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Chemical compositions and antimicrobial activities of oregano and thyme essential oils

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The aim of this study was to evaluate the chemical composition and antimicrobial activity of essential oils of thyme and oregano. Gas chromatography with mass spectrometry (GC-MS) was used to determine the chemical structure of essential oils and their dominant components. Antimicrobial activity of essential oils was tested against standardized bacterial and fungal cultures: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 24433, *Enterococcus faecalis* ATCC 25929, using the agar diffusion method with wells. Minimum inhibitory concentration (MIC) for essential oils determined by the broth dilution method and valued in the range of 3 - 5 μl/ml, depends on the essential oil and bacteria and *C.albicans* tested. Both essential oils provided strong antibacterial activity for the tested microorganisms. The essential oil of thyme was especially recognized. These experiments aimed at showing the *in vitro* antimicrobial activity of the selected essential oils in order to find out the most potential essential oil that would be an objective in further investigation on chitosan drug delivery system with controlled release of antimicrobial essential oil.

Key words: essential oils, antimicrobial activity, oregano, thyme

INTRODUCTION

There is an increasing interest in the examination of the antimicrobial activity of phyto-therapeutics in the past few years, especially considering the increased bacteria resistance to implemented antibiotics, which becomes a global problem.

Among all alternative natural antimicrobial agents, essential oils display significant antimicrobial activity. It is important to note that no resistance or tolerance to essential oils has been discovered yet. This can be explained by the great complexity of their structure which allows the essential oils to act on several target places at the same time, rather than convential antibiotics, which act on one specific target place. Essential oils are secondary metabolites of plants. They are defined as complex mixtures of lipophilic liquid, fragrant and volatile components included in the secretory structures of aromatic plants. Main active components of essential oils are terpenoids (dominant and economically most significant components), aliphatic volatile components and substances which include nitrogen and sulfur [1]. Numerous studies of the antimicrobial activity and the mechanisms of essential oils action argued that resistance does occur due to the large number of different compounds with ultimate strong antimicrobial action. However, specifity of their synergistic effects is very important. These effects actually prevent emergence of resistance [2].

As hydrophobic substances, essential oils have high affinity for the lipids of bacterial cell membranes and their antibacterial effect is mostly related to their lypophilic property [3]. Such strong antimicrobial activity of essential oils against pathogenic bacteria is based on the high level of phenol components such as carvacrol, eugenol (2-methoxy-4-(2-propenyl) phenol) and thymol (4-6).

However, despite numerous studies of the antimicrobial activity of the natural products, the number of tested microorganisms is relatively small and it does not include newer multiple resistant species. Nowadays, aromatic plants from the *Lamiaceae* family present very important sources of biologically and pharmacologically active substances which are widely used and whose effects are very well documented. Antioxidant and antimicrobial activity of 100 volatile components of essential oils from *Lamiaceae* species, especially thyme and oregano, showed that these essential oils are very important for regulation of oxido-reduction potential and normal bacterial flora [7].There is a great number of studies which confirmed the strong antibacterial effect of essential oils of thyme and oregano [8-12].

The objective of this study was to prove the antimicrobial effect of thyme and oregano essential

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oils in order to select the essential oil with the strongest antibacterial effect. The selected essential oil would be the subject of subsequent investigations on designing an adequate *drug delivery* system based on chitosan particles with encapsulated essential oil in them. The chitosan particles with essential oil would be an optimal solution for therapeutic purposes of these phyto-antibiotics by keeping the pharmacological properties of the volatile and photosensitive essential oils and enhancing their therapeutic effect.

MATERIAL AND METHODS

Plant material

Leaves of thyme and oregano were obtained from the Institute for the Study of Medicinal Herbs "Dr. Josif Pančić" in Belgrade in 2010. The plant material was kept in double paper bags in a dark and dry place until hydrodistillation. Immediately prior to hydrodistillation, the fruits of thyme (*Thimis vulgaris*) and oregano (*Origanum vulgare*) were chopped to the size of 0.75[13].

Hydrodistillation (HD)

The essential oils were isolated through hydrodistillation as per Ph. Eur. IV, with *n*-hexane as the collective solvent. An amount of 500 ml of distilled water was poured over the plant material (100 g). The obtained essential oils (EO) were initially dried over anhydrous Na_2SO_4 for 24 h, and then the drying process continued in a desiccator for another hour. The n-hexane was removed in a rotational vacuum-boiler, the obtained EO was measured three times and its quantity was expressed as per mass of dry plant material (g/100 g). Dried oils were preserved in cuvettes with Teflon stoppers at +4°C until use.

Gas chromatography with mass spectrometry (GC-MS)

Gas-chromatographic analysis of essential oils was conducted in a Hewlett Packard 5973-689 GC-MS system in EI mode on 70 eV with spectrometric mass detection (GC-MS). Initial temperature of the capillary column HP 5MS (30 m × 0.25 mm; film thickness 0.25 μm) was 60°C. Using a heating speed of 3°C/min, it was heated to 280°C. Helium was the gas carrier at a flow of 1 ml/min. An amount of 1 μl of each investigated sample was injected in the GC column in proportion of 1:10.

Identification of components was based on calculated retention indexes (RI) [14] and mass spectra compared with standard substances and/or with NIS/NBS Wiley library of mass spectra, including literature data or data from the free database (http//www. flavornet.org/iowtv. pherobase.com) [15]. Experimental values of retention indexes were defined using "calibrated Automated Mass Spectral Deconvolution and Identification System software" (AMDIS ver.2.1., DTRA/NIST, 2002). Results were compared with retention indexes from literature data and internet available database.

The microorganism cultures

For the purpose of *in vitro* testing of the antimicrobial activity of the thyme and oregano essential oils, the following standardized bacterial cultures were used (ATCC – American Type Culture Collection): *Staphylococcus aureus* ATCC 25923, *Escherichia coli A*TCC 25922, *Candida albicans* ATCC 24433, *Enterococcus faecalis* ATCC 29212. These microorganism cultures were supplied from the Collection of bacteria cultures of the Department for Microbiology at the Faculty of Technology, Belgrade, and the bacteria cultures – from the Institute of Virology and Immunology, Torlak.

Determination of antimicrobial activity

Antibacterial activity was determined by the agar well diffusion method and the agar dilution method.

Agar well diffusion method was employed for the determination of the antimicrobial activity of the essential oils. Tubules with diameter of 6 mm were placed on Petri plates with prepared sterile Miller-Hinton TSA (tryptonesoyagar-Torlak) surface, impregnated with soft agar (0.60 % of agar) with the same surface, inoculated with indicator pathogenic strain (0.2 ml of 24-h broth culture for 6 ml of soft agar). After firming of the agar, the tubules were removed and each of the formed wells was filled with 20 μl of the investigated essential oil. Plates were incubated at 37°C for 24 h. In the study of the antimicrobial activity of essential oils, the antimicrobial activity of lactic acid (20 μl) and that of the individual essential oil and lactic acid was investigated in order to determine their synergistic effect (the compound included 50 ppm of lactic acid for 20 μl of essential oil). As a positive control of antibacterial activity the standard antibiotic – clyndamicin (10 μg/ml) and the antimycotic nystatine (30 μg/ml) were taken.

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

Minimum inhibitory concentration (MIC) evaluation using the agar dilution method

For determining the minimal inhibitory concentration (MIC) of the tested bacterial strains, the agar dilution method was performed in a Mueller Hinton broth. Each test tube with 2.997 ml of base was filled with 3 µl of essential oil. Due to the difference in antimicrobial activity by applying the agar dilution method, concentrations of 10, 30, 50 and 100 µl/ml were used for essential oils of thyme and oregano. Indicator breeds of microorganism (1% of inoculum) were inoculated in the prepared test tubes with diluted essential oils and in the control test tube. The tubes were incubated at 37 °C.

At certain time intervals, after 1, 3, 8, 16 and 24 h, the change in optic density (OD) was followed on a colorimeter (MA 9504. Metrix), using a yellow filter (575 nm). Increasing of optic density or blurring was observed on increasing the microorganism biomass in the liquid base. MIC is defined as the first concentration of essential oil with no visible growth of bacteria registered.

RESULTS

Results on the qualitative and quantitative analysis of the chemical composition of essential oils of thyme and oregano are presented in Table 1. Chemical profile of essential oils is presented by names of the components while amounts of these components are presented in percentages (%).

GC-MS analysis conducted for essential oils of thyme and oregano provided definition of 25 compounds or 94.53% for thyme oil and 41 compounds or 97.98% of oregano oil (Table 1, Figure 1). Essential oil of thyme mostly includes monoterpenes (94.08%), especially oxidized monoterpenes (55.86%). There are hydrocarbon monoterpenes (38.67%) as well. The amount of sesquiterpenes is much lower.

Table 1. Chemical composition of essential oils of thyme and oregano.

Component	Oregano	Thyme
Monoterpenic hydrocarbons	**9.40**	**15.38**
Pinene	0.31	1.2
Phellandrene	0.27	0.08
Terpinene	0.88	1.75
Pinene	1.54	0.39
Camphene	0.2	2.09
Carene	-	-
Terpinene	4.64	6.98
Limonene	0.69	0.7
Myrcene	0.53	1.64
Sabinene	-	-
Terpinolene	0.12	0.46
L-Fenchone	-	-
Elemene	0.22	-
Pseudolimonene	-	0.09
Aromatic monoterpene hydrocarbons	**9.66**	**21.15**
p-Cymene	5.02	21.15
p-Anisaldehyde	-	-
-Caryophyllene	4.38	-
o-Cymene	-	-
Cuminaldehyde	0.26	-
Oxidized monoterpenes	**75.76**	**53.51**
Terpineol	1.04	0.95
Camphor	0.39	-
Eucalyptol	1.59	4.74
Isopropenyltoluene	-	-
Linalol	2.69	5.9
3-Octanol	0.1	-
3-Octanone	0.2	-
4-Carvomenthenol	1.47	0.61
4-Thujanol, stereoisomer	0.07	-
Copaene	0.17	-
-ELEMENE	0.03	-
Fenchyl alcohol	0.38	-
Carvacrol	59.03	2.34
CARVACROL METHYL ETHER	0.63	-
Carvone	0.11	-
Eugenol	0.42	-
Isoborneol (Isomer 2)	1.67	0.96
p-Isopropenyl toluene	0.08	-
Thymol	5.69	36.12
Isoborneol (isomer 1)	-	0.58
Menthone	-	-
Geraniol	-	0.32
Geranyl acetate	-	0.69
Nerol	-	0.3
Monocyclic sesquiterpenes	**2.72**	**4.41**
Amorphene	0.22	-
Humulene	1.34	0.44
Bisabolene	0.29	-
Cadinene	0.5	-
Humulene Oxide	0.07	-
Isoledene	0.08	-
Naphthalene, 1,2,3,4,6,8a--hexahydro-1-isopropyl-4,7-dimethyl-CADINA-1,4-DIENE	0.09	-
Caryophyllene	-	3.38
Caryophyllene oxide	-	0.59
Bornyl acetate		
Valencene	0.13	-
Bicyclic sesquiterpenes	**0.44**	**0.00**
Caryophyllene oxide	0.38	-
-Cadinene	0.06	-
Tricyclic sesquiterpene	**0.00**	**0.08**
-Copaene	-	0.08
TOTAL:	**97.98**	**94.53**

More than half of the amount of thyme essential oil consists of 6 dominant compounds. Oxidized monoterpene thymol (36.12%) and monoterpene hydrocarbon p-cymene (21.15%) are mostly included. Other dominant components of this essential oil are -terpinene (6.98%), linalool (5.90%), carvacrol (4.54 %) and eucalyptol (4.74%). (Figure 1).

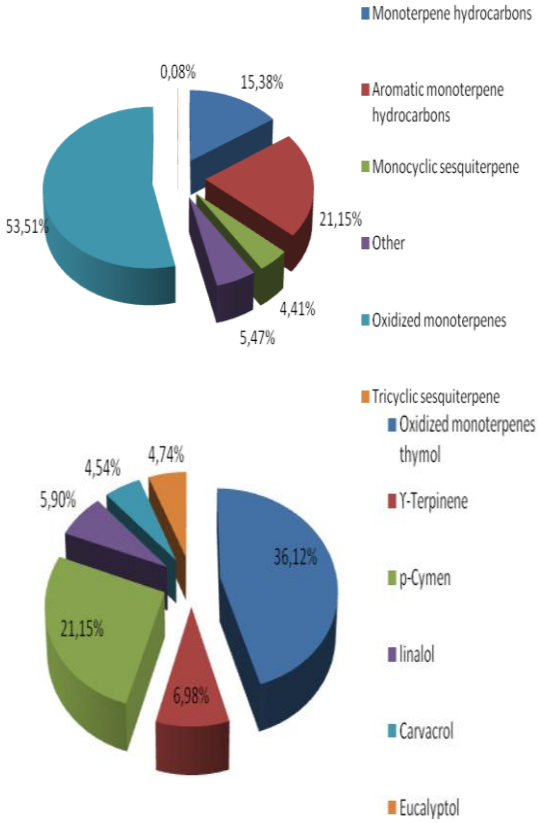

Fig. 1. Main classes of compounds (%) in the essential oil of thyme.

More than half of the total amount of essential oil of oregano consists of oxidized monoterpene carvacrol (59.03%); significantly lower amount of thymol (5.69%) and monoterpene hydrocarbons p-cymene (5.02%) and-terpinene (4.64 %). There is a large number of sesquiterpenes but their quantity is significantly lower (Figure 2).

Antimicrobial activity of essential oils

Results of the study of essential oil activity using the disk diffusion method are presented in Table 2 as inhibition zone diameter (mm) and decrease in bacterial growth (Figure 3).

According to the results of the bacteriological analysis *S.aureus* (11-12 mm) showed higher sensitivity towards Gram-positive bacteria than *E. faecalis* (0.5-0.8 mm). Slightly lower sensitivity was noted in Gram-negative bacteria *E. coli* ATCC 25922 (8 mm).

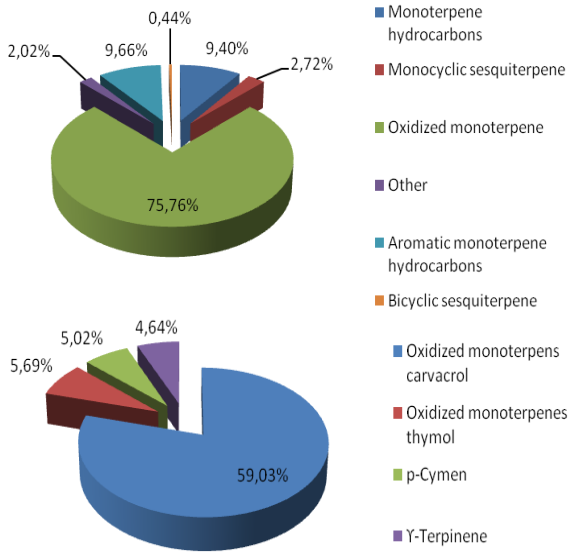

Fig. 2. Main classes of compounds (%) in the essential oil of oregano

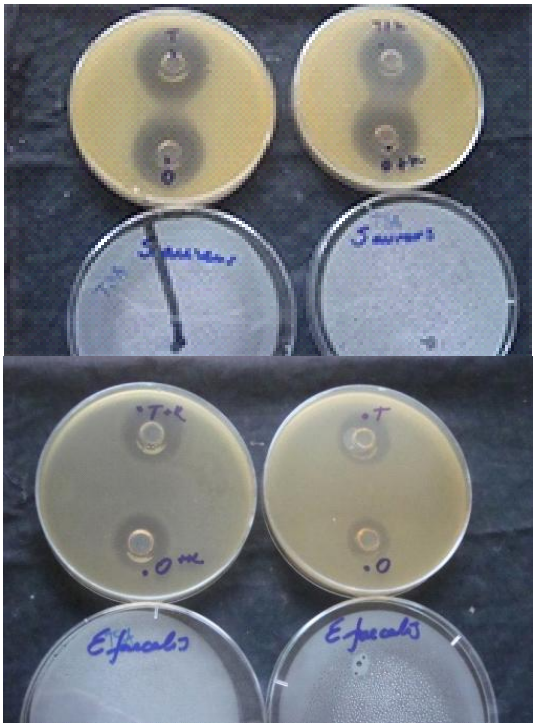

Fig. 3. Antibacterial effect of thyme (T), oregano (O) to *S. aureus* and *E. faecalis*

Table 2. Antimicrobial activity of thyme and oregano, combination of essential oils and lactic acid, clindamycin and nystatin towards investigated bacteria

Essential Oil	Inhibition zone diameter, mm			
	S. aureus	*E. coli*	*C. albicans*	*E. faecalis*
Oregano	11	8	1	0.5
Thyme	12	8	1	0.8
Oregano+acid	11	10	1.25	0.7
Thyme+acid	13	10	1.3	0.9
Acid	-	-	-	-
Nystatin (30µl/ml)	-	-	4	-
Clindamycin (30µl/ml)*	2	2	-	2.5

Results obtained by the dilution method for determination of MIC values showed that essential oils of oregano and thyme in concentrations of 1 to 5 μl/ml act bacteriostatic and bactericidal.

DISCUSSION

The main components of thyme and oregano essential oils detected by GC-MS analyses are phenolic compounds, thymol and carvacrol, which define the biological and pharmacologic properties of these oils. Therefore, the ratio between chemical composition and antimicrobial activity will be crucial for the antibacterial effect of the essential oil. Regarding the strength of antibacterial effect of phenolic compounds, hydroxyl groups have no important role, as expected. This was proven by the similar antibacterial effect of carvacrol and thymol to *S.aureus* and *P.aeruginosa* [5]. The importance of phenolic ring (system of destabilized electrons) can be observed in the significantly lower activity of menthol relating to carvacrol [1]. There are many earlier studies that confirmed the antibacterial activity of the main constituents of thyme and oregano essential oils [4, 6-8].

Low sensitivity of Gram-negative bacteria in the antimicrobial analyses of the tested essential oils can be a consequence of the construction of their cell wall which includes an additional outer membrane around the peptidoglycan layer [16]. It decreases the diffusion of hydrophobic components through their lipopolysaccharide cover [17].

Although both essential oils include the strongest antimicrobial components thymol and carvacrol, thyme oil contains 36.12% of thymolol and 2.34% of carvacrol while oregano oil – 59.63% of carvacrol and 5,69% of thymolol. The antibacterial effect of thyme oil is stronger than the effect of oregano oil. This is aligned with data on the higher activity of thymol relating to carvacrol [4] and the fact that antimicrobial activity is affected by the amount of certain components in the oil and their synergistic action. Both oils showed antimycotic activity to *C.albicans*. The strong antimicrobial effect of the tested essential oils is also explained by the fact that the zones of inhibition for the tested antibiotics are significantly smaller relating to the essential oils of thyme and oregano.

Also, significant synergism between the essential oil of thyme and lactic acid can be noted. This is aligned with literature data on the antimicrobial effect of lactic acid [18]. It is very significant from the aspect of formulation of mycoadhesive products of essential oils in chitosan particles whose optimal effect necessarily includes acid medium.

Significant inhibition of the growth of bacteria *E. coli* was shown in concentrations of 5 μl/ml for oregano, which can be taken as its MIC value. There is no significant difference in the antimicrobial action of the two oils to *E. coli*. The antimicrobial effect of the essential oils of oregano and thyme at the investigated concentrations of 1, 3, 5 and 10 μl/ml is sufficient for efficient inhibition of the growth of *E. faecalis*. MIC value for thyme is 1 μl/ml. Thyme acts bactericidal relating to oregano which acts bacteriostatic with a MIC value of 3 μl/ml.

Significant antimicrobial action of the essential oils of oregano and thyme can be observed for the species of *S. aureus* where the MIC values for oregano and thyme are 3 μl/ml.

The essential oils of thyme and oregano display a lower antimicrobial effect to the pathogenic fungi *C.albicans* in the applied concentrations. However, thyme oil showed a slightly better effect, decreasing the development of cells of *C.albicans* to 50% at a concentration of 10 μl/ml (MIC_{50} value).

Summarizing, one can imply that the results of these investigations are aligned with those of preliminary investigations on the antimicrobial effect of essential oils using the disk diffusion method in wells [9-11]. These results are partially expected having in mind the preliminary results of inhibition zones of the tested microorganisms.

CONCLUSIONS

According to the obtained results of chemical and microbiological analysis, aligned with literature data, the essential oil of thyme showed a stronger antibacterial effect to the tested bacteria and *C.albicans*. Regarding the natural origin, numerous biochemical and pharmacologic investigations and wide antibacterial spectrum of thyme, the possibility of production and use of antimicrobial phyto-products for therapeutic and prophylactic purposes is taken into account. This biologically active natural product would be an ideal replacement for conventional antimicrobial products, especially if we consider the increasing resistance to implemented antibiotics.

REFERENCES

1. R. Croteau, T.M. Kutchan, N.G. Lewis, Natural products (secondary metabolites). Biochemistry and Molecular Biology of Plants. American Society of Plant Biologists, Rockville, MD, 2000, p. 1250.

2. S. Prabuseenivasan, M. Jayakumar, S. Ignacimuthu, *Compl. Altern. Med.*, **12**, 6 (2006).
3. Tacamovic, J.D.Brooker, Proceedings of the Nutrition Society, **64**, 403 (2005).
4. R. J. W. Lambert, P. N. Skandamis, P. J. Coote, G.-J.E. Nychas, *Journal of Applied Microbiology* , **91**, 453 (2001).
5. A. Ultee, M. H. Bennik, R. Moezelaar, *Appl Environ Microbiol.*, **68**: 1561 (2002).
6. A. Sivropoulou, E. Papanikolaou, C. Nikolaou, S. Kokkini, *Journal of Agricultural and Food Chemistry*, **44**: 1202 (1996).
7. M. Marino, C. Bersani, G. Comi, *International Journal of Food Microbiology*, **67**, 187 (2001).
8. S. Cosentino, C. I. G. Tuberoso, B. Pisano, M. Satta, V. Mascia, E. Arzedi, *Letters in Applied Microbiology*, **29**: 130 (1999).
9. M. Marino, C. Bersani, G. Comi, *Journal of Food Protection*,**62**: 1017 (1999).
10. C. Juliano, A. Mattana, M. Usai, *Journal of Essential Oil Research*, **12**: 516 (2000).
11. C. F. Bagamboula, M. Uyttendaele, J. Debevere, *Food Microbiol*, **21**: 33 (2004).
12. H.J. D. Dorman, S F. Deans, *J. Appl. Microbiol.* , **88**, 308 (2000).
13. Pharmacopoea Jugoslavica IV, The Federal Institute for Health Protection, Belgrade, 1984.
14. H. Van den Dool, D. P. A. Kratz, *J. Chromatograph*, **11**, 463 (1963).
15. R.P. Adams, Identification of Essential Oil Component by Gas Chromatography/Quadrupole Mass Spectrometry, Allured Publishing Corporation, Carol Stream: IL, 2007.
16. C. Ratledge, S.G. Wilkinson, An overview of microbial lipids. In: Ratledge, C.,Wilkinson, S.G. (Eds.), Microbial Lipids, vol. 1. Academic Press, London, 1988, p. 3.
17. A. Salam, S.A. Ibrahima, H.Y. Chung, W. Seoa, *Food Chemistry*, **109**: 137 (2008).
18. V. Dodane, V. D. Vilivalam, *Pharm. Sci. Technol. Today*, **1**, 246 (1998).

ХИМИЧЕСКИ СЪСТАВ И АНТИМИКРОБНИ ДЕЙНОСТИ НА РИГАН И МАЩЕРКА ЕТЕРИЧНИ МАСЛА

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(Резюме)

Целта на това проучване е да се оцени химичен състав и antimicrobal активност на етерични масла от мащерка и риган. Газова хроматография с мас спектрометрия (GC-MS) се използва за определяне на химическата структура на етерични масла и техните доминиращи елементи. Antimicrobal активност на етерични масла е тествана срещу стандартизирани и Fungy бактериални култури: *Staphylococcus aureus* ATCC 25923, E *Esherichia coli* ATCC 25922, *Candida Albicans* ATCC 24433, *Enterococcus faecalis* видове ATCC 25929., използвайки метода на дифузия в агар с кладенци. Минималната инхибираща концентрация (MIC) за етерични масла е била определена по метода на разреждане на бульон и ценен в диапазон от 3 µL/ml -. 5 µL/ml, Зависи от етерично масло и бактерии и тествани за *C.albicans*. И двете етерични масла, предвидени силна антибактериална активност за изследваните микроорганизми, а етеричното масло от мащерка е особено признат. Тези експерименти запишат гол на които е показано ин витро антимикробна активност на подбрани етерични масла направи подбор на най-голям потенциал етерично масло, че ще бъде обективен и по-нататъшно разследване и хитозан система за доставка на наркотици с контролирано освобождаване на антимикробната етерично. масло.