LEK. SIROV.	God.XXXVI	Broj 36	Str. 3 – 25	Beograd 2016.
LEK. SIROV.	Vol.XXXVI	No. 36	Pp. 3 – 25	Belgrade 2016.

Pregledni rad – Review paper ***Manuscript received:*** *27.12.2016.*
UDC: 665.53; 615.322.07:665.5 ***Accepted for publication:*** *30.12.2016.*
COBISS.SR-ID 228489996

ANALYSIS OF CHEMICAL COMPOSITION OF THE MOST EFFICIENT ESSENTIAL OILS TOWARDS *ENTEROCOCCUS FAECALIS* REFERENT STRAIN ATCC 29212 AND CLINICAL ISOLATES

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SUMMARY

Twenty one essential oils (EOs) documented their significant antimicrobial effect with regard to our pre–set criterion of the Minimal Inhibitory Concentration (MIC ≤ 200 μL / mL) of EOs towards *Enterococcus faecalis* (ATCC 29212 and or clinical isolates); the best effect MIC 0.4 μL / mL (approx. 0.26 μg / mL) achieved *Satureja horvatii* L. EO, while the EOs with the lowest antimicrobial efficacy were *Rosmarinus officinalis* L. and *Achilea milefolium* L., both with MIC s160.0 μg / mL. Analysis of the MIC values within the groups revealed that ATCC strain of *E. faecalis* is generally lower, ranging from 0.26 to 156 μg / mL, in comparison to those for clinical isolateswhich ranged from 10 to 160 μg / mL. Twelve 12 components that are common in EOs whith MIC s ≤ 200 μg / mL in testings towards both, the clinical and referent strains are given in descending order according to number of oils they are present in: *trans*–β–caryophyllene (13) > myrcene (8) > α–pinene (8) > linalool (7) > *p*–cymene (7) > borneol (7) > geraniol (6) > camphene (6) > limonene (5) > 1,8– cineol (5) > *γ* –terpinene (5) > *α*–terpinene (4). Comparison of EO constituents reviled that only, geraniol and 1,8–cineol, contributed with ≥ 10 % to more than one EO (MIC 0.3–200 μg / mL) efficient against both *E. faecalis* strains. Thirteen components in 11 EOs with MIC ≤ 200 μg / mL towards ATCC 29212 were representative based on their contents in EOs: eugenol 82.9 % > thymol 63.7 % > hexadecanoic acid

47.8 % > menthol 46.6 % > *cis*–β–ocimene 44.2 % > geranial 42.1 % > *trans*–β–caryophyllene 40.8 % > citronellal 36.7 % > α–pinene 31.2 % > neral 30.5 % > α–eudesmol 22.4 % > citronellol 13.1 % > menthone 11.3 %. Following seven components, representative in 10 EOs with MIC ≤ 200 μg / mL towards clinical isolates, are presented in order of their contribution to EOs: phenylethyl alcohol 57.7 % > geranial 32.9 % > neral 22.2 % > *p*–cymene 20 % > carvacrol 14 % > *α*–pinene 11.5 % > linalool 11.4 %. Out of 21 highly efficient EOs selected in this study, six EOs proved to be the most efficient (MIC ≤ 30 μg / mL); three oils in control of *E.faecalis* ATCC strain (*Satureja horvatii, Mentha pulegium* and *Rosmarinus officinalis*) and other three in control of *E. faecalis* clinical isolates (*Leptospermum petersonii, Thymus algeriensis, Thymus serpyllum*). Thymol is a major component in three out of the six aforementioned most efficient EOs.

The aim of our study was to investigate differences in efficacy of selected EOs that proved to possess great antimicrobial activity, towards the referent strain ATCC 29212 and clinical isolates of *E. faecalis* on, and to estimate which of their constituents might contribute to desired activity, as "markers compunds".

Key words: Essential oil, *Enterococcus faecalis*, clinical isolates, ATCC 29212, antimicrobials.

INTRODUCTION

Allergy caused by antibiotics and antibiotic resistance have increased rapidly in recent years, causing a lot of concern in medical community [1]. Knowing the fact that nearly 60 % of all drugs introduced in therapy between 1981 and 2006, were first identified as natural products [2], it is easy to understand why researchers are seeking for alternatives in natural products, such as essential oils [3].

According to different authors, approximately 3000 plants species contain essential oils, among which only 300 are considered as commercially important [4, 5, 6]. Essential oils (EOs) are complex mixtures of volatile constituents, biosynthesized by plants [7]. They are frequently comprising 20 to 60 components at concentrations ranging from the fairly high (20–70 %) to the trace amounts [8]. The main group of constituents in the most EOs used to be terpenoids, which are, according to Maguna et al., [9], molecules capable of causing death of bacterial cells by following mechanisms; increasing the membrane permeability, affecting structural stability of the membrane or disrupting the lipid bilayer packing.

Enterococcus faecalis is a gram–positive, spherical bacterium, and one of the most resistant bacteria in infected human teeth root canals, whose presence is

detected in teeth with periapical lesions [10], and particularly in persistent apical periodontitis [11,12]. Ability to invade dentinal tubules and survive harsh canal conditions, together with adaptability to lethal challenges, makes this pathogen very persistent to root canal treatments [13].

According to Stuart et al., [14], incidence of *E. faecalis* in infected root canals and in re–treatment cases of apical periodontitis ranges from 24 % to 77 %. Achieving the root canal treatment is highly related to the degree of reduction of the intracanal bacterial population. Although complete elimination of bacteria is challenging and might not always be successful, it generally might be achieved by chemo–mechanical root canal preparation with the aid of antimicrobial irrigation solutions and intracanal dressings between the treatment visits.

Since a standard endodontic procedure includes use of irrigation solutions, as a canal disinfectant, the most effective in removing endodontic biofilm, including *E. faecalis*, appears to be a 1–6 % sodium hypochlorite solution (NaOCl). However, if it is not used properly (with regard to concentration, pH and exposure time), NaOCl may easily create many problems outside the endodontic space, including inflammation, severe pain, extensive swelling, necrosis and cell destruction of exposed tissues, except the epithelium which is strongly keratinized [15].

Apart from its role in endodontic biofilm formation, the other difficulty with *E. faecalis* is its resistance to $Ca(OH)_2$–based medicaments [13] used as an intersession remedy. In addition to this, a number of researchers also devoted their research to find out best sealer against this pathogen; Al Shwaimi et al., [16] in their systemic review, reported that there is no such a canal sealer that possesses satisfactory antimicrobial effect towards *E. faecalis.*

Knowing all disadvantages that follow *E. faecalis*, it's easy to understand why it is important to find something that will effectively control this pathogen, the same time being harmless to human tissues. As the standard antimicrobial agents in endodontic treatment seems to lack in efficacy toward *E. faecalis* [15, 16, 17], in addition to the fact that great efficiency of EOs towards many pathogenic oral microorganisms are already well–documented [1, 4, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28], we assumed the EOs might be a good source to search for efficient alternatives.

The aim of this review is to present EOs with the most significant activity against *Enterococcus faecalis*, to mark their common constituents and discuss their responsibility in the achieved antimicrobial effect.

MATERIAL AND METHODS

Original papers and /or reviews (521 selected bibliographic units) have been collected from the index base Web of Science (WoS) and screened according

to pre–selected criteria in order to select appropriate results of their investigation and include them in this review. There were no time span limitations; all scientific manuscripts with pre–set criteria available from 1996 to 2016 were included.

The main key words used in searching procedure were *Enterococcus faecalis* and essential oil. Criteria for the final selection were as follows:

Original papers presenting antimicrobial effect of EOs on *E. faecalis* with

- presented chemical composition of tested EOs
- the use of microdilution method for determination of MIC values
- the MIC values in accordance to our pre–set criterion: MIC ≤ 200 μL of EO / mL of growing medium (Müeller–Hinton Broth or Tryptone Soya Broth)
- MIC values expressed only in μg or μL / mL
- ATCC 29212 referent strain and /or clinical isolates of *E. faecalis.*

Screening also included results of previous investigation of the author of this rewiev [26].

RESULTS AND DISCISSION

Observation of EOs efficacy towards *Enterococcus faecalis*

As to the best of our knowledge, 21 EOs in available scientific literature documented their significant antimicrobial effect with regard to our pre–set criterion (MIC ≤ 200 μL / mL) towards *E. faecalis* (ATCC 29212 and or clinical isolates); the best effect MIC 0.4 μL / mL (approx. 0.26 μg / mL) achieved *Satureja horvatii* L. EO [18], while the EOs with the lowest antimicrobial efficacy were *Rosmarinus officinalis* L. and *Achilea milefolium* L., both with MIC 160.0 μg / mL [26].

During analysis of reported MIC values with desired efficacy and chemical composition of corresponding EOs, first we have observed the differences in susceptibility towards EOs between the reference strain ATCC 29212 and clinical isolates of *E. faecalis*, so we grouped them in accordance to this; the group that showed the most efficiant EOs towards ACTT 29212 included 11 EOs while the other one included 10 most efficient EOs towards clinical isolates of *E. faecalis*.

Analysis of the MIC values within the groups revealed that those for ATCC strain of *E. faecalis* are generally lower, ranging from 0.26 to 156 μg / mL , in comparison to those for clinical isolates, which ranged from 10 to 160 μg / mL . Similar observation reported Jaradat et al. [29] with *Thymus bovei* EO, which was tested towards clinicaly isolated MRSA *Staphylococcus aureus* and *S. aureus* ATCC 25923; the MIC value for the clinical isolate was two times higher. On the other hand, Subbiya at al. [30], reported difference in sensitivity between *E. faecalis* clinical isolate and the referent ATCC 29212; double concentration of the RC Solve (Prime Dental., Thane, Maharashtra, India) containing orange EO was required for ATCC 292121 in comparison to clinical isolates, implying that clinical

isolates are more sensitive than the referent strain. In addition, Lysakowska at al., [31] investigated sensitivity of 21 clinical isolates of endodontic *Enterococcus* spp., and two referent *E. faecalis* strains (ATCC 29212 and ATCC 51299) to geranium EO; the MIC values for the referent strains were equal or lower then those for the most clinical isolates (only one proved to be more sensitive).

Such variability in results shows that complete understanding of the efficient MIC values of selected EOs towards *E. faecalis* should always include simultaneous testing on clinical isolates and referent strains with the use of the same EO of known chemic al composition, so that the outcome of investigation would be less confusing.

EOs constituents

The most common components contributing to the EOs with ≥ 1.0 %, range of their incidence in selected EOs, and a number of oils in which they were found, are presented in Tables 1 and 2.

While comparing presented components, we noticed following 12 that are common in EOs which MIC s were up to 200 μg / mL in testings towars both, the clinical and referent strains: *trans*–β–caryophyllene (13) > myrcene (8) > *α*–pinene (8) > linalool (7) > *p*–cymene (7) > borneol (7) > geraniol (6) > camphene (6) > limonene (5) > 1,8– cineol (5) > *γ* –terpinene (5) > *α*–terpinene (4).

Germacrene D, eugenol, cadinene, *t*–cadinol and *δ*–cadinene are components that are present in more than two EOs effective against ATCC, but not in EOs affective against clinical isolates, while camphor, citronellol, *β*–pinene, *α*–terpineol, thymol, carvacrol, caryophyllene oxide, *cis*–caryophyllene, terpinene–4–ol, bornyl acetate, *β*–elemene, citronellal and *β*–bisabolene were present in two or more EOs effective against clinical isolates but not in two or more EOs that are effective against ATCC 29212 strain.

Major constituents, common in the EOs

Comparison of EO constituents presented in Charts 1 and 2., reviled that only two of them, geraniol and 1,8–cineol, contributed with ≥ 10 % to more than one EO (MIC range 0.3–200 μg / mL) that showed efficiency against both, the ATCC and the clinical strains. Bearing in mind the fact that the EOs included in this rewiev proved highly efficient antimicrobial effect towards both, the clinical isolates and the referent strain, it cound be generally implied that all EOs containing geraniol and 1,8–cineol in amounts ≥ 10 % would probably achieve antimicrobial effect, on both *E. faecalis*.

Geraniol is an acyclic monoterpene alcohol [32]. According to previous reports geraniol exhibit high antimicrobial activities against various Gram–positive and Gram–negative bacteria and *Candida* spp. [25, 33, 34].

Table 1. The range of common constituents in EOs that are efficient on *Enterococcus faecalis* ATCC 29212 (MIC ≤ 200 μg / mL).
Tabela 1. Opseg variranja zajedničkih komponenti etarskih ulja koja su ispoljila efikasnost (MIC ≤ 200 μg / mL) na *Enterococcus faecalis* ATCC 29212.

Component	**Number of EOs in which it is present with ≥ 1,0 %**	**Range of its contribution to the EOs**
trans–*β*–Caryophyllene	7	1,6 – 40,8
Myrcene	4	1,1 – 3,0
Germacrene D	4	1,7 – 39,1
Limonene	3	3,3 – 5,1
Linalool	3	1,3 – 3,1
Eugenol	3	1,1 – 82,9
Geraniol	3	7,2 – 79,7
1, 8–cineol	2	15,9 – 21,6
p–Cymene	2	2,7 – 4,5
α–Pinene	2	3,7 – 31,2
γ–Terpinene	2	6,0 – 7,5
α–Terpinene	2	1,5 – 1,8
Camphene	2	1,4 – 5,0
Borneol	2	1,6 – 4,2
Cadinene	2	1,2 – 4,9
t–Cadinol	2	1,2 – 3,2
δ–Cadinene	2	1,1 – 1,3

Deeper analysis of its content in EOs that we investigated, suggests that it is generally higher in EOs that are more effective against ATCC strains, with following content trend 79.9 % > 21,8 % > 7,2 %; it is maximal in the most effective oil that contains it (MIC 63 μg / mL) and decreases as the EOs efficacy decrease (MIC 125 μg / mL) [23], while in the case of EOs with efficiency against clinical isolates, the trend of geraniol content proved to be just the opposite, 3.3 % > 12.1 % > 19.2 %; content of geraniol was the lowest in most effective EO containing it (MIC 10 μg / mL) and raised in less effective ones (MIC 130 μg / mL) [26]. This may lead to conclusion that geraniol may play important role in antimicrobial activity towards both, clinical and referent *E. faecalis* strains, but it is obvious that the EOs containing this compound also contain some other compounds that interfere in the achieved activity, either enhancing it or retarding.

Table 2. The range of common constituents in EOs efficient on clinical isolates of *Enterococcus faecalis* (MIC ≤ 200 μg / mL).
Tabela 2. Opseg variranja zajedničkih komponenti etarskih ulja koja su ispoljila efikasnost (MIC ≤ 200 μg / mL) na kliničke izolate *Enterococcus faecalis*.

Component	Number of EOs in which it is present with ≥ 1,0 %	Range of its contribution to the EOs
trans–β –Caryophyllene	6	1.3 – 3.9
α–Pinene	6	1.1 – 11.5
p–Cymene	5	1.2 – 20.0
Borneol	5	1.4 – 6.0
Linalool	4	1.3 – 11.4
Myrcene	4	1 – 4.3
Camphene	4	2.4 – 7.5
Camphor	4	1.1 – 42.7
Citronellol	4	5.4 – 27.0
1, 8 –cineol	3	3.0 – 49.3
β –Pinene	3	3.1 – 8.2
γ –Terpinene	3	4.2 – 7.2
α –Terpineol	3	1.1 – 2.3
Thymol	3	38.5 – 56.0
Carvacrol	3	3.5 – 14.0
Geraniol	3	3.3 – 19.2
Caryophyllene oxide	2	1.0
cis–Caryophyllene	2	1.2 – 1.4
Limonene	2	1.9 – 2.8
Terpinene–4–ol	2	1.1 – 2.0
α–Terpinene	2	1.1 – 1.6
Bornyl acetate	2	1.1 – 7.0
β–Elemene	2	1.0 – 3.8
Citronellal	2	21.1 – 73.5
β–Bisabolene	2	1.0 – 4.0

It is already documented that eugenol /geraniol, geraniol / menthol [35] and geraniol / linalool [36], while in mixtures, interfere to each others activity; they acts synergisticaly towards *B. cereus* and *S. aureus* [35], and towards *Candida* strains [36].

1,8–cineol is oxygenated monoterpene [37]. Mehanizm of its antibacterial effect is demonstrated towards *E. coli* (Gram–negative bacterium) and *S. aureus* (Gram–positive coccal bacterium). Antibacterial effect of 1,8–cineol is explained by cell deformation, breakage of cell wall and membrane and also condensation of cellular material in *E.coli*; while it activity towards *S. aureus*, leads to leaking of the contents of the cells, by making cell iregular and shriveled [38]. Its antimicrobial effect towards *E. faecalis* is also investigated in the study where 1,8–cineol, together with *α*–pinene, is the main constituent in amount ranging from 49.07 to 83.59 % in EOs of several *Eucalyptus* species (*E. lehmani*, *E. leucoxylon*, *E. astrengens*, *E. cinerea*, *E. maiden*, *E. sideroxylon* and *E. bicostata*). Results of the study implicated that *E. faecalis* ATCC 29212 showed sensitivity on all examined oils with zone diameters between 10 and 12 mm [39].

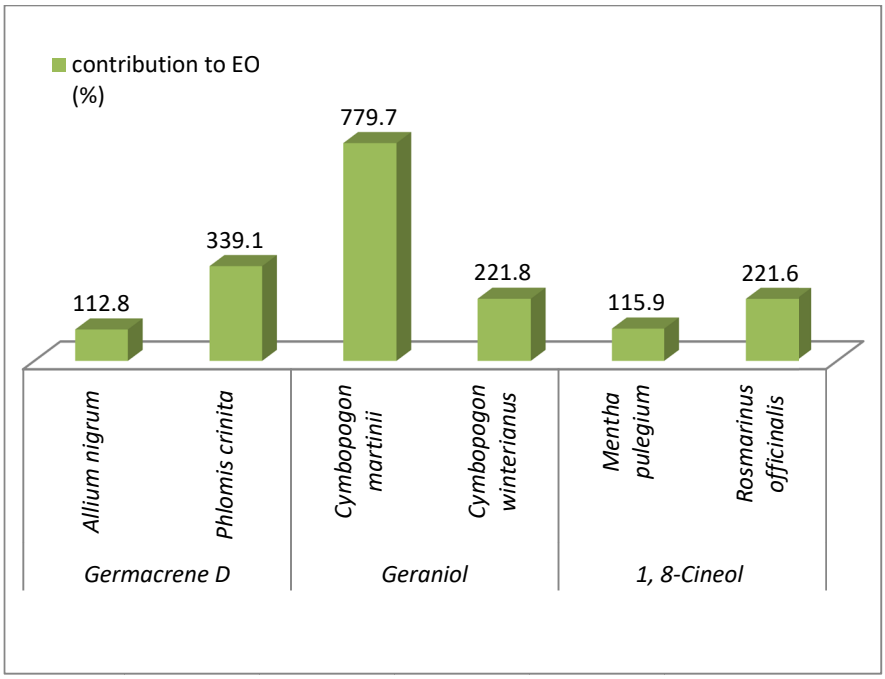


Chart 1. Constituents present with ≥ 10 % in more then one EOs effective towards *E. faecalis* ATCC 29212 (MIC 0.3 – 200 µg / mL).
Grafikon 1. Komponente sa učešćem ≥ 10 % u više od jednom etarskom ulju efikasnom (MIC 0.3 – 200 µg / mL) na ATCC 29212 *E. faecalis*.

Its trend in EOs effective against ATCC strain of *E. faecalis,* that we analysed was opposite to the trend of MIC values, 15.0 % for the most efficient EO

(MIC 0,3 µl / mL (approx. 0.28 µg / mL) raised to 21,6 % for the less efficient ones MIC 25 µl / mL (approx. 22.65 µg / mL) [19, 40], while in the case of clinical strains, content of 1,8– cineol followed the EO efficacy trend; it was the highest in the most efficient EO 49.3 % (MIC 60 µg / mL) and deacred as the EO efficacy decreased, > 43.8 % > 3.0 %, (MIC 160 µg / mL) [26].

Such contradictory results of the EOs efficacy with regard to the content of the major constituents, geraniol and 1,8–cineol, again highlight differences in susceptibility between the referent and clinical strains of *E. faecalis,* thus confirming necessity to perform comparative testing of EOs on both, as a best testing model for EOs efficacy, which would result in more precise conclusions with regard to pre–selection of EOs according to their favourable chemical composition.

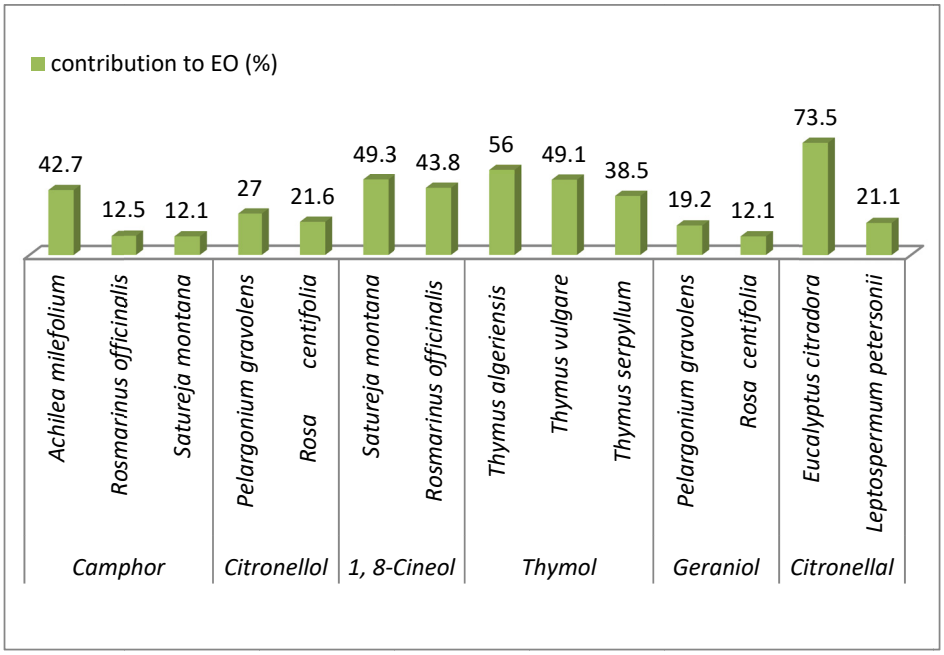


Chart 2. Constituents present with ≥ 10 % in more then one EOs effective (MIC 0.3 – 200 µg / mL) towards *E. faecalis* clinical isolates.
Grafikon 2. Komponente sa učešćem ≥ 10 % u više od jednom etarskom ulju efikasnom (MIC 0.3 – 200 µg / mL) na kliničke izolate *E. faecalis*.

1,8–Cineol (also geraniol), may interfere with other components in EO. For example, Mulyaningsih at al. [41], showed that 1,8–cineol and aromadendrene act additive or synergistic towards four tested bacteria (MRSA, *B. subtilis, S. aureus*

and *S. pyogenes*), while Faleiro et al., [42] reported that *E. coli*, which was susceptible to pure linalool, became highly resistant to a mixture containing linalool and 1,8–cineole (1:1).

Savele et al., [43] investigated the *in vitro* anticholinesterase activities of eight commercially available terpenoid constituents (1,8–cineole, camphor, 5 *α*–pinene, *β*–pinene, borneol, caryophyllene oxide, linalool and bornyl acetate). They found a minor synergy in combinations of 1,8–cineole / *α*–pinene, and 1,8–cineole / caryophyllene oxide, applied at higher concentrations, and an antagonism effect of 1,8–cineole /camphor mixture.

Major constituents specific for the EOs

While comparing the content of EO constituents, we notice that there are several components whose percentage exceeded 10 %, also being specific for only one oil with efficacy towards *E. faecalis* ATCC or clinicaly isolated.

Thirteen components in eleven EOs with MIC ≤ 200 μg / mL towards ATCC 29212 are representative compomponent, and they are listed in the Chart 3, in descending order, according to their percentual contribution to the oil: eugenol > tymol > hexadecanoic acid > menthol > *cis*–*β*–ocimene > geranial > *trans*–*β*–caryophyllene > citronellal > *α*–pinene > neral > *α*–eudesmol > citronellol > menthone.

No correlation was observed between the content of major components presented in the Chart 3, and the MIC values of their corresponding EOs.

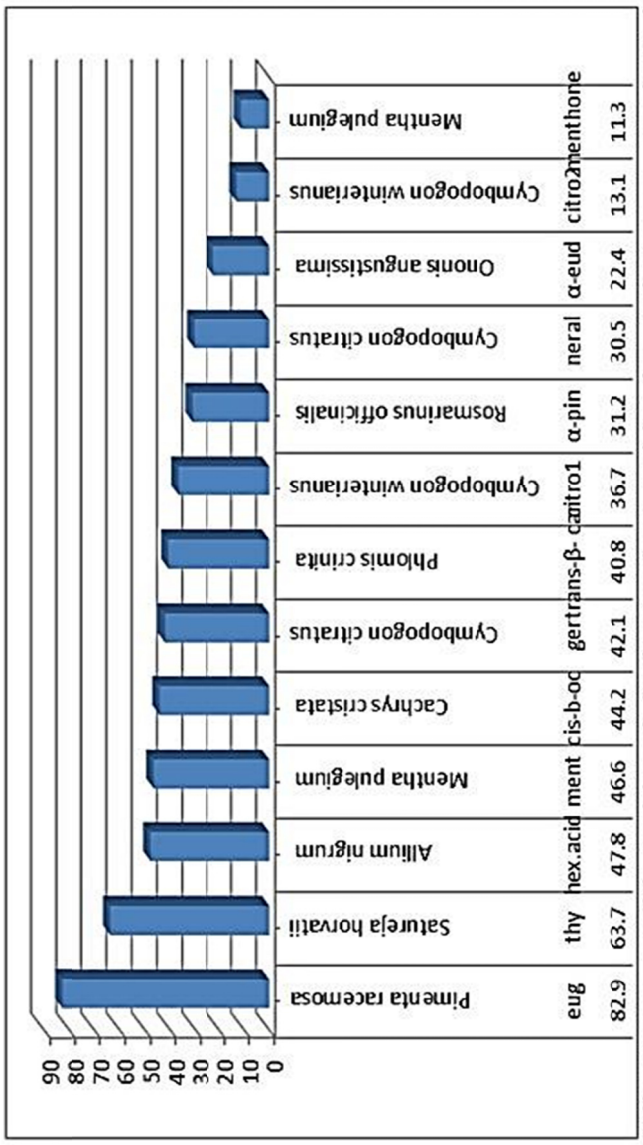
Seven components in 10 EOs with MIC ≤ 200 μg / mL effective towards clinical isolates are representative compomponents also listed in descending order (Chart 4), according to their contribution to the EO: phenylethyl alcohol > geranial > neral > *p*–cymene > carvacrol > *α*–pinene > linalool.

Also, no correlation was observed between the content of major components presented in the Chart 4, and the MIC values of their corresponding EOs.

It's quite known that major EO constituents are commonly charged for biological activities of complete EOs, such as intensity of antimicrobial activity [44]. Majority of compounds found in EOs presentd in Charts 3 and 4., belong to either phenoles or alcohols. Monoterpenes with phenolic structures, such as carvacrol, eugenol and thymol, are already known as highly active against many microorganisms [45, 46, 47], while the alcoholic monoterpenes (menthol, *α*–eudesmol, citronellol, linalool, phenylethyl alcohol) are known to possess rather bactericidal than bacteriostatic activity [46]. Acording to Lakusic et al. [18], phenolic compounds are capable to increase cellular membrane permeability, which appear to be related to the loss of the cellular pH gradient, decreased ATP levels, and loss of the proton motive force, which finally causes cellular death.

Although phenols and alcohols are known by their antimicrobial activity, the other terpenes, for example, oxygenated monoterpenes, such as neral and geranial, may also achieve strong antibacterial effect; according to De Jesus et al.,

[49], these two aldehydes are capable to diffuse into cell membrane and damage its structure. In our EOs, geranial and neral are present in the content that ranges from 22.2 to 42.1 % (Charts 3 and 4); geranial in amount of 42.1 % in EO towards *E. faecalis* ATCC and 32.9 % in EOs towards clinical isolates; while the contents of neral are 30.5 % and 22.2 %, respectively (Charts 3 and 4.)



* *Abbrevations: eug – eugenol, thy – tymol, hex.acid– hexadecanoic acid, ment – menthol, cis–β–oc – cis–β–ocimene, ger – geranial., trans–β–car– trans–β– caryophyllene, citro1– citronellal , α–pin – α–pinene , neral – neral., α–eud – α–eudesmol, citro2 – citronellol, menthone – menthone*

Chart 3. Major components, specific just for one EO effective (MIC 0 -200 µg / mL) on *E. faecalis* ATCC 29212.
Grafikon 3. Glavne komponte, specifične samo za jedno etarsko ulje (MIC 0 -200 µg / mL) koje je ispoljio efikasnost na *E. faecalis* ATCC 29212.

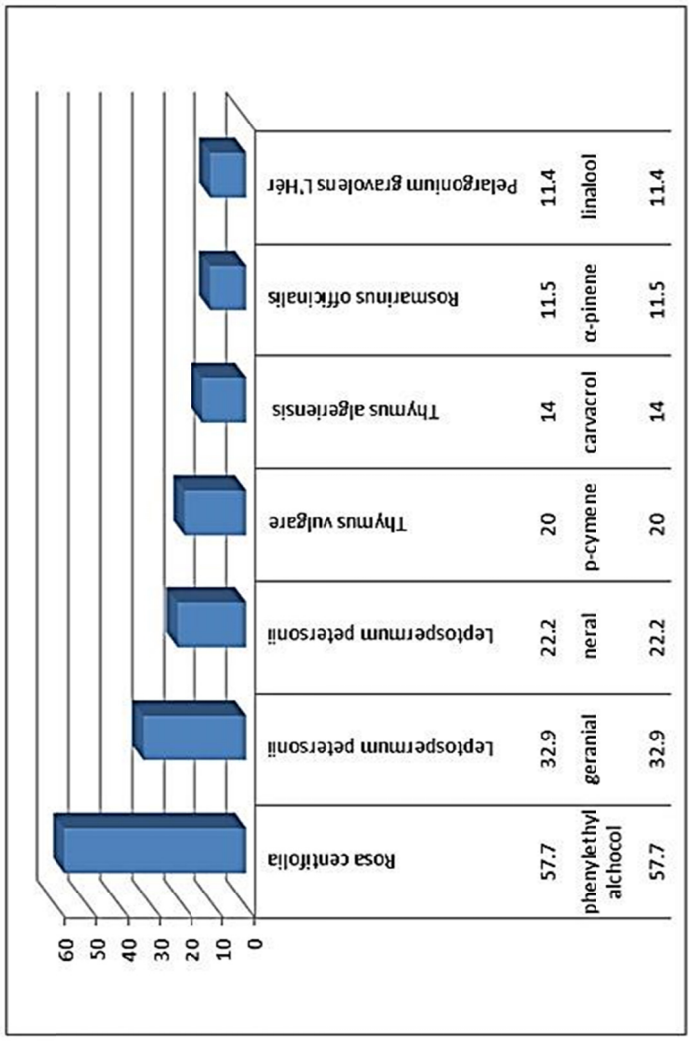


Chart 4. Major components – specific for one EO effective (MIC 0 – 200 µg / mL) on clinical isolates of *E. faecalis*.
Grafikon 4. Velike komponte – specifične za jedno etarsko ulje koje je ispoljio efikasnost (MIC 0 – 200 µg / mL) na kliničke isolate *E. faecalis*.

Beside the phenolic structure, antimicrobial effect also depends on the position of hydroxyl group, or alkyl substitution into the phenol nucleus, which is known to enhance the antimicrobial activity of phenols [45, 46, 48]. In our results for *E. faecalis* ATCC (Chart 3), the highest percentage among all major components show eugenol and thymol, while in case of *E. faecalis* clinical isolates (Chart 4), it was phenylethyl alcohol.

In inestigation of antimicrobial effect of aldehydes, it has been proposed that an aldehyde group conjugated to a carbon to carbon double bond is a highly electronegative arrangement, which may explain the achieved activity [50]; an increase in electronegativity could increase the antibacterial activity [51]. The aldehyde citronellal was tested against the 25 test microorganisms (16 Gram–negative bacteria and nine Gram–positive, among them *E. faecalis*) and was only active against few investigated bacteria *B. subtilis, Clostridium sporogenes, Flavobacterium suaveolens, Micrococcus luteus* and *Pseudomonas aeruginosa* [48]. In our results citronellal content is 36.7 % and it is one of three main componenets in EO *Cymbopogon winterianus* with MIC 125 μg / mL. Menthone is a ketone, and in our study its content was 11.3 %, in second the best EO with MIC 0.3 μL / mL (approx. 0.28 μg / mL). In the study of Dorman and Deans [48], menthone provided no inhibition growth of *E. faecalis*.

Pinene is a monoterpenes hydrocarbon, capable to destroy cellular integrity by inhibiting the respiration and ion transport processes, and may also increase the membrane permeability in isolated mitochondria [52]. *α*–Pinene in our sudy is a component with high content (31.2 % in EO towards clinical isolates of *E. faecalis*) (Chart 4), but is also a common component in six EOs effective towards clinicaly isolated *E. faecalis* in range of 1.1 – 11.5 %, and two EOs effective agenst *E. faecalis* ATCC with range of 3.7 – 31.2 % (Tables 1. and 2).

Similar situation is with *p*–cymene; its content, as a major component, is 20 %, in EOos effective against *E. faecalis* clinical isolates, but is also present in five EOs effective agenst *E. faecalis* in range 1.2 – 20 %, clinicaly isolated and two *E. faecalis* ATCC in range 2.7 – 4.5 % (Tables 1. and 2).

Major constituents, common in the most efficient EOs

Out of 21 highly efficient EOs selected in this study, six proved to be the most efficient (MIC ≤ 30 μg / mL); three oils in control of *E. faecalis* ATCC strain (*Satureja horvatii, Mentha pulegium* and *Rosmarinus officinalis*) and other three in control of clinical isolates (*Leptospermum petersonii, Thymus algeriensis* and *T. serpyllum*). During analysis of their major constituents, certain regularity was noticed.

Thymol is a major component in three out of the six aforementioned most efficient EOs. Comparative observation of the MIC values for the oils that contain thymol (Table 3), reveals the highest thymol content (63 %) in EO with the smallest MIC (*Satureja horvatii* EO), while the EOs of *T. algeriensis* and *T. serpyllum* contained it in even lower amounts, 56 % and 38 %, respectively [18, 26]. The observation that the lower thymol content correlates with the higher EO MIC value, points out that tymol might be used as a “marker constituent” for EOs with a solid potential against *E. faecalis*.

In addition, it is important to sress that thymol was the only component in selected EOs contributing to the oils with more then 30 %, the same time being

common in even four out of thirteen EOs with MICs ≤ 100 μg / mL. None of other components contributed with such a high percentage (≥ 30 %) in more than one EO (Table 4).

Table 3. Major constituents (≥ 30 %) of the most efficient EOs (MIC 0.3 – 30 μg / mL) tested on ATCC 29212 and *E. faecalis*, clinical isolates.
Tabela 3. Glavne komponente (≥ 30 %) etarskih ulja koja su ispoljila najveću efikasnost na referentni *E. faecalis* soj ATCC 29212 i kliničke izolate.

EOs	**Mp**	**Sh**	**Lp**	**Ta**	**Ro**	**Ts**
MIC (μg / mL)	**0.3**	**0.4**	**10.0**	**20.0**	**25.0**	**30.0**
Thymol		63.7		56.0		38.5
α–Pinene					31.2	
Geranial			32.9			
Menthol	46.6					

*Abbreviations: *Mp – Mentha pulegium, Sh – Satureja horvatii, Lp – Leptospermum petersonii, Ta – Thymus algeriensis, Ro – Rosmarinus officinalis, Ts – Thymus serpyllum.*

Table 4. Major constituents (≥ 30 %) of the most efficient EOs (MIC ≤ 100 μg / mL) tested on ATCC 29212 and *E. faecalis*, clinical isolates.
Tabela 4. Glavne komponente (≥ 30 %) etarskih ulja koja su ispoljila najveću efikasnost na referentni *E. faecalis* soj ATCC 29212 i kliničke izolate.

EOs	**Mp**	**Sh**	**Ta**	**Ro**	**Ts**	**Sm**	**Cm**	**A n**	**Tv**	**Ec**	**Pr**
MIC (μg /mL)	**0.3**	**0.4**	**20.0**	**25.0**	**30.0**	**60.0**	**63.0**	**62.5**	**80**		**100**
1, 8–Cineol						49.3					
α–Pinene				31.2							
Geraniol							79.7				
Thymol		63.7	56.0		38.5				49.1		
Eugenol											82.9
Citronellal										73.5	
Menthol	46.6										
Hexadecanoic acid								47.8			

*Abbreviations: *Mp – Mentha pulegium, Sh – Satureja horvatii, Ta – Thymus algeriensis, Ro – Rosmarinus officinalis, Ts – Thymus serpyllum, Sm – Satureja montana, Cm – Cymbopogon martinii, An – Allium nigrum, Tv – Thymus vulgare, Ec – Eucalyptus citradora, Pr – Pimenta racemosa*

Content of thymol in *Satureja horvatii* (MIC 0.26 μg / mL) is 63.7 %, in *T. algeriensis* (MIC 20 μg / mL) 56 % while in *T. serpyllum* L (MIC 30 μg / mL) and *T. vulgare* (MIC 80 μg / mL) it was 38.5 % and 49.1 %, respectively. High concentration of thymol in EOs seems to be generally responsable for good

antimicrobial effect of thymol rich essential oils. Ilic et al., [53] showed that only thymol, as a single component, showed antimicrobial activity almost 13 times stronger then those of *Thymus glabrescens* entire oil towards *Enterococcus faecium* ATCC 19433 (MIC of thymol was 195.2 μg / mL and MIC of *Thymus glabrescens* oil was 2508.4 µg / mL). However, this does not implicate that every single EO containing thymol in high percentage will be equally successful toward referent strain or clinically isolates of *E. faecalis*.

Even when it seems quite logical to attribute antibacterial effect of an EO to its one or few active components, it must not be forget that EOs are mixtures of so many different compounds [5, 54, 55, 56, 57] and their mutual interactions may be very variable; as the activity is dependent on the adequate number and the content of key bioactive compounds [19], the components may interact to either reduce or enforce it [45, 58].

According our investigation, it could be outlined that the most efficiant combination towards *E faecalis* would probably combine three most common components that exideded 10 %, geraniol and 1,8–cineol, with the most common major oil component, thymol. However, mixtures might be quite tricky, particularly in case of unexpected antagonism between them, as some of them are already proven in the available literature between thymol and geraniol [35] although in test on other bacterial species (*B. cereus, S. aureus* and *E. coli*). Researchers must always keep in mind the fact that the same mixture combination will not be equally efficiant in every bacterial species, and gold standard combination for *S. aureus* or *E. coli* must not nessery be efficiant towards *E. faecalis* [35]. Another question is, if clinical isolates and refferent strains will be equally susceptible to applied EOs or their mixtures, as it was not always the case, at least with oral flora microorganisms [27, 28]; will the thymol / geraniol mixture be equally indiferent to both, the referent and the isolated strains of *E. faecalis*, stays unknown until results of a new study reviel it.

CONCLUSIONS

Complete understanding of the efficient MIC values of selected EOs towards *E. faecalis* should always include simultaneous testing on clinical isolates and referent strains with the use of the same EO of known chemic al composition, so that the outcome of investigation would be less confusing.

Major common constituents, geraniol and 1,8–cineol, again highlight differences in susceptibility between the referent and clinical strains of *E. faecalis*, thus confirming necessity to perform comparative testing of EOs on both.

1.8 cineol and geraniol can interfere with other components in EO.

No correlation between percentage of major components specific for one EO effective (MIC 0 – 200 μg / mL) on *E. faecalis* clinical isolate / *E. faecalis* ATCC and MIC values could be noticed.

Tymol might be used as a "marker constituent" for EOs with a solid potential against *E. faecalis.*

According our investigation, it could be outlined that the most efficiant combination towards *E. faecalis* would probably combine two the most common components that exideded 10 %, geraniol and 1,8–cineol, with the most common major oil component, thymol.

AKNOWLEDGEMENTS

The authors are grateful to the Ministry of Education, Science and Technological Development of Serbia for financial support (Grants № 173032 and 41008).

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ANALIZA HEMIJSKOG SASTAVA ETARSKIH ULJA SNAŽNE EFIKASNOSTI NA REFERENTNI SOJ ATCC 29212 I KLINIČKE IZOLATE *ENTEROCOCCUS FAECALIS*

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REZIME

Pregledom literature odabrano je 21 etarsko ulje (EU) koje pokazuje izuzetno značajan antimikrobni efekat prema kriterijumima koje smo zadali (MIC ≤ 200 μL / mL) na *Enterococcus faecalis* (ATCC 29212 referentni soj ili klinički izolat); Najbolji antimikrobni efekat sa MIC 0.4 μL / mL (približno 0.26 μg / mL) pokazalo je etarsko ulje *Satureja horvatii* L., dok su sa najslabijim antimikrobnim efektom bila ulja *Rosmarinus officinalis* L. i *Achilea milefolium* L. (MIC 160.0 μg / mL). Međugrupnom analizom MIC vrednosti, utvrđeno je da se MIC vrednosti, i u grupi u kojoj su testirana ulja na referentne sojeve i u grupi na kliničkim izolatima, razlikuju. MIC vrednosti ulja koja su delovala na referentni soj *E. faecalis* ATCC 29212, variraju od 0.26 to 156 μg / mL , dok je opseg MIC vrednosti koja pokazuju ulja efikasna na kliničke izolate *E. faecalisa* bio između 10 to 160 μg / mL. Dvanaest komponenti koje su zajedničke za etarska ulja testirana i na referentnom soju i na kliničkim izolatima, a čiji MIC ≤ 200 μg / mL , iskazana su u opadajućem nizu u odnosu na broj ulja u kojima su sadržana: *trans*–β–kariofilen (13) > mircen (8) > *α*–pinen (8) > linalool (7) > *p*–cymen (7) > borneol (7) > geraniol (6) > kamfen (6) > limonen (5) > 1,8–cineol (5) > *γ*–terpinen (5) > *α*–terpinen (4). Poređenjem komponenti utvrđeno je da su samo dve komponente, geraniol i 1,8–cineol, prisutne u procentualnoj zastupljenosti ≥ 10 % u više od jednog etarskog ulja (MIC opseg ulja 0.3–200 μg / mL), a koja su pokazala efikasnost i na ATCC i na kliničke sojeve *E. faecalis*. Geraniol i 1,8–cineol su glavne komponente sadržaja ≥ 10 % u više ulja efikasnih i protiv ATCC soja i kliničkih izolata (MIC 0.3–200 μg / mL). U 11 ulja sa MIC ≤ 200 μg / mL na *E. faecalis* ATCC 29212, uočeno je

13 reprezentativnih komponenti, predstavljanih opadajućem nizu u odnosu na zastupljenost u uljima: eugenol 82.9 % > timol 63.7 % > heksadekanoanska kiselina 47.8 % > mentol 46.6 % > *cis–β–*ocimen 44.2 % > geranial 42.1 % > *trans–β–*kariofilen 40.8 % > citronelal 36.7 % > *α–*pinen 31.2 % > neral 30.5 % > *α–*eudesmol 22.4 % > citronelol 13.1 % > menton 11.3 %. U 10 etarskih ulja sa MIC ≤ 200 μg / mL efikasnim na klinički izolat, 7 je reprezentativnih i one su prikazane u opadajućem nizu u odnosu na njihovu zastupljenost u EU: feniletil alkohol 57.7 % > geranial 32.9 % > neral 22.2 % > *p–*cimen 20 % > karvakrol, 14 % > *α–*pinen 11.5 % > linalool 11.4 %. Od 21 EU iz ovog pregleda, šest su se izdvojila kao najefikasnija (MIC ≤ 30 μg / mL); 3 na referentni *E. faecalis* ATCC soj *(Satureja horvatii, Mentha pulegium* and *Rosmarinus officinalis*) i druga 3 na *E faecalis* klinički izolat (*Leptospermum petersonii, Thymus algeriensis, Thymus serpyllum*). Timol je bio procentualno najzastupljenija komponenta u 3 od 6 najefikasnijih EU.

Cilj ovog istraživanja bio je da se utvrde razlike u efikasnosti etarskih ulja koja su odabrana zbog njihove jake antimikrobne aktivnosti na kliničke izolate *E. faecalis* kao i na referentni soj ATCC 29212, i da se izvrši procena njihovog hemijskog sastava vezano za komponente koje svojim učešćem doprinose antimikrobnoj aktivnosti i mogu poslužiti kao “markeri efikasnosti”.

Ključne reči: Etarska ulja, *Enterococcus faecalis*, klinički izolati, ATCC 29212, antimikrobno dejstvo.