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Research Article

Chemical composition and biological activity of *Salvia officinalis* essential oil

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The study was aimed at analyzing chemical composition, and biological and antibiofilm activity of *Salvia officinalis* L. essential oil (EO) with MALDI-TOF MS Biotyper. The main compounds of *S. officinalis* EO were a-thujone 24.6%, camphor 20.6%, 1,8-cineole 12.1%, and a-humulene 5.8%. Free radical scavenging activity was medium high. The highest antimicrobial activity was observed against *Bacillus subtilis*. Changes in the biofilm structure confirmed the inhibitory action of *S. officinalis* and the most pronounced effect was observed in *B. subtilis* biofilm. The highest inhibition *in situ* in antimicrobial activity was 78.45% at 125 $\mu.L^{-1}$ on apple for *B. subtilis*.

Keywords: sage essential oil, antimicrobial activity, antibiofilm profile, flavonoids, microorganisms

1 Introduction

Salvia officinalis L. (sage) has been used for medical purposes for several thousand years. The plant is used for many medical conditions (e.g. insomnia, measles, sea sickness, sexually transmitted diseases, worms) and it has a historical background dating to ancient Greek and Roman times. Sage was used for medicinal purposes against itching in Ebers Papyrus (1500 BC) period. It was used for mental clarity and to strengthen memory toward the end of 17th century (Altindal & Altindal, 2016). Sage is used due to its diuretic, anti-inflammatory, antimicrobial, antiseptic effects, as an expectorant, and for hyperhidrosis. Sage, which was used against plague in the past, is used for various clinical conditions today; thanks to its sedative, antimicrobial, antioxidant, antitumor, antihypertensive effects, for perspiration, coronary heart disease, chronic bronchitis, asthma, chronic renal failure, cirrhosis, dysmenorrhea, insomnia, infantile colic, and dyspepsia (Arica et al., 2010). Sage has been the subject of various studies due to its phenolic substances. These studies reported that sage can be used as an antiperspirant, antifungal, antiseptic, antibiotic, astringent, antispasm, estrogenic, hypoglycemic, diuretic, carminative, and tonic agent (Cenic-Milosevic et al., 2013). Essential oil obtained from *S. officinalis* has medicinal effects against respiratory and digestive syndromes, heart and blood circulation, metabolic conditions, and endocrine diseases (Badiee et al., 2012; Oliveira et al., 2019). Moreover, sage leaves are used in medicine due to their antiseptic and anti-inflammatory effects (Bauner et al., 2012); in addition to this, sage has

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shown anticancer activity (Mohammad, 2011) and has inflammatory, estrogenic, and sedative effects. Sage can be helpful against muscle pains and chronic stress or mental tension. In addition, sage can be used as a mouthwash for sore throat, infected gingiva, mouth ulcers, and colds. Sage oil has aroma like camphor and thujone; therefore, it is used in the perfume industry. This plant has sedative effects on sweat glands, which decreases sweat secretion in the hand, foot, armpit, and the whole body (Mohammad, 2011). Sage treatment has shown promising and beneficial effects for slowing the progress of bone loss, thus indicating its usefulness as a potential therapeutic agent in humans (Abdallah et al., 2010). Moreover, it was reported that sage reduces the injury area and hastens the recovery period of the injury (e.g. skin breaks, muscle tears or bone fractures) (Anitha et al., 2013). Sage can be used in different forms such as tablets, as tea or droplets of essential oil. It can also be used for colouring carpets or rug yarn (Olmez & Kayabasi, 2002). The aim of this study was to investigate chemical composition, antimicrobial and antibiofilm activity of *Salvia officinalis* essential oil.

2 Material and methods

2.1 Essential oil

Salvia officinalis L. EO was purchased from Hanus, s.r.o. (Nitra, Slovakia), and previously prepared by steam distillation of dried flowering stalk. It was stored in the dark at 4 °C before the analyses.

2.2 Tested bacteria

The biofilm-forming bacteria *Bacillus subtilis* and *Stenotrophomonas maltophilia* were obtained from the dairy industry, identified with 16S rRNA sequencing and MALDI-TOF MS Biotyper. These bacterial species were tested for antimicrobial and antibiofilm activity.

2.3 Chemical characterization of the essential oil by gas chromatography/mass spectrometry (GC-MS) and gas chromatography (GC-FID)

GC-MS analyses of the selected essential oil sample was performed using Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to quadrupole mass spectrometer 5,975B (Agilent Technologies, Santa Clara, CA, USA). A HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm) was used. The temperature program was: 60 °C to 150 °C (increasing rate 3 °C.min^{-1}) and 150 °C to 280 °C (increasing rate 5 °C.min^{-1}). The total run time was 60 min. There was Helium 5.0 used as the carrier gas with flow rate of 1 mL.min^{-1}. The injection volume was 1 µL (EO sample was diluted in pentane), while the split/splitless injector temperature was set at 280 °C. The investigated sample with split ratio at 40.8 : 1 was injected in the split mode. Electron-impact mass spectrometric data (EI-MS; 70 eV) were acquired in scan mode over the m/z range 35–550. MS ion source and MS quadrupole temperatures were 230 °C and 150 °C, respectively. The acquisition of data started after the solvent delay time of 3 min. GC-FID analyses were performed on Agilent 6890N gas chromatograph coupled to FID detector. Column (HP-5MS) and chromatographic conditions were the same as for GC-MS. The temperature of the FID detector was set at 300 °C. The individual volatile constituents of injected essential oil samples were identified according to their retention indices (Adams, 2007) and were compared with the reference spectra (Wiley and NIST databases). The retention indices were experimentally determined by the standard method described in (Van Den Dool & Kratz, 1963) which included retention times of n-alkanes (C6-C34), injected under the same chromatographic conditions. The per-centages of the identified compounds (amounts higher than 0.1%) were derived from their GC peak areas.

2.4 Antioxidant activity of *S. officinalis* essential oil

The free radical scavenging activity was determined using the 2,2-diphenyl-1-picrylhydrazil (DPPH) radical as described in Kačániová et al. (2020).

2.5 Antimicrobial activity with disk diffusion method

Antimicrobial activity of *S. officinalis* EO was determined using the disc diffusion method. Bacteria were aerobically cultivated on Tryptone soya agar (TSA, Oxoid, Basingstoke, UK) at 37 °C for 24 h. An inoculum with an optical density of 0.5 McFarland standard (corresponded to 1.5 × 10^{8} CFU.mL^{-1}) was prepared and an amount of 100 µL was used for Mueller Hinton agar (MHA, Oxoid, Basingstoke, UK) inoculation. Clean discs with 6 mm diameter were saturated with 10 µL of *S. officinalis* EO and placed on the agar. Bacteria were incubated aerobically at 37 °C for 24 h. Each test was repeated 3 times.

2.6 Minimum inhibitory concentrations (MIC)

Bacteria were aerobically cultured for 24 h in a Mueller Hinton Broth (MHB, Oxoid, Basingstoke, UK) at 37 °C. The 50 µl of microbial suspension with optical density 0.5 McFarland standard was applied to a 96-well microtiter plate. 100 µL of MHB with *S. officinalis* EO in concentrations from 400 µL.mL^{-1} to 0.2 µL.mL^{-1}, prepared with serial dilution, was added to sample. The contents of the wells were mixed by pipetting. MHB and EO were used as a negative control, and MHB with inoculum was used as a positive control of maximal growth. The MIC of biofilms was measured after 24 with use of a crystal

violet. Suspension with non-attached cells was discarded and wells were washed with distilled water three times, dried at room temperature, stained with crystal violet (200 µL 0.1% (w/v)) for 15 min, and repeatedly washed and dried. Stained biofilms were resolubilized with 200 µL of 33% acetic acid (Hassan et al., 2011). Absorbance was measured at 570 nm (Glomax spectrophotometer, Promega Inc., Madison, WI, USA). Each test had three replications.

2.7 Analysis of differences in biofilm development with MALDI-TOF MS Biotyper

The various phases of biofilm development were evaluated with MALDI-TOF MS Biotyper. The goal was to monitor changes in the structure of the biofilm on glass and wooden surfaces after treatment with *S. officinalis* EO. Experimental and control samples were prepared in 50 mL polypropylene tubes with 20 mL of MHB, a glass slide and a wooden toothpick. The experimental groups contained MHB enriched with 0.5% *S. officinalis* EO and inoculated samples were incubated at 37 °C on a slope 45° shaker at 170 rpm. Biofilm and planktonic cell samples were collected on days 3, 5, 7, 9, 12, and 14. Subsequently, the analysis of developmental phases and molecular differences of biofilms and dendrogram was performed with MALDI-TOF MS Biotyper (Kačániová et al., 2020).

2.8 *In situ* antimicrobial analysis on apples, carrots, and potatoes

The antimicrobial analysis *in situ* was tested in the vapor phase on biofilm-forming bacteria *B. subtilis* and *S. maltophilia*. Warm MHA was poured into 60 mm Petri dishes (PD) and the lid. Sliced carrot, apple and potatoe fruits (0.5 mm) were placed on agar. Inoculum was prepared as described previously. *S. officinalis* EO was diluted twice in ethyl acetate to obtain concentration of 500, 250, 125, and 62.5 µL.L^{-1} and used for sterile filter paper inoculation. The filter paper was placed in for 1 min to evaporate the remaining ethyl acetate, sealed and incubated for 7 days at 37 °C and for fungi at 25 °C for 14 days. The growth assessment was performed as in the *in situ* antimicrobial activity method. *In situ* bacterial growth was determined using stereological methods. In this concept, the volume density (*Vv*) of bacterial colonies was firstly estimated using ImageJ software counting the points of the stereological grid hitting the colonies (*P*) and those (*p*) falling to the reference space (growth substrate used). The volume density of bacterial colonies was consequently calculated as follows:

$$Vv\ (\%) = P/p \qquad (1)$$

The antibacterial activity of EO was defined as percentage of bacterial growth inhibition (*BGI*):

$$BGI = [(C - T)/C] \times 100 \qquad (2)$$

where:
C – bacterial growth (expressed as *Vv*) in the control group; *T* – in the treatment group. The negative results represented the growth stimulation

2.9 Statistical data evaluation

Microsoft-Excel® software was used for data processing. Results of the MIC value (concentration that caused 50% and 90% inhibition in bacterial growth) were determined by logit analysis.

Results and discussion

3.1 Chemical composition of *Salvia officinalis* EO

Table 1 Chemical composition of essential oil from *Salvia officinalis*

No	RI*	Compound**	%***
1	926	α-thujene	0.1
2	938	α-pinene	3.1
3	948	camphene	2.0
4	977	sabinene	0.1
5	980	β-pinene	1.9
6	992	β-myrcene	0.6
7	1016	α-terpinene	0.1
8	1023	*p*-cymene	1.7
9	1028	α-limonene	2.1
10	1033	1,8-cineole	12.1
11	1047	(*E*)-b-ocimene	tr
12	1060	ɤ-terpinene	0.4
13	1016	α-terpinene	1.1
14	1101	α-thujone	24.6
15	1114	β-thujone	5.4
16	1148	camphor	20.6
17	1170	borneol	3.6
18	1178	4-terpinenol	0.6
19	1189	α-terpineol	0.7
20	1255	linalool acetate	0.2
21	1286	bornyl acetate	1.9
22	1289	*trans*-sabinyl acetate	0.1
26	1302	carvacrol	0.1
27	1379	α-copaene	tr
28	1422	(*E*)-caryophyllene	5.2

Continuation of table 1

No	RI*	Compound**	%***
29	1440	α-guaiene	tr
30	1443	aromadendrene	0.2
31	1456	α-humulene	5.8
32	1485	α-amorphene	tr
33	1490	β-selinene	0.2
34	1492	α-selinene	tr
35	1498	ledene	0.5
36	1583	caryophyllene oxide	0.7
37	1593	viridiflorol	3.4
	Total		99.1

* values of retention indices on HP-5MS column; ** identified compounds; *** tr – compounds identified in amounts less than 0.1%

There was used gas chromatography/mass spectrometry (GC/MS) and gas chromatography (GC-FID) of *S. officinalis* EO detected for detection of α-thujone 24.6%, camphor 20.6%, 1,8-cineole 12.1%, and α-humulene 5.8% as the major compounds (Table 1). In different study of Damyanova et al. (2016) the main compounds of *Salvia officinalis* essential oil were as follows: α-thujone (26.68%), (E)-β-caryophyllene (7.47%), 1,8-cineole (7.19%), α-humulene (6.11%), β-pinene (5.44%), β-thujone (5.35%), camphor (4.84%), allo-aromadendrene (4.55%), borneol (3.69%), and α-pinene (3.58%).

3.2 Antioxidant and antimicrobial activity, minimum inhibitory concentrations (MIC)

The antioxidant activity of *S. officinalis* EO measured by the DPPH method was determined at 30.7 ±1.3% of inhibition that corresponds to 171.93 ±1.90 TEAC. The antioxidant activity of essential oils depends on part of plant, solvent and chosen technique of extraction as it was found out for other Salvia species (Ozcan & Al Juhaimi, 2011). Our results are in line with the above mentioned data. The highest antimicrobial activity was found against *B. subtilis* by the use of both methods (Table 2). Studies on antimicrobial properties of *Salvia officinalis* EO revealed its activity against *Klebsiella pneumonia*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Shigella flexneri*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Salmonella* spp., *Bacillus subtilis* and *Aspergillus niger* (Miladinovic & Miladinovic, 2000; Rota et al., 2004). Oliviera et al. (2018) set MICs 50 and 90 of *Salvia officinalis* essential oil for *C. albicans* at 6.25 and 6.25, respectively 12.5 μg.mL^{-1}, for *C. tropicalis* 12.5, resp. 25 μg.mL^{-1}, for *C. glabrata* 6.25 resp. 12.5 μg.mL^{-1} and for *S. aureus* 5.28 resp. 10.56 μg.mL^{-1}. Tafi et al. (2020) established a minimum bactericidal concentration of the essential oil of 5.185 μg.mL^{-1}. Tadi et al. (2020) established MICs of 50 and 90 for *S. aureus* and *S. typhimurium* 15.62 and 31.14 μg.mL^{-1}, and 19.5 and 39 μg.mL^{-1}, respectively. Pavić et al. (2019) determined the minimum inhibitory concentrations for *B. subtilis* 15.6 μL.mL^{-1}, for *S. aureus* 62.5 μL.mL^{-1} and for *P. aeruginosa* 10.82 ±0.02 μL.mL^{-1}.

3.3 Antibiofilm activity of *S. officinalis* EO

Mass spectra analysis of *B. subtilis* biofilm showed the similarity of the experimental spectra and the control planktonic spectrum on the 3rd day of the experiment (Fig. 1A) which indicated that bacterial biofilm developed equally due to the protein production. The changes in mass spectra on 5th day were more visible on biofilm formed on wood than one on glass surface (Fig. 3B). The changes in the mass spectra were observed in biofilm on both surfaces from 7th day in comparison to the control planktonic cells (Fig. 3C–F). The changes in protein profile of *B. subtilis* biofilm treated with *S. officinalis* EO was analysed. However, the effect of *S. officinalis* EO on protein production can be confirmed in biofilm forming bacteria *B. subtilis* compared to untreated control cells. The dendrogram constructed according to mass spectra also confirmed the similarity of young biofilms with planktonic cells and control cells. The distance of MSP growth was during the experiment progression which indicates the differences in protein production caused by infl uence of *S. officinalis* EO addition.

The difference between the mass spectra of biofilms on glass and wooden surface and control sample occurred from the 5th day (Fig. 1B–F). There were visible changes in protein profile of biofilm treated with *S. officinalis* EO. It seems that *S. officinalis* EO influences the homeostasis of bacterial biofilm formed on the wooden and the glass surface. The dendrogram was constructed as a visualization of mass spectra for determination of some similarities of biofilm structure regarding to the distance

Table 2 Antibacterial activity of *S. officinalis*

Microorganism	Zone inhibition (mm)	Activity of EO	MIC 50 (μL.mL^{-1})	MIC 90 (μL.mL^{-1})	Activity of EO
Stenotrophomonas maltophilia	16.47 ±0.58	***	0.39	0.78	***
Bacillus subtilis	22.43 ±0.58	***	0.20	0.39	***

*** very strong inhibitory activity

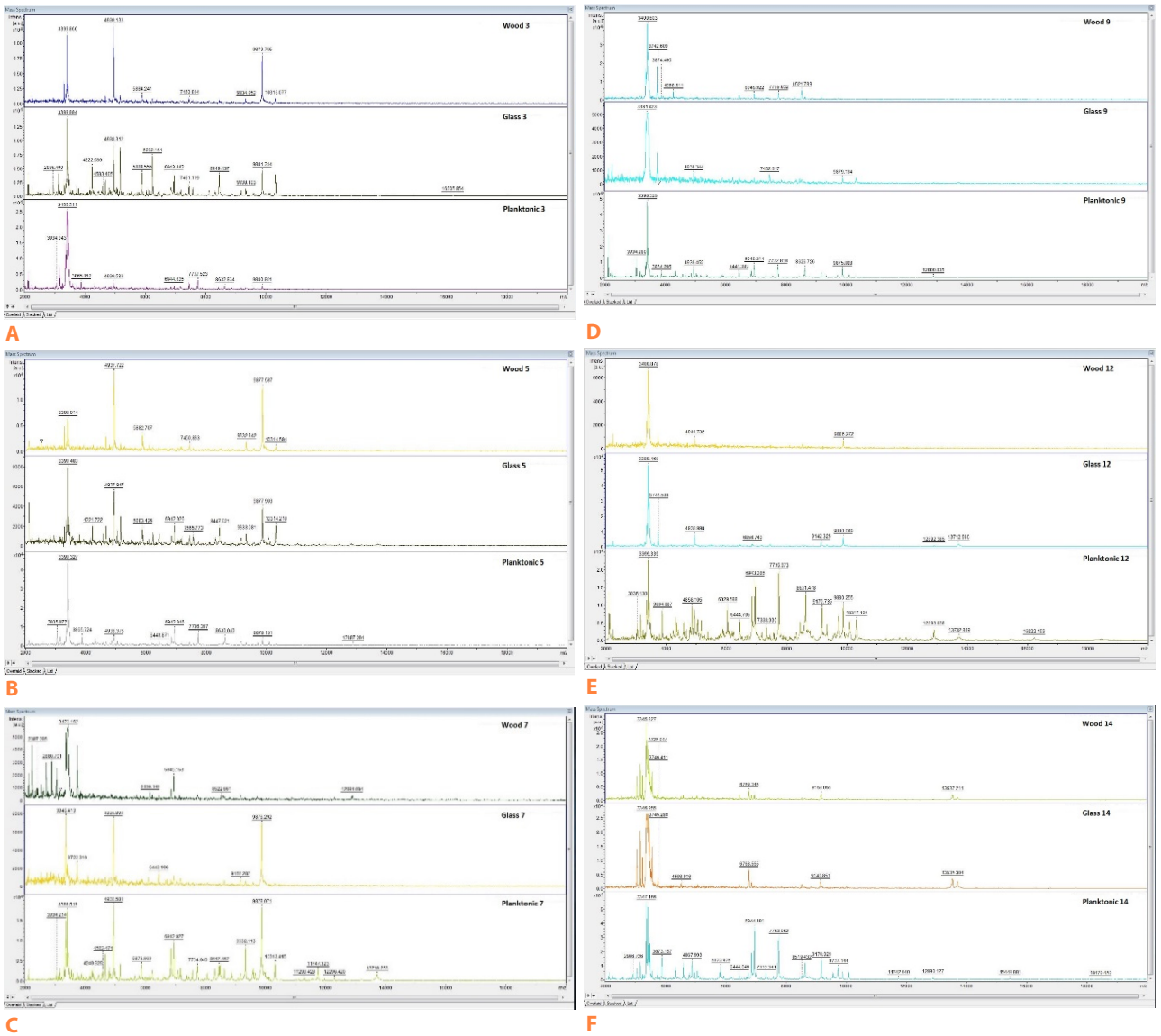

Figure 1 Representative MALDI-TOF mass spectra of *B. subtilis*
A – 3rd day; B – 5th day; C – 7th day; D – 9th day; E - 12th day; F – 14th day

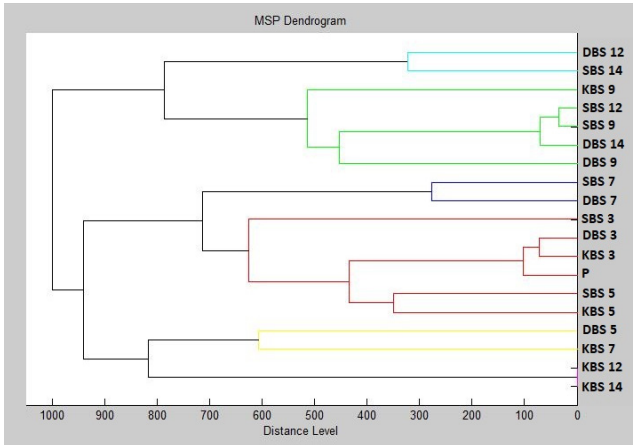

Figure 2 Dendrogram of *B. subtilis* generated using MSPs for planktonic cells and the control
BS – *B. subtilis*; K – control; S – glass; D – wood; P – planktonic cells

of MSP. It can be stated from the constructed dendrogram (Fig. 2), that the planktonic stage (P) had the shortest distance together with control groups and with young biofilms from the 3rd day when it grew on the wood and the glass (SSM3, DSM3). The similarity in protein profile of the control groups was confirmed by short distances of MSP (Fig. 3). The young biofilms and control planktonic cells also had short distances of MSP which corresponds with mass spectra. The distance of MSP experimental groups increased gradually with days. Mass spectra prepared on the 12th and 14th day of the experiment had the longest distance of MSP which indicates the changes in bacterial biofilm protein profile of *S. maltophilia* (Fig. 4). The use of MALDI-TOF for detection of degradation of biofilm has been previously less reported. Kırmusaoğlu (2019) described various methods for biofilm detection

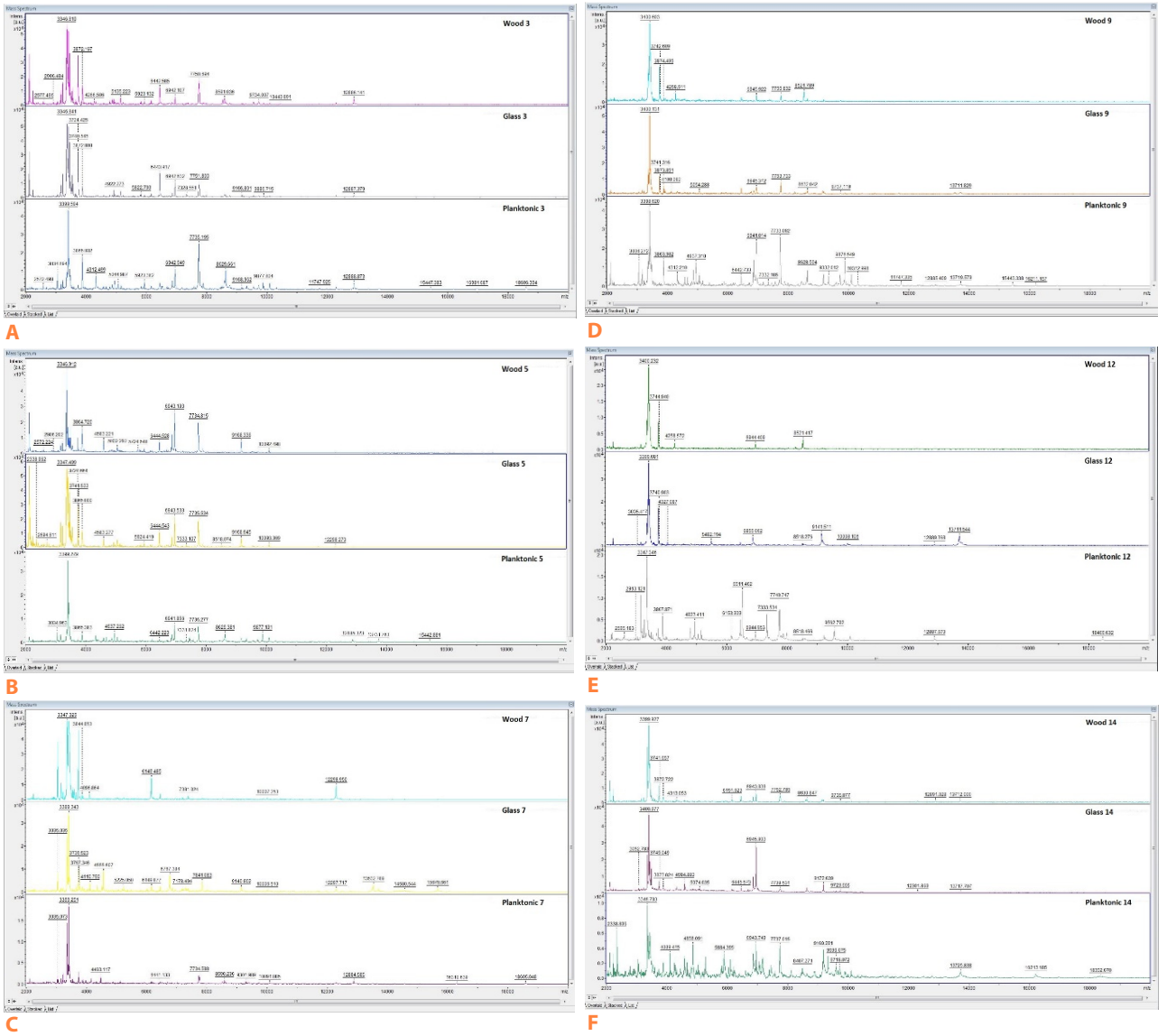

Figure 3 MALDI-TOF mass spectra of *S. maltophilia* biofilm during development
A – 3rd day; B – 5th day; C – 7th day; D – 9th day; E – 12th day; F – 14th day

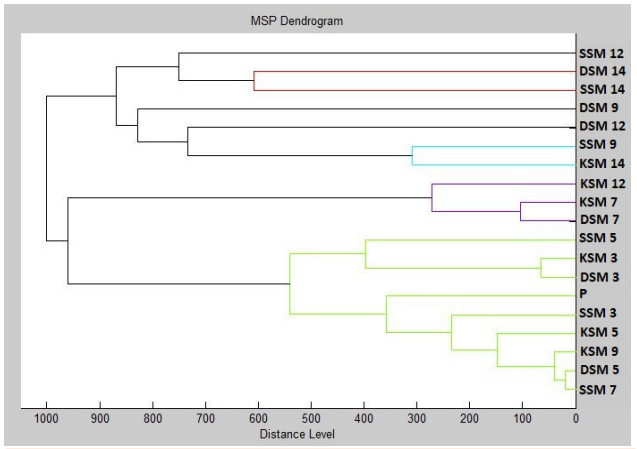

Figure 4 Dendrogram of *S. maltophilia* generated using MSPs of planktonic cells and control
SM – *S. maltophilia*; K – control; S – glass; D – wood; P – planktonic cells

and stated that the mass spectrometry is a less common but a very suitable method for biofilm research. Stîngu et al. (2008) described the accuracy of identification biofilm forming bacteria by MALDI-TOF MS compared to 16S rRNA sequencing and confirmed that MALDI-TOF MS can distinguish the differences in the mass spectra of closely related biofilms.

3.4 *In situ* antimicrobial analysis on apples, carrots, and potatoes

The growth of *B. subtilis* was inhibited on apples, carrots, and potatoes as it is shown in Table 3. The highest inhibition was 78.45% at 125 $\mu L.L^{-1}$ on apple and the lowest of 1.89% at 125 $\mu L.L^{-1}$ on carrot. The inhibition of *S. maltophilia* was recorded on potato at 125 $\mu L.L^{-1}$ concentrations with the inhibition rate of 51.16% and the

Table 3 Results of *in situ* analysis of antibacterial activity of the vapor phase of *S. officinalis* essential oil on apples, carrots, and potatoes

Bacteria strains	**MBI (%) – apple *Salvia* (μL.L^{-1})**			
	62.5	**125**	**250**	**500**
B. subtilis	14.17	78.45	5.45	-21.45
S. maltophilia	12.60	-13.33	-5.37	-17.01
	62.5	**125**	**250**	**500**
B. subtilis	65.44	1.89	-3.85	72.60
S. maltophilia	14.40	14.59	15.97	8.50
	62.5	**125**	**250**	**500**
B. subtilis	11.18	54.18	9.24	30.79
S. maltophilia	20.60	51.16	26.73	10.25

lowest of 8.50% at 500 μL.L^{-1} (Table 3). *Salvia officinalis* EO was evaluated in the liquid and vapor phases against *Haemophilus influenza*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa* and *Streptococcus pneumonia*. Moreover, the MIC of the essential oil in the vapor phase against *Streptococcus pneumonia* was around 690 μL.L^{-1} for bulk oil (Moghimi et al., 2017).

4 Conclusions

The results of our study show that the major component of *Salvia officinalis* essential oils was α-thujone, camphor, 1,8-cineole, and α-humulene. The antioxidant activity was evaluated as the medium high. *S. officinalis* EO had very good antimicrobial effects as well as antibiofilm effects observed on various surfaces and detected by MALDI-TOF MS Biotyper. MALDI-TOF MS Biotyper was a suitable method for evaluating phases of biofilms development. *S. officinalis* EO demonstrated inhibitory activity on microorganisms in a food model in the vapor phase.

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