, and *P. graveolens* essential oils at 1 mg/mL concentration initially appeared as relatively ineffective against the tested human pathogenic microorganisms, as shown in Table 2. However, for the *S. mutans*, the Fractional Inhibitory Concentration Index (FICI) value of the combination of *M. x piperita* and Chlorhexidine against *S. mutans* bacteria was 1.4166, resulting in an additive effect. The FICI value of the combination of *T. serpyllum* and Chlorhexidine was 3.3643 and it was determined that it provided an antagonist effect accordingly. The FICI value of the combination of *P. graveolens* and Chlorhexidine was 0.4326, and it was determined that this combination showed a synergistic effect (Table 3).

The FICI value of the combination of *M. x piperita* and Chlorhexidine against *S. aureus* bacteria was 1.002, and this result showed that the combination has an additive effect. The FICI value of the combination of *T. serpyllum* and Chlorhexidine was 0.501, and it was revealed that this combination showed a partial synergistic effect for *S. aureus*. The FICI value of the combination of *P. graveolens* and Chlorhexidine was 0.252, showing a synergistic effect. In line with the experiments and subsequent calculations, a simple mouthwash formulation was prepared based on the synergistic and additive effect rates (Table 3).

After the disc diffusion antimicrobial study, the antimicrobial effects of the formulations against two different bacteria were determined. As a result of the determination, it was determined that the combination of *Pelargonium graveolens* x Chlorhexidine provided a more significant antimicrobial effect against *S. mutans* bacteria than other combinations (Table 4).

The antimicrobial activity of *M. x piperita* essential oils was also reported in previous studies against a broad spectrum of pathogens (5,22). Additionally, menthol, one of the major components, and two different *Mentha* essential oil combinations were investigated for their antimicrobial activity with different combined formulations (23). Also, other menthol-rich essential oils were reported for their antimicrobial effects against different pathogens (24,25). *M. x piperita* essential oil was studied in combination with different antibiotics where a synergistic effect against different human pathogenic bacteria was determined (26). When compared with previous work, in the present study, a systematic checkerboard assay with a panel of different microbial strains was studied. According to the findings obtained in this study, combinations of *M. x piperita* and chlorhexidine showed additive effects at different concentrations against both microorganisms tested. However, it was determined that this combination is effective against *S. aureus* microorganisms (1 and 0.002 mg/mL) at lower concentrations than *S. mutans* (1 and 0,4166 mg/mL).

The antimicrobial activity of *T. serpyllum* essential oil was also previously studied against various Gram-negative and Gram-positive bacteria (10). In addition, thymol and carvacrol, which are specific markers of *Thymus* species, are known for their strong antimicrobial properties (27). According to the results obtained from this study, *T. serpyllum* essential oil showed antagonism against *S. mutans* in combination with chlorhexidine, while a partial synergistic effect was detected in the combination tested against *S. aereus* microorganism. An interesting results pattern was observed, where both antagonism and synergistic effects against different microorganisms were the case.

P. graveolens essential oil was evaluated for its antimicrobial activity against food-borne pathogens (28). Only limited work on synergistic effects was reported previously where *P. graveolens* essential oil-ciprofloxacin

Table 1. Chemical Compositions of the Essential Oils Used in the Experiments.

KI	RRI	Component	*M. x piperita*[c]	*T. serpyllum*[c]	*P. graveolens*[c]
1025[a]	1032	α-Pinene	0.7	1.3	.5
1026[a]	1035	α-Thujene	tr	0.5	–
1043–1086[a]	1076	Camphene	–	1.9	–
1110[a]	1118	β-Pinene	0.8	0.5	tr
1122[a]	1132	Sabinene	0.3	0.2	–
1160[a]	1174	Myrcene	0.1	0.7	.1
1154–1195[a]	1188	α-Terpinene	tr	0.9	–
1178–1219[a]	1203	Limonene	2.1	0.5	.1
1186–1231[a]	1213	1,8-Cineole	5.2	1.2	
1188–1233[a]	1218	β-Phellandrene	–	–	tr
1211–1251[a]	1246	(*Z*)-β-Ocimene	tr	–	tr
1222–1266[a]	1255	γ–Terpinene	0.1	2.9	-
1230–1280[a]	1265	3-Octanone	–	0.2	–
1232–1267[a]	1266	(*E*)–β-Ocimene	tr	–	tr
1246-1291[a]	1280	*p*–Cymene	0.4	13.7	.1
1261–1300[a]	1290	Terpinolene	tr	0.2	–
1301–1375[b]	1348	6-Methyl-5-hepten-2-one	–	tr	tr
1331–1369[a]	1362	*cis*-Rose oxide	–	–	.9
1341–1386[a]	1377	*trans*-Rose oxide	–	–	.4
1372–1408[a]	1393	3-Octanol	0.1	–	–
1405–1431[a]	1429	Perillene	–	–	tr
1385–1441[a]	1437	α-Thujone	–	5.8	–
1400–1452[a]	1451	β-Thujone	–	2.1	–
1411–1465[a]	1452	1-Octen-3-ol	–	0.1	–
1438–1480[a]	1466	α-Cubebene	–	–	.1
1443–1479[a]	1475	Menthone	23.4	–	3.1
1410–1478[a]	1478	*cis*-Linalool oxide (*Furanoid*)	–	–	.1
1457–1495[a]	1487	Citronellal	–	–	tr
1459–1500[a]	1493	α-Ylangene	–	–	.1
1458–1502[a]	1497	Menthofuran	1.7	–	–
1462–1522[a]	1497	α-Copaene	–	–	.5
1464-1506 [a]	1503	Isomenthone	3.8	–	4.6
	1528	α-Bourbonene	–	–	.1
1515[a]	1532	Camphor	–	3.7	–
1496–1546[a]	1535	β-Bourbonene	0.2	–	1.1
	1541	Neomenthyl acetate	0.2	–	–
1507–1564[a]	1553	Linalool	0.2	6.4	4.6
1532–1570[a]	1565	Linalyl acetate	–	0.4	–
1535–1585[a]	1574	Menthyl acetate	4.8	–	.2
1547–1589[a]	1589	β-Ylangene	–	–	tr
1549–1597[a]	1591	Bornyl acetate	–	0.5	–
550–1603[a]	1597	β-Copaene	–	–	tr
1563–1607[a]	1604	Thymol methyl ether	–	0.5	–
1583–1668[a]	1607	α-Guaiene	–	–	.6
1564–1630[a]	1611	Terpinen-4-ol	3.0	0.9	
1569–1632[a]	1612	β-Caryophyllene	2.3	3.8	.8
1576–1614[a]	1614	Carvacrol methyl ether	–	1.9	–
1617–1626[b]	1617	6,9-Guaiadiene	–	–	5.7
1588–1644[a]	1628	Citronellyl formate	–	–	13.6
1600–1650[a]	1624	*trans*-Dihydrocarvone	–	0.1	
1555–1645[a]	1638	*cis*-p-Menth-2-en-1-ol	–	0.2	–
1599–1651[a]	1638	Menthol	47.8	–	–
1624–1668[a]	1661	Alloaromadendrene	–	–	.2
1626–1663[a]	1662	Pulegone	0.4	–	–
1633–1671[a]	1668	Citronellyl acetate	–	–	.5
1641–1708[a]	1684	Neryl formate	–	–	.3
1637–1689[a]	1687	α-Humulene	–	1.7	.3
1641–1706[a]	1694	Neral	–	–	.3
1659–1724[a]	1706	α-Terpineol	–	0.9	–
1672–1718[a]	1709	α-Terpinyl acetate	–	0.4	–
1665–1727[a]	1715	Geranyl formate	–	4.1	6.5
1676–1726[a]	1726	Germacrene D	–	0.2	–
1700–1738[a]	1729	Citronellyl propionate	–	–	.9
	1733	γ-Amorphene	0.3	–	–
1680–1750[a]	1740	Geranial	–	tr	1.0
1698–1748[a]	1741	β-Bisabolene	–	1.0	–
1689–1748[a]	1748	Piperitone	0.4	–	–
1728–1772[a]	1765	Geranyl acetate	–	5.4	–
1734–1789[a]	1772	Citronellol	–	–	25.7
1722–1774[a]	1773	δ-Cadinene	–	tr	–
1763–1786[a]	1784	(*E*)-α-Bisabolene	–	1.0	–
1765–1811[a]	1809	Citronellyl butyrate	–	–	.4

(*Continued*)

Table 1. (Continued).

KI	RRI	Component	*M. x piperita*[c]	*T. serpyllum*[c]	*P. graveolens*[c]
1752–1832[a]	1808	Nerol	–	0.5	–
1795–1865[a]	1857	Geraniol	–	19.5	9.1
1833–1907[a]	1901	Geranyl butyrate	–	–	3.7
	1980	Furopelargone A	–	–	.3
1936–2023[a]	2008	Caryophyllene oxide	–	0.4	.5
	2020	(*E*)-Citronellyl tiglate	–	–	.4
2003–2071[a]	2071	Humulene epoxide-II	–	0.3	.1
2019–2090[a]	2080	Cubenol	–	–	.3
2022–2074[a]	2080	1,10-epi-Cubenol	–	–	.2
2041–2110[a]	2104	Viridiflorol	–	2.1	–
	2105	Furopelargone B	–	–	.7
2075–2105[b]	2105	Geranyl caproate	–	–	.2
	2122	Neryl tiglate	–	–	2.0
2100–2205[a]	2198	Thymol	–	3.3	–
	2209	Citronellyl caprylate	–	–	.1
2154–2227[a]	2214	Phenylethyl tiglate	–	–	.7
2140–2246[a]	2239	Carvacrol	–	5.5	–
		Total	**98.3**	**97.4**	91.7

KI retrieved from literature (a20,b21); RRI Relative retention indices calculated against *n*-alkanes.
[c]% calculated from FID data; tr Trace (<0.1 %).

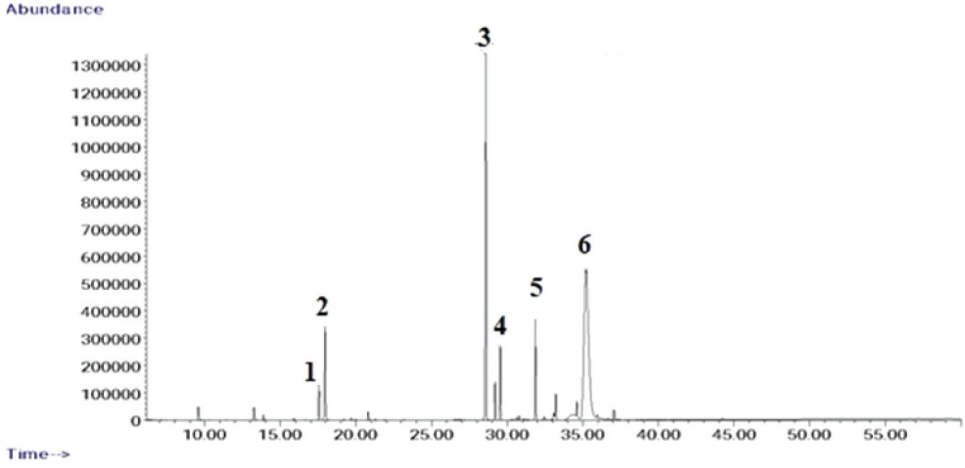

Figure 1. GC/MS Chromatogram of *mentha X piperita* essential oil (1: Limonene, 2: 1,8-Cineole, 3: Menthone, 4: Isomenthone, 5: Menthyl Acetate, 6: Menthol).

Table 2. Chlorhexidine and essential oil Inhibitions (MIC, µg/mL) by broth microdilution assay.

Samples	*S. mutans* MIC (mg/mL)	*S. aereus* MIC (mg/mL)
Mentha x piperita	>1	>1
Thymus serpyllum	>1	>1
Pelargonium graveolens	>1	>1
Chlorhexidine	0,00006	0,00012

antibiotic combination was tested against uropathogens (29). In another study, nystatin and *P. graveolens* essential oil was tested in binary combinations, where synergistic effects were observed for *Candida* strains, with FIC indices of 0.14–0.42, respectively (30). According to this study, the combination of *P. graveolens* and chlorhexidine was the most successful binary combination, showing a synergistic effect against both tested microorganisms. This combination, which allows chlorhexidine to be effective at very low concentrations, may be a good active ingredient choice for a successful mouthwash formulation that can be effective against both oral pathogens.

Based on the findings of this present study, synergistic, antagonistic, and additive effects using three essential oil and Chlorhexidine combinations against the pathogens *S. mutans* and *S. aureus* were observed.

As an overall result, promising results were obtained against resistant and human pathogenic

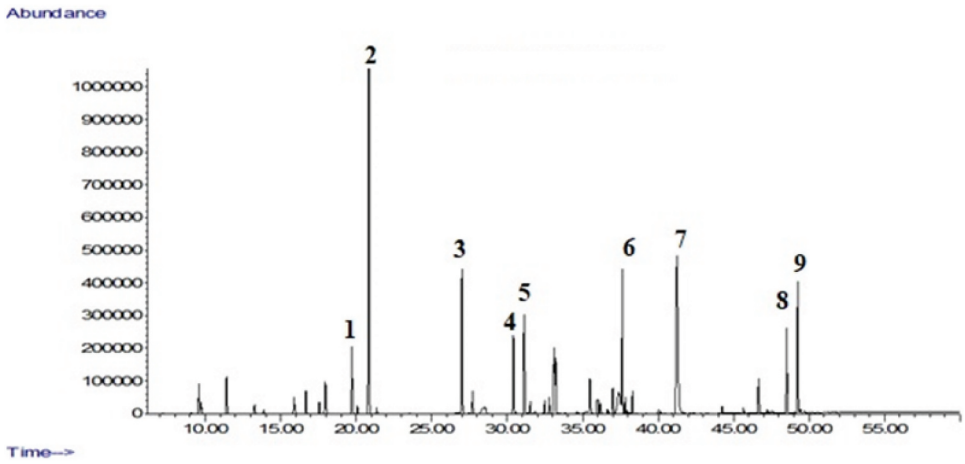

Figure 2. GC/MS Chromatogram of *thymus serpyllum* essential oil (1: γ-Terpinene, 2: p-Cymene, 3: α-Thujone, 4: Camphor, 5: Linalool, 6: Geranyl Acetate, 7: Geraniol, 8: Thymol, 9: Carvacrol).

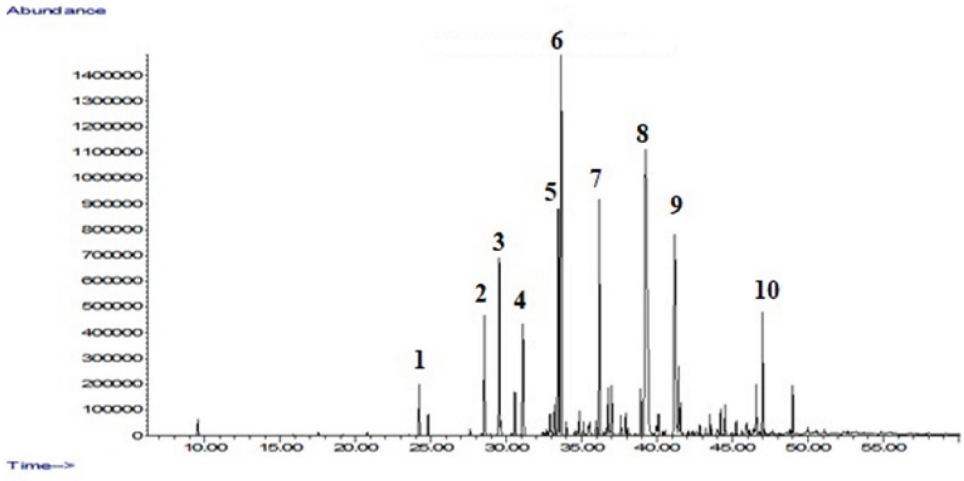

Figure 3. GC/MS Chromatogram of *pelargonium graveolens* essential oil (1: *Cis*-Rose Oxide, 2: Menthone, 3: Isomenthone, 4: Linalool, 5: 6,9-Guaiadiene, 6: Citronellyl Formate, 7: Geranyl Formate, 8: Citronellol, 9: Geraniol, 10: Neryl Tiglate).

Table 3. FIC and FICI values of binary combinations.

Bacteria	Combination	Oil and Chlorhexidine FIC value	Combination FICI value	Result
S. mutans	*M. x piperita* + Chlorhexidine	1 and 0.4166	1.4166	0.75≤FICI ≤2) Additive
	T. serpyllum + Chlorhexidine	0.031 and 3.333	3.3643	(FICI >2) Antagonism
	P.graveolens + Chlorhexidine	0.016 and 0.4166	0.4326	FICI ≤0.5 Synegism
S. aereus	*M. x piperita* + Chlorhexidine	1 and 0.002	1.002	0.75≤FICI ≤2) Additive
	T. serpyllum + Chlorhexidine	0.5 and 0.001	0.501	(0,5 ≤FICI ≤0.75) Partial synergism
	P. graveolens + Chlorhexidine	0.25 and 0.002	0.252	FICI ≤0.5 Synergism

microorganisms, using the binary combinations of both essential oils and Chlorhexidine, significantly increasing the *in vitro* antimicrobial activity. The synergistic effect of tested essential oils and Chlorhexidine can be justified as feasible, which may suggest a new insight against resistant oral pathogen microorganisms. However, further *in vivo* studies are needed using essential oil – antibiotic/antimicrobial agent combinations as well as formulations to confirm the efficacy and safety.

Table 4. Disc Diffusion Test Results.

Microorganism	Formulation	Diameter (mm)
S. mutans	*Mentha x piperita* + *Chlorhexidine*	12
S. aureus		10
S. mutans	*Thymus serpyllum* + *Chlorhexidine*	12
S.aureus		11
S.mutans	*Pelargonium graveolens* +	17
S.aureus	*Chlorhexidine*	10
	Chlorhexidine	18

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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