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Antimicrobial activity of the three commercial drug's essential oils: Chamomillae flos, Calendulae flos and Millefolii herba

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***Abstract*:**
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Commercial herbal drugs of the three plants belonging to family Asteraceae were used for the extraction of the essential oils (hydrodistillation method). Since various factors during the processing of the plant material can affect several characteristics of essential oils such as yield, chemical composition and, thus, biological activities of essential oils, comparison of the antimicrobial activity of the herein isolated oils was done with avaiable literature sources dealing with this subject. In order to evaluate antimicrobial potential of the isolated essential oils, a broth microdilution method was employed. The results pointed to very high activity of the tested oils, in all cases much stronger in comparison to the previous results. As a conclusion, the adverse effects of plant material processing did not affect its antimicrobial potential, while the obtained high antimicrobial activity can be explained by sinergistic action of the herein used solvent with essential oils.

Key words: *Achillea millefolium*, antimicrobial activity, *Calendula officinalis*, *Chamomilla recutita*, herbal drug

Introduction

Essential oils are complex mixtures of compounds, synthetized in plant tissues as secondary metabolites. The role of essential oil in plant is multiple: defence of pathogens and herbovores, attraction of insects, inhibition of other plant's seed germination, formation of protective layer which reduces transpiration and formation of specific microclimate. Due to their lipophylic character, essential oils can interact with microbial membranes and achieve significant antimicrobial effect. For this reason, during the last several decades, much attention has been devoted to plant essential oils, especially to their antimicrobial activity against various pathogenic and non-pathogenic species. During the research of some plant's medicinal properties, very important indicator of antimicrobial potential is ethnopharmacological use for the treatment of infective diseases. Owing to this, many studies have been focused to ethnopharmacological plants and their antimicrobial activity, as a novel source of antimicrobial compounds. In Serbia, plants belonging to family Asteraceae are very significant medicinal plants and are used extensively in both traditional medicine and current phytotherapy. Among them, very commonly used ones are chamomile, yarrow and calendule.

Yarrow (***Achillea millefolium***) is an aromatic, perennial herbaceous plant, widely distributed in Serbia. Healing properties of yarrow are well known from the ancient times and it is used in both official and alternative medicine. According to Ph. Eur. IV, biological source of the yarrow herbal drug is strictly the species *Achillea millefolium*. However, some authors (Božin *et al*., 2008) stated that *Achillea millefolium*, in wider sence, represents a cytogenetic and chemically polymorphous aggregate

of 12 species, characterized by a well defined morphological, anatomical and caryological features, while the existence of the naturally occurring hybrids increases the number of the representatives in the aggregate. Herbal drug used in medicinal purposes is the above-ground part of the plant in bloom (*Millefolii herba*). Rarely, some parts can be used as individual drugs – inflorescence (*Millefolii flos*) or leaves (*Millefolii folium*) (Sarić, 1989). The main utilization is connected to the treatment of the various gastrointestinal disorders owing to its carminative, holagogue and spasmolytic effects. Also, it can be used to treat hemorrhoids, hypertension, thrombosis, inflammations and respiratory infections (Sarić, 1989; Kojić *et al*., 1998; Tajik *et al*., 2008; Sant'Anna *et al*., 2009). Applied externally, yarrow can heal inflammations of skin and mucosal surfaces; it promotes epitelization, heals the wounds and stops the bleeding process by constricting the blood vessels. Essential oil of this plant yields from 0.2-1% and can contain up to 50% of chamazulenes (Ross, 2003). Investigations of its chemical composition showed that it is very variable, depending on factors such as geographical origin, duration of inflorescence and level of polyploidy – the oil has characteristic blue color only in the case of its isolation from tetraploid specimens (Kubelka *et al*., 1999; Rohloff *et al*., 2000; Bošković *et al*., 2005), and it is connected to the presence of the sesquiterpene chamazulene.

Literature data on antimicrobial activity of *Achillea millefolium* are relatively poor, since the most number of investigations studied antimicrobial effects of its various extracts (Kojić *et al*., 1998; Nascimento *et al*., 2000; Holetz *et al*., 2002; Candan *et al*., 2003; Stojanović *et al*., 2005; Tajik *et al*., 2008). Only one of these studies investigated antimicrobial potential of the yarrow essential oil (Candan et al., 2003), where moderate antimicrobial activity was detected.

***Calendula officinalis* L.** is used extensively in ethnopharmacology. The most frequent application of this plant is in the form of tincture or creme for curing the wounds, abscesses and decubitus. It has beneficial effect to gastrointestinal and urogenital disorders, and it is also used for sedative purposes (Willfort, 1959). The famous wound healing properties of this plant prompted investigations which proved its proliferative effects (Chandran & Kutton, 2008; Leach, 2008). Marigold contains small amounts of essential oil: 0.06-0.3% (Naguib *et al*., 2005), but due to its significant pharmacological activities, it has been studied well. In the most papers, its main component is α-cadinol (Chalchat *et al*., 1991, Okoh *et al*., 2007, Gazim *et al*., 2008a, Petrović *et al*., 2010). Investigations of marigold's essential oil antimicrobial activity inhibited growth of *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans* (Janssen *et al*., 1986). Also, it has been shown that it possess potent activity against *Candida albicans* and *Staphylococcus aureus* clinical isolates (Gazim *et al*., 2008b; Fit *et al*., 2009).

Chamomilla recutita is an annual, perennial plant, native in southeastern Europe, but today widely distributed and can be found in North Africa, Asia, America and New Zealand (Pirzad *et al*., 2006). Chamomille is used in therapy since an ancient times (Owlia *et al*., 2007). Owing to its medicinal properties, it is included as a drug in pharmacopeia of 26 countries (Pirzad *et al*., 2006; Owlia *et al*., 2007; Shikov *et al*., 2008). This plant possesses anti-inflammatory, anesthetic, antiseptic, sedative, carminative, antidiarrhoeic and vulnerary properties (Pirzad *et al*., 2006; Miller, 2009). Scientifically investigated and confirmed pharmacological properties of chamomile are multiple - antioxidant (Asgary *et al*., 2002; Owlia *et al*., 2007; Romeilah, 2009; Ayoughi *et al*., 2011), antiulcer (Karbalay-Dost & Noorafshan, 2009), antiinflammatory (Presibella *et al*., 2006; McKay & Blumberg, 2006), vulnerary (McKay & Blumberg, 2006; Martins *et al*., 2009), anticoagulant, antitumor, antigenotoxic, hypocholesterolemic, antispasmolitic and sedative (McKay & Blumberg, 2006). The most pharmacologically active compounds are those found in chamomile essential oil – chamazulene, α-bisabolol, bisabolol oxides, farnesene and cyclic esters (Orav *et al*., 2001; Martins *et al*., 2009), but flavonoids, coumarins and pyrones are also significantly active compounds, found in chamomille (Grgešina *et al*., 1995; Pirzad *et al*., 2006). Antimicrobial activity of chamomile is confirmed against 25 different gram positive and gram negative bacterial strains, as well as against 20 *Lysteria monocytogenes* strains (Lis-Balhin *et al*., 1998). The same study showed antifungal activity against *Aspergillus niger, A. ochraceus* and *Fusarium moniliforme.* Study of Owlia *et al.* (2007) showed antistreptococcal activity of chamomile essential oil, which was also active against *Mycobacterium tuberculosis, Salmonella typhymurium* and *Staphylococcus aureus* (Pirzad *et al*., 2006). Beside antibacterial activity, it was confirmed that chamomile essential oil possess antiviral activity against polio and herpes viruses (McKay & Blumberg, 2006).

Since most of the literature data about antimicrobial activity of these plants is based on unprecise method (disc diffusion) or the data are relatively old, the main goal of this paper was to investigate antimicrobial properties of these three plants, but using more precise microdilution method and to compare the obtained results with the previous ones on this subject. Owing to the fact that commercial plant material (for medicinal purposes) can be found at the markets nowdays, there is high possibility that it possess different properties than the same material collected by botanical experts. The processing after the collection such as drying, time of grounding and storage period before the isolation are very important since they can significantly affect chemical composition of essential oil and, thus, antimicrobial and other biological activities. In the light of these facts, this investigation used commercial plant material which had a lot of potential to differe when compared to personally collected material by relevant experts. The paper provides updated and more precise data on these essential oil's antimicrobial activity, as well as information whether usage of commercial material can significantly affect expected beneficial properties.

Materials and methods

Plant material

Plant material used for isolation of the essential oils was puchased in the form of mono-component herbal drug from the following commercial sources: Jeligor, Svrljig (yarrow), Montes, Leskovac (chamomille) and „Josif Pančić" Institute, Belgrade (marigold).

Isolation of the essential oil

Air-dried, to constant weight, the commercial sample of *Carlinae radix* drug (three batches of 100-150 g) were subjected to hydrodistillation with *ca*. 1 L of distilled water for 3.5 h using the original Clevenger-type apparatus (Clevenger, 1928). The obtained oils were separated by extraction with freshly distilled diethyl ether and dried over anhydrous magnesium sulphate. The solvent was evaporated under a gentle stream of nitrogen at room temperature in order to exclude any loss of the essential oil and immediately analyzed. When the oil yields were determined, after the bulk of ether was removed under a stream of N_2, the residue was exposed to *vacuum* at room temperature for a short period to eliminate the solvent completely. The pure oil was then measured on an analytical balance and multiple gravimetric measurements were taken during 24 h to ensure that all of the solvent had evaporated.

Microorganisms and culture conditions

Antimicrobial activity assays were performed against seven American Type Culture Collection (ATCC) strains: Gram positive *Staphylococcus aureus* 6538, Gram negative *Escherichia coli* 8739, *E. coli* 25922, *Proteus vulgaris* ATCC 8427, *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumoniae* 10031 and the yeast *Candida albicans* ATCC 10231.

Bacterial strains were maintained on Nutrient agar (NA), while *C. albicans* was maintained on Sabouraud Dextrose Agar (SDA) in the culture collection of the Microbiology laboratory, Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš.

Determination of MIC and MBC/MFC (minimal inhibitory/microbicidal) concentrations

Antimicrobial activity determination was performed by a microdilution method as described previously (Stojanović-Radić *et al*., 2012). Briefly, overnight cultures of microorganisms were used for the preparation of suspension. Final size of the bacterial inoculum was 5 x 10^5 CFU mL^{-1}. Stock solutions of the essential oil were prepared in 50% aqueous ethanol, whose final concentration never exceeded 5% (v/v) in a well. Dilution series were prepared in 96-well microtitre plates in the concentration range from 0.001-50 μL mL^{-1} (essential oil). After attaining the right dilutions, the inoculums were added to all wells. The plates were incubated for 24 h at 37 ºC (bacteria) and 48 h at 30 ºC (yeast). Bacterial growth was determined by adding 20 μL of 0.5% triphenyl tetrazolium chloride (TTC) aqueous solution. One inoculated well was included to allow control of the broth suitability for organism growth. One non-inoculated well, free of antimicrobial agents, was also included to ensure medium sterility. Positive controls were tetracycline and nystatine, while the solvent for the oil dilution (ethanol) was used as negative control. Minimal inhibitory concentration (MIC) was defined as the lowest concentration of the oil inhibiting visible

Table 1. Yield and features of the essential oils isolated from commercial material of herbal drugs belonging to three species from family Asteraceae

Herbal drug	Plant species stated as the only one component of the herbal drug	Features of isolated essential oil	Yield of isolated essential oil	Range of yields according to literature data
Chamomilae flos	*Chamomila recutita*	Liquid, specific blue color, intensive pleasant aroma	0. 100%	0.20-1.90%
Milefolii herba	*Achillea millefolium*	Liquid, specific blue color, intensive pleasant aroma	0. 430%	0.20-1.00%
Calendulae flos	*Calendula officinalis*	Liquid, colorless, intensive pleasant aroma	0. 063%	0.06-0.30%

growth (red colored pellet on the bottom of wells after the addition of TTC), while the minimal microbicidal (bactericidal and fungicidal) concentration (MBC/MFC) was defined as the lowest oil concentration killing 99.9% of bacterial/fungal cells. To determine MBC/MFC, the broth was taken from each well without visible growth and inoculated in Mueller Hinton Agar (MHA) for 24 h at 37 ºC and in Sabouraud Dextrose Agar (SDA) for 48 h at 30 ºC in the case of the tested yeast. Experiments were done in quintuplicate.

Results and discussion

Essential oils were isolated and yields, together with features are presented in the Table 1. The highest yield of essential oil was in the case of yarrow, which classifies this plant into the category of aromatic plants. Considering literature data about content of essential oils in the investigated plants, the only one which had different amount is chamomile. In the present work, it yielded only 0.1%, which is lower than the up to now determined range from 0.2-1.9%. This can be explained by material processing before purchasing and distillation, where evaporation of the oil during long storage could contribute to the herein obtained lower yield.

The results of antimicrobial activity determination showed activity of the three essential oils in the range from 0.09-50.00 μL mL^{-1}. The most active essential oil was the one isolated from Milefolii herba drug, while the essential oil from Calendulae flos exhibited the lowest activity among the tested ones. Negative control (solvent = ethanol) showed no activity against any of the tested microbial strains. Considering activity in comparison to the reference antibiotics (positive controls), all tested oils showed lower activity than the antibiotic (Figures 1-3).

Considering essential oil of *Chamomilla recutita*, the results of microdilution method revealed very significant activity, which was higher than those reported in previous studies on this subject. The range of the active concentrations was from 0.10-1.73 μL mL^{-1} (Table 2, Figure 1). Among the tested microbial species, *Candida albicans* showed the highest sensitivity, with inhibited growth at only 0.10 μL mL^{-1}, while concentration of 0.32 μL mL^{-1} showed fungicidal effect. On the other hand, the most resistant strain was *Escherichia coli* (ATCC 8739), where active concentrations were MIC=1.73 μL mL^{-1} and MBC=2.60 μL mL^{-1}.

Table 2. Minimal inhibitory and bactericidal concentrations of essential oils from commercial *Milefolii herba, Calendulae flos* and *Chamomillae flos* herbal drugs (μL mL^{-1})

Bacterial/fungal species	ATCC number	*Milefolii herba*		*Calendulae flos*		*Chamomillae flos*		AB
		MIC	MBC	MIC	MBC	MIC	MBC	MIC
Staphylococcus aureus	6538	0.29	0.78	0.14	0.39	0.16	0.16	0.39
Escherichia coli	25922	0.78	3.12	3.12	6.25	0.12	0.32	1.56
Escherichia coli	8739	0.65	1.56	18.7	50.0	1.73	2.60	1.56
Klebsiella pneumoniae	10031	0.32	12.50	0.09	0.19	0.13	2.60	0.78
Pseudomonas aeruginosa	9027	0.09	0.39	0.09	0.19	0.13	0.32	1.56
Proteus vulgaris	8427	0.32	3.12	0.10	0.78	0.10	1.30	1.56
Candida albicans	10231	0.12	0.39	0.19	0.19	0.10	0.32	6.25

AB- reference antibiotic (Tetracycline and Nystatin), given in μg mL^{-1}

Previous investigations on chamomile antimicrobial activity were numerous (Lis-Balhin et al., 1998; Al-Ismail & Talal, 2003; Pirzad et al., 2006; Owlia et al., 2007; Shikov et al., 2008; Pereira et al., 2008) and it is considered as a plant with moderate antimicrobial activity. It possesses activity against both Gram positive and Gram negative strains, as well as against fungi (Romeilah, 2009). Essential oil isolated from this plant species showed antibacterial activity at concentration of 25 mg mL^{-1} against *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus mutans* and *Streptococcus salivarius* as well as against *Candida albicans* (Aggag & Yousef, 1972; Berry, 1995). In the study of Lis-Balchin *et al*. (1998), it has been reported that this oil has effect against 25 different bacterial species and 20 strains of *Lysteria monocytogenes*. The same study revealed significant antifungal activity against *Aspergillus niger, A. ochraceus* and *Fusarium culmorum.* Different streptococcal species - *Streptococcus pyogenes*, *S. mutans*, *S. salivarius*, *S. faecalis* and *S. sanguis* were inhibited by chamomile essential oil in the concentration range from 0.10 – 4.0 μL mL^{-1} (Owlia *et al*., 2007), which is similar to the active concentration range in the present work. The mechanism of chamomile essential oil's activity was investigated against fungal species, where it was reported that it increases permeability of cytoplasmic membrane, which leads to osmotic pressure disturbance and death of the cell as a consequence (Tolouee *et al*., 2010). However, due to different methods used in the mentioned investigations (disc diffusion in most cases), as well as different solvents used (dimethyl sulfoxide), data from the present paper cannot be compared properly to the previously obtained results (Aggag & Yousef, 1972; Berry, 1995; Lis-Balhin *et al.*, 1998). In some of the existing papers, α-bisabolol is considered as the main carrier of the activity (Owlia *et al*., 2007; Romeilah, 2009), which is mentioned in some studies as antifungal compound (Pauli, 2006; Tolouee *et al*., 2010). It inhibits the synthesis of ergosterol, which would explain the highest activity against fungal organism obtained in the present investigation.

Results of Calendulae flos essential oil's antimicrobial activity testing showed very high effect against all tested microbial strains. Obtained active concentration were in the range from 0.09-18.7 μL mL^{-1} (Table 2, Figure 2). This essential oil exhibited the highest activity against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, while *Escherichia coli* 8739 showed the highest resistance, where higher concentration of 18.7 μL mL^{-1} was necessary for inhibition of its growth. Yeast *Candida albicans* showed very significant sensitivity to this oil, which points to the potential of this plant in the treatment of fungal infections.

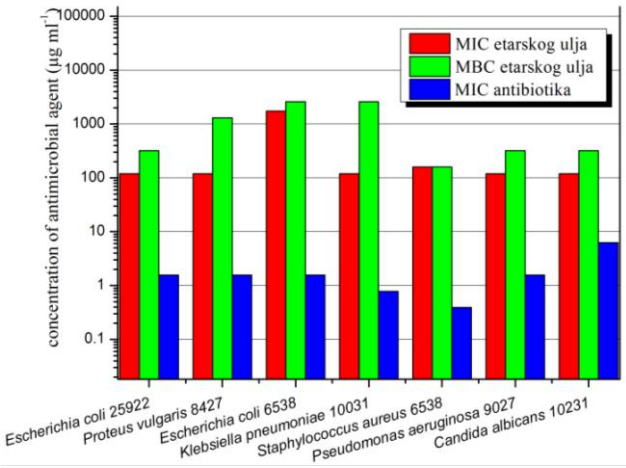

Figure 1. Antimicrobial activity of Chamomillae flos essential oil compared to the reference antibiotic (μg mL^{-1})

Marigold is mentioned in the literature data as a plant with high antimicrobial potential with reported activity against Gram positive, Gram negative and fungal species (Janssen *et al*., 1986; Iauk *et al*., 2003). Studies dealing with investigation of antimicrobial effect of marigold's essential oil isolated from flowers (Janssen *et al*. 1986; Gazim *et al*., 2008b; Fit *et al*., 2009) reported its activity against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* (Janssen *et al*. 1986). Also, this essential oil showed extraordinary effect against 35 different isolates from skin lesions, with average zone of inhibition of 21.3 mm (at concentration of 30 µL/disc), which was the second strongest activity among ten tested essential oils (aloe vera, rattle, fir, savory, marigold, coconut, eucalyptus, lavender, mint and pine), isolated from medicinal plants (Fit *et al*., 2009). Study on antimicrobial activity of marigold's essential oil against 23 clinical isolates of *Candida albicans* showed intensive antifungal activity, comparable or sometimes even several times higher than nystatin (Gazim *et al*., 2008b). All reported studies pointed to very high antimicrobial potential of marigold, where we must highlight that the lack of results obtained by microdilution method makes the comparison with the present ones very difficult. Certainly, according to the previous results, the herein obtained activity was expected. Previous studies on chemical composition of this essential oil reported the presence of many compounds already reported as antimicrobial such as monoterpenes α-thujone, α-pinene, limonene or 1,8-cineole. Therefore, we can suppose that the obtained activity might be a result of their synergistic effect.

Essential oil isolated from commercial drug of yarrow showed unexpected, very high activity, showing antimicrobial effect against all tested strains. Inhibitory concentrations were in the range from 0.09-0.78 µL mL^{-1}, while concentrations from 0.39-12.50 µL mL^{-1} exhibited microbicidal effect (Table 1, Figure 3). This oil showed the highest activity against bacterial species *Pseudomonas aeruginosa* (MIC=0.09 µL mL^{-1}, MBC=0.39 µL mL^{-1}), followed by *Candida albicans* with MIC at 0.12 µL mL^{-1} and MBC at 0.39 µL mL^{-1}. The highest tolerance to the essential oil presence showed both species of *Escherichia coli*, where *E. coli* 25923 was more resistant and showed sensitivity at 0.78 µL mL^{-1} (MIC) and 3.12 µL mL^{-1} (MBC).

Previous investigation of this plant essential oil's antimicrobial activity (Candan *et al*., 2003; Božin *et al*., 2008) pointed to moderate effect, while different extracts in most cases showed no activity. Comparison of the present results with the previous one obtained by the same antimicrobial testing method (Candan *et al*., 2003) showed extremely higher activity of the herein tested essential oil. The oil from the mentioned investigation showed antimicrobial activity in the range from 4.50-72.00 mg mL^{-1}, while the oil in the present study was active at concentrations from 0.08-0.60 mg mL^{-1}, which can be explained by eventual qualitative differences of the compared essential oils, as well as by different solvents used. Candan *et al*. (2003) used 0.5% Tween 80 for solution and dispersion of the essential oil, while the present study used ethanol as solvent. Beside this, the authors of the previous study added solvent directly into the medium and then performed the dilution of essential oil. In the present study, due to

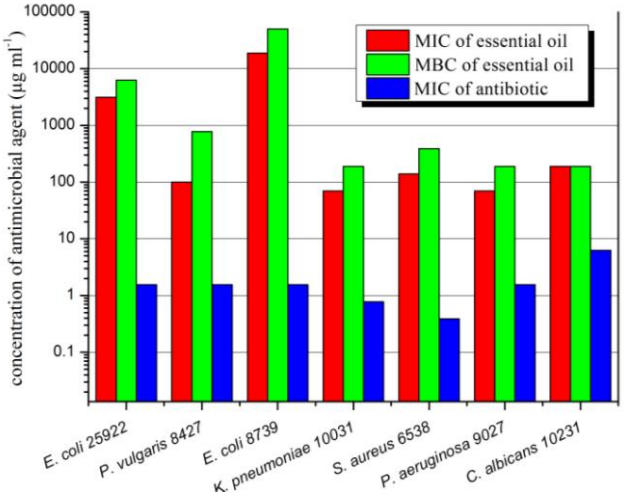

Figure 2. Antimicrobial activity of Calendulae flos essential oil compared to the reference antibiotic (µg mL^{-1})

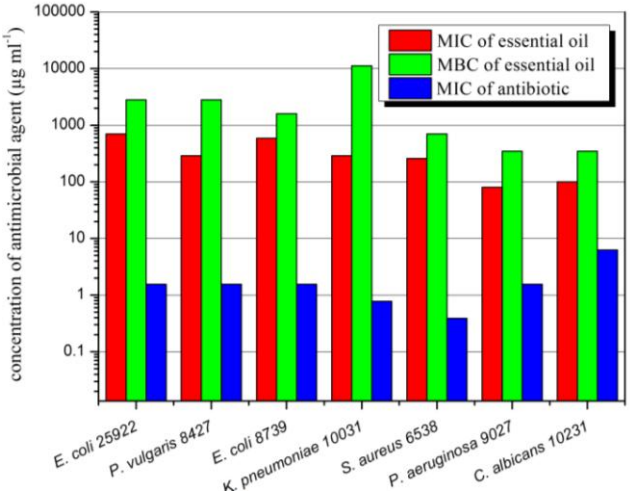

Figure 3. Antimicrobial activity of Milefolii herba essential oil compared to the reference antibiotic (µg mL^{-1})

very weak solubility, the oil was first dissolved in 50% ethanol. After that, dilutions were made in the same solvent, which was followed by adding of these solutions into the wells of the microtiter plate in order to achieve appropriate concentrations. Owing to the low solubility of the yarrow essential oil, it is possible that the previous study did not have completely dissolved oil, which affected its activity due to lower final concentration of the oil in the wells. Also, better activity obtained in this study might be connected to the synergistic activity with ethanol, which does not affect the growth of bacteria in the final concentration, but might increase the permeability of the bacterial membrane and facilitate the passage of the active compounds to its target place.

Conclusion

As a final conclusion, we can state that commercial plant material does not have significantly altered biological properties as a consequence of the plant material processing. The only difference was yield of chamomile essential oil, which can be related to evaporation of oil during a longer period of storage. Antimicrobial activity of the tested essential oils was, in all three cases very significant and in the range or even lower than the previously investigated essential oil. Higher activity obtained in the present investigation is probably due to more precise and quantitative method used herein (microdilution method), while used solvent might contribute by synergistic action with essential oils. Synergism of these mixtures might be very promising and future studies should be focused on this object of investigation.

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