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ARTICLE

Antistaphylococcal activity of *Inula helenium* L. root essential oil: eudesmane sesquiterpene lactones induce cell membrane damage

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Abstract The purpose of this study was to investigate the inhibitory/bactericidal activity and cell membrane effects of the hydrodistilled essential oil of *Inula helenium* L. roots against *Staphylococcus aureus*. Additionally, detailed chemical investigation was done in order to pinpoint the most active oil constituents and also the parts of these molecules responsible for their antimicrobial effect. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the broth microdilution method. The membrane-active nature of this oil was investigated by measuring the culture turbidity, leakage of phosphates, and 260-nm-absorbing material, together with lysis of the exposed cells. Finally, the effect of the oil on the cells was visualized using scanning electron microscopy (SEM). The chemical composition of the essential oil was analyzed using gas chromatography-mass spectrometry (GC-MS) and preparative medium-pressure liquid chromatography (MPLC). Chemical modification of the oil was performed using catalytic hydrogenation (H_2, Pd/C) and reduction with $NaBH_4$. The MIC and MBC values were 0.01 μl mL^{-1} and 0.02 μl mL^{-1}, respectively. Membrane damage was demonstrated through increased permeability (phosphates and nucleic acid leakage), followed by lysis of the exposed cells, captured on SEM images. The most active constituents were alantolactone, isoalantolactone, and diplophyllin. The essential oil showed very potent antistaphylococcal activity, with obvious membrane-damaging effects. Sesquiterpene lactones were found to be the most active principles of the oil, whose eudesmane core olefinic bonds, along with the α,β-methylene-lactone ring, are essential structural parts responsible for the exhibited antimicrobial activity.

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Introduction

Inula helenium L. (elecampane inula, oman in Serbian), family Compositae, is a perennial plant species native to Europe and East Asia. In Serbia, it is a widespread plant, used in folk medicine mostly for the treatment of respiratory conditions such as asthma, bronchitis and whooping cough, disorders of digestion, urinary infections, and also for skin disorders. Although only a limited number of studies deals with the antimicrobial activity of *I. helenium* root essential oil, it was found that it was active against some Gram-positive and Gram-negative bacteria and *Candida* species [1]. Previous chemical studies of *I. helenium* have showed that the dominant root volatiles are eudesmane-type sesquiterpene lactones [1–4]. It is known that a number of these compounds have strong antihelmintic, anti-inflammatory,

and antimicrobial activity, and the potential to induce detoxifying enzymes [1, 5, 6]. The roots contain up to 5% of the essential oil, thymol derivatives, triterpenes, sterols, and up to 44% of the polysaccharide inulin [5].

The potential of the major compounds that constitute the essential oil and extracts of this plant species to cause cell death in a variety of cytotoxicity models (against both prokaryotic and eukaryotic cells [1, 2, 7], as well as the lack of more detailed knowledge of the composition of its rather abundant in yield essential oil, prompted us to work more in this direction. With an idea that has been put forward more intensely recently on the fact that the net activity of a plant extract is, in fact, the result of the work of several and only rarely one principle (several minor constituents on their own or working in unison with the major compounds [8, 9]), it provoked us to analyze meticulously the chemical composition of *I. helenium* root oil. A number of investigations dealing with the antimicrobial activity of essential oils and their constituents alone pointed to the common antimicrobial mode of action by targeting the cell membrane. Since Gram-positive bacteria, due to the lack of the outer membrane, are expected to be more susceptible to such antimicrobial agents, we decided to probe the activity of the *I. helenium* oil, as well as its effects on the cell membrane, by assaying its antistaphylococcal activity (*Staphylococcus aureus*). Four spectrophotometric methods were employed in order to investigate the membrane-active nature of this essential oil, assessed through the measurement of the culture turbidity, leakage of phosphates, and 260-nm-absorbing material, and, finally, lysis of the exposed cells. The role of minor oil constituents in the net antistaphylococcal activity was investigated by determining the activity of the oil fractions (consisting of either pure compounds or representing mixtures of only a few oil constituents) obtained by means of medium-pressure liquid chromatography (MPLC). The investigation was deepened further in order to "locate" the active pharmacophore of the dominant oil constituents by chemically modifying (catalytic hydrogenation (H_2, C/Pd) and reducing with $NaBH_4$) the fractions and pure constituents. The prepared reduction products were tested for their antimicrobial activity as well. Finally, in order to visualize the noted effect of the oil on the cells, scanning electron microscopy (SEM) was employed.

Materials and methods

Isolation, GC, GC-MS, and MPLC analyses, and chemical modifications of the essential oil

Dried roots of *I. helenium* were purchased from a local herb shop in Niš, Serbia (manufactured by the Institute Josif Pančić, Belgrade, Serbia). The essential oil was isolated from the roots of *I. helenium* using the original Clevenger-type apparatus [10] (a detailed description can be found in the Online Resource 1). Reduction of the essential oil (catalytic hydrogenation [H_2, Pd/C] and reduction with $NaBH_4$), as well as gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), and MPLC analyses of the obtained sample (IH sample) were done according to the previously described methods [8, 9]. A more extensive description of the used methods can be found in the Online Resource 2.

Microdilution method

The bacterial strain used in all experiments was *S. aureus* ATCC 6538, maintained on nutrient agar (NA) in the culture collection of the Microbiology Laboratory, Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš. Antimicrobial activity determination was performed by a microdilution method as described previously [11]. An overnight culture of *S. aureus* was used for the preparation of suspension. Stock solutions of the essential oil and its fractions and reduction products were prepared in 70% aqueous ethanol, whose final concentration never exceeded 5% (v/v) in the well. Dilution series of the *I. helenium* essential oil and its fractions and reduction product samples were prepared in 96-well microtiter plates. After that, inoculums were added to all wells. The final size of the bacterial inoculum was $\sim 1 \times 10^6$ CFU ml^{-1} (colony forming units per milliliter). Bacterial growth was determined by adding 20 μL of 0.5% triphenyl tetrazolium chloride (TTC) aqueous solution. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the oil inhibiting visible growth (red-colored pellet on the bottom of the wells after the addition of TTC), while the minimum bactericidal concentration (MBC) was defined as the lowest oil concentration killing 99.9% of bacterial cells. To determine the MBC, the broth was taken from each well without visible growth and inoculated in Mueller–Hinton agar (MHA) for 24 h at 37°C. Experiments were done in triplicate and the mean value is presented.

Effect of the essential oil on the growth of *S. aureus*

The effect of *I. helenium* essential oil on the growth of *S. aureus* was determined for periods of 6 and 24 h. Exponential-phase cultures of *S. aureus* were used to determine turbidity changes in the bacterial culture, exposed to the activity of the oil [12, 13]. Bacterial suspensions of *S. aureus* were prepared by adjusting the turbidity using a spectrophotometer (UV-VIS 1650, Shimadzu, Japan), so that the final concentration in the medium was $\sim 1 \times 10^6$ (determined by viable counting of the same suspensions). Stock solutions of the oil were prepared in ethanol (70%). *I.*

helenium essential oil was added to the bacterial suspensions at MIC and MBC concentrations. The cultures were incubated at 37°C and at regular time intervals, and samples were taken for turbidity measurements at the wavelength of 600 nm. An untreated suspension, exposed only to the solvent, was used as a negative control. Experiments were done in triplicate and the mean values are presented.

Determination of phosphate leakage

Phosphate leakage was investigated according to a modified method of Johnston et al. [14]. Overnight cultures of *S. aureus* were harvested by centrifugation at 10,000×g for 10 min at 4°C and washed twice with physiological saline. Finally, the cells were resuspended in physiological saline and the suspensions' concentration adjusted to the appropriate cell number (1×10^{10} CFU/mL^{-1}). The suspensions were treated with MIC and MBC concentrations of the *I. helenium* essential oil. At regular time intervals, samples were taken from the cultures, centrifuged for 5 min at 3 500×g, and treated with acidic ammonium molybdate solution (2.4 mL) to form the phosphomolybdate. After the addition of the reducer, 1-amino-2-naphthol-4-sulfonic acid (2.4 mL of the working ANSA solution), the samples were mixed on a vortex mixer and heated for 10 min in a boiling water bath to produce the blue phosphomolybdenum complex. The intensity of the color was proportional to the phosphate concentration and was measured at 830 nm on a UV-VIS 1650 Shimadzu spectrophotometer (Tokyo, Japan). A more extensive description of this method can be found in the Online Resource 3.

Determination of cell lysis induced by the essential oil

The overnight culture of *S. aureus* was used to inoculate Mueller–Hinton broth (MHB). This culture was incubated for 18 h and separated from the growth medium by centrifugation at 10,000×g for 10 min at 4°C, washed twice with phosphate-buffered saline (PBS) (pH 7.2) and resuspended in PBS. The suspensions were adjusted so that the absorbance of a 1 in 100 dilution was~0.3 (corresponding to the cell number of 3×10^{10} CFU ml^{-1}). After the addition of the oil in MIC and MBC concentrations, samples were taken at regular time intervals and their absorbance at 600 nm was measured.

Determination of the cell leakage of 260-nm-absorbing material

The leakage of 260-nm-absorbing material was determined by measuring the absorbance of the culture filtrates, taken at regular time intervals during a period of 6 h. The filtration was done through 0.2-μm pore size filters (Cronus Syringe Filters, 25 mm, 0.2 μm). Each filtrate was measured against a blank and the mean values of three replicates are presented.

Scanning electron microscopy (SEM) analysis

The cells, after being treated with the *I. helenium* essential oil in MIC and MBC concentrations (2 and 6 h), were harvested by centrifugation for 15 min at 3,500×g. The cells were then fixed with 2.5% glutaraldehyde solution, dehydrated with ethanol solutions of increasing concentrations, and dried on a critical point dryer. The now-dried cells were then sputter-coated with gold under vacuum (JFC 1100 E, JEOL, Japan) and observed under a scanning electron microscope (JSM 5300, JEOL, Japan) [15, 16].

Results

The results of the chemical analyses (GC and GC-MS) of *I. helenium* root essential oil (IH) are listed in Table 1. The yield of the essential oil was 1.4% (w/w). Forty-five different compounds, representing 91.5% of the total oil, were identified. The oil was characterized by a relatively high amount of sesquiterpenoids (90.2%), while monoterpenes represented only 0.2% of the oil. The dominant constituents were three isomeric eudesmane-type sesquiterpene lactones (**1**–**3**): alantolactone (55.8%), isoalantolactone (26.1%), and diplophyllin (5.1%) (Fig. 1). The obtained results are in general agreement with the previous studies of *I. helenium* oil [1, 3]. Nevertheless, some of the minor compounds (e.g., dihydro-2-methyl-3-furanone, geranyl 2-methylbutyrate, dehydro-1,8-cineole, 1-decene, etc.) have not been previously reported as *I. helenium* (or any other *Inula* taxa) volatiles.

Investigation of the MICs and MBCs against *S. aureus* demonstrated activity at very low concentrations of the *I. helenium* essential oil. The oil exhibited inhibitory activity (MIC) at 0.01 μl mL^{-1} (13.00 μg mL^{-1}), while the MBC was 0.02 μl mL^{-1} (26.00 μg mL^{-1}).

I. helenium essential oil was fractioned using preparative MPLC and MIC values of the obtained fractions (nine in total) were also determined. Table 2 lists, alongside the corresponding MIC values, the main (classes of) components of the oil fractions. The chromatographic separation of the IH oil enabled the detection (and identification) of some additional minor *I. helenium* volatiles, undetected in the GC and GC-MS runs of the unfractioned oil. Most probably, their relative amount in the oil was below the detection limits of the mass selective detector. Worth noting is the case of a sesquiterpene aldehyde (MS resembled mostly that of bicyclogermacrenal, as well as its refractive index value, but it was not identical), which was the dominant constituent of a fraction that exhibited very high

Table 1 Chemical composition of *Inula helenium* L. essential oil

Rt/ min	Constituent class	Percentage	Constituent
2.63	o	tr	Hexanal
2.70	o	tr	Dihydro-2-methyl-3-furanone
2.90	o	tr	3-Methylbutanoic acid
3.52	o	tr	1-Nonene
4.22	m	tr	α-Pinene
4.52	m	tr	Camphene
4.87	m	tr	Sabinene
5.00	m	tr	β-Pinene
5.11	o	tr	1-Decene
5.15	o	tr	2-Pentyl furan
5.18	m	tr	Dehydro-1,8-cineole
5.88	m	tr	p-Cymene
5.98	m	tr	Limonene
6.04	m	tr	1,8-Cineole
6.84	m	tr	*cis*-Linalool oxide (furanoid)
7.24	o	tr	1-Undecene
7.47	m	tr	Linalool
7.71	m	tr	α-Thujone
7.98	m	tr	β-Thujone
8.74	m	tr	Camphor
8.92	m	tr	Menthone
9.06	s	tr	Albene
9.15	m	tr	*iso*-Menthone
9.55	m	tr	Terpinen-4-ol
9.92	m	tr	α-Terpineol
12.13	m	tr	Isobornyl acetate
12.25	o	tr	1-Tridecene
13.74	m	tr	Citronellyl acetate
13.87	s	tr	α-Longipinene
14.32	m	tr	Neryl acetate
14.83	s	0.1	β-Elemene
15.08	s	tr	Petasitene
15.63	s	0.1	β-Caryophyllene
15.90	s	tr	*cis*-α-Ambrinol
16.26	s	tr	*epi*-β-Santalene
16.86	s	0.2	4,5-Di-*epi*-aristolochene
16.92	s	0.3	Selina-4,11-diene
16.99	m	tr	Citronellol isobutanoate
17.10	m	tr	Neryl isobutanoate
17.18	s	tr	γ-Humulene
17.29	s	0.3	Eremophilene
17.34	s	0.1	β-Selinene
17.51	s	0.1	α-Selinene
17.67	m	0.1	Geranyl isobutanoate
17.71	s	tr	Eremophila-1(10),8,11-triene
17.77	s	0.3	Germacrene A
19.17	m	tr	Neryl isovalerate
19.57	s	0.1	Caryophyllene oxide
19.79	m	tr	Geranyl 2-methylbutyrate
20.00	s	tr	Rosifoliol
20.86	s	tr	Eudesm-3,11-dien-5-ol
20.93	s	0.2	*epi*-α-Cadinol (syn. τ-cadinol)
21.17	s	0.1	β-Eudesmol
21.33	s	0.4	Selin-11-en-4-ol
22.11	o	tr	2-Pentadecanone
23.22	s	tr	α-Cyperone
24.67	s	0.1	Callitrin
24.94	s	tr	Dihydroinunolide
25.82	s	tr	Dihydroisoalantolactone
26.49	s	55.8	Alantolactone
26.99	s	0.5	Dihydroalantolactone
27.31	s	5.1	Diplophyllin
27.55	s	26.3	Isoalantolactone
28.59	m	0.1	10-Acetoxy-8,9-epoxythymolisobutyrate (syn. 2-(3-[(acetyloxy)methyl]-2-oxiranyl)-5-methylphenyl 2-methylpropanoate)
30.77	o	0.3	(*Z*,*Z*)-9,12-Octadecadienoic acid (syn. linoleic acid)
30.89	o	0.9	(9*Z*)-9-Octadecenoic acid (syn. oleic acid)
31.36	o	tr	Octadecanoic acid (syn. stearic acid)
		90.1	Sesquiterpenes
		0.2	Monoterpenes
		1.2	Others
		91.5	Total

tr – trace (<0.05%), s – sesquiterpene, m – monoterpene, o – other, syn. – synonym

activity against *S. aureus*, IH-Fr2 (more than 50%), and one of the major constituents of IH-Fr1 (8.0%) (Table 2). The only oil fraction that had a lower MIC value than that for IH-Fr2 was the fraction IH-Fr4 (a mixture of alantolactone, isoalantolactone, and diplophyllin). The third most active oil fraction was IH-Fr8, dominated by sesquiterpene lactones as well, but this time comprising the isomeric dihydroalantolactones (minor oil components).

As can be concluded from the activity data from Table 2, the major antistaphylococcal principles of the *I. helenium* oil (with the lowest MIC value) were the three major contributors, alantolactone, isoalantolactone, and diplophyllin (IH-Fr4). The structures of all three compounds possess a lactone functionality and two double bonds (one of which is conjugated to the carbonyl of the lactone ring, Fig. 1). In order to "locate" the structural parts of the mentioned

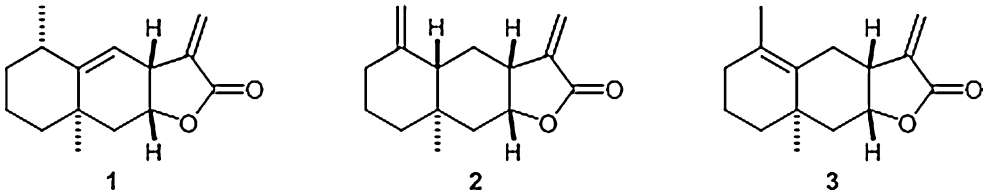

Fig. 1 The structures of the major constituents of *Inula helenium* root essential oil: **1** alantolactone, **2** isoalantolactone, and **3** diplophyllin

compounds (**1**–**3**) that are carriers (pharmacophores) of the observed strong antibacterial activity, the oil was subjected to catalytic hydrogenation (which leads to the reduction of the two olefinic double bonds; sample IH-H_2/Pd) and reduction with $NaBH_4$ (this leads to the reduction of aldehyde/ketone groups and/or olefinic bonds of the α,β-unsaturated carbonyls; sample IH-$NaBH_4$). The main (classes of) constituents of the samples obtained by such transformations of *I. helenium* root oil and their MIC values are given in Table 2. The structures of the expected dominant products of reduction (both methods) of alantolactone are depicted in Fig. 2 (the analogous stands for isoalantolactone and diplophyllin). The major product of the catalytic hydrogenation of the IH sample was tetrahydroalantolactone (71.1%) (molecular weight [MW] 236). This compound was most probably formed as the product of the total hydrogenation of all three main oil constituents (**1**–**3**; MW 232). The second most abundant compound (10.4%) was a saturated sesquiterpene acid (MW 238), 12-eudesmic acid, resulting from the further reduction of tetrahydroalantolactone. Under the same conditions, dihydroisoalantolactone quantitatively gives tetrahydroalantolactone [17]. The presence of the carboxylic group in the mentioned acid and in three other products of hydrogenation was confirmed via their extraction from IH-H_2/Pd by a water solution of NaOH. In this way, the IH-H_2/Pd fraction

Table 2 Major (classes of) constituents and antistaphylococcal activity of *I. helenium* essential oil, transformed oils, and obtained medium-pressure liquid chromatography (MPLC) fractions

Sample	Main (classes of) constituents	MIC (mg mL^{-1})
IH (pure oil)	Alantolactone (55.8%), isoalantolactone (26.1%), diplophyllin (5.1%)	0.0130
IH-Fr1 (1% Et_2O in hexane; 82.6 mg)	β-Elemene (24.1%), (*E*)-caryophyllene (4.3%), 4,5-di-*epi*-aristolochene (4.1%), selina-4,11-diene (4.1%), eremophilene (9.4%), sesquiterpene aldehyde (8.0%)	0.2580
IH-Fr2 (5% Et_2O in hexane; 1.8 mg)	Sesquiterpene aldehyde (53.4%)	0.0008
IH-Fr3 (10% Et_2O in hexane; 5.2 mg)	Acyclic/sesquiterpene ketones/esters	0.0020
IH-Fr4 (20% Et_2O in hexane; 100.0 mg)	Alantolactone (45.6%), isoalantolactone (23.8%), diplophyllin (11.6%)	0.0002
IH-Fr5 (30% Et_2O in hexane; 73.1 mg)	Alantolactone (63.8%), isoalantolactone (30.0%), diplophyllin (1.1%)	0.0280
IH-Fr6 (40% Et_2O in hexane; 46.1 mg)	Alantolactone (33.1%), isoalantolactone (52.7%), diplophyllin (6.8%)	0.0125
IH-Fr7 (50% Et_2O in hexane; 10.2 mg)	Dihydroisoalantolactone (6.8%), dihydrodiplophyllin (72.5%)	0.0420
IH-Fr8 (60% Et_2O in hexane; 8.0 mg)	Sesquiterpene lactones (dihydroalantolactone isomers), fatty acids	0.0070
IH-Fr9 (100% Et_2O; 4.5 mg)	Sesquiterpene lactones (dihydroalantolactone isomers), palmitic acid	0.0140
IH-H_2/Pd	12-Eudesmic acid (10.4%), tetrahydroalantolactone isomer (71.1%)	0.0280
IH-H_2/Pd-acid	Dehydro-12-eudesmic acid isomers (21.5%), 12-eudesmic acid (73.1%)	>0.2580
IH-H_2/Pd-acid-ester	Methyl 12-eudesmate (70.8%), methyl dehydro-12-eudesmate isomers (15.2%)	n.t.
IH-H_2/Pd-lactone	Tetrahydroalantolactone isomer (91.3%)	0.0008
IH-$NaBH_4$	Dihydroisoalantolactone (52.0%), dihydroisoalantolactone isomer (2.7%), eudesmen-8,12-diol I (8.1%), dihydroalantolactone (26.2%)	n.t.
IH-$NaBH_4$-Fr1 (10% Et_2O in hexane; 61.0 mg)	β-Elemene (13.6%), (*E*)-caryophyllene (3.7%), 4,5-di-*epi*-aristolochene (4.4%), selina-4,11-diene (4.0%), eremophilene (9.2%), germacrene A (7.5%)	0.3800
IH-$NaBH_4$-Fr2 (30% Et_2O in hexane; 48.0 mg)	Dihydroisoalantolactone (61.0%), dihydroisoalantolactone isomer (5.3%), dihydrodiplophyllin (28.7%)	0.0250
IH-$NaBH_4$-Fr3 (50% Et_2O in hexane; 42.5 mg)	Dihydroisoalantolactone (43.0%), dihydroisoalantolactone isomer (1.3%), dihydro diplophyllin (43.4%)	0.0001
IH-$NaBH_4$-Fr4 (100% Et_2O; 102.8 mg)	Eudesmen-8,12-diol I (44.6%), eudesmen-8,12-diol II (8.7%), eudesmen-8,12-diol III (20.1%)	0.0100

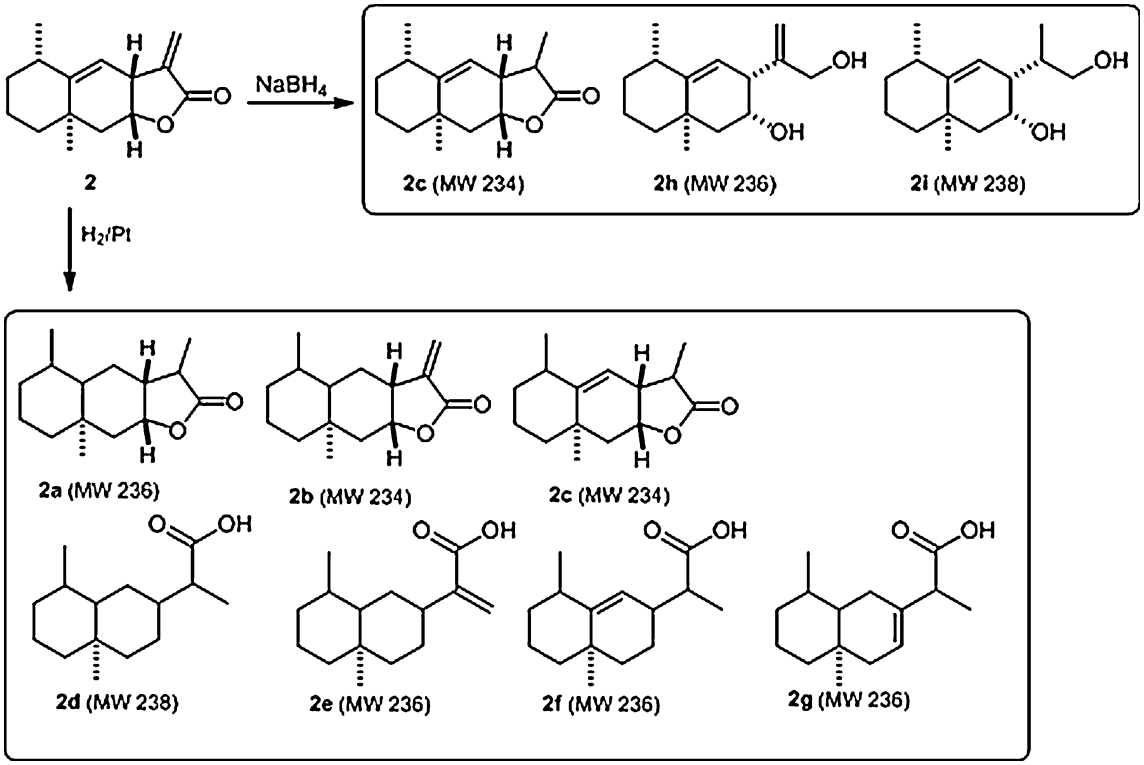

Fig. 2 The reduction products of alantolactone (**2**): tetrahydroalantolactone isomer (**2a**), dihydroalantolactone isomers (**2b** and **2c**), 12-eudesmic acid (**2d**), dehydro-12-eudesmic acid (**2e**–**2g**), eudesmadien-8,12-diol (**2h**), and eudesmen-8,12-diol (**2i**)

was further separated into two fractions: (a) the "acid" (compounds extractable with a water alkali solution) and (b) the "lactone" (non-acidic compounds). As a means of additional confirmation, the acid fraction was quantitatively esterified using diazomethane and, as expected, the increment of 14 amu (atomic mass unit) in the molecular ion peaks of obtained products (MW 250 and 252; starting acids had MW 236 and 238) was observed.

The dominant constituents (80.9% in total) of IH-$NaBH_4$ were the 1,4-reduction products of IH main root volatiles (**1**–**3**): (isomers of) dihydroalantolactone, dihydroisoalantolactone, and dihydrodiplophyllin. Another compound, present in a high relative amount (8.1%) in IH-$NaBH_4$ was an eudesmen-8,12-diol isomer (obtained by the complete reduction of the α,β-unsaturated carbonyl functionality). The sample IH-$NaBH_4$ was subjected to MPLC and the antibacterial activity (MIC values) of the obtained fractions (four in total) was determined (Table 2).

In order to investigate the effect of the essential oil on the growth of *S. aureus*, turbidity measurements of the growing *S. aureus* cultures were performed. The turbidity measurements were done during a 24-h period and a high growth inhibition was confirmed for the inhibitory concentration (MIC) when compared to the control (Fig. 3), while the MBC concentration decreased the absorbance to undetectable values of the spectrophotometer 3.5 h after the addition of the oil. After this time, no growth in the broth was observed at this concentration.

The phosphate leakage from *S. aureus* cells treated with *I. helenium* essential oil was monitored for 6 h in order to evaluate whether the oil affects the membrane permeability. A significant leakage, that was time- and concentration-

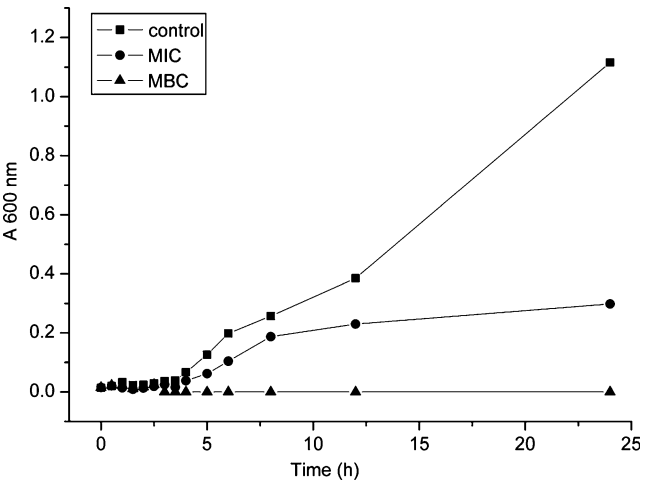

Fig. 3 Growth of *Staphylococcus aureus* at 37°C in Mueller-Hinton broth (MHB) containing minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of *I. helenium* L. essential oil during 24 h of incubation

dependent, was observed for all of the tested concentrations, demonstrating increased membrane permeability (Fig. 4).

To determine whether *I. helenium* root essential oil stimulates the autolysis of the treated cells in suspension, measurements of the turbidity changes were performed at 600 nm. The results showed a decrease in absorbance, and, when compared to the control, the decrease was higher than that for untreated suspensions (Fig. 5). After 4 h, the percentage of the initial absorbance of *S. aureus* cells treated with *I. helenium* oil dropped by 12.4% and 14.0% in the presence of MIC and MBC oil concentrations, respectively. The absorbance of the control decreased by only 3.8% during a 4-h period.

The absorbance of cell-free suspensions (filtrates) at 260 nm had higher values for the treated suspensions (the control suspensions showed no significant difference in the absorbance during the 4-h period), indicating the release of 260-nm-absorbing material from the damaged cells (Fig. 6).

SEM analysis was carried out to visualize the effects of *I. helenium* root oil on the morphology of *S. aureus* ATCC 6538. The untreated cells showed a regular, smooth surface and were uniform in size. The cells treated with the MIC concentration of the oil for 2 and 6 h showed very marked effects of the oil on the cell morphology. After the treatment with the MIC concentration of the oil, the cells showed deformation of the shape, severe disruption of the membrane integrity, and lytic effect as a consequence. Six hours after the treatment with the MIC concentration of the oil, cell debris formed after the cells' collapsed can be noted, together with a small number of living and regular cells in the culture. On the other hand, the MBC concentration of the oil, after 6 h of contact, caused an intensive lysis of the cells, where the "bursting" of the cells and the loss of their content can be clearly seen (Fig. 7).

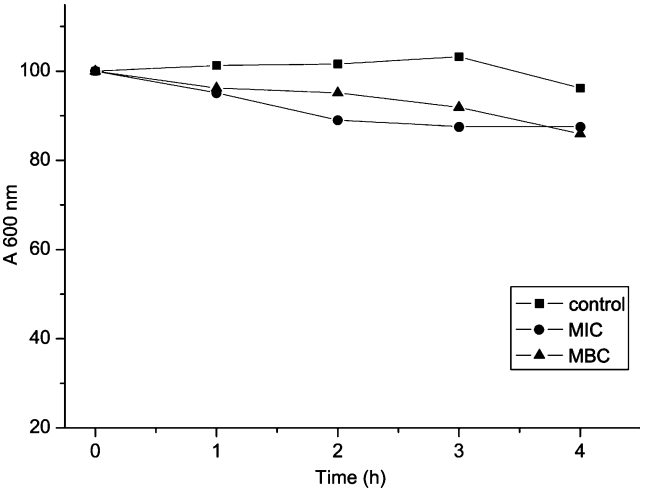

Fig. 5 Autolysis of *S. aureus* cells in the presence of MIC and MBC concentrations of *I. helenium* L. essential oil

Discussion

The essential oil of *I. helenium* has been previously tested against *S. aureus* and the activity (MIC) of 0.600 mg mL^{-1} was reported [1]. The current study indicated a much higher activity with an MIC of 0.013 mg mL^{-1}. The difference in the chemical compositions of the herein and previously studied [1] oils (the levels of alantolactone and isoalantolactone were higher in the IH sample) could be, perhaps, used to explain the higher antibacterial activity of the IH sample. Alantolactone and isoalantolactone were reported to be strong antimicrobials, which even gave rise to MIC values of 0.032 mg mL^{-1} [3, 18–20]. In addition, and to the best of our knowledge, this is the first report of diplophyllin as the *I. helenium* root volatile. This compound was not previously investigated for its antimicrobial activity, but

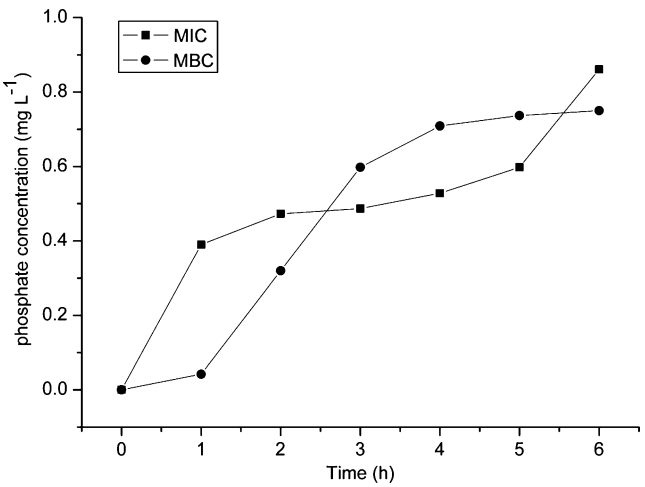

Fig. 4 Phosphate leakage from *S. aureus* cells induced by *I. helenium* L. essential oil treatment

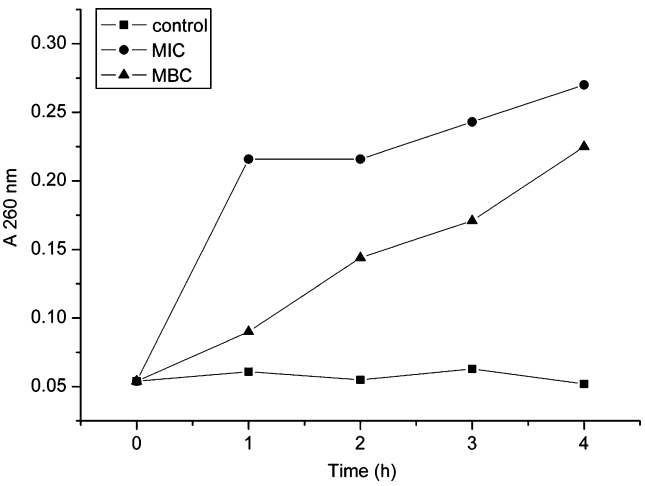

Fig. 6 Appearance of 260-nm-absorbing material in the filtrates of *S. aureus* suspensions in phosphate-buffered saline (PBS) treated with MIC and MBC concentrations of *I. helenium* L. essential oil

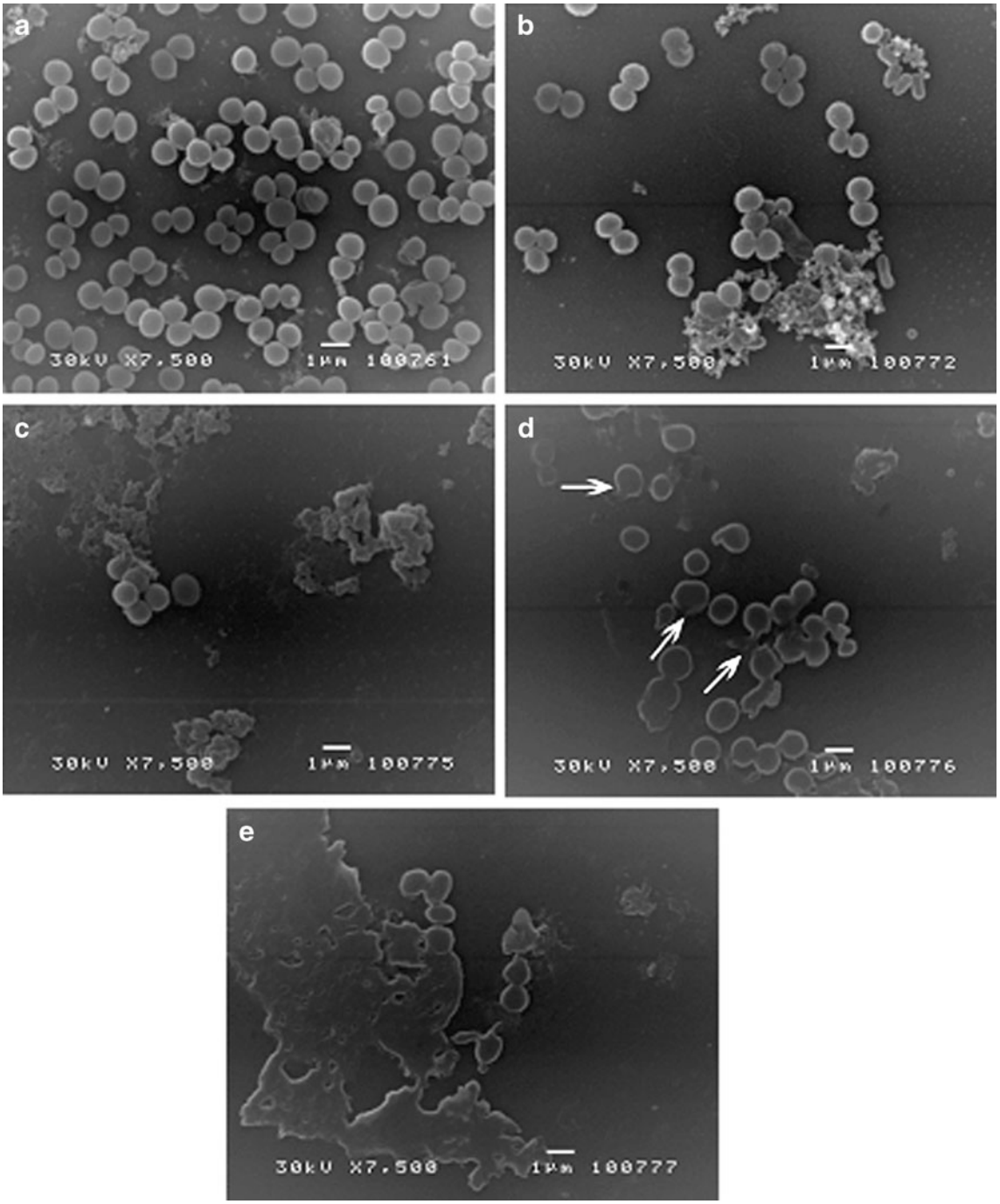

Fig. 7 Scanning electron micrographs of *S. aureus* ATCC 6538 treated with root essential oil of *I. helenium* L. in MIC and MBC oil concentrations (**a**) control; (**b**) disruption of membrane integrity after 2 h of treatment with MIC oil concentration; (**c**) cells after 6 h of treatment with MIC oil concentration, collapsed cells forming amorphous debris; on the left, survivor cells with regular shape and smooth surface can be noted. (**d**, **e**) Lysis caused by MBC oil concentration after 6 h of treatment (the *arrows* indicate rupturing cells)

since it possesses the structural motif in common with the active (iso)alantolactone, the observed high activity of the presently analyzed *I. helenium* oil could be, at least partially, connected to the fact that the mentioned compound was one of its major contributors. It must be stressed that the GC and GC-MS analyses of the IH sample enabled the positive identification of 45 compounds in total. The number of the herein identified components was (at least) twice as high as the number of components identified in any of the previous studies [1, 3].

In order to identify, with a higher degree of certainty, the active principles of the IH sample, some of which could

even be minor oil constituents, *I. helenium* oil was subjected to chromatographic separation by MPLC. As can be seen from Table 2, the fraction with the lowest MIC was mainly comprised of three sesquiterpene lactones (**1**–**3**; 81.0% in total; sample IH-Fr2) (Table 2). The activity of this fraction (MIC=0.0002 mg mL^{-1}) was significantly higher than that of the IH oil (MIC=0.0130 mg mL^{-1}; 87.0% of compounds **1**–**3**). It is interesting to note that an additional two fractions (IH-Fr5 and IH-Fr6) were also dominated with alantolactone, isoalantolactone, and diplophyllin. In fact, the sum of the relative amounts of these three compounds was even higher in the fractions IH-Fr5 (94.9%) and IH-Fr6 (92.6%). Nevertheless, the antimicrobial activities of IH-Fr5 (MIC=0.0280 mg mL^{-1}) and IH-Fr6 (MIC=0.0125 mg mL^{-1}) were comparable to the activity of original oil (IH) and lower than that of IH-Fr4. One could even speculate that the highest relative amount of diplophyllin in IH-Fr4 (11.6%) is responsible for the observed decrease of the MIC value (the amounts of diplophyllin were much lower in IH [5.1%], IH-Fr5 [1.1%], and IH-Fr6 [6.1%]). As mentioned previously, the fraction IH-Fr2, mostly comprised of the sesquiterpene aldehyde (unfortunately, incompletely unidentified), had a very low MIC value (Table 2). The similar is true for IH-Fr3 and IH-Fr8. These two fractions were now made up of minor oil constituents, some of which were not even detected during the GC and GC-MS analyses of IH, but are, clearly, potent antistaphylococcal agents.

With a goal to "locate" the pharmacophore of the major *I. helenium* oil constituents, the oil was chemically modified. In general, it is thought that the occurrence of an α,β-unsaturated lactone moiety is responsible for the high antimicrobial activity of a number of sesquiterpene lactones [21]. In order to test whether the loss of this Michael acceptor of the IH main constituents will result in a reduction/loss of the oil activity, IH was subjected to two chemoselective reductions: catalytic hydrogenation (IH-H_2/Pd) and reaction with $NaBH_4$ (IH-$NaBH_4$). As can be seen from Table 2, there was only a slight increase in the MIC (loss of activity) for the sample IH-H_2/Pd, in comparison to the untransformed oil. This sample was additionally separated via an acid-base extraction. The obtained "acid" fraction (the main constituent [73.1%] was 12-eudesmic acid) showed no antistaphylococcal activity in the tested concentrations. This is in agreement with a previous study on the antifungal activity of the 12-eudesmic acid and structurally related compounds [22]. This points to the fact that the main IH-H_2/Pd constituent (71.1%), tetrahydroalantolactone isomer (with no α,β-unsaturated carbonyl moiety or an isolated double bond [Fig. 2]), retains antimicrobial activity to a certain degree. It should be mentioned that it is not reasonable to expect that the minor IH-H_2/Pd constituents (in the relative amount in which they are present in the sample) should exhibit such a high activity.

The sample obtained after the reduction of the oil with $NaBH_4$ was subjected to MPLC as mentioned. Two of the obtained fractions had (isomers of) dihydroalantolactone, dihydroisoalantolactone, and dihydrodiplophyllin (1,4-reduction products of **1**–**3**) as their major contributors (IH-$NaBH_4$-Fr2 and IH-$NaBH_4$-Fr3). However, the determined MIC values of these fractions were mutually significantly different (Table 2). The relative amounts of the constituting isomeric dihydrolactones differed significantly in the two mentioned fractions, and this being the most probable reason for their different antistaphylococcal activities. In fact, the MIC value of IH-$NaBH_4$-Fr3 was much lower than the MIC value determined for the pure oil. Bearing this in mind, one can conclude that, although the α,β-methylene lactone moiety could be considered as the operative pharmacophore in the case of the (iso)alantolactones, it is not the only structural fragment of the lactones that carries the high antimicrobial activity of this and similar compounds/mixtures of compounds. The fraction IH-$NaBH_4$-Fr4, also noted to be a very strong inhibitor of bacterial growth, was comprised of isomeric eudesmen-8,12-diols (complete reduction of the α,β-unsaturated carbonyl system), and also demonstrates that the lactone ring itself might not be the essential antimicrobial structural fragment of this class of compounds. It must be stressed, however, that the presence of two alcoholic groups in eudesmen-8,12-diols is at least partially responsible for the observed strong antistaphylococcal activity of the corresponding fraction.

In the present paper, assays based on spectrophotometric measurements were employed in order to determine the membrane damage effects of the investigated essential oil [15]. Absorbance (turbidity) measurements of the growing culture showed high inhibition at the MIC concentration, while the MBC concentration of the oil decreased the turbidity to zero after a period of 3.5 h. The previously investigated essential oils [15, 23–26] and components [24, 25, 27–29] exhibited bactericidal activity, mostly during the first 2 h of contact with the bacterial cells. Although our results do not show a fast bactericidal effect, it must be stressed that, in the previous investigations, these effects were brought about by the action of monoterpenes, components with very high bactericidal effects. In this respect, the current study represents the first report on the antibacterial nature of a sesquiterpene-rich essential oil.

The lysis of *S. aureus* cells during the first 6 h of contact with the essential oil is a significant one when compared to the tea tree oil effects against the same bacteria. Carson et al. [15] reported that, after 6.5 h of contact with the oil, the cell lysis was 89.6% and 85.5% (MIC and 2×MIC, respectively), while *I. helenium* essential oil caused a decrease of absorbance to 87.56% (MIC) and 85.95% (MBC, which is also 2×MIC) after a much shorter period of 4 h. Additional tests, performed in order to detect disturbed membrane

integrity, showed the release of phosphates, followed by large molecules (DNA and RNA). *S. aureus* suspensions lost a significant amount of 260-nm-absorbing material, indicating severe damage of the cytoplasmic membrane of the treated cells. The results from the phosphate and nucleic acid investigations, as well as from the SEM analysis, demonstrated a faster effect of the MIC oil concentration in comparison to the other tested concentrations.

The SEM analysis showed that, after only 2 h post-addition of the MIC concentration, marked cell clumping and shrinkage was noted. On the other hand, after 6 h, the condition of the cells treated with an MBC oil concentration was worse when compared to those treated with the MIC oil concentration. The presented SEM images show the lysis of the cells being very intensive at this length of time of treatment with MBC and that all cells were affected.

The presented results showed obvious membrane damage effects in the form of increased membrane permeability, which leads to cell autolysis, but it is also clear that the essential oil of *I. helenium* does not exhibit a fast bactericidal action. This suggests that changes in some other mechanisms (aside from the membrane action), such as energy transducing (ATP pool, membrane potential, pH gradient across the cytoplasmic membrane or potassium gradient) or synthetic processes inside the bacterial cells may also be involved. Further investigations on the *I. helenium* root essential oil mode of action are necessary in order to confirm or exclude these other mechanisms. The antimicrobial activity of the herein obtained fractions pointed to alantolactone, isoalantolactone, diplophyllin, and the still completely unidentified sesquiterpene aldehyde as the carriers of the observed activity against *S. aureus*. The structure–activity relationship study showed that the eudesmane core olefinic bonds, alongside the α,β-methylene-lactone ring, are essential structural parts of the molecules responsible for their antimicrobial activity.

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