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Antioxidant, Antimicrobial, and Anti-Insect Properties of *Boswellia carterii* Essential Oil for Food Preservation Improvement

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Abstract: *Boswellia carterii*, known as frankincense, is a fragrant medicinal plant. The essential oil from this plant is often used in traditional medicine or aromatherapy. Due to its positive properties, it has potential applications as an antimicrobial agent in medicine and the food industry. The aim of this study was to evaluate the antimicrobial, anti-insect, and antioxidant activities of frankincense essential oil (FEO). The composition of volatile compounds was determined by GC/MS, and the main components were found to be α-pinene (37.0%), α-limonene (19.8%), and *p*-cymene (6.3%). The antioxidant activity was evaluated with DPPH and ABTS methods with the resulting inhibition of 73.88 ± 0.35% of DPPH radical (6.27 ± 0.17 TEAC) and 97.09 ± 0.48% of ABTS radical cation (5.21 ± 0.07 TEAC). The antimicrobial activity was the strongest against *Candida* species with inhibition zones in the range of 38.00–44.00 mm and MIC 50 and MIC 90 values of 11.72 and 12.58 µL/mL, respectively. The inhibition activity of the vapor phase was the highest against G^+ and G^- bacteria growing on a carrot with inhibition ranging from 65.14 to 93.67%. The anti-insect activity against *O. lavaterae* was determined as 100% at 100% concentration and 50% at 25% concentration. The degradation of biofilm was tested with MALDI-TOF MS, and changes in the protein profile were observed.

Keywords: *Boswellia carterii*; frankincense; essential oil; antioxidant; antimicrobial; antibiofilm; insecticidal activity

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1. Introduction

Frankincense essential oil (FEO) is produced from trees of the genus *Boswellia* that are native to the Arabian Peninsula, northeast Africa, and India. The *Boswellia* genus contains about 20 different species [1]. Popular types include *B. carterii*, *B. sacra*, *B. serrata*, or *B. neglecta* which occur in different locations and show different compositions of oils [2]. Some authors claim *B. carterii* and *B. sacra* to be the same species, but differences in chemical composition and the ratio of some enantiomers between these plants have been observed [3].

Most of the FEOs are obtained by steam distillation of an oleo gum resin of *Boswellia* species. They contain volatile compounds that cause a characteristic woody and spicy aroma [4]. FEO often contains components such as α-pinene, α-thujene, β-pinene, limonene, *p*-cymene, myrcene, or sabinene [4–6]. The resin has a protective role in plants; thus, the essential oil also manifests many positive activities. Its good antimicrobial and anti-insect activity protects the tree from [2]. FEO can inhibit the growth of human pathogens such as *S. aureus* or *P. aeruginosa* [7,8]. FEO has good antimicrobial properties against bacterial (*P. acnes*) and fungal (*C. albicans*, *Malassezia* spp., and *Trichophyton* spp.) pathogens that cause skin-associated infections [9]. Moreover, FEO prevents the formation of biofilms by *Staphylococcus* and *Candida* microorganisms [10]. The antiparasitic activity of FEO against *Leishmania* was also reported [11].

Frankincense has been used in traditional medicine due to its positive medicinal properties [12]. The resin from *Boswellia* has a positive impact on patients with diseases of the gastrointestinal tract [13]. Due to the specific aroma, FEO has stress-relieving properties [14]. FEO also shows potential for beneficial effects in neurodegenerative disorders [15]. Moreover, the anticancer activity of FEO towards pancreatic cancer cells was reported in in vitro studies [16]. FEO was also found to induce tumor cell-specific apoptosis in the cell lines of bladder [17] and breast cancer [18].

Nowadays, an increasing trend to substitute artificial substances with natural alternatives is observed. Due to the overuse of antibiotics, some microorganisms have become more resistant. Research has thus become more focused on the search for bioactive compounds that inhibit pathogenic microorganisms in medicine or the food industry. Moreover, the extended use of synthetic pesticides has led to increased concerns related to the condition of the environment and human health. This has promoted research on the anti-insect properties of natural substances.

According to previous research, FEOs have antimicrobial and anti-insect potential. On the other hand, there is not enough research that would provide information about the use of FEO as an antimicrobial agent in food preservation against pathogenic microorganisms or insects. These potential positive properties of FEO have resulted in its selection for analysis from among the plethora of readily available essential oils.

The aim of this study was to evaluate the positive properties of FEO. The antioxidant potential of this substance was determined. Antimicrobial and antibiofilm activities were tested in order to assess the potential of this oil in inhibiting pathogenic bacteria which are common risk factors in the food industry. Moreover, anti-insect activity against *Oxycarenus lavaterae* was tested and the chemical composition of volatile compounds of FEO was determined.

2. Materials and Methods

2.1. Essential Oil

Frankincense essential oil (FEO) was purchased from Slovak company Hanus s.r.o. The provider stated that essential oil was extracted by steam distillation from *Boswellia carterii* resin obtained from Somalia. The essential oil was stored in the dark at 4 °C during the analyses.

2.2. Gas Chromatography–Mass Spectrometry and Gas Chromatography Analyses

The identification of volatile compounds in the FEO sample was performed using an Agilent Technologies (Palo Alto, Santa Clara, CA, USA) 6890 N gas chromatograph equipped with a quadrupole mass spectrometer 5975 B (Agilent Technologies, Santa Clara, CA, USA). The Agilent Technologies gas chromatograph was operated by an interfaced HP Enhanced ChemStation software (Agilent Technologies, Santa Clara, CA, USA). Using an HP-5MS (30 m × 0.25 mm × 0.25 µm) capillary column separation of volatiles has been achieved. The essential oil was diluted in hexane (10% solution) prior to analysis and injected in a volume of 1 µL. With the flow rate of 1 mL/min as a carrier gas helium 5.0 was used. The temperature of the split/splitless injector was set at 280 °C, while the temperature

of the MS source and MS quadruple were set at 230 °C and 150 °C, respectively. The mass scan was in the range of 35–550 amu at 70 eV. The solvent delay time was 3.00 min. The temperature program of GC and GC-MS analysis was as follows: temperature program of 60 °C to 150 °C (rate of increase 3 °C/min), and 150 °C to 280 °C (rate of increase 5 °C/min), held 4 min at 280 °C. The total run time was 60 min. The split ratio was 40.8:1.

The identification of volatile constituents was performed by the comparison of their retention indices (RI) as well as the reference spectra reported in the literature and the ones stored in the MS library (Wiley7Nist) [19,20]. Using the data collected with GC-FID equipped with the same HP-5MS capillary column, semiquantification of the components was performed. Only compounds in amounts higher than 0.1% were taken into consideration.

2.3. Antioxidant Activity

2.3.1. DPPH Assay

The antioxidant potential of FEO was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. An aliquot of 190 µL of DPPH radical solution in methanol (prepared so absorbance is adjusted at 0.8 at 515 nm) was mixed with 10 µL FEO in a 96-well microtiter plate. The reaction mixture was incubated at room temperature for 30 min in darkness by continuous shaking at 1000 rpm, after which the absorbance of the sample was measured spectrophotometrically at 517 nm. All measurements were performed in triplicate. Methanol was used as the control solution, whereas Trolox (1–5 mg/L in methanol) was used as the reference compound. Antioxidant activity was expressed as a percentage of $DPPH^{\bullet}$ inhibition. The calculation was performed using the equation:

$$(A0 - AA)/A0 \times 100 \quad (1)$$

where A0 was the absorbance of $DPPH^{\bullet}$ and AA was the absorbance of the sample.

The total radical scavenging capacity was expressed according to the calibration curve of Trolox (TEAC). The results were presented as mean values ± standard deviation (SD) of three independent measurements.

2.3.2. ABTS Assay

ABTS [2,20-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium] radical cation was generated according to an already described procedure [21]. The prepared radical cation was diluted prior to the analysis up to an absorbance value of 0.7 at 744 nm. An aliquot of 190 µL of this solution was mixed with 10 µL of FEO (in a 96-well microtiter plate). This was followed by a 30 min incubation at room temperature, in darkness, and with shaking at 1000 rpm. A decrease in absorbance at 744 nm was registered and the results are presented as a percentage of ABTS inhibition using the previous Equation (1). All measurements were performed in triplicate. Methanol was used as the blank sample, and Trolox was the standard reference substance. The results were expressed as the percent of inhibition as well as an equivalent of Trolox (TEAC) calculated based on the standard curve. The results were presented as mean values ± standard deviation (SD) of three independent measurements.

2.4. Antimicrobial Activity

The antimicrobial activity of FEO was tested against the following microorganisms *Pseudomonas aeruginosa* CCM 3955, *Yersinia enterocolitica* CCM 7204, *Salmonella enterica* subsp. *enterica* ser. Enteritidis CCM 4420, *Bacillus subtilis* CCM 1999, *Staphylococcus aureus* subsp. *aureus* CCM 2461, *Enterococcus faecalis* CCM 4224, *Candida krusei* CCM 8271, *Candida albicans* CCM 8261, *Candida tropicalis* CCM 8223, *Candida glabrata* CCM 8270 purchased from the Czech Collection of Microorganisms (Brno, Czech Republic).

Three microscopic filamentous fungi (*Aspergillus flavus*, *Botrytis cinerea*, and *Penicillium citrinum*) previously obtained from grapes and identified with MALDI-TOF MS Biotyper and 16S rRNA sequencing were used for the analyses of antifungal activity. A biofilm-

forming *S. enterica* was obtained and cultivated from chicken meat, analyzed by 16S rRNA sequencing, and identified using MALDI-TOF MS with a score higher than 2.0.

The inocula of bacteria and microscopic filamentous fungi were incubated for 24 h. Bacterial samples were incubated in Mueller Hinton Broth (MHB, Oxoid, Basingstoke, UK) at 37 °C and microscopic filamentous fungi samples were incubated in Sabouraud dextrose broth (SDB, Oxoid, Basingstoke, UK) at 25 °C.

2.4.1. Disk Diffusion Method

The disc diffusion method was used for the analyses of the antimicrobial activity of FEO. The inoculum was diluted to 0.5 McFarland (1.5×10^8 CFU/mL) and 100 µL of it was spread onto a Petri dish with Mueller Hinton agar (MHA, Oxoid, Basingstoke, UK) for bacteria and Sabouraud dextrose agar (SDA, Oxoid, Basingstoke, UK) for microscopic filamentous fungi. Discs with a 6 mm diameter were placed in a Petri dish and 10 µL of non-diluted FEO was added. Petri dishes were incubated for 24 h at 37 °C (bacteria) or 25 °C (microscopic filamentous fungi).

The antimicrobial activity was evaluated according to the Clinical and Laboratory Standards Institute—Performance Standards for Antimicrobial Susceptibility Testing [22]. Antibiotics (Oxoid, Basingstoke, UK) were used as a control: cefoxitin for G^- bacteria, gentamicin for G^+ bacteria, and fluconazole for microscopic filamentous fungi [23]. Analyses were performed in triplicate.

2.4.2. Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) was evaluated by the broth microdilution method. The inoculum was adjusted to 0.5 McFarland and an aliquot of 50 µL was added to a well in a 96-well plate. FEO was distributed into the wells by two-fold serial dilution to final concentration values from 500 µL/mL to 0.2 µL/mL. Samples were incubated for 24 h at 37 °C (bacteria) and 25 °C (yeasts). As a negative control, MHB with FEO was used and MHB with inoculum served as a positive control. Absorbance was measured at 570 nm in time 0 h and after 24 h. Analyses were prepared in triplicate [23].

The minimal inhibition concentration of FEO against fungi was measured in four concentrations (500, 250, 125, and 62.5 µL/mL) of the oil diluted in 0.1% DMSO solution. The inoculum was diluted to 0.5 McFarland (1.5×10^8 CFU/mL). A total of 100 µL of inoculum was spread to SDA. Discs with 6 mm were placed on the Petri dish and 10 µL of the appropriate concentration of FEO was added. Petri dishes were incubated for 5 days at 25 °C.

2.4.3. In Situ Inhibition of Vapor Phase FEO on Carrot

An antimicrobial analysis in situ was performed on the carrot. A 0.5 mm slice of carrot was washed in distilled water, left to dry, and subsequently transferred to a Petri dish with MHA. Bacteria and microscopic filamentous fungi were diluted to 0.5 McFarland and the carrot was inoculated with three stabs. The FEO was diluted with ethyl acetate to final concentrations of 500, 250, 125, and 62.5 µL/L. A total of 100 µL of each diluted solution was added to filter paper and ethyl acetate was allowed to evaporate. A total of 100% ethyl acetate was used as the negative control. The samples were incubated for 7 days at 37 °C (bacteria) and 25 °C (microscopic filamentous fungi). The inhibitory activity was calculated using the stereological method. The bulk density was calculated according to the formula $Vv = P/p \times 100$ (P = stereological lattice of the colonies, p = substrate). Growth inhibition was expressed as $GI = [(C - T)/C] \times 100$ (C = growth density of control group, T = growth density in the group with FEO) [24]. The activity was measured in triplicate.

2.4.4. Antibiofilm Activity

A 50 mL tube was filled with 20 mL of MHB and 100 µL of biofilm-forming *S. enterica* inoculum. Subsequently, a plastic and stainless-steel piece were added to the tube. FEO was added to experimental groups in the final concentration of 0.1%. Control samples were

left untreated. The incubation was carried out at 37 °C with shaking at 170 rpm. Samples were analyzed on days 3, 5, 7, 9, 12, and 14.

Changes in protein spectra during biofilm development in the presence of FEO were monitored using MALDI-TOF MS Biotyper. Biofilm samples were taken from plastic and stainless-steel surfaces by a sterile cotton swab and were directly transferred to a MALDI-TOF plate. A 300 µL of culture medium was taken for analysis of planktonic cell spectra. A suspension with planktonic cells was centrifuged for 1 min at 12,000 rpm. The pellet was washed three times in ultrapure water and resuspended. A total of 1 µL was applied to a target plate.

A matrix α-Cyano-4-hydroxycinnamic acid matrix (10 mg/mL) was applied to the dried target plate. MALDI-TOF MicroFlex (Bruker Daltonics) was used for the analysis of biofilm protein spectra. Spectra in the range of mass to charge ratio 200–2000 were recorded in the linear and positive mode. The protein spectra were obtained, and the similarities of the spectra were used to generate the standard global spectrum (MSP). Nineteen MSP were generated from the spectra with MALDI Biotyper 3.0 and were grouped into dendrograms using Euclidean distance [25].

2.5. Insecticidal Activity of FEO Vapor Phase

Oxycarenus lavaterae was used for the analysis of insecticidal activity. Thirty specimens were added to Petri dishes with vented lids. Subsequently, 100 µL was added to filter paper which was placed inside the Petri. Petri plates were sealed with parafilm. Concentrations of 100%, 50%, 25%, 12.5%, 6.25%, and 3.125% of FEO were prepared in 0.1% polysorbate. A negative control sample was treated with 100 µL of 0.1% polysorbate. Specimens were exposed for 24 h at room temperature. Live and dead subjects were counted, and the percentage of insecticidal activity was calculated [24]. The insecticidal activity was measured in triplicate.

2.6. Statistical Analysis

One-way analysis of variance (ANOVA) was performed with Prism 8.0.1 (GraphPad Software, San Diego, CA, USA) and by Tukey's test at $p < 0.05$. SAS® version 8 software (SAS Institute, Cary, NC, USA) was used to process the data. MIC 50 and MIC 90 values (50% and 90% inhibition of microbial growth) were determined by logit analysis.

3. Results

3.1. Volatile Composition of Examined Essential Oil

The results of the chemical composition analysis of FEO are presented in Table 1. In Table 2, percentage amounts of each class of identified compounds are presented. Overall, this essential oil is characterized by monoterpene hydrocarbons that constitute 79.3% of the total. Among this class of compounds, major detected were α-pinene (37.0%) and α-limonene (19.8%), followed by a notable amount of *p*-cymene (6.3%), α-thujene (4.2%), sabinene (4.1%), and β-myrcene (2.6%). Sesquiterpene hydrocarbons (10.4%) were the second most represented class of compounds, with β-elemene (2.2%) and (*E*)-caryophyllene (2.5%) found in significant quantities. Other identified volatiles were found in amounts smaller than 2%.

3.2. Antioxidant Activity

The antioxidant potential of FEO was determined by the means of neutralization of the stable DPPH radical and the ABTS radical cation. The obtained results are presented in Table 3. As can be seen, the radical scavenging capacity of the tested FEO is found to be stronger compared to the IC50 value of the reference compound Trolox. A total of 10 µL of this FEO was able to neutralize 73.88 ± 0.35% of DPPH radical, which is equivalent to the 6.27 ± 0.17 TEAC, and 97.09 ± 0.48% of ABTS radical cation (5.21 ± 0.07 TEAC).

Table 1. Chemical composition of FEO.

No	RI [a]	Compound [b]	% [c]
1	926	α-thujene	4.2
2	938	α-pinene	37.0
3	948	camphene	1.3
4	969	verbenene	0.4
5	977	sabinene	4.1
6	980	β-pinene	1.9
7	992	β-myrcene	2.6
8	1004	α-phellandrene	0.8
9	1009	δ-3-carene	0.9
10	1023	*p*-cymene	6.3
11	1028	α-limonene	19.8
12	1098	Linalool	0.3
13	1101	α-thujone	tr
14	1140	*trans*-pinocarveol	1.0
15	1145	*trans*-verbenol	0.9
16	1178	4-terpinenol	0.6
17	1183	*p*-cymen-8-ol	0.5
18	1208	verbenone	0.6
19	1217	*trans*-carveol	0.4
20	1229	*cis*-carveol	tr
21	1241	Carvone	0.4
22	1286	bornyl acetate	1.0
26	1353	α-cubebene	0.4
27	1379	α-copaene	0.7
28	1385	β-bourbonene	tr
29	1388	β-elemene	2.2
30	1408	α-gurjunene	0.4
31	1422	(*E*)-caryophyllene	2.5
32	1437	α-*trans*-bergamotene	tr
33	1443	aromadendrene	tr
34	1485	α-amorphene	1.1
35	1490	β-selinene	1.5
36	1492	α-selinene	0.7
37	1525	δ-cadinene	0.9
38	1542	α-cadinene	tr
39	1545	α-calacorene	tr
40	1583	caryophyllene oxide	1.6
41	1593	viridiflorol	0.3
	total		97.3

[a] Values of retention indices on HP-5MS column; [b] Identified compounds; [c] tr—compounds identified in amounts less than 0.1%.

Table 2. The volatiles presented in percentage for each class of compounds.

Class of Compounds	%
Monoterpenes	85.0
monoterpene hydrocarbons	79.3
oxygenated monoterpenes	5.7
monoterpene alcohols	3.7
monoterpene ketones	1.0
monoterpene esters	1.0
Sesquiterpenes	12.3
sesquiterpene hydrocarbons	10.4
oxygenated sesquiterpenes	1.9
sesquiterpene alcohols	0.3
sesquiterpene epoxides	1.6
Total	97.3

Table 3. In vitro antioxidant activity of FEO.

	% of Inhibition	TEAC (mg/L)	Trolox (IC_{50}) (mg/L)
$DPPH^{\bullet}$	73.88 ± 0.35	6.27 ± 0.17	4.39 ± 0.13
$ABTS^{\bullet+}$	97.09 ± 0.48	5.21 ± 0.07	2.96 ± 0.01

3.3. Antimicrobial Activity

Overall, the FEO showed good antimicrobial activity against the tested microorganisms (Table 4). The evaluation was performed according to the CLSI-M100 guideline. The microorganisms for which an inhibition zone larger than 20 mm in diameter was observed were considered susceptible to FEO. All the chosen microorganisms were thus susceptible to the tested essential oil. The most pronounced activity was noted for yeasts. The inhibition zone diameters for *C. glabrata*, *C. tropicalis*, and *C. albicans* were 38.00, 40.67, and 44.00 mm, respectively. High activity was also observed against Gram-positive bacteria *B. subtilis* and *S. aureus* with inhibition zones of 37.34 and 31.34 mm, respectively. Strong activity was also determined for Gram-negative bacteria *Y. enterocolitica* and biofilm-forming *S. enterica*. In the case of filamentous fungi, the biggest inhibition zone was noted for *A. flavus*.

Table 4. Antimicrobial activity of FEO measured by disk diffusion method.

Microorganism		Inhibition Zone Diameter (mm)	Control (mm)	Activity of FEO
G^- bacteria	*P. aeruginosa*	26.00 ± 2.00	25.00 ± 0.03	***
	Y. enterocolitica	28.00 ± 2.00	24.00 ± 0.08	***
	S. enterica ser. Enteritidis	26.00 ± 2.00	28.00 ± 0.06	***
	S. enterica biofilm	28.00 ± 2.00	25.00 ± 0.02	***
G^+ bacteria	*B. subtilis*	37.34 ± 1.15	26.00 ± 0.05	***
	S. aureus subsp. *aureus*	31.34 ± 1.15	24.00 ± 0.08	***
	E. faecalis	21.34 ± 1.15	25.00 ± 0.08	***
Yeasts	*C. krusei*	23.34 ± 1.15	24.00 ± 0.09	***
	C. albicans	38.00 ± 2.00	26.00 ± 0.08	***
	C. tropicalis	40.67 ± 4.16	25.00 ± 0.02	***
	C. glabrata	44.00 ± 2.00	28.00 ± 0.04	***
Fungi	*A. flavus*	29.34 ± 1.15	31.50 ± 0.58	***
	B. cinerea	24.66 ± 1.15	32.00 ± 1.00	***
	P. citrinum	25.34 ± 1.15	33.00 ± 0.58	***

*** susceptible ≥ 20 mm; antibiotics used as a control: cefoxitin for G^- bacteria, gentamicin for G^+ bacteria, fluconazole for microscopic filamentous fungi.

The microdilution method (Table 5) also showed the highest activity against the yeasts of *Candida* spp. The lowest MIC 50 and MIC 90 were determined for *C. krusei* with values of 11.72 and 12.58 µL/mL, respectively. MIC 50 and MIC 90 values for *C. tropicalis* were 11.83 and 28.19 µL/mL, respectively, while for *C. albicans* 50% and 90% inhibition was found at 23.44 and 25.10 µL/mL, respectively. Among bacteria, the best results were observed against *S. aureus* with MIC 50 and MIC 90 values of 23.44 and 25.10 µL/mL, respectively.

The antifungal activity was performed against microscopic filamentous fungi *B. cinerea*, *A. flavus*, and *P. citrinum* for four different concentrations of FEO (Table 6). The strongest antifungal activity of FEO against all three tested microorganisms was observed at the highest concentration of 500 µL/mL. The effect of FEO against *B. cinerea* appeared to be dependent on the concentration as the size of inhibition increased with it. In contrast, the inhibitory activity against *A. flavus* was lower at the concentrations of 125 and 250 µL/mL compared to the value observed for the concentration of 62.5 µL/mL.

Table 5. Minimum inhibitory concentration of FEO against microorganisms.

Microorganism		MIC 50 (µL/mL)	MIC 90 (µL/mL)
G^- bacteria	*P. aeruginosa*	93.80 ± 0.64	99.91 ± 1.41
	Y. enterocolitica	46.89 ± 0.56	50.07 ± 1.23
	S. enterica ser. Enteritidis	39.69 ± 1.64	79.18 ± 4.98
	S. enterica biofilm	374.02 ± 3.42	397.64 ± 4.68
G^+ bacteria	*B. subtilis*	108.98 ± 0.14	347.46 ± 1.56
	S. aureus subsp. *aureus*	23.44 ± 0.14	25.10 ± 0.90
	E. faecalis	248.24 ± 2.98	413.93 ± 3.72
Yeasts	*C. krusei*	11.72 ± 0.32	12.58 ± 0.53
	C. albicans	23.44 ± 1.30	25.10 ± 2.10
	C. tropicalis	11.83 ± 0.19	28.19 ± 0.21
	C. glabrata	245.02 ± 0.58	295.79 ± 1.81

Table 6. Antifungal activity of FEO. Inhibition zones measured in mm.

Concentration (µL/L)	*B. cinerea*	*A. flavus*	*P. citrinum*
500	8.33 ± 0.58 ^{a}	9.33 ± 0.58 ^{b}	6.67 ± 0.58 ^{a}
250	7.67 ± 0.58 ^{a}	6.00 ± 1.00 ^{a}	6.33 ± 1.15 ^{a}
125	5.00 ± 1.00 ^{b}	6.67 ± 1.15 ^{a}	6.33 ± 0.58 ^{a}
62.5	4.33 ± 0.58 ^{b}	9.00 ± 1.00 ^{b}	4.33 ± 0.58 ^{b}

One-Way ANOVA, Individual letters (^{a,b}) in upper case indicate the statistical differences between the concentrations; $p < 0.05$.

3.4. In Situ Inhibition of Vapor Phase FEO on Carrot

The activity of the vapor phase of FEO was evaluated on carrots inoculated with selected microorganisms (Table 7). Compared to the untreated control, the inhibition of bacteria was the strongest at the highest concentration of 500 µL/L of FEO for which inhibition percentage values ranged between 65.14 and 93.67%. The highest bacterial inhibition was observed for *B. subtilis* at the mentioned concentration. Strong inhibition of the bacterial growth was also noticeable at 250 µL/L against *Y. enterocolitica* and *B. subtilis* with percentages of inhibition of 74.82 and 75.92%, respectively. Moreover, *E. faecalis* was strongly inhibited at the concentration of 125 µL/L.

Table 7. Bacterial growth inhibition after FEO addition (%).

Concentration (µL/L)	*P. aeruginosa*	*Y. enterocolitica*	*S. enterica*	*S. enterica* Biofilm	*B. subtilis*	*E. faecalis*	*S. aureus*
62.5	45.87 ± 2.04 ^{b}	4.64 ± 1.32 ^{a}	73.63 ± 0.95 ^{c}	56.17 ± 2.42 ^{b}	7.76 ± 1.11 ^{a}	15.63 ± 2.07 ^{a}	6.63 ± 0.97 ^{a}
125	7.14 ± 1.68 ^{a}	13.44 ± 2.07 ^{b}	14.74 ± 1.01 ^{a}	5.33 ± 0.87 ^{a}	14.30 ± 2.18 ^{b}	74.39 ± 2.56 ^{c}	13.10 ± 1.93 ^{b}
250	54.00 ± 2.67 ^{c}	74.82 ± 2.06 ^{c}	36.83 ± 2.14 ^{b}	56.25 ± 2.03 ^{b}	75.92 ± 2.90 ^{c}	25.82 ± 1.00 ^{b}	57.14 ± 1.68 ^{c}
500	75.73 ± 2.87 ^{d}	86.14 ± 0.54 ^{d}	85.33 ± 2.14 ^{d}	65.14 ± 2.09 ^{c}	93.67 ± 2.11 ^{d}	85.70 ± 2.68 ^{d}	75.22 ± 1.53 ^{d}

One-Way ANOVA, Individual letters ($^{a–d}$) in upper case indicate the statistical differences between the concentrations; $p \leq 0.05$.

The antifungal activity (Table 8) was not as strong as the antibacterial activity. The percentual inhibition of all the tested fungi at the highest concentration of 500 µL/L ranged from 15.23 to 95.63%. Strong inhibition was noted only for *C. krusei* and *C. glabrata* with values of 76.33 and 95.63%, respectively.

Table 8. Microscopic filamentous fungi growth inhibition after FEO addition (%).

Concentration (µL/L)	*C. albicans*	*C. glabrata*	*C. krusei*	*C. tropicalis*	*A. flavus*	*B. cinerea*	*P. citrinum*
62.5	15.10 ± 2.15 ^{a}	13.74 ± 2.83 ^{a}	54.17 ± 0.52 ^{c}	15.04 ± 2.51 ^{a}	8.66 ± 1.07 ^{a}	43.77 ± 2.10 ^{d}	23.75 ± 1.78 ^{b}
125	34.55 ± 0.99 ^{b}	36.83 ± 1.03 ^{b}	14.07 ± 1.57 ^{a}	24.14 ± 2.47 ^{b}	15.84 ± 1.00 ^{b}	34.37 ± 2.24 ^{c}	46.03 ± 2.68 ^{c}
250	46.26 ± 1.74 ^{c}	47.17 ± 1.20 ^{c}	24.14 ± 3.32 ^{b}	55.67 ± 1.65 ^{d}	23.68 ± 0.99 ^{c}	24.11 ± 0.51 ^{b}	17.37 ± 0.70 ^{a}
500	56.29 ± 2.62 ^{d}	76.33 ± 2.71 ^{d}	95.63 ± 2.69 ^{d}	45.85 ± 2.05 ^{c}	34.77 ± 1.91 ^{d}	15.23 ± 1.57 ^{a}	23.22 ± 2.26 ^{b}

One-Way ANOVA, Individual letters ($^{a–d}$) in upper case indicate the statistical differences between the concentrations; $p \leq 0.05$.

3.5. Antibiofilm Activity

The inhibitory effect of FEO against *S. enterica* biofilm developing on plastic and stainless-steel surfaces was evaluated with a MALDI-TOF MS Biotyper mass spectrometer. Control mass spectra for both tested surfaces were developed identically with control planktonic spectra. For better comprehensibility of the results, a planktonic spectrum was used as the control for each day of the experiment. Experimental spectra of stainless-steel and plastic surfaces, and control planktonic spectra are grouped in the individual images prepared on selected days of the experiment (day 3, 5, 7, 9, 12, and 14).

Since day 3 (Figure 1A), differences in the development of the experimental and control groups were noticed, while similarity was preserved between experimental groups treated with FEO. On the 5th day of the experiment (Figure 1B), a difference in biofilm development was recorded between the control and experimental groups. However, a more pronounced effect of FEO was visible against the biofilm developing on the plastic surface as reflected by the lower number of peaks in the mass spectra compared to the mass spectra of biofilm developing on stainless steel. This trend persisted until the 7th day of the experiment (Figure 1C), which suggests that the antibiofilm activity of FEO on *S. enterica* with has a stronger effect on a biofilm created on a plastic surface. During the following days of the experiment (Figure 1D–F), the differences in mass spectra of the experimental and control groups persisted. This suggests that FEO affects the creation of *S. enterica* biofilm and causes deterioration of homeostasis, thus inhibiting biofilm development.

To express the similarity of mass spectra profiles of biofilm after FEO addition, a dendrogram was constructed based on the main peaks of the mass spectra (MSP) (Figure 2). The shortest distances in MSP were visible for the control groups (CSE) and biofilms of the experimental group during days 3 and 5 (SES 3, SEP 3, SES 5, and SEP 5). During the following days of the experiment, the MSP were extended, with the most significant changes on the 12th and 14th days of the experiment (SES 12, SEP 12, SES 14, SEP 14). During the whole experiment, the distance of the control group MSP was clearly shorter, compared to the distance of the experimental groups. Based on these observations, we can suggest that the addition of FEO resulted in changes of the protein profile, leading to the deterioration of biofilm homeostasis and the inhibition of the development of *S. enterica* biofilm. These findings are in accordance with the results of the mass spectra analyses.

3.6. Insecticidal Activity

The activity of FEO against insects was evaluated in triplicate by testing with *Oxycarenus lavaterae* (Table 9). With a growing concentration of FEO, the mortality of individuals increased. At a 100% concentration of FEO, 100% of the tested subjects were found to be dead. At a concentration of 25% of FEO, only 50% of the insects seemed to be alive. The concentration of 3.125% of FEO did not affect the viability of tested specimens.

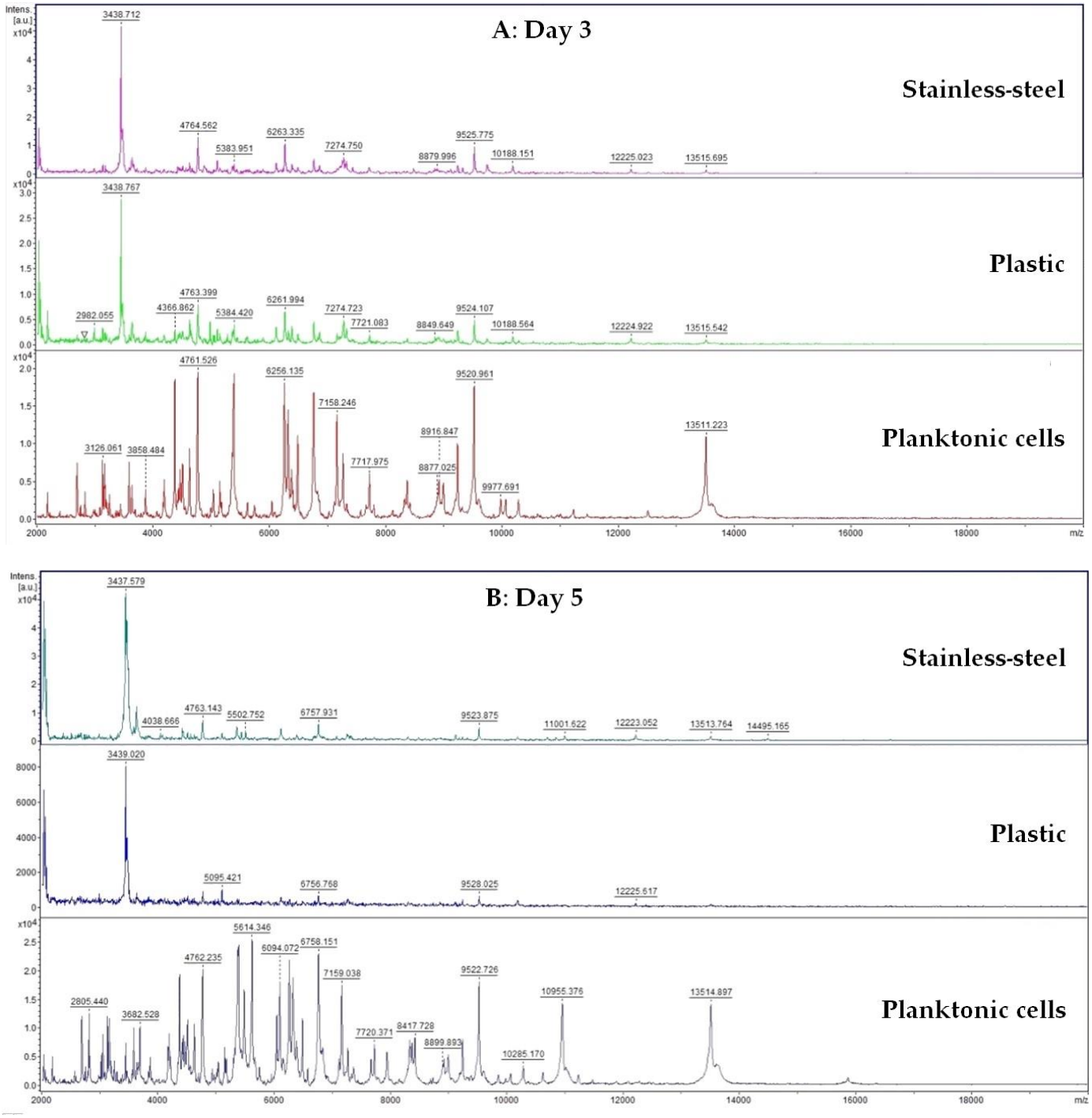
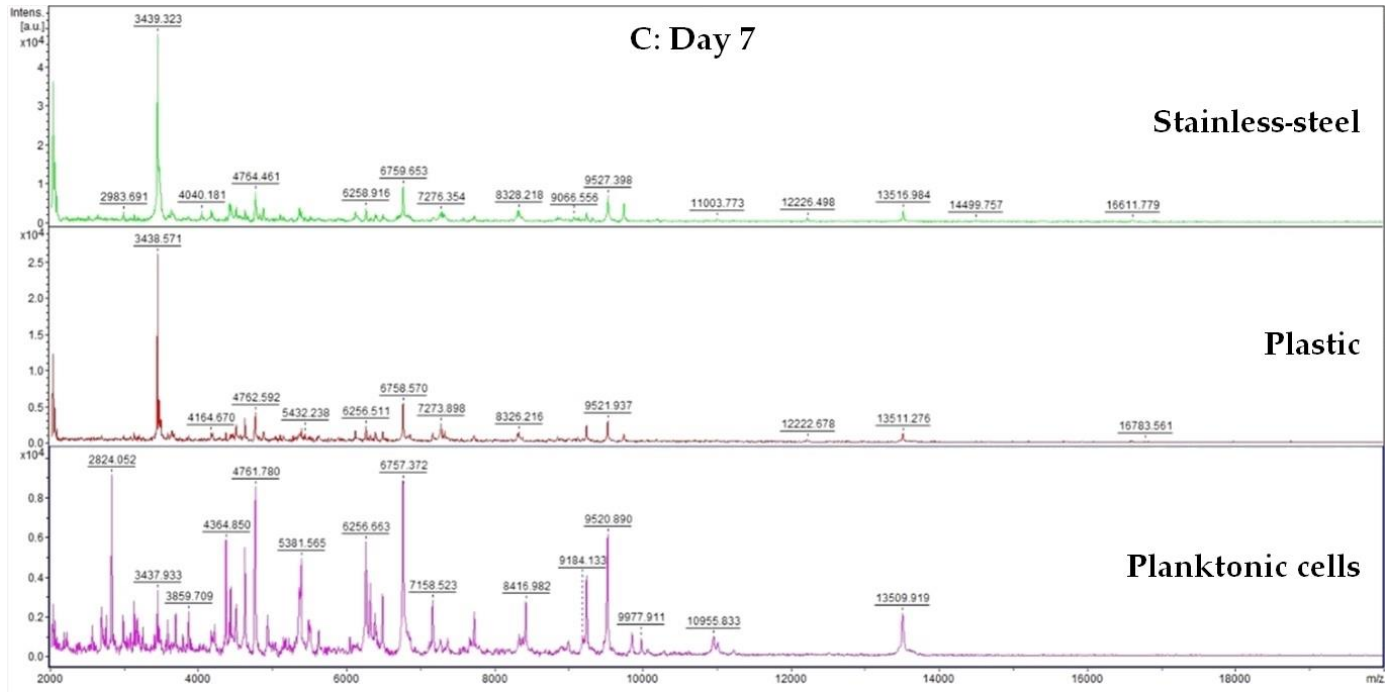


Figure 1. *Cont.*



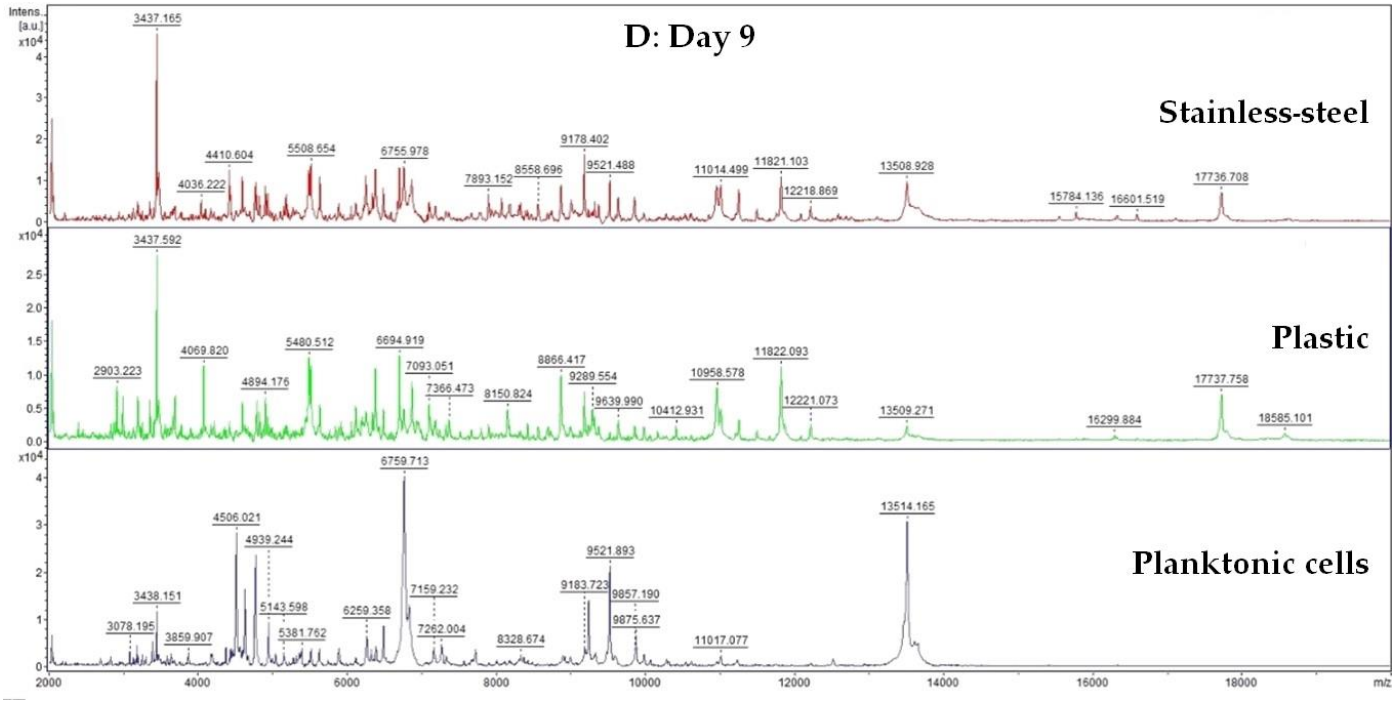


Figure 1. *Cont*.

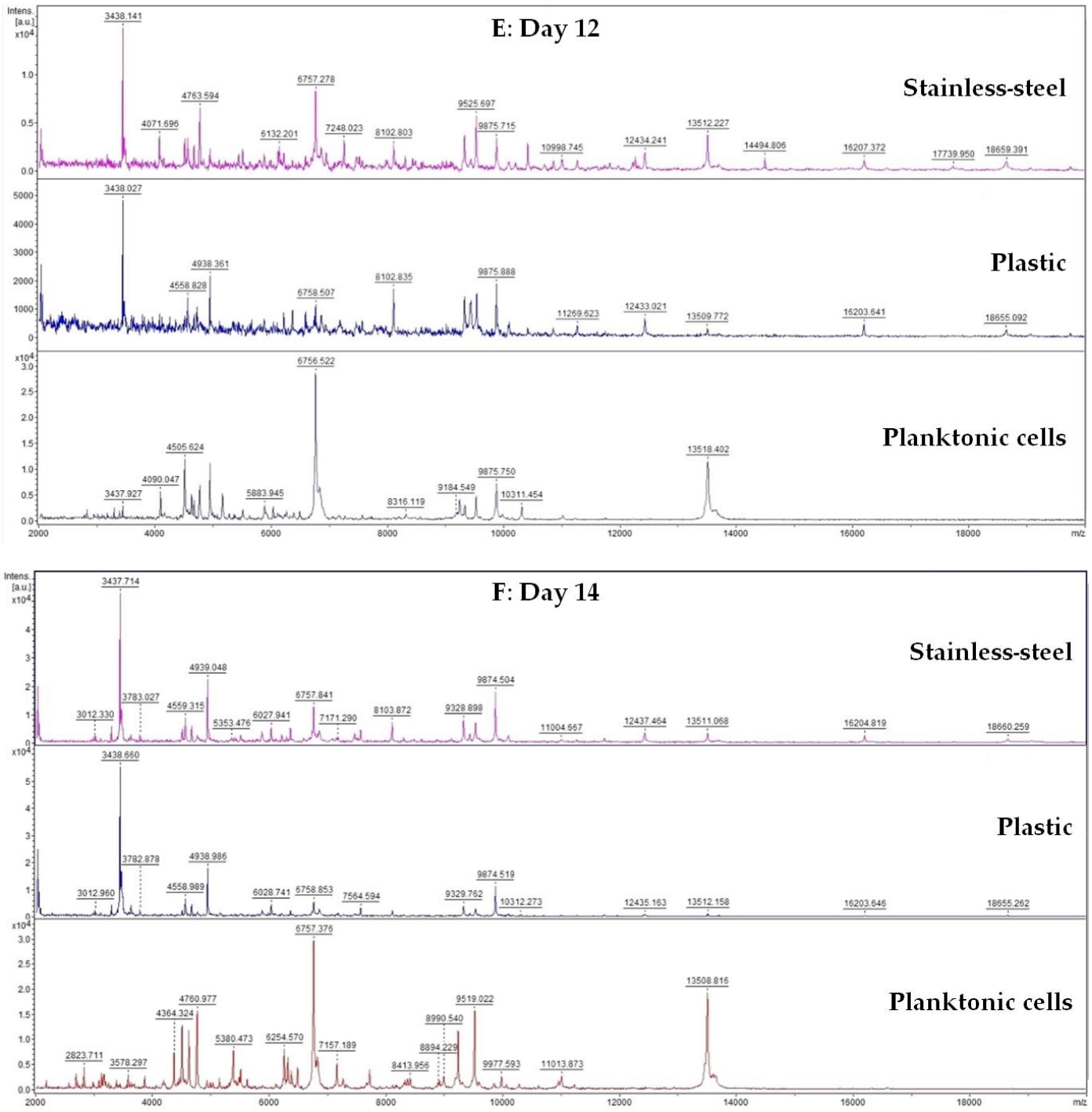


Figure 1. Changes in MALDI-TOF mass spectra of *S. enterica biofilm* after FEO addition: (**A**) 3rd day; (**B**) 5th day; (**C**) 7th day; (**D**) 9th day; (**E**) 12th day; (**F**) 14th day.

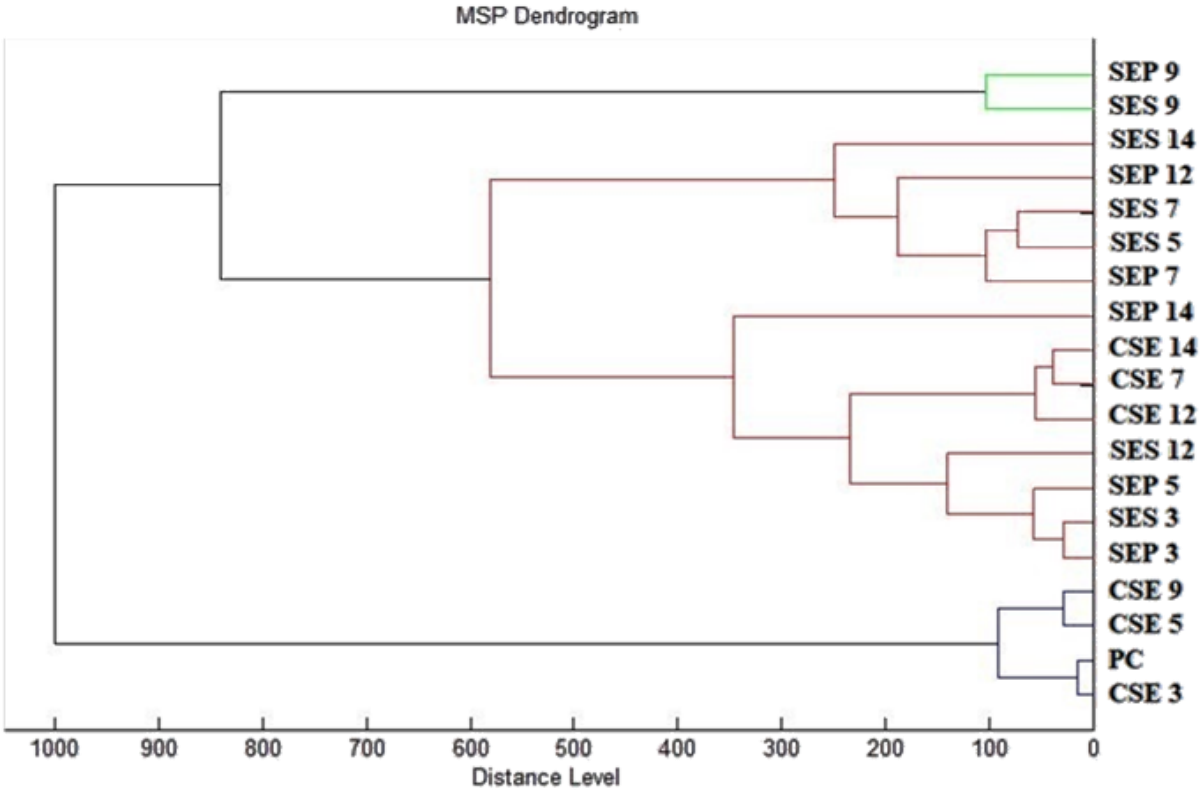


Figure 2. Dendrogram of *S. enterica* biofilm progress after FEO exposition. SE—*S. enterica*; C—control; S—stainless steel; P—plastic; PC—planktonic cells.

Table 9. Anti-insect activity of FEO.

Concentration (%)	Number of Living Individuals	Number of Dead Individuals	Insecticidal Activity (%)
100	0	30	100.00
50	7	23	76.66
25	15	15	50.00
12.5	22	8	26.66
6.25	27	3	10.00
3.125	30	0	0.00
control	30	0	0.00

4. Discussion

The findings observed in the present study show that commercial FEO is characterized by a high abundance of monoterpene hydrocarbons (79.3% of the total), with α-pinene (37.0%) and α-limonene (19.8%) being the major compounds. These results are in agreement with a previously published study on the chemical composition of 21 samples of commercial FEO as well as two laboratory-distilled oils [6]. Moreover, a report by DeCarlo et al. [26] indicates at least three different chemotypes defined by a high abundance of α-pinene, α-thujene, and methoxydecan. According to the same report, as well as many others, the α-pinene chemotype is also rich in limonene [4,6,26–28]. We can thus conclude that FEO investigated in this study belonged to the previously described α-pinene chemotype. Moreover, some previous examinations showed FEOs rich in octyl acetate, a compound not identified in the sample investigated in this study [29,30]. Observed differences in the chemical composition of this FEO could be explained by distinct environmental factors, such as harvesting season, geographical location, climate, as well as the part of the plant and the method used for FEO extraction, etc. Even though there are a number of previously

published results on the biological activity of this species, the variations in chemical composition can seriously influence the observed results.

DPPH and ABTS assays are commonly used for the evaluation of the antioxidant potential of essential oils. Our study showed that 10 µL of FEO can neutralize 73.88 ± 0.35% of DPPH radical and 97.09 ± 0.48% of ABTS radical cation. Considering these results, we can imply a high potential of this FEO as an antioxidant. The observed differences in the results of the two performed assays can be attributed to the differences in the mechanisms involved in the reactions responsible for neutralization. It is well known that ABTS radical cation reacts much faster, including the electron transfer reaction, compared to DPPH radical, whose neutralization is dependent on the antioxidant's ability to donate hydrogen. In previously published results, this FEO showed a weak to moderate antioxidant potential [31–33]. Essential oils are known to be complex mixtures of compounds with different functional groups, and mainly the synergistic effect of two or more of its components is responsible for the observed effects. With that in mind, the diversity of the results obtained in various studies can be explained. Nevertheless, the results can also vary due to the assay employed in the determination of the antioxidant activity.

In our study, the antimicrobial activity of FEO was the strongest against *Candida* species. Strong activity of FEO was also determined against G^+ and G^- bacteria. Abers et al. [34] analyzed the antimicrobial activity by disc diffusion method and determined a moderate activity of FEO with inhibition zone diameters exceeding 30 mm against G^+ bacteria *S. pyogenes* and *M. smegmatis* and G^- bacteria *P. aeruginosa*. Al-Saidi et al. [35] determined the activity against G^- *P. aeruginosa* to result in diameters of 23.3–29.7 mm. Among G^+ bacteria, a comparable activity was determined for *B. subtilis* and *S. aureus* with ranges of inhibition zones 14.7–16.0 mm and 7.0–24.3 mm, respectively. Almutairi et al. [36] observed the antimicrobial properties of FEO against antibiotic-resistant strains and showed mild activity against methicillin-resistant *S. aureus* and multi-drug-resistant *P. aeruginosa*.

Di Stefano et al. [7] compared the growth-inhibition activity of three FEOs. They determined MIC value against bacteria *S. epidermidis*, *S. hominis*, and *P. acnes* ranging from 0.264 to 6.16 mg/mL. MIC for *S. aureus* and *P. aeruginosa* ranged from 52 to 400 mg/mL, which appeared to reflect the weak activity. Notable activity was determined against *C. albicans* and *M. furfur* with a MIC range of 0.03–3.10 mg/mL. On the other hand, Man et al. [37] tested six essential oils (Frankincense, myrtle, thyme, lemon, oregano, and lavender) and determined the weakest activity compared to the essential oil with MIC of 50% *v*/*v* or higher against G^+ and G^- bacteria. De Rapper et al. [38] tested the activity of various *Boswellia* species and found MIC values to be the lowest against fungi *C. neoformans* (0.8 mg/mL) and the highest against *S. aureus* (6.0 mg/mL). Van Vuuren et al. [4] tested various FEOs and reported mean MIC values against G^+ bacteria, *S. aureus* and *B. cereus*, of 8.1 and 3.4 mg/mL, respectively. The reported MIC values against G^- bacteria, *E. coli* and *P. vulgaris*, were 6.2 and 4.0 mg /mL, respectively, and against the yeast *C. albicans*, the value was 7.4 mg/mL. These values were lower compared to our study. Bogavac et al. [39] determined the activity of FEO against a *C. albicans* strain at 12.5 µL/mL which suggest a stronger activity compared to our study.

The activity of FEO together with other essential oils against fungal pathogens was evaluated as mild. Against *A. niger*, an MIC value of 625 µg/mL was determined [40]. Antifungal activity of FEO against *Aspergillus*, *Penicillium*, *Fusarium*, or *Alternaria* species was found at low concentrations, which are suitable for the inhibition of pathogenic fungi to preserve plant-based products [32]. FEO showed promising antifungal properties against 15 different fungal species with a value of mycelial inhibition ranging from 15.9 to 56.3% at 1 µL/mL. MIC was determined in a range between 0.039 and 0.625 µL/mL [41]. Ljaljević Grbić et al. [42] determined MIC for *Aspergillus* species in the range from 10.0 to 120.0 mg/mL and for *Penicillium* species, in the range from 10 to 30 mg/mL. These results on the antifungal properties of FEO are in agreement with our study.

Many factors affect the activity of the FEOs. Generally, the changes in biological activity are a result of differences in chemical composition [4]. Alpha-pinene, which was

the most abundant molecule of our FEO, did not show any significant activity alone, but the antimicrobial activity was stronger when combined with other substances, which suggests synergism with other components in FEO [43,44]. On the contrary, limonene, the second most abundant compound, has shown better activity than the essential oil containing it [45,46]. Overall, no correlation was observed between the number of major constituents in essential oils [47]. Therefore, the antimicrobial activity cannot be attributed only to the major constituents of essential oils, as minor compounds contribute to activity against potentially pathogenic microorganisms as well [48].

FEO showed good inhibitory activity against G^+ and G^- bacteria on the carrot used as a model food. For microscopic filamentous fungi, determined activity was weaker compared to bacteria. Prakash et al. [32] evaluated the potential of FEO for the preservation of *Piper nigrum* L. fruits and reported that the growth of a storage mold, *A. flavus*, was decreased by 65.38%. Not enough research has been focused on the application of FEO for the preservation of fruits or vegetables from potential pathogenic bacteria. On the other hand, some research has been conducted in our laboratories which aimed at the observation of the inhibition activity of essential oils against pathogenic microorganisms that grew on fruits, vegetables, and bread samples [23,49,50]. In other reports the inhibitory activity of eucalyptus essential oils on sweet potatoes and bread [51], or thyme oil against *Botrytis* in grapes [52] were investigated. Generally, essential oil components are considered to be good preservatives of food products [53]. Various mechanisms of action have been described, including interactions of compounds with cell membranes, cell walls, intracellular proteins, enzymes, or nucleic acids [54]. Some components of FEO have been described as sufficient inhibitors of pathogenic microorganisms. For example, α-pinene was used in the prolongation of the storage of wine [55]. Limonene coating was found to decrease fungal growth on cucumbers [56]. We can suggest that the chemical composition of FEO increases the potential for its use as a food preservative.

Bacteria in the form of biofilm have a better ability to survive compared to single-cell forms due to the ability to adhere and the quorum sensing mechanisms. Analyses of biofilm degradation showed the ability of FEO to inhibit growth and deteriorate the formation of biofilm. There has not been enough research on the changes in biofilm resulting from the addition of FEO. Moreover, this is the first time that biofilm degradation was analyzed on different surfaces. The antibiofilm activity of the essential oils isolated from *Boswellia* against *Staphylococcus* species and *Candida* was reported, with FEO showing the ability to prevent the adhesion of biofilm [10]. The properties of terpenes, which are found in essential oils, were described with regard to biofilm formation [57]. Boudiba et al. [58] and Soyocak et al. [59] observed the disruption of biofilms by the inhibition of quorum sensing in bacteria after the treatment with essential oils with a similar composition.

The activity of FEO against *Oxycarenus lavaterae* showed 50% mortality at the concentration of 25%. The anti-insect activity of FEO against female forms of the fly *Musca domestica* was tested by Pavela et al. [60] who rported the toxicity values of LD50 and LD90 of 72.5 and 269.3 μg/individual, respectively. The activity against the mosquito *C. quinquefasciatus* larvae was found to be significant. Yang et al. [61] observed insecticidal activity of various essential oils against the weevil *Sitophilus zeamais*. Even the contact and fumigant activity of FEO was not significant, the repellent activity of FEO was moderate even after 24 h of incubation. Metayi et al. [62] tested a nanoemulsion of FEO against the cotton bollworm *Earias insulana* and found out that 80% mortality of larvae was reached at a concentration of 1800 μL. Due to the activity of FEO against pests, it can potentially serve as a repellent or pesticide. More research is necessary in order to determine the mechanisms of action.

5. Conclusions

The FEO tested in our study showed good properties that enable it to be used as an antimicrobial agent. The main volatile compounds were α-pinene, α-limonene, and *p*-cymene. The antioxidant activity was evaluated as strong. Antimicrobial activity de-

termined by the disk diffusion method was the strongest against *Candida* species. The inhibition activity of the vapor phase on carrot was more pronounced against G^+ and G^- bacteria than against microscopic filamentous fungi. Visible changes in the protein profile suggest the degradation of bacterial biofilm forming on plastic and stainless-steel surfaces. The anti-insect activity against *O. lavaterae* was found to increase with increasing concentration of FEO. We can thus conclude that the analyzed essential oil could serve as an agent against pathogenic microorganisms and can be used as a potential preservative of food and agricultural crops.

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