

COMPARATIVE ANALYSIS OF THE CHEMICAL COMPOSITION AND ANTIMICROBIAL ACTIVITIES OF SOME OF LAMIACEAE FAMILY SPECIES AND EUCALIPTUS (*Eucalyptus globules M*)

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*The aim of this study was to evaluate and compare the antimicrobial activity of four essential oils that belong to Lamiaceae family (sage, oregano, thyme) and eucalyptus oil. Gas chromatography coupled with mass spectrometry (GC-MS) revealed that the highest percent of essential oils 98.93% include three classes of compounds – monoterpene hydrocarbons, aromatic hydrocarbons and oxidized monoterpenes. The main components are oxidized monoterpenes: carvacrol (59.03%); thymol (36.12%), eucalyptol (20.66%), hydrocarbon monoterpenes: limonene (30.96%) and α -pinene (12.21%) and aromatic monoterpene, p-cymene (22.25%) All essential oils showed great potential of antimicrobial activity against several bacteria and yeast *C. albicans*, using the agar diffusion method with wells. Minimum inhibitory concentration (MIC) for the essential oils has been determined by the broth dilution method and valued in the range from 1 to 5 μ L/m, depending on the essential oil and bacteria tested, and up to 100 μ L/ml for *C. albicans*. The essential oils of Lamiaceae family exhibited a strong antibacterial activity for tested microorganisms, while the essential oils of thyme were especially recognized.*

KEY WORDS: Lamiaceae, essential oils, eucalyptus, antibacterial activity

INTRODUCTION

The increasing bacterial resistance to applied antibiotics presents a global health problem nowadays. Therefore, there is a great interest in finding new alternative herbal products with antimicrobial activity that could be used in antimicrobial therapy instead of the commercial ones.

Aromatic plants from the *Lamiaceae* family present very important potential resources of biologically and pharmacologically active substances, whose activities are proven in numerous scientific studies. Ever since ancient times, these herbs are used for their spasmolytic, antioxidant, antimycotic and many other activities (1). Antioxidant and anti-

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bacterial activity developed by 100 volatiles of essential oils of *Lamiaceae* family has been proven in (2). The plant secondary metabolites, especially essential oils have been known and used by people thousands years ago.

Essential oils are very complex natural mixtures which include 20 to 60 components contained in different concentrations (3). Each essential oil is defined by two to three components with high concentration (20-70%) related to the other components which are presented in traces. These main components of essential oils define the biological and pharmacologic features of the oil itself. The essential oil components include two main groups of biosynthetic substances. To the first group belong terpenes or terpenoids, while the second group includes aromatic and aliphatic constituents. There are numerous studies that prove antimicrobial activity of essential oils (4). One of the greatest studies was conducted by Morris et al. who investigated 521 essential oils and their components. They concluded that 44% of the investigated essential oils inhibited growth of at least one tested microorganism (*Candida albicans*, *Esherichia coli*, *Staphylococcus aureus*, etc.) and the essential oils of *Lamiaceae* family showed the highest antimicrobial efficiency (5).

Regarding the numerous components contained in certain essential oils, antibacterial activity of the oil can be explained by the specific reactions which have multiple target places in bacterial cells (1, 6). Dorman and Deans noticed that this mechanism of antibacterial activity is based on the lipophilic properties of the constituents of essential oils and their functional groups (7). Such strong antimicrobial activity of essential oils on pathogenic bacteria is based on a high level of phenolic components such as carvacrol, eugenol (2-methoxy-4-(2-propenyl) phenol) and thymol (7, 8). The mechanism of their antimicrobial activity is similar to that of the other phenolic compounds: they react with the cytoplasmic membrane, causing its lesions and change its permeability, which results in a change of the membrane potential, ATP-pool, pH of the intracellular medium, i.e. the pH-gradient end efflux of Na^+ -ions (9, 10). Some terpenoid molecules with functional groups such as the phenol, alcohol and aldehyde group influence the membranes proteins by decreasing or completely inhibiting their activity (11). There is no yet scientific evidence of the resistance to essential oils. This can be explained by a great complexity of the structure of essential oils and the fact that the essential oils are active for several target places on the bacterial cells at the same time (7).

Considering the above, the aim of the present study was to investigate: a) the potential antimicrobial activity of sage, thyme, oregano and eucalyptus essential oils, and b) to choose the most potent antimicrobial essential oil for the further development of the available drug delivery system with selected essential oils.

EXPERIMENTAL

Essential oils

The study encompassed the investigation of the commercial essential oils of oregano, thyme, sage and eucalyptus obtained from *Sanoflore*, France.

The selection of essential oils was made according to the individual assessment and literature data on previously confirmed antibacterial activity of the used essential oils.

Microorganisms

For the purpose of *in vitro* testing of the antimicrobial activity of essential oils, the following standardised bacterial and yeast cultures were used (ATCC - American Type Culture Collection): *Staphylococcus aureus* ATCC 25923, *Esherichia coli* ATCC 25922, *Candida albicans* ATCC 24433, *Enterococcus faecalis* ATCC 29212. These cultures of microorganism were deposited in the Collection of the bacteria cultures of the Department for Microbiology at Faculty of Technology, Belgrade, and the bacteria cultures of the Institute of Virology and Immunology, Torlak.

Gas chromatography with mass spectrometry (GC-MS)

Gas-chromatographic analysis of essential oils was conducted on a Hewlett Packard 5973-689 GC-MS system in the EI mode at 70eV, with spectrometric detection of masses (GC-MS-Gas Chromatography-Mass Spectroscopy). The initial temperature of the capillary column HP 5MS (30m x 0,25mm; film thickness 0,25µm) was 60°C. Using the heating speed of 3°C/min, it was heated to 280°C. Helium, at a flow rate of 1 ml/min served as the carrier gas. The amount of 1µl of each investigated sample was injected into the GC column in the proportion of 1:10.

The identification of the components was based on the calculated retention indexes (RI) (12) and mass spectra compared to those of the standard substances and/or with NIS/NBS Wiley library of mass spectra, including the literature data or data from the free database (<http://www.flavornet.org/iowtv.pherobase.com>) (13). The experimental values of the retention indexes are defined using the calibrated Automated Mass Spectral Deconvolution and Identification System software' (AMDIS ver. 2.1, DTRA/NIST, 2002) and the results were compared with the retention indexes from the literature data and from the internet available database.

Methods of the determination of antimicrobial activity

The antimicrobial activity of the essential oils was determined by the agar-well diffusion method. The tubules with a diameter of 6 mm were placed on Petri plates with the prepared sterile Miller-Hinton TSA (tryptone soy agar -Torlak) impregnated with soft agar (0.60 % of agar), which was inoculated with indicator pathogenic strain (0.2 ml of the 24-hours broth culture for 6 ml of soft agar). After forming of agar, the tubules were removed and each of formed wells was filled with 20 µl of tested essential oil. The plates were incubated at 37°C during 24 hours. The antimicrobial activity of lactic acid in combination with the essential oils was also tested in order to determine their synergistic effect (the compound included 50 ppm of lactic acid for 20 µl of essential oil). The antibiotics clindamycin (10 µg/ml) and antimycotic nystatine (30µg/ml) were used as a positive control of the antibacterial activity.

Antimicrobial activity of essential oils was present all over the inhibition zone, which was measured and expressed in mm.

Evaluation of the minimum inhibitory concentration (MIC) using broth dilution method

The determination of the minimal inhibitory concentrations (MIC) of the tested bacterial strains was conducted by broth dilution method on Miller-Hinton broth 14. To each test tube containing 2.997 ml of base were added 3 μ l of the essential oil. Due to the difference in the antimicrobial activity of tested essential oils, proved by the agar dilution method, the concentrations of 1, 3, 5 and 10 μ l/ml of thyme and oregano were used, the corresponding concentrations for the essential oils of thyme and oregano being 10, 30, 50 and 100 μ l/ml. The indicator breeds of microorganism (1% of inoculum) were inoculated in the prepared test tubes with diluted essential oils and in the control test tube and incubated at 37°C.

In the time intervals of 1, 3, 8, 16 and 24 h, the changes of the optical density (OD) were followed on an MA 9504 Metrix colorimeter, using yellow filter (575 nm). The increase in the OD or blurring indicates an increase of the microorganism biomass in the liquid base. In the presence of inhibition substances, MIC is defined as the first concentration of the essential oil and in its presence no blurring appears. In other words, there is no visible growth of bacteria.

RESULTS AND DISCUSSION

The results of the qualitative and quantitative analyses of the chemical composition of essential oils are shown in Figure 1 and Figure 2. The main components of the tested essential oils determine biological and pharmacological features of the oil itself. Therefore, the proportion of chemical composition and antimicrobial activities of a certain essential oil would be very important for its antibacterial activity.

From the results of the chemical analysis of the tested essential oils it can be noticed that the highest percent of essential oils include three classes of compounds – monoterpene hydrocarbons, aromatic hydrocarbons and oxidized monoterpene monoterpenes (98.93%). The other groups of compounds are present in 1% of them (Figures 1-2). The most dominant components of tested essential oils were oxidized monoterpenes: carvacrol (59.03%); thymol (36.12%), eucalyptol (20.66%); hydrocarbon monoterpenes: limonene (30.96%) and α -pinene (12.21%) and aromatic monoterpene, *p*-cymene (22.25%).

More than one half of the total composition of the essential oil of oregano was made of the dominant compound - oxidized monoterpene carvacrol (59.03%), followed by thymol (5.69%), and monoterpene hydrocarbons *p*-cymene (5.02%) and γ -terpinene (4.64%). Sesquiterpenes were also present, but less than the other terpenoid groups (Figure 1).

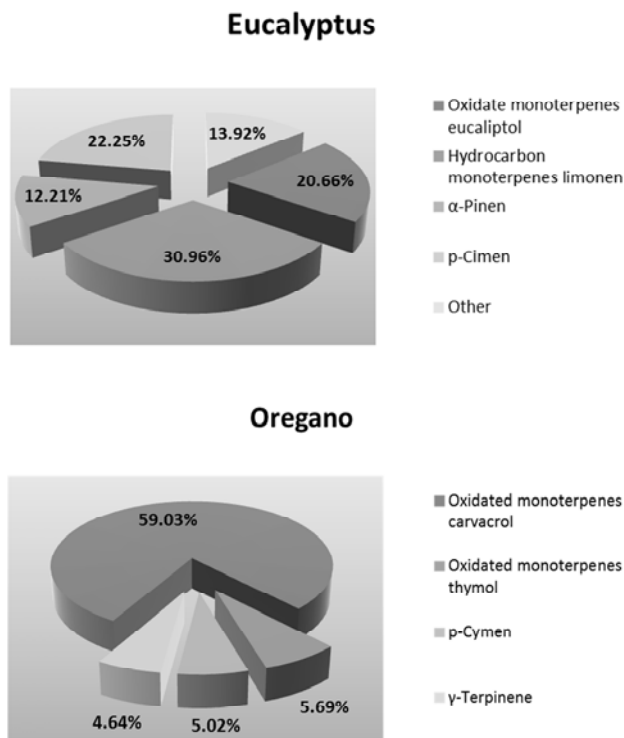


Figure 1. Contents of the basic compound classes (%) in the eucalyptus and oregano essential oils

Dominant components in the sage essential oil were oxidized monoterpene α -thujone (29.9%), camphor (15.47) and β -thujone (13.68%), carvacrol (3.32 5), and monoterpene hydrocarbons camphene (3.04%) and α -pinene (2.63%) (Figure2). The content of the sesquiterpene hydrocarbon 1- α -bornyl acetate (2.23%) was also significant.

More than one half of the total content of essential oils of thyme included six dominant compounds. Among them, most dominant was the oxidized monoterpene thymol (36.12%), then the monoterpene hydrocarbon *p*-cymene (21.15%). The other dominant components of this essential oil were: γ -terpinene (6.98%), linalool (5.90%), carvacrol (4.54 %) and eucalyptol (4.74%) (Figure 2).

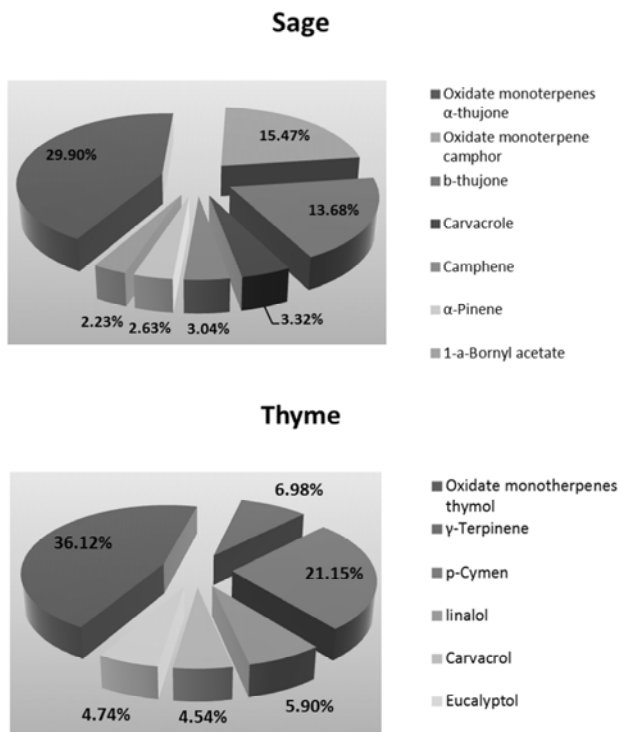


Figure 2. Contents of the basic compound classes (%) in the sage and thyme essential oils

Based on our results, it can be concluded that most of essential oils from *Lamiacea* family included the following dominant components: carvacrol, thymol, *p*-cymene, γ -terpinene, linalool, α -pinene, eucalyptol and camphor.

The *in vitro* antimicrobial activity of essential oils against tested microorganisms and their potential activities were assessed qualitatively and quantitatively by the presence or absence of inhibition zones, and MIC values. According to the results presented in Table 1 and Figure 3, all tested essential oils showed significant antibacterial and antifungal activity against all bacteria tested.

Table 1. Antimicrobial activities of the tested essential oils

Essential oil	Zone of inhibition (mm)			
	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>	<i>E. faecalis</i>
Eucaliptus	3	1	2	2
Sage	10	3	1	1
Oregano	11	8	1	0.5
Thyme	12	8	1	0.8
Nystatin (30 µl/ml)	-	-	4	-
Clindamycin (10 µl/ml)	2	2	-	2.5

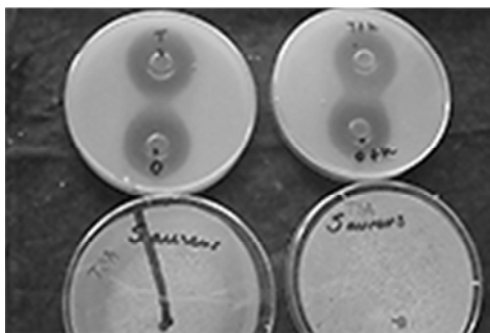


Figure 3. Antibacterial activity of the thyme (T) and oregano (O) essential oils against *S. aureus*

Among Gram-positive bacteria, *S. aureus* (0.1-12 mm) was more sensitive than *E. faecalis* (2-8 mm). A slightly less sensitivity exhibited the Gram-negative bacteria *E. coli* ATCC 25922 (1-8 mm). The low sensitivity of the Gram-negative bacteria to the essential oils can be a consequence of the structure of their cell walls which includes external membrane around the peptidoglycan layer, which also inhibits the diffusion of hydrophobic components through their lipopolysaccharide layer (14).

The best antibacterial activity showed the essential oils of plant species with highest content of thymol and carvacrol: thyme (thymolol 36.12% and carvacrol 2.34) and oregano (carvacrol 59.63% and thymolol 5.69%). The thyme oil was slightly stronger than the oregano essential oil, which is aligned with data on the higher activity of thymol related to carvacrol [9]. Although the essential oils of sage and eucalyptus include both components (thymol and carvacrol), they are less stable and weaker than above mentioned essential oils, which implies that the antimicrobial activity is also influenced by the amount of certain component present in oil, and their synergistic activity. All tested essential oils showed significant antifungal activity of the yeast *C. albicans* and different levels of antibacterial activity shown in the following order: thyme > oregano > sage > eucalyptus. The results obtained using broth dilution method, MIC values and of the following of the kinetics of growth of tested microorganisms are presented in the form of graphs.

The essential oil of oregano and thyme in concentration of 5 $\mu\text{L/ml}$ and 3 $\mu\text{L/ml}$ were considered as bacteriostatic. The results presented in Figure 4 show that the essential oils of sage and eucalyptus showed a more significant antimicrobial activity for *E. coli* in the concentrations higher than 50 $\mu\text{L/ml}$. They exhibited total inhibition of the growth in the concentration of 100 $\mu\text{L/ml}$, which was considered as MIC value. A slightly stronger inhibition activity showed the essential oil of sage compared to that of eucalyptus.

Figure 4 shows that the bacteria *E. coli* was more sensitive to the activity of the essential oils of oregano and thyme than to the essential oils of sage and eucalyptus. A significant inhibition of the growth was observed for the concentrations of 5 $\mu\text{L/ml}$, and it was considered as MIC value. There was no significant difference in the antimicrobial activity of these two essential oils for bacteria *E. coli*.

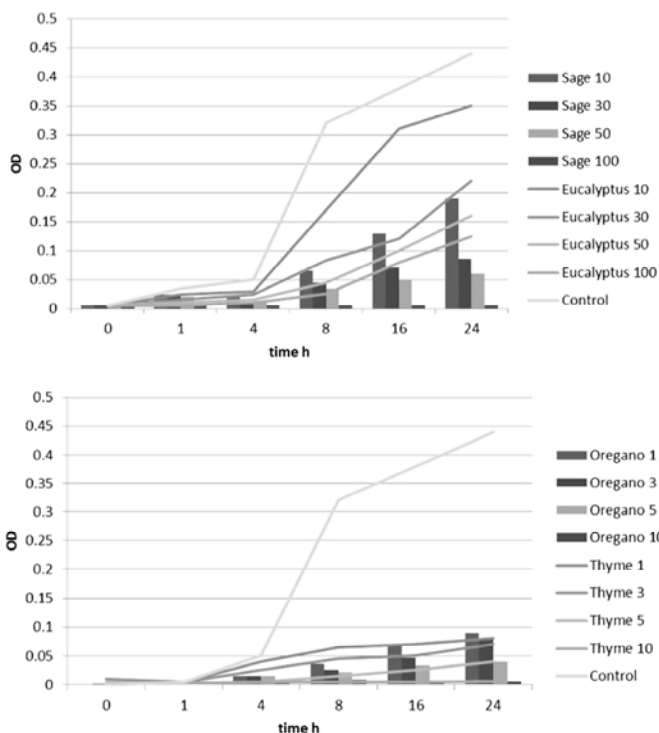


Figure 4. Effect of the eucalyptus, sage, oregano and thyme oils on the kinetics of the growth of *E. coli*

The essential oils of sage and eucalyptus showed poor antibacterial activity on *E. faecalis* in low concentrations (10 $\mu\text{L/ml}$ and 30 $\mu\text{L/ml}$) (Figure 5). Much stronger antibacterial activity these oils showed in concentrations of 50 $\mu\text{L/ml}$, while concentration of 100 $\mu\text{L/ml}$ is not enough for total inhibition of growth of *E. faecalis*. The eucalyptus

essential oil showed slightly better antimicrobial activity for *E. faecalis*, which was probably due to content of monoterpene hydrocarbon *p*-cymene (22.25%) in eucalyptus [15].

Antimicrobial activity of essential oils of oregano and thyme for tested concentrations of 1, 3, 5 and 10 $\mu\text{L}/\text{ml}$ was enough for efficient inhibition of growth of *E. faecalis*, while MIC has the value of 1 $\mu\text{L}/\text{ml}$ (Figure 5.). Slightly stronger activity is showed by essential oil of thyme, rather than essential oil of oregano.

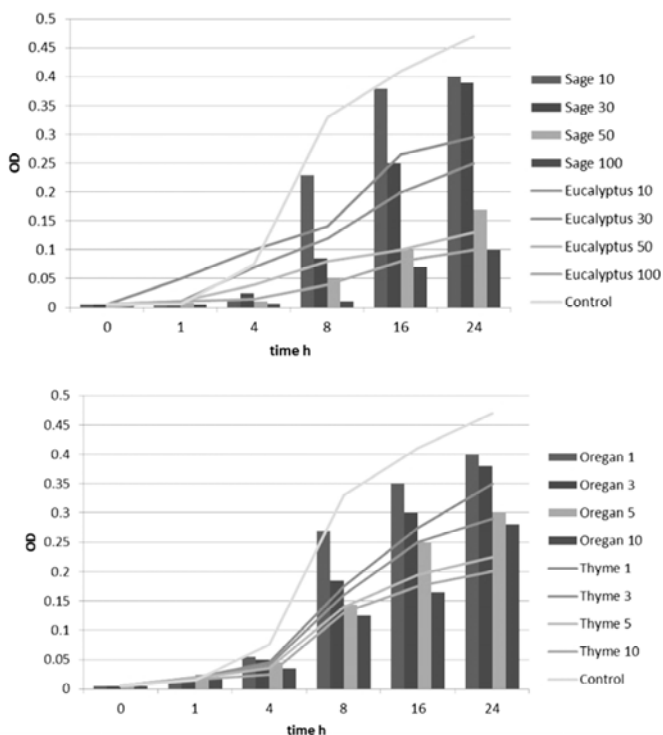


Figure 5. Effect of the eucalyptus, sage, oregano and thyme oils on the kinetics of the growth of *E. faecalis*

Figure 6 showed significant inhibition of growth of *S. aureus* using the essential oils of sage and eucalyptus in concentrations higher than 30 $\mu\text{L}/\text{ml}$. MIC values of these oils for *S. aureus* were 50 $\mu\text{L}/\text{ml}$ for the essential oil of sage and 100 $\mu\text{L}/\text{ml}$ for the essential oil of eucalyptus.

A significant antimicrobial activity of the essential oils of oregano and thyme for *S. aureus* is evident from the results shown in Figure 6. The MIC values for thyme and oregano in this case were 3 $\mu\text{L}/\text{ml}$.

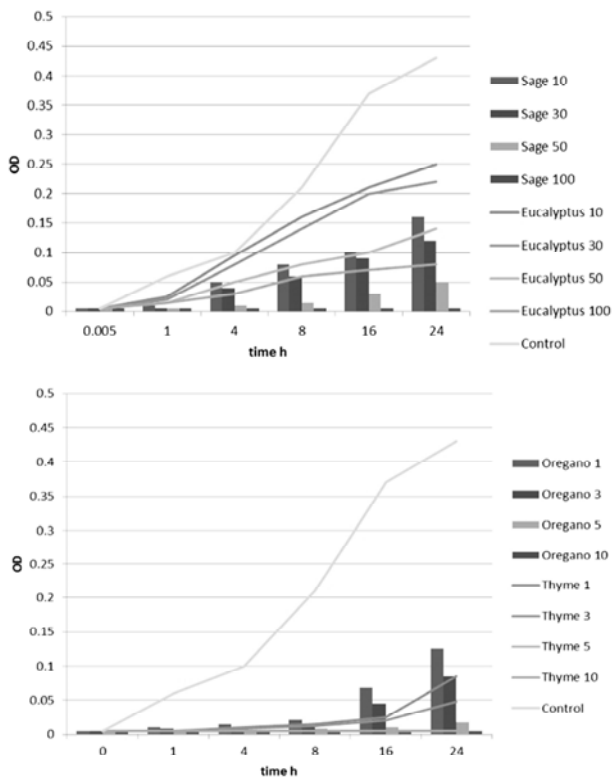


Figure 6. Effect of the eucalyptus, sage, oregano and thyme oils on the kinetics of the growth of *S. aureus*

The yeast *C. albicans* showed a lower sensitivity to the eucalyptus essential oil activity, and in the tested concentrations this essential oil did not show total inhibition of the growth of this yeast (Figure 7). A slightly stronger antimicrobial activity in the applied concentrations showed the essential oil of sage; its MIC value for the yeast *C. albicans* is slightly higher than 100 μL/ml.

The essential oil of thyme and oregano in the applied concentrations showed a lower antimicrobial activity for the pathogenic yeast *C. albicans* (Figure 7). A better antimicrobial effect showed the essential oil of thyme, which decreased the growth of cells of *C. albicans* by 50% in the concentration of 10 μL/ml (MIC₅₀ value).

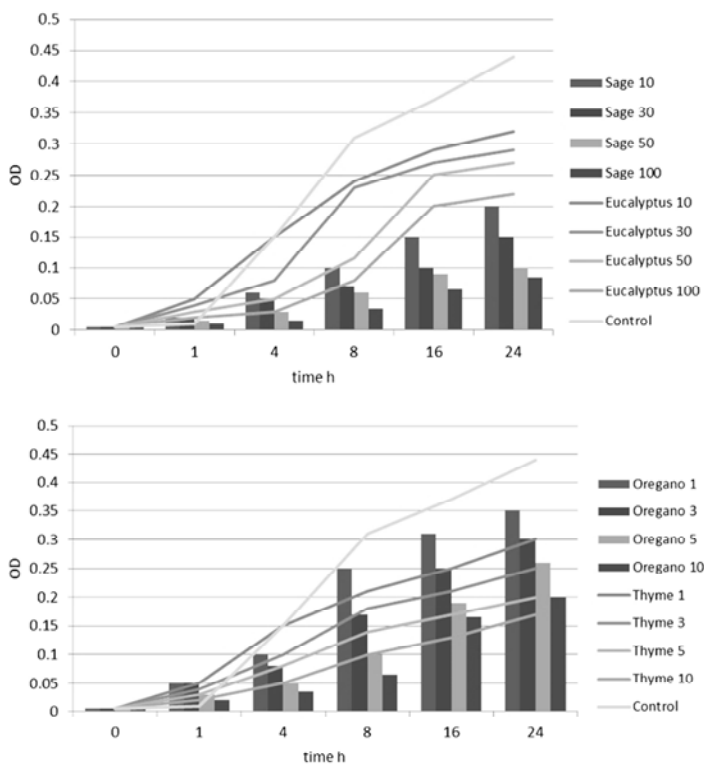


Figure 7. Effect of the eucalyptus, sage, oregano and thyme oils on the kinetics of the growth of *C. albicans*

The presented results of the determination of MIC values of these four essential oils were aligned with the results of the preliminary investigations of antimicrobial activity of the essential oils, using well-diffusion method (17).

It was observed that the essential oils of sage in lower concentrations are mostly bacteriostatic for the tested microorganisms. At the concentration of 100 $\mu\text{L}/\text{ml}$, the sage oil stopped completely the growth of *E. coli* and *S. aureus* (MIC value of 100 $\mu\text{L}/\text{ml}$). *E. faecalis* and *C. albicans* showed higher resistance for the applied concentrations of sage essential oil. The essential oil of eucalyptus used in the concentration of 100 $\mu\text{L}/\text{ml}$ showed significant activity only for the Gram-positive bacteria *S. aureus*, while the other breeds survived at certain level.

The essential oils of sage and thyme in the concentrations of 5 $\mu\text{L}/\text{ml}$ and 3 $\mu\text{L}/\text{ml}$ were bactericidal for *E. coli* and *S. aureus*, respectively. The bacteriostatic activity of these essential oils in the applied concentrations was observed for *E. faecalis* and *C. albicans*. These results are partially expected considering the preliminary results of the determination of the antibacterial activity by agar-well diffusion method.

CONCLUSION

Based on the results of the chemical and microbiological analyses, the essential oils of thyme showed the highest antimicrobial activity. Considering its origin and numerous biochemical and pharmacological studies, the probability for this essential oil to be toxic during pharmacological tests is very low, which is very important for its potential application as an antimicrobial agent. However, in designing an appropriate formulation incorporating this violet essential oil would be a subject of the future studies.

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КОМПАРАТИВНА АНАЛИЗА ХЕМИЈСКОГ САСТАВА И АНТИМИКРОБНЕ АКТИВНОСТИ НЕКИХ ЕТАРСКИХ УЉА ФАМИЛИЈЕ ЛАМИАСЕАЕ И ЕТАРСКОГ УЉА ЕУКАЛИПТУСА (*Eucalyptus globules* M)

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Ова студија се бави испитивањем и упоређивањем антимикробног дејства етарских уља фамилије Lamiaceae: тимијана, жалфије, оригана и етарског уља еукалиптуса. Гасном хроматографијом са масеном спектрометријом (GC-MS) је одређена хемијска структура и заступљеност најважнијих компонената, при чему 98,92% садржаја етарског уља заузимају три класе једињења, угљоводонични монотерпени, ароматични угљоводоници и оксидовани монотерпени, док осталим групама једињења припада мање од 1% садржаја. Главне компоненте испитаних етарских уља су оксидовани монотерпени: карвакрол (59,03%), тимол (36,12%), еукалиптол (20,66%), и угљоводонични монотерпени: лимонен (30,96%), α -пинен (12,21%), као и ароматични монотерпен *p*-цимен (22,25%). Сва етарска уља су показала значајну антимикробну активност против свих тестираних бактерија и *C. albicans*, методом дифузије у бунарчићима на агарној подлози. Минимална инхибиторна концентрација је одређена дилуционом методом, и утврђено је да се вредности за МИС крећу у опсегу од 1 μ L/ml - 5 μ L/ml за тестиране бактерије, зависно од испитиваног етарског уља и бактерије која се тестира, и до 100 μ L/ml за *C. albicans*. Етарска уља фамилије Lamiaceae показују снажно антимикробно дејство, међу којима се посебно издваја етарско уље тимијана.

Кључне речи: Lamiaceae, етарска уља, еукалиптус, антибактеријска активност

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