Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.apjtb.com

Document heading doi:10.12980/APJTB.4.2014C1203

Antimicrobial activity of some essential oils against oral multidrug-resistant *Enterococcus faecalis* in both planktonic and biofilm state

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PEER REVIEW

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Comments

This study evaluated some EOs in treatment of intractable oral infections, principally caused by biofilm of multidrug-resistant *E. faecalis*. The results of this study is useful for *E. faecalis* infection treatment. The high yield and strong antimicrobial activity of three Algerian medicinal plants EOs used in eradication of MDR pathogens from oral ecosystem may contribut to the medical treatment for oral intractable infections caused by *E. faecalis*.

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ABSTRACT

Objective: To evaluate some essential oils in treatment of intractable oral infections, principally caused by biofilm of multidrug-resistant *Enterococcus faecalis* (*E. faecalis*), such as persistent endodontic infections in which their treatment exhibits a real challenge for dentists.

Methods: Ten chemically analyzed essential oils by gas chromatography-mass spectrometry were evaluated for antimicrobial activity against sensitive and resistant clinical strains of *E. faecalis* in both planktonic and biofilm state using two methods, disk diffusion and broth micro-dilution.

Results: Studied essential oils showed a good antimicrobial activity and high ability in *E. faecalis* biofilm eradication, whether for sensitive or multidrug-resistant strains, especially those of *Origanum glandulosum* and *Thymbra capitata* with interesting minimum inhibitory concentration, biofilm inhibitory concentration, and biofilm eradication concentration values which doesn't exceed 0.063%, 0.75%, and 1.5%, respectively.

Conclusions: Findings of this study indicate that essential oils extracted from aromatic plants can be used in treatment of intractable oral infections, especially caused by biofilm of multidrug-resistant *E. faecalis*.

KEYWORDS

Bacterial infections, Biofilm, *Enterococcus faecalis*, Essential oils, Multidrug-resistance

1. Introduction

Enterococci are commensal Gram-positive bacteria that inhabit in oral cavity, gastrointestinal tract, and vagina of humans and animals[1]. These bacteria can cause a wide variety of diseases in humans, especially, nosocomial infections and they now rank among the leading causative pathogens in the world[2]. *Enterococcus faecalis* (*E. faecalis*) is responsible for up to 90% of human enterococcal infections[3], its pathogenicity ranges from life threatening

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Foundation Project: Supported by the Ministry of Higher Education and Scientific Research of People's Democratic Republic of Algeria (Grant No. B05/N125).

Article history:
Received 13 Feb 2014
Received in revised form 20 Feb, 2nd revised form 25 Feb, 3rd revised form 3 Mar 2014
Accepted 12 Apr 2014
Available online 28 Jun 2014

diseases in compromised individuals to less severe conditions particularly due to many virulence factors[4]. Enterococci are multidrug-resistant (MDR) bacteria to most antimicrobial drugs used to treat human infections which exhibit a considerable therapeutic challenges[5].

In oral cavity, *E. faecalis* is not considered to be part of the normal oral microbiota[6]. *E. faecalis* is mainly responsible for several oral pathologies, particularly, dental caries[7], dental abscess[8], periodontal infections[9], apical periodontitis[10], and persistent endodontic infections, also known as post-treatment endodontic diseases, in which *E. faecalis* is the etiological causative agent and responsible for serious complications[11,12]. This can be explained by the fact that this bacterium possesses not only many virulence factors, but also an endogenous resistance to extreme ecological conditions and antimicrobials[8], allowing *E. faecalis* to tolerate harsh environmental conditions in some sites within oral cavity, especially in root canal[11].

The resistance of microorganisms to harsh conditions is due to biofilm formation[13], a complex of lifestyle that allowing bacteria displaying specific properties, including an increase in resistance to antibiotics and antiseptic chemicals[14]. In fact, formation of these sessile communities and their inherent resistance to biocides are the origin of many persistent and chronic bacterial infections[15]. In dental root canal, eradication of *E. faecalis* with chemo-mechanical preparations and using antiseptics is difficult[11]. Even the most used antiseptics in endodontic treatments, sodium hypochlorite and chlorhexidine showed low ability to eliminate *E. faecalis*[16].

The lack of strategies for *E. faecalis* biofilm elimination requires trying other substances except antiseptics and antibiotics, such as secondary metabolites of plants, especially, essential oils (EOs) one of the most important bioactive substances in medicinal plants[17]. Possessing a good antimicrobial activity[18], EOs can replace treatments with antibiotics and disinfection using antiseptics. Furthermore, EOs have many interesting medicinal properties which can contribute to the treatment of intractable oral infections such as anti-inflammatory[19,20], anti-oxidant and stimulating the immune system response activities[21,22].

Treatment of oral infections by plant preparations, such as decoctions and infusions, is very popular among Arab peoples. Major reason of using those herbs extracts is their effectiveness and availability. In Algeria, many herbs especially from Lamiaceae family are widely used in treatment of oral diseases such as candidiasis, dental caries and periodontal diseases. Present principally in wild, species of thyme, lavender, oregano and rosemary are even applied by local population as antiseptics and for oral cavity aromatization because of their refreshing scent.

In the lack of studies that evaluate EOs as treatments of intractable oral infections, such as persistent endodontic infections, the aim of this study was to evaluate some Algerian EOs as natural antiseptics and antimicrobials against MDR *E. faecalis*, one of the principal oral pathogens, in both planktonic and biofilm state.

2. Materials and methods

2.1. Plant material

We have selected ten medicinal plants for this study which are presented in Table 1. The choice of plant species is based on their use by the local population against oral infections, such as periodontal infections and dental caries. All species have been harvested from the region of

Table 1

Data on the studied plant material.

Scientific name	Family	Studied organs	Harvest station			Harvest date
			Name (Municipality)	Location	Altitude (m)	
Ammi visnaga (L.) Lam.	Apiaceae	Leaves, Stems, Flowers & Seeds	Bouhannak (Mansoura)	+34°88′19″ − 1°37′07″	711	Jul-11
Ammoides verticillata (Desf.) Briq.	Apiaceae	Leaves, Stems, Flowers & Seeds	Atar (Mansoura)	+34°88′51″ −1°37′75″	980	Jul-11
Artemisia arborescens (Vaill.) L.	Asteraceae	Leaves, Stems & Flowers	Sidi Yahyia (Sidi Medjahed)	+34°46′45″ −1°38′12″	380	Jul-11
Dittrichia graveolens (L.) Greuter	Asteraceae	Leaves, Stems & Flowers	Bouhannak (Mansoura)	+34°88′14″ −1°36′38″	725	Aug-11
Lavandula dentata L.	Lamiaceae	Leaves, Stems & Flowers	Sidi Yahyia (Sidi Medjahed)	+34°88′14″ −1°36′38″	580	Jul-11
Lavandula multifida L.	Lamiaceae	Leaves, Stems & Flowers	Bouhannak (Mansoura)	+34°88′51″ −1°37′75″	700	Oct-11
Mentha piperita L.	Lamiaceae	Leaves, Stems & Flowers	Ouled charef (Maghnia)	+34 °49′57″ −1°42′4″	400	May-12
Origanum vulgare subsp. *glandulosum* (Desf.) Ietsw.	Lamiaceae	Leaves, Stems & Flowers	Atar (Mansoura)	+34°88′51″ −1°37′75″	980	Jun-11
Rosmarinus eriocalyx Jord. & Fourr.	Lamiaceae	Leaves, Stems & Flowers	Honaine	+34°88′14″ −1°36′38″	100	Jun-11
Thymbra capitata (L.) Cav.	Lamiaceae	Leaves, Stems & Flowers	El Koudia	+34°53′59″ −1°21′55″	690	Jul-11

Tlemcen located in the northwestern Algeria, from Jun 2011 to May 2012, in the wild and others cultivated. Specimens of all species in this study were identified by Laboratory of Ecology and Management of Natural Ecosystems, University of Tlemcen. All voucher specimens were deposited in our laboratory.

2.2. Obtaining EOs

For this purpose, we have used hydrodistillation with Clevenger-type apparatus of the fresh plant material, as recommended by Benbelaïd *et al*[23]. Recovered EOs were dried using magnesium sulfate and stored in smoked vials at 4 °C until analysis.

2.3. EOs analysis with GC and GC/MS

Gas chromatography (GC) analysis was performed using a Perkin Elmer Autosystem GC-type chromatograph, equipped with two flame ionization detectors, for the detection of volatile compounds, one injector/splitter, and two polar (Rtx-Wax, polyethylene glycol) and nonpolar (Rtx-1, polydimethylsiloxane) columns (60 m×0.22 mm inner diameter, film thickness 0.25 μm). The carrier gas was helium (1 mL/min) with a column head pressure of 25 psi. The injector temperature was 250 °C and that of the detector was 280 °C. The temperature was programmed to increase from 60 to 230 °C at the rate of 2 °C/min, and then maintained constant for 45 min at a level of 230 °C. The injection was done by split mode with a split ratio of 1/50. The amount of EO injected was 0.2 μL. Quantification was made by direct electronic integration of peak areas.

For the gas chromatography-mass spectrometry (GC/MS), analysis was performed using a Perkin Elmer Autosystem XL chromatograph, with an automatic injector and two polar (Rtx-Wax) and nonpolar (Rtx-1) columns (60 m×0.22 mm inner diameter, film thickness 0.25 μm), coupled with a Perkin Elmer TurboMass mass detector. The carrier gas was helium (1 mL/min) with a column head pressure of 25 psi. The injector temperature was 250 °C. The temperature was programmed to rise from 60 to 230 °C at the rate of 2 °C/min, and then kept constant for 35 min at a level of 230 °C. The injection was done by split mode with a split ratio of 1/80. The amount of EO injected was 0.2 μL. Detection was carried out by a quadrupole analyzer which consisted of an assembly of four parallel electrodes with cylindrical section. The source temperature was 150 °C. The device functioned in electron impact and fragmentation was performed at an electric field of 70 eV. The resulting mass spectra were acquired over the mass range of 35-350 Da.

To identify the constituents of the studied EOs, identification made by Kovats index was used, where the polar and nonpolar retention indices were calculated from the retention times of a series of *n*-alkanes, and from databases of mass spectra, where the obtained mass spectra were compared with those of computerized libraries[24].

2.4. Microbial strains

Seven strains of *E. faecalis* have been selected for this study; two of them are American Type Culture Collection strains with codes ATCC 29212 and ATCC 49452 (sensitive to antibiotics), while the rest are multidrug-resistant strains, selected from a collection of clinical *E. faecalis* strains obtained from patients with various oral infections, including, apical periodontitis, chronic periodontitis, aggressive periodontitis, and cervicofacial cellulitis which are summarized in Table 2. Strain samples were taken in the service of stomatology at University Health Center of Tlemcen from December 2011 to June 2012. At the first time, samples were enriched in Roth broth (Conda Pronadisa™, Spain) at 37 °C for 18 h. Thus, positive culture was inoculated in bile esculin agar (Fluka®, Switzerland) and incubated at 37 °C for 24 h, in order to isolate pure colonies. After purification, all isolated strains of *E. faecalis* were firstly identified by conventional microbiological methods, while the final identification confirmation was carried out by API Strep® gallery (BioMérieux®, France). Finally, all *E. faecalis* strains were conserved in brain heart infusion broth (Conda Pronadisa™, Spain) with glycerol (Fluka®, France) (8:2,v/v) at -20 °C.

Table 2

Data on studied *E. faecalis* strains.

Strains	Origin	Antibiotics						
		GN	C	VA	E	CIP	AX	TE
1	Apical periodontitis	R	R	R	R	R	S	R
2	Apical periodontitis	R	R	R	R	R	S	R
3	Cervicofacial cellulitis	R	S	R	R	S	S	R
4	Aggressive periodontitis	R	R	R	R	S	S	R
5	Chronic periodontitis	R	R	R	S	S	S	R

GN: Gentamicin; C: Chloramphenicol; VA: Vancomycin; E: Erythromycin; CIP: Ciprofloxacin; AX: Amoxicillin; TE: Tetracycline. R: Resistant; S: Sensitive.

2.5. Antibiogram

For selection of *E. faecalis* multidrug-resistant strains from clinical collection, we have performed an antibiogram according to Clinical and Laboratory Standards Institute (CLSI) recommendations[25]. Antibiogram was determined with the following antimicrobial agent-containing disks: amoxicillin (25 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (15 μg), tetracycline (30 μg) and vancomycin (30 μg) (Oxoid®, England).

2.6. Antimicrobial assay

2.6.1. Inocula preparation

Previously identified and conserved strains were taken and

inoculated in Mueller-Hinton broth (Fluka®, India). After incubation at 37 °C for 24 h, suspensions were taken and shaken well using the vortex then diluted for standardizing. Inocula were set to 0.5 McFarland or an optical density from 0.08 to 0.13 at 625 nm wavelength, which corresponds to 10^8 CFU/mL[25].

2.6.2. Antimicrobial activity of EOs against planktonic E. faecalis strains

2.6.2.1. Disk diffusion method

We have used Kirby-Bauer's agar disk diffusion modified method[26], where antimicrobial activity of EOs against *E. faecalis* strains was evaluated in plates with Mueller-Hinton agar (Fluka®, India) pre-inoculated by swabbing of standardized microbial suspension at 10^8 CFU/mL. Whatman No. 3 paper disks impregnated with 10 μL of EO, were placed on the surface of agar, each disk has a 6 mm diameter. After incubation at 37 °C for 24 h, the results were read by measuring the diameter of inhibition zones in millimeters (mm) by vernier scale. All tests were performed in triplicate.

2.6.2.2. MIC determination

The minimum inhibitory concentrations (MICs) of EOs were determined by modified broth micro-dilution method from Wiegand *et al*[27]. Briefly, concentrations of each EO were prepared by 1/2 dilution series in Mueller-Hinton broth with 1% of Tween 80, starting from 40.00% to 0.08%. After that, 96-well microplate was filled by distributing 90 μL of 5×10^5 CFU/mL inoculum (prepared by 1/200 dilution of 10^8 CFU/mL inoculum) with 10 μL of each concentration. The final concentration of EOs in wells was ranging from 4% to 0.008%, and the final concentration of Tween 80 was 0.1% in each well. After 24 h incubation at 37 °C, the MIC was defined as the lowest concentration of EO inhibiting visible growth. In addition, two wells of every range of microplate were filled with inoculum and inoculum with 0.1% of Tween 80, and served as positive controls for each strain. All tests were performed in triplicate.

2.6.3. Antimicrobial activity of EOs against E. faecalis strains in biofilm

2.6.3.1. Biofilm inhibitory concentration

The biofilm inhibitory concentrations (BICs) of studied EOs against *E. faecalis* strains in biofilm case were determined as described by Nostro *et al.*[28] with modification. Firstly, 96-well microplate was filled by distributing 100 μL of inoculum at 10^8 CFU/mL in each well. After 24 h of incubation at 37 °C, medium was gently removed and all wells were washed three times with sterile phosphate buffered saline. In the same moment, ten concentrations of each EO were prepared by 1/2 dilution series in Mueller-Hinton broth with 3.33% of Tween 80, starting from 40.00% to 0.08%. After removing of planktonic cells from microplate, all wells were filled with 70 μL of sterile Mueller-Hinton broth with 30 μL of each concentration, the final concentrations of EO in wells were ranging from 12% to 0.02%, and the final concentration of Tween 80 was about 1% in each well. Biofilm inhibitory concentration was determined after 24 h incubation at 37 °C, as the lowest concentration with no culture in well, visually determined and confirmed by no increase in optical density compared with the initial reading. Two ranges of wells for each microplate were filled with sterile Mueller-Hinton broth and serve as positive controls. All tests were performed in triplicate.

2.6.3.2. Biofilm eradication concentration

Biofilm eradication concentration (BEC) is the lowest concentration of EO that kills all viable cells of *E. faecalis* present and protected in biofilm. BEC was defined also as described by Nostro *et al.*[28]with modification. Protocol was continued in the same 96-well microplate used in determination of BIC, in the same day where the result of BIC was read. Supernatant fluid was gently removed and the wells were well washed three times with sterile phosphate buffered saline and one time with 20% sterile ethanol solution (Scharlau®, Spain) (in order to eliminate remaining traces of EOs). Then, microplate wells were filled with sterile tryptic soy agar (Conda Pronadia™, Spain) and incubated for 72 h at 37 °C. BEC was determined as the lowest concentration with no culture visually determined and confirmed by no increase in optical density compared with the initial reading. Positive control was performed to verify that 20% of ethanol has no effect on strains. All tests were performed in triplicate.

2.7. Statistical analysis

Statistical analyses used in this study have been carried out using Microsoft® Excel. Where, comparison between antimicrobial activity against reference and clinical strains was performed by Student *t*-test at 95% level ($P<0.05$) in both planktonic and biofilm state.

3. Results

3.1. Chemical composition of studied EOs

Quantitative and qualitative analytical results of the studied EOs by GC and GC/MS are shown in Table 3. In which we notice variability in composition of EOs between studied plants. EOs of species which belong to Lamiaceae and Apiaceae family are mainly rich in oxygenated monoterpenes, especially alcohols, such as thymol, carvacrol, and linalool with a high percentage. *Thymbra*

Table 3

Chemical composition of studied EOs.

#	Component	nRI	pRI	Chemical composition (%)									ID
				1	2	3	4	5	6	7	8	9	10	
1	Santolina triene	901	1018				0.70							RI, MS
2	Isobutyl isobutyrate	902	1090	2.27										RI, MS
3	Tricylene	921	1020									0.68		RI, MS
4	alpha-Thujene	922	1023	1.55	0.33					0.33	1.02		2.07	RI, MS
5	alpha-Pinene	931	1022	1.90	1.04	0.90	0.88	4.86	1.12		0.74	11.68	1.07	RI, MS
6	Camphene	943	1066				6.03	0.91			0.11	11.64	0.48	RI, MS
7	1-Octen-3-ol	959	1446						0.56		0.17		0.99	RI, MS
8	Octan-3-one	963	1253								0.13			RI, MS
9	Sabinene	964	1120	2.25		1.87		1.51		0.38				RI, MS
10	beta-Pinene	970	1110		0.11		1.19	13.89	0.42	0.51	0.19	2.77	0.54	RI, MS
11	Myrcene	979	1159		0.57	2.28			0.93	0.57	1.98	0.71	2.39	RI, MS
12	Dehydro-1,8-cineole	979	1197				1.29							RI, MS
13	Isobutyl isovalerate	993	1175	3.23										RI, MS
14	alpha-Phellandrene	997	1164								0.26	0.26	0.54	RI, MS
15	2-Methyl butyl isobutyrate	1004	1176	10.27										RI, MS
16	delta-3-Carene	1005	1147								0.1	0.48		RI, MS
17	alpha-Terpinene	1008	1178		0.13	0.64					2.76		1.54	RI, MS
18	para-Cymene	1011	1268	1.71	15.58				2.77		17.07	1.83	8.58	RI, MS
19	Limonene	1020	1199	1.80	15.02		0.58				0.58			RI, MS
20	1,8-Cineole	1020	1209			0.51		36.72	1.26	5.57		15.31		RI, MS
21	cis-beta-Ocimene	1024	1230	1.59							0.11			RI, MS
22	trans-beta-Ocimene	1034	1247	1.78							0.13			RI, MS
23	gamma-Terpinene	1047	1243		6.63	1.14					27.03	0.35	5.67	RI, MS
24	trans-Sabinene hydrate	1051	1451			1.75					0.17	0.11	1.80	RI, MS
25	Linalool oxide	1057	1435					1.34		0.53				RI, MS
26	alpha-Thujone	1067	1395			1.44								RI, MS
27	Fenchone	1071	1401	3.78				0.83				0.32		RI, MS
28	Terpinolene	1078	1280		0.13	1.10			0.56		0.10	0.30		RI, MS
29	Linalool	1081	1544	35.57	0.11	0.80		3.11		51.59	0.66	0.30	0.57	RI, MS
30	2-Methyl butyl isovalerate	1098	1274	14.14										RI, MS
31	beta-Thujone	1103	1422			47.58								RI, MS
32	Camphor	1123	1517			0.91						35.50		RI, MS
33	trans-Pinocarveol	1125	1651					5.76						RI, MS
34	trans-Verbenol	1129	1676					2.02						RI, MS
35	Pinocarvone	1136	1558					2.35						RI, MS
36	delta-Terpineol	1143	1658					1.53						RI, MS
37	Borneol	1148	1698			1.05	19.48	2.04			0.12	2.03	1.07	RI, MS
38	Cryptone	1157	1667					2.30						RI, MS
39	Terpinen-4-ol	1161	1600		0.18	3.26		0.96			0.11	2.11	5.16	RI, MS
40	Myrtenal	1172	1628					4.12						RI, MS
41	Estragole	1176	1670	4.49										RI, MS
42	Myrtenol	1177	1789					3						RI, MS
43	alpha-Terpineol	1179	1700		0.14	0.83		1.82	1.51	6.87	0.16	0.47		RI, MS
44	trans-Carveol	1196	1832					1.09						RI, MS
45	Cuminaldehyde	1217	1782					1.14						RI, MS
46	Carvone	1222	1739					1.17						RI, MS
47	Carvacrol methyl ether	1231	1603						0.84					RI, MS
48	Geraniol	1232	1844							3.15				RI, MS
49	Linalyl acetate	1240	1557							21.12				RI, MS
50	Thymol	1266	2189		50.13						41.62			RI, MS
51	Bornyl acetate	1269	1515				56.16					0.33		RI, MS
52	Perillyl alcohol	1276	2005					0.86						RI, MS
53	Carvacrol	1278	2219		8.81	0.92			57.75		2.15		58.68	RI, MS
54	Eugenol	1330	2171									0.41		RI, MS
55	alpha-Terpinyl acetate	1334	1695						0.15					RI, MS
56	Neryl acetate	1342	1725							2.26				RI, MS
57	Methyl eugenol	1367	2009						0.16					RI, MS

Results are in percentage (%) of components for EOs of (1) *A. visnaga*, (2) *A. verticillata*, (3) *A. arborescens*, (4) *D. graveolens*, (5) *L. dentata*, (6) *L. multifida*, (7) *M. piperita*, (8) *O. vulgare* subsp. *glandulosum*, (9) *R. eriocalyx*, and (10) *T. capitata*. Percentages and elution order of individual components are given on nonpolar column. Retention indices nRI and pRI are given respectively on nonpolar (Rtx-1) and polar (Rtx-Wax) columns. ID: identification method by comparison of RI and MS. RI: Retention indices; MS: Mass spectra.

Table 3, continued

Chemical composition of studied EOs.

#	Component	nRI	pRI	Chemical composition (%)										ID
				1	2	3	4	5	6	7	8	9	10	
58	alpha-Ylangene	1375	1476									0.16		RI, MS
59	gamma-Caryophyllene	1407	1571				1.91						1.70	RI, MS
60	Neryl acetone	1410	1825									0.51		RI, MS
61	3-Buten-1-ol, 3-methyl-, benzoate	1422	1979				0.88							RI, MS
62	beta-Caryophyllene	1424	1591					0.58	1.86		0.96			RI, MS
63	beta-Farnesene	1448	1661						0.09					RI, MS
64	alpha-Humulene	1456	1665								0.12			RI, MS
65	Germacrene D	1478	1710			13.85						0.10		RI, MS
66	beta-Bisabolene	1500	1720						22.91		0.14			RI, MS
67	Cadinene D	1516	1725									0.30		RI, MS
68	beta-Sesquiphellandrene	1516	1765								0.57			RI, MS
69	Spathulenol	1560	2119						1.58					RI, MS
70	Caryophyllene oxide	1576	1980				2.56		1.24		0.18	0.46		RI, MS
71	Caryophyllen-4(14),8(15)-dien-5α-ol	1624	2155				1.06							RI, MS
72	T-Cadinol	1632	2169				0.93					0.20		RI, MS
73	beta-Eudesmol	1640	2230					0.81						RI, MS
74	alpha-Cadinol	1645	2231									0.94		RI, MS
75	Intermedeol	1647	2215				0.63							RI, MS
76	alpha-Bisabolol	1672	2217									0.50		RI, MS
77	Chamazulene	1713	2410			13.39								RI, MS
78	Geranyl linalool	2026	2444	6.06										RI, MS
	Yield (% v/w)			0.7	1.7	1.3	0.8	1.8	0.4	1.5	2.3	1.9	1.9	
	Total identified (%)			92.39	98.91	94.22	94.28	94.72	95.71	92.88	99.44	90.76	92.85	
	Monoterpene hydrocarbons			12.58	39.54	7.93	9.38	21.17	5.8	1.79	52.18	33.70	22.88	
	Oxygenated monoterpenes			39.35	59.37	59.05	76.93	72.16	62.07	91.09	44.99	56.76	67.28	
	Sesquiterpene hydrocarbons					27.24	5.53	0.58	24.86		1.79	0.56	1.70	
	Oxygenated sesquiterpenes						1.56	0.81	2.86		0.18	2.61		
	Others			40.46			0.88		0.16		0.30	0.41	0.99	

Results are in percentage (%) of components for EOs of (1) *A. visnaga*, (2) *A. verticillata*, (3) *A. arborescens*, (4) *D. graveolens*, (5) *L. dentata*, (6) *L. multifida*, (7) *M. piperita*, (8) *O. vulgare* subsp. *glandulosum*, (9) *R. eriocalyx*, and (10) *T. capitata*. Percentages and elution order of individual components are given on nonpolar column. Retention indices nRI and pRI are given respectively on nonpolar (Rtx-1) and polar (Rtx-Wax) columns. ID: identification method by comparison of RI and MS. RI: Retention indices; MS: Mass spectra.

Table 4

Inhibition effect of studied EOs against planktonic *E. faecalis* strains. Expressed by the diameter inhibition zones (IZ in mm) and minimum inhibitory concentration (MIC in % v/v) methods.

Species		R1	R2	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5
A. visnaga	IZ	10±1	11±0	11±1	11± 0	10±1	11±0	10±1
	MIC	4.000±0.000	4.000±0.000	4.000±0.000	2.000±0.000	4.000±0.000	4.000±0.000	4.000±0.000
A. verticillata	IZ	35±1	34±1	36±1	35±1	35±2	35±1	34±1
	MIC	0.250±0.000	0.208±0.072	0.250±0.000	0.250±0.000	0.250±0.000	0.208±0.072	0.250±0.000
A. arborescens	IZ	10±1	11±0	11±1	11±1	10±0	11±1	11±0
	MIC	4.000±0.000	4.000±0.000	4.000±0.000	3.333±1.154	4.000±0.000	4.000±0.000	4.000±0.000
D. graveolens	IZ	12±1	13±1	13±0	13±1	13±0	13±1	12±1
	MIC	4.000±0.000	4.000±0.000	2.000±0.000	2.000±0.000	4.000±0.000	3.333±0.154	4.000±0.000
L. dentata	IZ	15±0	16±1	15±1	16±1	15±1	16±1	15±1
	MIC	1.000±0.000	0.833±0.288	0.666±0.288	0.500±0.000	1.000±0.000	1.000±0.000	1.000±0.000
L. multifida	IZ	26±1	26±1	27±1	27±1	26±1	26±1	26±1
	MIC	0.250±0.000	0.250±0.000	0.250±0.000	0.208±0.072	0.250±0.000	0.250±0.000	0.250±0.000
M. piperita	IZ	9±1	9±0	10±0	11±1	10±0	10±1	9±0
	MIC	2.000±0.000	2.000±0.000	2.000±0.000	1.666±0.057	2.000±0.000	2.000±0.000	2.000±0.000
O. vulgare subsp.	IZ	28±1	29±1	29±1	29±1	28±1	29±1	28±1
glandulosum	MIC	0.063±0.000	0.063±0.000	0.026±0.009	0.026±0.009	0.063±0.000	0.032±0.000	0.063±0.000
R. eriocalyx	IZ	13±1	13±0	13±1	13±2	13±0	13±0	12±1
	MIC	1.000±0.000	1.000±0.000	1.000±0.000	0.833±0.288	1.000±0.000	1.000±0.000	1.333±0.057
T. capitata	IZ	30±1	31±1	32±1	32±1	31±1	31±1	30±1
	MIC	0.052±0.018	0.063±0.000	0.063±0.000	0.052±0.018	0.063±0.036	0.063±0.000	0.063±0.000

All results are mean±SD of three repeats. R1: *E. faecalis* ATCC 29212; R2: *E. faecalis* ATCC 49452. -: not determined.

capitata (*T. capitata*) and *Lavandula multifida* (*L. multifida*) are constituted principally by carvacrol (58.68% and 57.75%, respectively), beta-bisabolene (22.91%) is the second major component in *L. multifida* EOs. Thymol followed by gamma-terpinene and para-cymene are the main constituents in EO of *Origanum glandulosum* (*O. glandulosum*) (41.62%, 27.03%, and 17.07%, respectively), and thymol followed by para-cymene and limonene in EO of *Ammoides verticillata* (*A. verticillata*) (50.13%, 15.58%, and 15.02%, respectively). Linalool and linalyl acetate (51.59% and 21.12%, respectively) are the major constituents of *Mentha piperita* (*M. piperita*). While the rest of studied EOs are constituted principally by non-alcoholic terpenes, such as beta-thujone (47.58%) in *Artemisia arborescens* (*A. arborescens*), bornyl acetate (56.16%) in *Dittrichia graveolens* (*D. graveolens*), 1,8-cineole (36.72%) in *Lavandula dentata* (*L. dentata*), and camphor (35.50%) in *Rosmarinus eriocalyx* (*R. eriocalyx*).

3.2. Antimicrobial activity of EOs against MDR *E. faecalis*

During sampling at the Service of Dentistry at University Health Center of Tlemcen, we have remarked that *E. faecalis* was responsible for several oral complications, especially, apical periodontitis. Antibiogram results of *E. faecalis* strains have shown that almost all of these bacteria are multidrug-resistant to majority of antibiotics, especially vancomycin and gentamicin, only amoxicillin was effective on all these *E. faecalis* strains.

Antimicrobial activity evaluation of studied EOs against *E. faecalis* strains was very interesting, whether in planktonic or biofilm state. In planktonic, both methods, agar disk diffusion and MIC determination (Table 4), have shown that EOs of *T. capitata* and *O. glandulosum* were the most active, with inhibition zone larger than 27 mm and MIC less than 0.07% whether for susceptible or resistant strains. At less degree, *A. verticillata*, *L. multifida*, *L. dentata*, and *M. piperita* have a good antimicrobial activity on all strains, while the rest of EOs have shown a moderate activity.

In biofilm state (Table 5), both EOs of *T. capitata* and *O. glandulosum* were also the effective oils, which can inhibit biofilm growth of all *E. faecalis* strains at 0.75%, and can eradicate all viable microbial cells protected in biofilm at 1.50% only. Other EOs have shown an interesting antimicrobial activity against *E. faecalis* strains in biofilm, notably *A. verticillata* and *L. multifida* with their BECs range between 1.50% and 3.00%.

Table 5

Inhibition effect of studied EOs against strains of *E. faecalis* in biofilm. Expressed by biofilm inhibitory concentration (BIC in %v/v) and biofilm eradication concentration (BEC in %v/v) methods.

Species		R1	R2	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5
A. visnaga	BIC	6.00±0.00	6.00±0.00	12.00±0.00	12.00±0.00	6.00±0.00	12.00±0.00	6.00±0.00
	BEC	6.00±0.00	6.00±0.00	–	–	12.00±0.00	–	12.00±0.00
A. verticillata	BIC	0.38±0.00	0.38±0.00	0.75±0.00	0.75±0.00	0.75±0.00	0.75±0.00	0.75±0.00
	BEC	0.75±0.00	0.75±0.00	2.00±0.86	3.00±0.00	1.50±0.00	3.00±0.00	1.50±0.00
A. arborescens	BIC	6.00±0.00	6.00±0.00	–	–	12.00±0.00	–	12.00±0.00
	BEC	12.00±0.00	12.00±0.00	–	–	–	–	–
D. graveolens	BIC	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00
	BEC	6.00±0.00	6.00±0.00	8.00±3.46	12.00±0.00	6.00±0.00	12.00±0.00	6.00±0.00
L. dentata	BIC	1.50±0.00	1.50±0.00	3.00±0.00	4.00±1.73	3.00±0.00	3.00±0.00	3.00±0.00
	BEC	3.00±0.00	3.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00
L. multifida	BIC	0.38±0.00	0.50±0.21	0.75±0.00	0.75±0.00	0.75±0.00	0.75±0.00	0.75±0.00
	BEC	0.75±0.00	0.75±0.00	2.00±0.86	3.00±0.00	2.50±0.86	3.00±0.00	1.50±0.00
M. piperita	BIC	1.50±0.00	1.50±0.00	3.00±0.00	3.00±0.00	3.00±0.00	3.00±0.00	3.00±0.00
	BEC	3.00±0.00	3.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	3.00±0.00
O. vulgare subsp. *glandulosum*	BIC	0.38±0.00	0.38±0.00	0.75±0.00	0.75±0.00	0.75±0.00	0.75±0.00	0.75±0.00
	BEC	0.75±0.00	0.63±0.22	1.50±0.00	1.50±0.00	1.00±0.43	1.50±0.00	0.75±0.00
R. eriocalyx	BIC	3.00±0.00	3.00±0.00	6.00±0.00	6.00±0.00	3.00±0.00	6.00±0.00	3.00±0.00
	BEC	6.00±0.00	6.00±0.00	–	–	12.00±0.00	–	12.00±0.00
T. capitata	BIC	0.38±0.00	0.38±0.00	0.75±0.00	0.75±0.00	0.75±0.00	0.75±0.00	0.75±0.00
	BEC	0.63±0.21	0.75±0.00	1.50±0.00	1.50±0.00	1.25±0.43	1.50±0.00	1.00±0.43

All results are mean±SD of three repeats. R1: *E. faecalis* ATCC 29212; R2: *E. faecalis* ATCC 49452. –: not determined.

4. Discussion

In the present study, we have evaluated some Algerian EOs against oral MDR *E. faecalis*. The choice of this species is based on many factors. Firstly, because of its multiple virulence factors[29], the presence of this Gram-positive bacterium in the oral cavity has, in fact, a relation with several dental diseases, especially apical periodontitis[10]. In a research released by Salah *et al.*[30] all *E. faecalis* isolates in their study were recovered only from patients with dental diseases, especially necrotic pulps, while no *E. faecalis* strains were found in healthy patients. Secondly, *E. faecalis* is a nosocomial bacterium which can resist against many antibiotics[31], chemo-mechanical preparations, and

antiseptics, which represent a real challenge for dentists in some intractable infections, as seen in post-treatment endodontic diseases[11]. Thirdly, *E. faecalis* has an ability to form biofilm which seems to be a key factor in many bacterial infections and resistance of microorganisms to disinfections[13,32,33]. So, all these factors make *E. faecalis* as one of the major pathogens in the oral cavity, with a high risk of treatment failure because of its resistance to both antibiotics and antiseptics. Therefore, elimination of this harmful bacterium from oral ecosystem needs other solutions.

As alternative to antibiotics and antiseptics, we have tested some EOs against oral multidrug-resistant *E. faecalis*, especially in biofilm state, which is the real existence state of this harmful bacterium in the oral cavity, and the reason for its ability to survive under severe conditions causing pathogenesis during chronic infections. Antimicrobial activity evaluation of studied EOs against *E. faecalis* strains was very interesting, whether in planktonic or biofilm state. When we compare the ability to total *E. faecalis* eradication of studied EOs with well-used endodontic antiseptics, we find these EOs are competitive, especially in biofilm state. In study realized by Sena *et al.*[34], it was found that chlorhexidine at 2% and NaOCl at 5.25% can eradicate *E. faecalis*, on condition when supplemented with mechanical agitation. While, some authors indicate that chlorhexidine and NaOCl show a low ability to eliminate *E. faecalis*[16]. For example, Arias-Moliz *et al.*[35] found that chlorhexidine at 4% did not eradicate *E. faecalis* biofilm. So, we conclude that studied EOs are good alternative antiseptic which can be used instead of chlorhexidine or NaOCl, especially against *E. faecalis* biofilm.

Comparison of the antimicrobial effect of studied EOs, using Student *t*-test at 95% confidence level ($P<0.05$), between sensitive and resistant strains have shown no difference in activity at planktonic state. While in biofilm state, Student *t*-test at 95% confidence level ($P<0.05$) show a significant difference between strains, where studied EOs were more effective against sensitive strains of *E. faecalis* than clinical ones. This could explain that biofilm formation was prevalent among isolates with MDR phenotype[36], as well as the slow metabolic rate of microorganisms in biofilms[37], and the extracellular matrix of the biofilm impede the effectiveness of many antimicrobials, which deters these agents to gain the protected cell inside[38]. We concluded that MDR strains of *E. faecalis* were less sensitive to EOs mainly due to their high ability to biofilm formation. But even if there is a mild resistance of biofilm to EOs, these antimicrobial agents remained effective, especially those of *T. capitata* and *O. glandulosum*.

A huge number of studies that evaluate the antimicrobial activity of many EOs were published in recent years, from which some authors concluded that antimicrobial activity of EOs was strongly correlated with the content of terpenoid phenols such as carvacrol, eugenol and thymol and some other oxygenated monoterpenes such as nerol, linalool, α-terpineol, fenchol and terpinen-4-ol[18,39,40]. In this study, we have found the same remark, where chemical analyses of studied EOs showed that among the most active EOs against MDR *E. faecalis* strains, *O. glandulosum*, *T. capitata*, *L. multifida*, and *A. verticillata* EOs are constituted principally by terpenoid phenols, 41.62% thymol, 58.68% carvacrol, 57.75% carvacrol, and 50.13% thymol, respectively. While *M. piperita* was constituted principally by oxygenated monoterpenes, 51.59% linalool. In the other hand, synergetic effect between all major compounds of EOs was reported in some studies, where EO was more antimicrobially active than its major compound that was responsible for activity. For example, in study realized by Veras *et al.*[41] they found that EO of *Lippia sidoides* was more effective against *Staphylococcus aureus* than thymol alone, its major compound. In addition, Mulyaningsih *et al.*[42] found a good synergetic effect between compounds of *Eucalyptus globulus* EO against multidrug-resistant bacteria, *Staphylococcus aureus* and *Enterococcus faecalis*. As well as, multiple combinations between terpenes were effective against microorganisms[43], the use of the entire EO as antiseptic in mouthwash seems to be better than use of those terpenes alone, in both antimicrobial activity and natural treatment.

In summary, the findings of this study indicate that EOs extracted from aromatic plants can be used in treatment of oral intractable infections caused by *E. faecalis*, especially persistent endodontic infections. Because of their high yield and strong antimicrobial activity in biofilm state, the activity of three Algerian medicinal plants *O. glandulosum*, *T. capitata* and *A. verticillata* as solution used in eradication of MDR pathogens from oral ecosystem seems to be very important in both medical and economical point of view.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The Authors profusely thank Dr. Boumediene Medjahdi, Department of Agronomy and Forestry, Aboubekr Belkaïd University of Tlemcen, for botanical assistance. This work was financially supported by the Ministry of Higher Education and Scientific Research of People's Democratic Republic of Algeria (Grant No. B05/N125).

Comments

Background

Enterococci are the commensal Gram-positive bacteria that inhabit in oral cavity, gastrointestinal tract and vagina of humans and animals. Enterococci are multidrug-resistant bacteria to most antimicrobial drugs used to treat human infections and they exhibit a considerable therapeutic challenges. *E. faecalis* is responsible for several oral pathologies, particularly, dental caries, dental abscess, periodontal infections *etc*. The secondary metabolites of plants, especially, EOs are one of the most important bioactive substances in medicinal plants. Possessing a good antimicrobial activity, EOs can replace treatments with antibiotics and disinfection using antiseptics.

Research frontiers

Ten medicinal plants were studied in this research. The choice of plant species was based on their use by the local population against oral infections, such as periodontal infections and dental caries. Seven strains of *E. faecalis* have been selected for this study; two of them were American Type Culture Collection strains with codes ATCC 29212 and ATCC 49452 (sensitive to antibiotics). While the rest were multidrug-resistant strains, selected from a collection of clinical *E. faecalis* strains obtained from patients with various oral infections, including apical periodontitis, chronic periodontitis, aggressive periodontitis, and cervicofacial cellulitis. Antimicrobial activity of EOs against planktonic *E. faecalis* strains assay and antimicrobial activity of EOs against *E. faecalis* strains in biofilm assay were done.

Related reports

E. faecalis is the etiological causative agent where it's responsible for serious complications. It is the fact that this bacterium possesses not only many virulence factors, but also an endogenous resistance to extreme ecological conditions and antimicrobials, allowing E.faecalis to tolerate harsh environmental conditions in some sites within oral cavity, especially in root canal. EOs have many interesting medicinal properties which can contribute to the treatment of intractable oral infections such as anti-inflammatory, anti-oxidant and stimulating the immune system response activities.

Innovations and breakthroughs

EOs extracted from aromatic plants in this study can be used in treatment of oral intractable infections caused by *E. faecalis*, especially persistent endodontic infections.

Applications

EOs are good alternative antiseptics which can be used instead of chlorhexidine or NaOCl, especially against MDR *E. faecalis* biofilm. It might be applied to use in other virulence bacterial strains.

Peer review

This study evaluated some EOs in treatment of intractable oral infections, principally caused by biofilm of multidrug-resistant *E. faecalis*. The results of this study is useful for *E. faecalis* infection treatment. The high yield and strong antimicrobial activity of three Algerian medicinal plants EOs used in eradication of MDR pathogens from oral ecosystem may contribut to the medical treatment for oral intractable infections caused by *E. faecalis*.

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