JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

INHIBITION EFFECTS OF SOME ANTIMICROBIAL AGENTS FROM *SALVIA OFFICINALIS* L. ON THE GROWTH OF SELECTED GRAM-NEGATIVE AND GRAM-POSITIVE BACTERIAL STRAINS

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doi: 10.15414/jmbfs.2018-19.8.3.960-964

ARTICLE INFO

Received 9. 8. 2018
Revised 11. 9. 2018
Accepted 25. 10. 2018
Published 1. 12. 2018

Regular article

OPEN ACCESS

ABSTRACT

In the present study, the antimicrobial activity of sage oil, some of its pure components (1,8-cineole, borneol and α-ß thujone) and sage extract were investigated against selected strains of Gram-positive bacteria (*Enterococcus faecalis*, *Staphylococcus aureus*) and Gram-negative bacteria (*Klebsiella pneumoniae*, *Escherichia coli*). The antimicrobial activities of the used antimicrobials agents were determined by using the micro broth dilution method according to Clinical and Laboratory Standards Institute. The percentage of bacterial growth was determined by measured absorbance on a Microplate Reader Opsys MRTM, Dynex (Chantilly, USA). The best minimum inhibitory concentration (MIC) was found against Gram-positive bacteria *S. aureus* CCM 4223 by sage essential oil with MIC_{50} 96.05 µg/mL. The results from the percentage growth of tested bacteria showed that antimicrobial agents inhibit more Gram-positive, than Gram-negative bacterial strains.
The chemical composition of sage essential oil (EO) was analysed using the gas chromatography-mass spectrometry (GC-MS). Sage EO contains primarily α -thujone (14.00 %), borneol (12.90%), camphor (12.90%), (−)-Isopulegol (10.10%), ß-thujone (8.40%) and 1,8-cineole (8.00%). The antioxidant properties of sage oil (71.55%) and sage propylene glycol extract (60.93%) were measured by testing their scavenging effect on DPPH radical activities. Due to antibacterial activity of the tested antimicrobials from sage, the problem of microbial resistance to conventional antibiotics could be treated by pharmaceutical industries to manufacture antibacterial drugs.

Keywords: essential oils, extract, antimicrobial activity, Gram-positive and Gram-negative bacteria

INTRODUCTION

Antibiotics are probably the most used drugs in human medicine, and over the last few years, abuse in the use of these drugs has created multidrug resistance (MDR), what puts the effective treatment of a growing number of infections caused by pathogenic microorganisms at serious risk (**Barreto *et al*., 2014**). The battle between antimicrobial-resistant pathogens and antibiotic therapy is an evolutionary arms race-one that we are currently losing. Consequently, antimicrobial resistance (AMR) - related deaths have reached alarming rates throughout the world. Estimates suggest that at least 700,000 people die annually from drug-resistance infections; this number could rise to 10 million by 2050, far surpassing cancer as the major cause of death worldwide (**O'Neill, 2014**). Nosocomial infections are an important factor in the emergence and spread of multidrug-resistant (MDR) bacteria. Broadspectrum antibiotics, such as vancomycin, third-generation cephalosporins and carbapenems, are often used for empirical treatment of infected patients, thereby selecting for and favouring the persistence of MDR pathogens (**Jenkins, 2017**). The most critical group of all includes multidrug resistant bacteria that pose a particular threat in hospitals, nursing homes, and among patients whose care requires such devices as ventilators and blood catheters. They include *Acinetobacter*, *Pseudomonas* and various *Enterobacteriaceae* (including *Enterbacter* spp., *Klebsiella pneumoniae*, *E. coli*, *Serratia*, and *Proteus*) (**WHO, 2017**). The highest ranked Gram-positive bacteria (high priority) were vancomycin-resistant *Enterococcus faecium*, *Enterococcus faecalis* and meticillin-resistant *Staphylococcus aureus* (**Tacconelli *et al*., 2018**). Most efforts to resolve AMR are geared toward the development of novel antibiotics, yet resistance has arisen to every antibiotic used in the clinic. Innovative strategies to reduce the rise of drug-resistant pathogens are therefore a necessary public health concern (**Ragheb *et al*., 2018**). This situation requires some effort to help combat such problems. One possible solution is to search for alternative therapies to control these diseases. An alternative is the use of essential oils to achieve control over antibiotic-resistant microorganisms (**Veras *et al*., 2012; Raut and Karuppayil, 2014**). In fact, many studies of essential oils have been carried to develop bio-products and safer drugs of industrial interest (**Hsouna *et al*., 2011**). Undeniably, the technological application of a specific essential oil must be supported by scientific researches in order to prove its effectiveness as antimicrobial agent and to elucidate health outcomes linked with its future uses (**Lahmar *et al*., 2016**).
In this investigation, we aimed to determine the antimicrobial activity of Sage oil, some of its pure components (1,8-cineole, borneol and α-ß thujone) and sage extract against selected strains of Gram-positive bacteria (*Enterococcus faecalis*, *Staphylococcus aureus*) and strains of Gram-negative bacteria (*Klebsiella pneumoniae*, *Escherichia coli*).

MATERIAL AND METHODS

Antimicrobial samples

The essential oil of *Salvia officinalis* L. and propylene glycol extract of *Salvia officinalis* L. (leaf) used in this study were supplied by Calendula company a.s. (Nová Ľubovňa, 238 A, Slovakia). The phenolic compounds standards, concretely: 1,8-cineole, borneol and α-ß thujone were of analytical grade purity and were obtained from Merck (Germany). Sage essential oil and extract were stored in air-tight sealed glass bottles at 4 °C; used standards were stored at 4°C until the analysis.

GC-MS analysis of Sage essential oil

Essential oils constituents were identified and the relative composition of the oil was determined by gas chromatography followed by mass spectrometry (GC-MS) as described by **Císarová *et al.* (2018)**. The identification of constituents was performed by matching their mass spectra and retention indices with those obtained from authentic samples and/or NIST/Wiley spectra libraries and available literature data (**Adams, 2007**). Relative proportions were calculated by dividing individual peak area by total area of all peaks. Only compounds over 1% were included.

Free radical scavenging assays

The antioxidant activity of sage essential oil and propylene glycol extract was determined by the radical 2,2-Diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, German). Determination of the antioxidant activity by the DPPH method (scavenging of DPPH radical) *in vitro* was carried out by the method of **Hatano *et al.*** (**1988**) with modification for microplate form. Fifty µL of tested samples were added into 100µL of DPPH solution (0.012 g of DPPH dissolved in 100 mL of ethanol, 96%, v/v). The obtained solutions were shaken and left for incubation (30 min) at darkness at room temperature for any reaction to occur. Absorbance was recorded at 517 nm using Microplate Reader Opsys MRTM, Dynex (Chantilly, USA). Antioxidant activity was expressed as percentage (%) of scavenging activity:

$$\text{DPPH scavenging activity (\%)} = [(A_0 - A_1)*100]/A_0$$

where A_0 is the absorbance of the blank sample and A_1 is the absorbance of the test sample at 30 min. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. The analyses were performed in triplicate.

Bacterial strains

To determine the antibacterial effects of Sage EO, sage extract and phenolic compounds, four kinds of bacteria were used in this study such as Gram-negative strains: *Escherichia coli* CCM 3954, *Klebsiella pneumoniae* CCM 2318 and *Enterobacter faecalis* CMM 4224, and Gram-positive strains: *Staphylococcus aureus* CCM 4223. The test strains were incubated in the Muller-Hinton broth medium (Hi-Medium, India) (MHB) at 37 °C for 16 h to obtain the active cultures.

Inoculum preparation

Overnight bacterial cultures were transferred to MHB and the suspension was adjusted to 0.5 McFarland standards. The suspension was subsequently diluted in cultivation medium to get the final concentration of 5×10^5 CFU/mL in the inoculated broth that was confirmed by colony count performed according to the CLSI guidelines (**CLSI, 2009**). The same inoculum preparation procedure was used for all experiments carried out in this study.

Determination of minimum inhibitory concentration

Determination of MIC (minimum inhibitory concentrations) was performed by serial microdilution technique using 96-well microtiter plates containing Mueller-Hinton broth. Briefly, the sage essential oil, 1,8-cineole and α-ß thujone were diluted in MHB with the addition of Tween 80 content 0.5%. Borneol was dissolved in dimethyl sulphoxide (DMSO) and following diluted in MHB where maximum DMSO content was 1%. Propylene glycol extract of Salvia officinalis L. was diluted directly in MHB until analysis. All used antimicrobials were prepared to give initial concentration of 512 µg/mL. The two-fold serially diluted concentrations of all antimicrobials ranging from 512 to 0.25 µg/mL. Final bacterial concentration was prepared as 0.5 °McF. In this test, the wells in the last column in all plates used for all tested strains were used as a positive control for optical density measurement (6 wells) and as sterility control of MHB (6 wells). The microplates were incubated at 37 °C during 24 h. The lowest concentration of the antimicrobial which prevents the growth of the bacterial was defined as the minimal inhibitory concentrations (MIC). The optical density was measured on a Microplate Reader Opsys MRTM, Dynex (Chantilly, USA) at 405 nm. The 96-well microtiter plates were measured before and after (24 h.) experiment. The results were expressed as a mean of three replicates in three independent tests.

Statistical analysis

The statistical analysis was performed by Probit analysis by using statistical program Statgraphic (Statpoint technologies, Warrenton, VA, USA) according to **Hleba *et al.* 2014** with some modifications.

RESULTS AND DISCUSSION

Sage (*Salvia officinalis* L.), which is one of the most important pharmaceutical herbs, has been exploited for many uses (**Vosoughi *et al.*, 2018**). EO from the sage is already known to present antimicrobial action against various bacteria and fungi (**Mandras *et al.*, 2016; Shahbazi *et al.*, 2015; Snoussi *et al.*, 2015**). According to the results of phytochemical analysis of Salvia species oils, the main constituents of the oils are monoterpenes (α–and β-thujone, 1,8-cineole, camphor, and linalool), sesquiterpenes (α-humulene), and phenolics (**Roby *et al.*, 2013; Martins *et al.*, 2015**). For borneol and 1,8-cineole have been reported antimicrobial activities. The antimicrobial activity of both α- and β-thujones was also confirmed (**Tsiri *et al.*, 2009; Rashid *et al.*, 2013**) and a synergistic interaction was observed when the two isomers were tested in a mixture (**Viljoen *et al.*, 2006**). In this research we tested sage EO, sage extract and some phenolic compounds present in sage. The minimum inhibitory concentration (MIC) MIC_{50} and MIC_{90} (expressed as µg/mL) of five antimicrobials against four strains of bacteria are summarized in Table 1.

Table 1 The minimum inhibitory concentration (MIC) of tested antimicrobials expressed as µg/mL

Tested antimicrobials	Gram-negative				Gram-positive			
	E. coli		***K. pneumoniae***		***E. faecalis***		***S. aureus***	
	MIC 50	**MIC 90**	**MIC 50**	**MIC 90**	**MIC 50**	**MIC 90**	**MIC 50**	**MIC 90**
	p Value		***p Value***		***p Value***		***p Value***	
α-β thujone	>512	>512	>512	>512	>512	>512	>512	>512
	nd		*nd*		*nd*		*nd*	
1,8-cineole	>512	>512	383.00	407.13	383.00	407.13	>512	>512
	nd		*0.0002*		*0.0002*		*nd*	
Borneol	>512	>512	553.48	928.02	451.94	890.42	2726.25	6219.10
	nd		*0.1179*		*0.1261*		*0.8657*	
Salvia extract	773.24	1662.83	553.48	928.02	503.07	1270.30	1385.70	2574.31
	0.4613		*0.1179*		*0.3596*		*0.6606*	
Salvia EO	191.83	203.97	383.00	407.13	>512	>512	96.05	102.31
	0.0001		*0.0002*		*nd*		*0.0001*	

Legend: nd-antimicrobial effect not determined

We did not detect any antimicrobial effect of α-β thujone in this study (MIC_{50} and MIC_{90} >512). All tested strains were resistant to α-β thujone. The strains *E. faecalis* CCM 4224 were found to be more susceptible to the borneol with a MIC_{50} value of 451.94 µg/mL and *K. pneumoniae* CCM 2318 with MIC_{50} value of 553.48 µg/mL. Both strains were sensitive to 1,8-cineole with the same MIC_{50} value of 383.00 µg/mL. *S. aureus* CCM 4223 with higher MIC_{50} values (2726.25 µg/mL) was found the least sensitive to borneol. But this strain was found to be most sensitive to salvia EO (MIC_{50} 96.05 µg/mL), followed by > *E. coli* CCM 3954 (191.83 µg/mL) > *K. pneumoniae* CCM 2318 (383.00 µg/mL). **Vetas *et al***. (**2017**) tested antibacterial activity of sage and spearmint essential oils against *S. aureus.* The results showed that both EOs presented MIC and MBC equal to 1.25 and 2.5%, respectively. *E. faecalis* CCM 4224 was resistant to salvia EO, in this study. 1,8-cineole had no antimicrobial effect on strains *E. coli* CCM 3954 and *S. aureus* CCM 4223. The best antimicrobial effect of tested salvia extract was shoved against strain *E. faecalis* CCM 4224 with MIC_{50} value 503.07 µg/mL, followed by *K. pneumoniae* CCM 2318 (553.48 µg/mL) > *E. coli* CCM 3954 (773.24 µg/mL) and *S. aureus* CCM 4223 (1385.70 µg/mL). In our study, the Gram-negative bacterial strains were more sensitive to 1,8-cineole than Gram-positive tested strains. In study of **Bosnić *et al.*** (**2006**), the tested compound 1,8-cineole seems to be more effective against Gram-positive strains (*S. aureus* and *B. subtillis*). They found no effect of this compound against Gram-negative strains (*E. coli* and *P. aeruginosa*).

Based on the measured absorbance on a Microplate Reader Opsys MRTM, Dynex (Chantilly, USA), we determined the percentage of bacterial growth. We established 100% bacterial growth in pure MHB and this value was compared against bacterial growth under treatments with antimicrobial agents, statistically. The results showed that antimicrobial agents inhibit more Gram-positive, than Gram-negative bacterial strains. The growth of *E. faecalis* CCM 4224 and *S. aureus* CCM 4223 was inhibited by all tested antimicrobials in comparison with control sets. The growth of *E. faecalis* (Fig. 1) was inhibited by sage extract and by borneol at concentration 128 µg/mL completely. The least inhibitory effect on the growth of this species was observed in treatment with α-β thujone, followed by sage EO. *S. aureus* (Fig. 2) was inhibited completely only by sage EO at concentration 128 µg/mL. Also, the least inhibitory effect for this strain was determined in a treatment with α-β thujone.

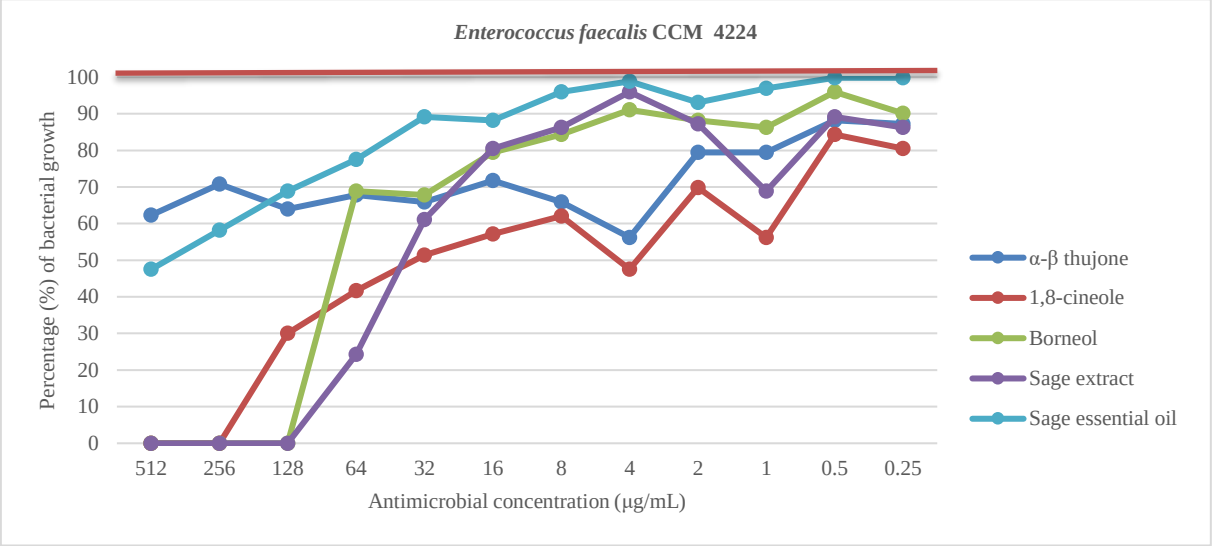

Figure 1 The percentage (%) growth of *E. faecalis* CCM 4224

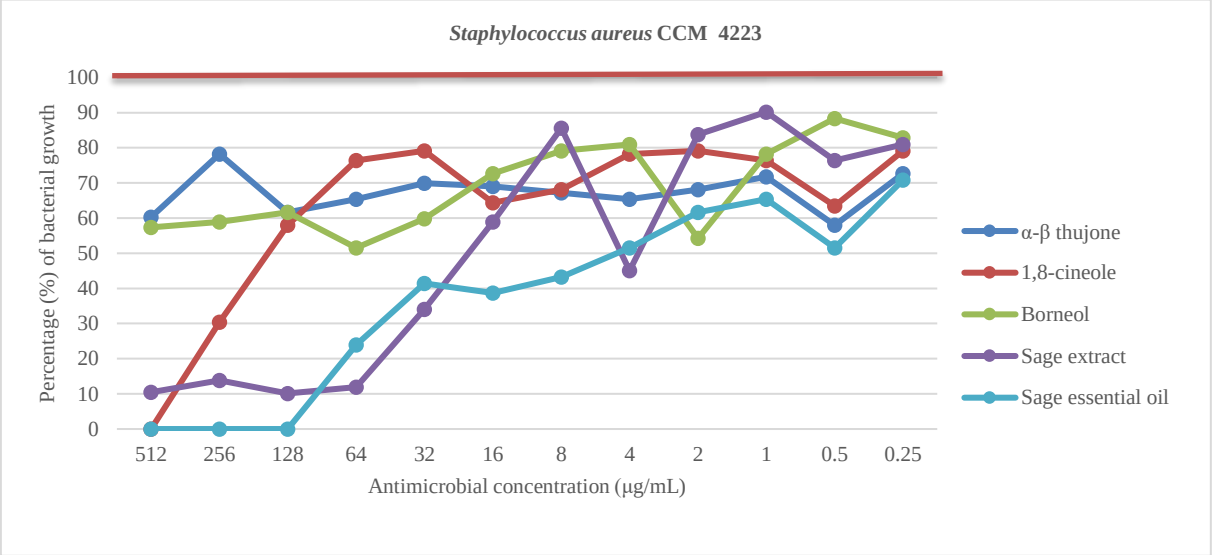

Figure 2 The percentage (%) growth of *S. aureus* CCM 4223

The Gram-negative bacteria *E. coli* CCM 3954 (Fig. 3) was more sensitive to antimicrobial agents such as *K. pneumoniae* CCM 2318 (Fig. 4). The growth of *E. coli* was inhibited by sage essential oil at the concentration 256 µg/mL, completely. The most effective tested antimicrobial was α-β thujone, which was able to inhibit the growth of *E. coli* below 100% throughout the cultivation period. The highest increase in its growth was observed at a concentration of 0.5 µg/mL (50%). *K. pneumoniae* CCM 2318 was the most resistant bacteria from all tested species. The sage extract was able to inhibit its growth up to a concentration of 256 µg/mL completely. Other tested antimicrobials were able to inhibit growth of *K. pneumoniae* completely, only at the initial concentration (500 µg/mL). The best results were observed in the treatment with α-β thujone that inhibited growth of this strain below 50% until the concentration of 16 µg/mL.

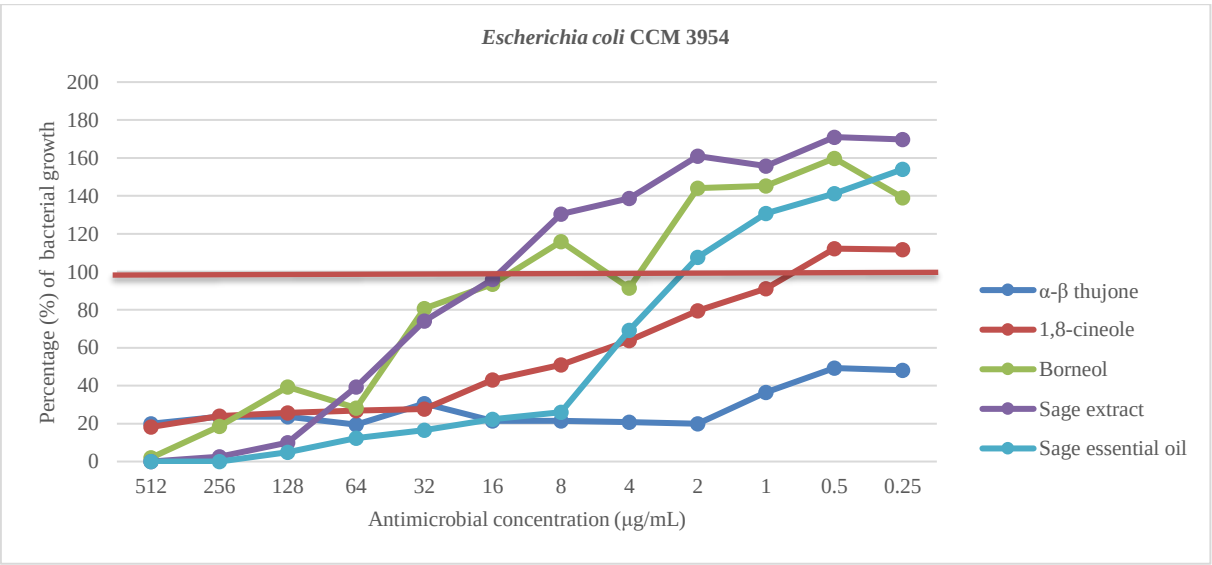

Figure 3 The percentage (%) growth of *E. coli* CCM 3954

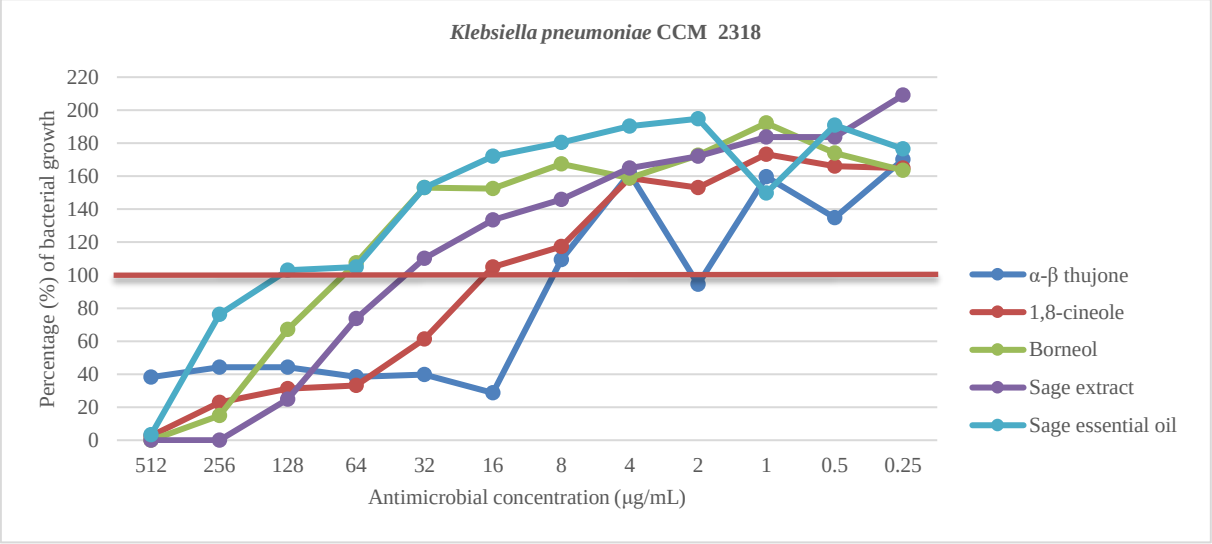

Figure 4 The percentage (%) growth of *K. pneumoniae* CCM 2318

Many authors investigated the antibacterial activity of essential oils or some compounds related with the MIC and MBC activities, mainly.

But for a determination of MIC concentration the bacterial growth is usually checked only visually. However, we also focused on monitoring the percentage growth of bacteria treated with antimicrobial agents and we compared the obtained data with the control samples, in this study.

We found some differences between the MIC values and percentage growth of bacteria. For example, the α-β thujone had no antimicrobial effect on the growth of tested strains in determination of MIC values. However, when we compared the data obtained from MIC determination and from the percentage growth of bacteria, we found that α-β thujone was particularly effective against Gram-negative bacteria. This compound was able to inhibit their growth under 50% throughout all cultivation time at relatively low concentrations. Based on the results, therefore, it is necessary to obtain more data from the experiments to compare them in assessing the antimicrobial activity.

To identify the various antimicrobial compounds that might be contained in each EO used in the present study, GC/MS analysis was applied. Sage EO contains primarily α-thujone (14 %) and the presence of other isoprenoids such as borneol, camphor, (−)-Isopulegol and 1,8-cineole (Table 2). These compounds may contribute to antibacterial effects. Also **Russo *et al.* (2013)** studied the chemical composition of different *S. officinalis* essential oils. Their results showed that the main components were α-thujone, camphor, borneol, c-Muurolene and sclareol for all the samples. These findings correspond with our results.

Table 2 Qualitative and quantitative analysis (%) of the sage essential oil by GC-MS

RI[b]	Component	Sage[c]
938	**α-pinene[a]**	5.40
953	Camphene	5.00
981	**β-Pinene[a]**	1.60
1034	1,8-cineole	8.00
1062	γ-Terpinene	1.10
1107	α-thujone	14.00
1116	ß-thujone	8.40
1143	camphor	12.90
1148	(−)-Isopulegol	10.10
1168	**borneol[a]**	12.90
1287	(−)-Bornyl acetate	5.20
1419	β-Caryophyllene	1.60
1436	γ-Elemene	3.40
1478	γ-Muurolene	5.40
1574	Caryophyllene oxide	1.20
total		**96.20**

Legend: [a] - Identification confirmed by co-injection of authentic standard; [b] - RI: identification based on Kovat's retention indices (HP-5MS capillary column) and mass spectra; [c] - relative proportion was calculated in % by dividing individual peaks area by total area of all peaks.

The sage EO and extract were subjected to screening for their possible antioxidant activity. Free radical scavenging capacities of the antimicrobials, measured by DPPH assay, are shown in Table 3. As the best scavenging solutions of DPPH radical were the oils at the highest concentration 50 µg/mL, with the decreasing concentration of oil solution, the antioxidant activity was reduced (not shown). DPPH radical-scavening activities of 15 EOs were studied in the work of **Kačániová *et al*. (2014)**. They observed the highest % of inhibition at *Origanum vulgare* (93%), *Satureia montana* (90.66%) and *Lavandula augustifolia* (90.22%). For *Salvia officinalis* was found a lower antioxidant activity (68.55%).

Our results showed higher antioxidant activity of sage EO (71.55%), but the sage propylene glycol extract showed the lowest antioxidant activity (60.93%). The better antioxidant activity of sage extracts was observed by **Süntar *et al.* (2011)** at *Salvia cryptantha* (85.47%) but at the highest concentration of 500 µg/mL.

Table 3 Antioxidant activity expressed as % inhibition of DPPH radical at 50 µg/mL concentration of tested antimicrobials

Antimicrobials	Antioxidant activity (%)
Sage essential oil	71.55
Sage propylene glycol extract	60.93

Legend: The values represent the average (standard deviations) for triplicate analyses

CONCLUSION

Salvia officinalis L. is a promising source of essential oil or extracts. According to the results of this study, it can be concluded that sage EO, and antimicrobials such as α-β thujone, 1,8-cineole and borneol showed promising antimicrobial effects. The best minimum inhibitory concentration (MIC) was found against Gram-positive bacteria *S. aureus* CCM 4223 by sage essential oil with MIC_{50} 96.05 µg/mL. That allows it to be used by the pharmaceutical and cosmetic industries as natural preservative. However, we also studied the percentage growth of bacteria treated with antimicrobial agents. Subsequently we compared the obtained data with the control samples and found some differences between the MIC values and percentage growth of bacteria. We found that α-β thujone had no antimicrobial effect in determination of MIC values, but it was particularly effective against Gram-negative bacteria in percentage (%) growth study. Therefore, there is necessary to obtain more data from the experiments to compare them in assessing the antimicrobial activity. Chromatographic analysis demonstrated that sage oil is rich in bioactive compounds, such as α-thujone, borneol, camphor, (−)-Isopulegol or 1,8-cineole. Our results showed higher antioxidant activity of sage EO (71.55%) and the less antioxidant activity for a sage extract (60.93%). Due to antibacterial activity of the tested antimicrobials from sage, the problem of microbial resistance to conventional antibiotics could be treated by pharmaceutical industries to manufacture antibacterial drugs.

Acknowledgements: This work was supported by project no. FPPV-09-2019 and by Slovak Research and Development Agency under Contract no. APVV-16-0173.

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