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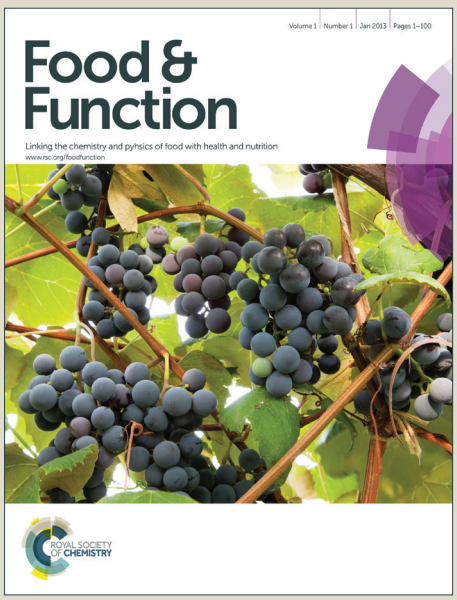

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Extracts of three *Laserpitium* L. species and their principal components laserpitine and sesquiterpene lactones inhibit microbial growth and biofilm formation by oral *Candida* isolates

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ABSTRACT

Antimicrobial properties of extracts of underground parts of three *Laserpitium* L. (Apiaceae) species, namely *Laserpitium latifolium* L., *Laserpitium zernyi* Hayek and *Laserpitium ochridanum* Micevski, were investigated. The investigated species are widely used as functional foods, as spices and for preparations in traditional medicine for treating the complaints connected with infection and inflammation. Furthermore, antimicrobial and antibiofilm effects of the laserpitine, the most abundant compound in the chloroform extract of *L.latifolium*, and guaianolide sesquiterpene lactones, such as, isomontanolide, montanolide and tarolide, principal components of the extracts of *L. zernyi* and *L. ochridanum* were assessed. Antimicrobial activity was tested using microdilution method against five pathogenic bacteria and five fungi, as well as in microplate biofilm assay on two *Candida* clinical isolates (*C. albicans* and *C. krusei*). Among the extracts, *L. latifolium* showed the most prominent activity. Isolated metabolites exerted higher effect against fungal than against bacterial strains, being isomontanolide the most active. Interestingly, all constituents showed higher potential on inhibition of biofilm formation than fluconazole, a reference compound. Tested metabolites may be good novel agents with high antifungal and antibacterial potentials that might find practical applications in food industry as food preservatives in order to retard the growth of food spoiling microbes, but only after detailed safety assessments.

Keywords: Laserpitium extracts, laserpitin, guaianolide lactones, antimicrobial activity, microdilution method, microplate biofilm assay

Introduction

The food spoilage by microorganisms is a problem that has not yet been brought under adequate control, despite the range of available techniques employed, so new natural sources i.e. plant secondary metabolites are in the scope of many research aiming for the discovery of novel and efficient food preservatives.[1] On the other hand, in humans fungal infections, *Candida albicans* is the most common cause, and third or fourth most commonly isolated pathogen in the blood stream of the patients in the US Hospitals.[2] *Candida* strains are known to form structured communities that are under the control of signalling molecules and attach to surfaces. Those specific and organised communities, commonly referred to as biofilms, are known to be more resistant to conventional therapy than free-floating, planktonic cells, which turns therapeutic approach towards the constant search for novel agents with biofilm inhibiting properties.[2, 3]

Genus *Laserpitium* L. (Apiaceae) comprises about 20 perennial aromatic species and some of the widely distributed such as *Laserpitium siler* L. and *L. latifolium* L., are being used in European traditional medicine to treat disorders connected to inflammation and infection.[4,5] In Alps region, roots and rhizomes of laserwort, *L. siler* were used to make tonics for refreshing and strengthening, but also to make mouthwash for the treating of toothache.[4] In Russian traditional medicine, underground parts of *L. latifolium* were used as diuretic, for treatment of various gastro-intestinal disorders, heart and liver failures, pulmonary tuberculosis, rheumatism and topically in pruritic dermatomicoses.[5]

In the scope of this investigation were three *Laserpitium* species: *L. latifolium*,[6] and two Balkan endemic species: *L. zernyi* Hayek, distributed in mountainous regions of Central Balkan previously treated as a subspecies of *L. siler* L.,

L. s. subsp. *zernyi* (Hayek) Tutin,[6,7] and *L. ochridanum* Micevski, originating only in Mountain Galičica, Former Yugoslav Republic of Macedonia.[8] Previous investigation of the extracts of the underground parts of those three *Laserpitium* species showed that *L. latifolium* extract was rich in daucane esters, particularly laserpitine,[9] while the most abundant compounds in two *Laserpitium* endemic species were guaianolide sesquiterpene lactones of a slovanolide type.[10] Sesquiterpene lactones are biologically active plant secondary metabolites, mainly investigated for their anti-inflammatory and antitumor properties;[11] nevertheless, some of them are gaining increasing scientific interest in the field of controlling resistant bacteria.[12] Daucane esters are relatively small group of sesquiterpene esters, limited to the species of Apiaceae family.[13] Even though this class of secondary metabolites is mostly tested for their pro-apoptotic and antiproliferative activity,[14] some of them are mentioned as antimicrobial principles of medicinal plants from the genus *Ferula* L.[15-18] Considering mentioned *Laserpitium* species, only essential oils were tested for their antimicrobial properties.[19-20]

Taken into account growing resistance of microorganisms in general and high resistance of common clinically isolated biofilms, we tested antimicrobial properties of extracts of the underground parts of three mentioned *Laserpitium* species and their highly abundant constituents (laserpitine, isolated from *L. latifolium,* and sesquiterpene lactones isomontanolide, montanolide and tarolide, isolated from *L. zernyi* and *L. ochridanum*, **Figure 1**), in order to discover novel natural agents that may have possible importance for the food industry as antimicrobial preservatives.

Experimental

Plant material

Underground parts (roots and rhizomes) of three *Laserpitium* species were collected at authentic localities in Serbia and Former Yugoslav Republic of Macedonia (for *L. latifolium*: Mt. Gučevo, north-western Serbia, October 2008; *L. zernyi* Mt. Ošljak, southern Serbia, July 2008; *L. ochridanum* Mt. Galičica, Former Yugoslav Republic of Macedonia, October 2009). Species were identified by botanist Dr. Marjan Niketićand representative herbarium specimens were deposited at the Natural History Museum, Belgrade, Serbia, under accession numbers ko03102008, ko20080709 and ko2008070215for *L. latifolium, L. zernyi* and *L. ochridanum,* respectively.

Extracts preparation, isolation and identification of compounds and their quantification in the extracts

Air dried, powdered plant material was extracted using chloroform as previously reported by Popović *et al*.[9-10] Briefly, extraction was carried out in dark bottles during 48 h, at room temperature. The ratio plant material : chloroform was 1:10 (*w/v*). After the extraction, the plant material was re-extracted (48 h) with new volume of chloroform under the same conditions. Chloroform was used as desirable solvent for extraction of sesquiterpene lactones and esters. Chloroform was evaporated under reduced pressure, and obtained gummy extracts were kept in fumehood for the next 4 weeks, in order to establish that the solvent is evaporated and to obtain dry extracts that were further used in the tests.

Full procedures considering isolation of the metabolites from the extracts are also previously reported. IR spectra of isolated compounds were recorded on a Perkin Elmer FT-IR Spectrometer SPECTRUM 1000. Mass spectra (HR-MS ESI^+) were obtained on a Waters LCT Premier XE Orthogonal Acceleration Time Of Flight (TOF) mass spectrometer. One- and two-dimensional NMR experiments were performed on a Varian Mercury 300 spectrometer. Dry chloroform extracts were quantified using gradient system optimised for terpenoids (chromatograms given in **Figure 1**) by an external standard method using HPLC-DAD as previously reported. Full procedures on isolation of the compounds and their quantification were reported previously.[9,10] The concentration of laserpitine, the most abundant component in *L. latifolium* extract, was 327.91 mg/g of extract. The concentrations of the principal sesquiterpene lactones in the extracts of *L. zernyi* and *L. ochridanum* were: 29.90 and 40.66 mg of isomontanolide/g of extract; 95.63 and 2.55 mg of montanolide/g of extract, and 24.65 and 205.11 mg of tarolide/g of extract, respectively.[9,10]

Compounds

Laserpitine (6α,10α-diangeloyloxy-4β-hydroxy-dihydrodaucane-9-one)

A white crystalline powder of the molecular formula $C_{25}H_{38}O_7$. HR-MS analyses: the molecular ion peak at *m/z* 451.2759 (calculated for $[M + H]^+$ 451.2690). IR analyses: ν_{max} 3368.6, 1711.1, 1228.7, 1144.0 cm^{-1}. NMR analyses: ^1H NMR δ: H-2α 1.65 *d* (4.8 *Hz*), H-2β 1.59 *d* (6.3 *Hz*); H-3α 1.99 *m*, H-3β 1.60 *m*; H-5 2.09 *d* (11.1 *Hz*); H-6 5.51 *dd* (11.1 *Hz*; 4.8 *Hz*); H-7α 2.26 *d* (16.9 *Hz*), H-7β 2.52 *dd* (17.0 *Hz*; 4.8 *Hz*); H-10 5.26 *s*; Me-11 1.42 *s*, H-12 1.61 *m*; Me-13 0.76 *d* (6.5 *Hz*); Me-14 0.88 *d* (6.7 *Hz*); Me-15 1.42 *s*; Ang: 6.16 *ql*, Me-2.02 *m,* 1.87 *m*; Ang: 6.13 *ql*, Me-2.02 *m*, 1.94 *m*.

Isomontanolide (8α-angeloyloxy,11α-acetoxy-10β-hydroxy-6αH,7αH-guaian-3-en-12,6-olide)

A transparent, crystalline substance of the molecular fomula $C_{22}H_{30}O_7$. HR-MS analyses: pseudomolecular ion peaks at *m/z* 429.1892 (calculated for $[M + Na]^+$ 429.1884) and 445.1619 (calculated for $[M + K]^+$445.1623). IR analyses: ν_{max} 3495.9, 1758.9, 1745.2, 1692.2, 1232.0, 1112.0 cm^{-1}. NMR analyses: ^1H NMR δ: H-1 2.42 *m*; H-2α 2.05 *m*, H-2β 2.11 *m*; H-3 5.57 *bs*; H-5 2.58 *q* (5.7 *Hz*); H-6 4.72 *dd* (11.4 *Hz*; 9.9 *Hz*); H-7 3.67 *dd* (11.4 *Hz*; 9.6 *Hz*); H-8 5.54 *dd* (11.4 *Hz*; 9.0 *Hz*), H-9α 2.10 *m*, H-9β 1.85 *m*; Me-13 1.55 *s*, Me-14 1.23 *s*; Me-15 1.89 *s*; Ang- 6.02 *m*; Me-2.03 *m*; Me-1.89 *m*; Ac-2.06 *s*.

Montanolide (8α-senecioyloxy,11α-acetoxy-10β-hydroxy-6αH,7αH-guaian-3-en-12,6-olide)

A transparent, sticky liquid of a molecular formula $C_{22}H_{30}O_7$. HR-MS analyses: pseudomolecular ion peaks at *m/z* 429.1910 (calculated for $[M + Na]^+$429.1884) and 445.1649 (calculated for $[M + K]^+$ 445.1623). IR analyses:ν_{max} 3464.1, 1784.6, 1711.6, 1229.3, 1144.4 cm^{-1}. NMR analyses: ^1H NMR δ: H-1 2.40 *m*; H-2α 2.04 *m*, H-2β 2.12 *d* (9.6 *Hz*); H-3 5.56 *bs*; H-5 2.58 *q* (5.7 *Hz*); H-6 4.71 *dd* (11.4 *Hz*; 10.2 *Hz*); H-7 3.61 *dd* (11.1 *Hz*; 10.2 *Hz*); H-8 5.52 *dd* (11.1 *Hz*; 9.6 *Hz*), H-9α 1.99 *d* (9.6 *Hz*), H-9β 1.82 *m*; Me-13 1.55 *s*, Me-14 1.22 *s*; Me-15 1.88 *s*; Sen- 5.62 *m*; Me-2.20 *m*; Me-1.94 *m*; Ac-2.08 *s*.

Tarolide (8α,11α-diangeloyloxy-10β-hydroxy-6αH,7αH-guaian-3-en-12,6-olide)

The transparent needle-like crystal of the molecular formula $C_{25}H_{34}O_7$. HR-MS analyses: molecular ion peak at *m/z* 447.2400 (calculated for $[M + H]^+$ 477.2377), as well as two pseudomolecular ion peaks at *m/z* 469.2214 and 485.1693 (calculated for $[M + Na]^+$ and $[M + K]^+$469.2197 and 485.1936, respectively). IR analyses: ν_{max} 3495.1, 2925.1, 1764.5, 1695.3, 1262.6, 1145.0 cm^{-1}. NMR analyses: ^1H NMR δ: H-1 2.43 *qui* (5.4 *Hz*); H-2α 2.13 *m*, H-2β 2.19 *m*; H-3 5.56 *bs*; H-5 2.61 *q* (5.7 *Hz*); H-6 4.78 *dd* (11.7 *Hz*; 10.2 *Hz*); H-7 3.77 *dd* (11.4 *Hz*; 10.2 *Hz*); H-8 5.60 *dd* (11.4 *Hz*; 9.0 *Hz*), H-9α 2.07 *m*, H-9β 1.85 *m*; Me-13 1.61 *s*, Me-14 1.23 *s*; Me-15 1.91 *s*; Ang- 6.18 *m*; Me-2.00 *m*; Me-1.84 *m*; Ang-6.13 *m*; Me-2.00 *m*; Me-1.86 *m*.

Microorganisms

The microorganisms tested included five bacterial and five fungal species: Gram-negative bacteria *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), and *Salmonella typhimurium* (ATCC 13311), Gram-positive bacteria *Listeria monocytogenes* (NCTC 7973) and *Staphylococcus aureus* (ATCC 6538), and fungi *Aspergillus fumigatus* (human isolate), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 10509), *Candida albicans* (ATCC 10231) and *Candida krusei* (clinical isolate). Bacterial species were maintained in Mueller Hinton Agar and Trptone Soy Agar (MHA, TSA, Torlak, Serbia). Fungi were maintained on Malt Agar and Potato Dextrose Agar (MA, PDA, Torlak, Serbia). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia.

Antimicrobial activity

The minimum inhibitory (MIC), minimum bactericidal (MBC) and minimum fungicidal (MFC) concentrations were determined by the microdilution method.[21]

The minimum inhibitory and bactericidal concentrations (MICs and MBCs) were determined using 96-well microtitre plates. The bacterial suspension was adjusted with sterile saline to a concentration of 1.0×10^5 CFU/ml. Dry chloroform extracts were dissolved in 5% DMSO solution containing 0.1% Tween 80 (*v/v*) (100 mg/ml) and added in Tryptic Soy broth (TSB) medium (100 µl) with bacterial inoculum (1.0×10^4 CFU per well) to achieve the wanted concentrations (0.25-25 mg/ml for extracts and 0.01-1 mg/ml for compounds). The MICs of the samples were detected following the addition of 40 µl of INT (0.2 mg/ml) and incubation at 37 °C for 30 min.[22] The MBCs were determined by serial sub-cultivation of 10 µl into microtitre plates containing 100 µl of broth per well and further incubation for 24 h. Streptomycin (Sigma, Steinheim, Germany) was used as a reference compound.

In case of the antifungal activity evaluation, a modified microdilution test was used. The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spores suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 µl per well. The inoculum was stored at 4 °C for further use. Dilutions of extract were carried out over the wells containing 100 µl of MA and afterwards, 10 µl of inoculum with fungal spores was added to all the wells. The microplates were incubated for 72 h at 28 °C. The lowest concentration without visible growth (checked on binocular microscope), was defined as MIC. The fungicidal concentrations (MFCs) were determined by serial sub-cultivation of 10 µl into microplates containing 100 µl of MA per well and further

incubation at the same conditions. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. Commercial fungicide ketoconazole (Zorkapharma, Šabac, Serbia) was used as a reference compound.

Biofilm susceptibility test

The effect of selected antimicrobial agents on biofilm of *C. albicans* and *C. krusei* were determined as previously described by Pierce et al.,[23] with some modifications. Briefly, fresh overnight cultures of *C. albicans* and *C. krusei* were harvested from liquid cultures for cells and resuspended for suspensions of 1×10^6 cells/ml final density. Biofilms was formed in 96-well polystyrene flat bottom TC treated microtitre plates (Sarstedt, USA). The plates were incubated at 37 °C for 48 h to allow biofilm formation. The culture media was removed, and the wells were washed three times with saline solution to remove the non-adhered cells. The extract and compounds solution (200 µl) was added to the wells. The microplates were incubated at 37 °C for 24 h. Three repetitions were performed. Fluconazole (Fluka, Newport News, VA, USA) was used as a reference compound. After 24 h at 37 °C, biofilm reduction was determined by following staining process[22] and measuring the UV absorbance of stains at 620 nm using a plate reader. MIC was defined as the minimum concentration of antimicrobial agent that inhibited further growth of the initial biofilm, and MBC was defined as the concentration that resulted in level of luminescence presenting no bacterial growth (empty well).

Results and discussion

The effect of dry extracts of the underground parts of three *Laserpitium* species (chromatograms with marked constituents obtained by HPLC analyses of extracts are given in the **Figure 1**) in microdilution method on pathogenic bacteria and fungi are given in the **Table 1**. Gram-positive bacterium *S. aureus* and two Gram-negative bacteria *E. coli* and *S. typhimurium* were generally more affected than other bacterial strains tested (MIC 1.25 mg/ml for all extracts, and MBC 1.25 mg/ml for *L. latifolium* extract, MBC 1.25-2.5 mg/ml for *L. ochridanum* extract and MBC 2.5 mg/ml for *L. zernyi* extract). Extract of *L. latifolium* showed high effect also on the strains of *L. monocytogenes* and *P. aeruginosa* (MIC 1.25 mg/ml, MBC 2.5 mg/ml), showing the highest overall effect among all extracts tested. However, activity was lower than streptomycin that was used as a reference compound. The antifungal effects (**Table 2**) observed in microdilution method were the highest for *L. latifolium* extract against all strains, and for *L. zernyi* extract against *A. fumigatus* and *P. funiculosum* (MIC 1.25 mg/ml and MFC 2.5 mg/ml). Still, exerted activities were weaker than those of ketokonazole, a reference compound.

All tested compounds showed promising antimicrobial effects being comparable to or even higher than reference compound used (**Table 3**). All compounds showed higher antibacterial activity against *E. coli* that the streptomycin and effects of laserpitine, isomontanolide and montanolide equal to that of streptomycin against *S. typhimurium*.

The highest antifungal effect was the one of isomontanolide (lowest MIC/MFC values among the tested compounds). Activity of this compound, as well as those of

montanolide and laserpitine, was higher against *A. fumigatus*, *A. niger* and *P. funiculosum* than those of ketoconazole (**Table 4**).

In the antibiofilm test on two *Candida* strains, extract of *L. latifolium* was the most effective among the tested extracts, and its MIC/MFC values were comparable to those of fluconazole (**Table 5**). All tested compounds showed high biofilm inhibiting properties in microplate biofilm assay, being isomontanolide the most active (MIC 0.25 mg/ml, MFC 0.5 mg/ml). Laserpitine and montanolide also exerted high activities; while activity of tarolide was slightly lower (**Table 6**). Interestingly, all compounds showed more pronounced effect than fluconazole, a reference compound.

Our results stand in line with some previously published data on antimicrobial activity of the compounds with a similar structure. Laserpitine (**Figure 2**), the dominant constituent of investigated *L. latifolium* chloroform extract, was the first isolated daucane ester from the roots of this species,[13] and its exact structure was established after several revisions. Cytotoxic activity of this compound was tested on two human breast adenocarcinoma cell lines and it did not cause significant effect, except in MTT test on invasive human breast adenocarcinoma cell line.[9] Apart from that, to our best knowledge, other activities of laserpitine were not investigated to date. Some daucane esters, mainly isolated from species of the genus *Ferula*, possessed antimicrobial properties. A daucane ester, 14-(*o*-hydroxycinnamoyloxy)-dauc-4,8-diene from the underground parts of *F. communis* Linn. exerted effect against four Gram-positive bacterial strains.[15] Another derivative of 14-dauc-4,8-diene, as well as jaescheanadiol ester from the roots of *F. hermonis* Boiss exerted strong effect (MIC 1.5-4.0 μg/ml) on non- and methicillin-resistant *S. aureus*.[17] Furthermore, three jaescheanadiol esters from roots of the same species exerted strong effect against two Gram-positive strains: *S.*

aureus and *B. subtilis*, and two *Mycobacterium* strains: *M. bovis* BCG and *M. tuberculosis* H37Rv.[16]

On the other hand, despite wide range of biological activities of sesquiterpene lactones, activity of guaianolides of slovanolide type was poorly investigated so far. Isomontanolide, montanolide and tarolide are slovanolides that are commonly distributed in different plant organs/parts of *Laserpitium* species.[25-27] Previously we have shown that those three lactones do not cause any significant cytotoxicity in human breast adenocarcinoma cell lines.[10] However, considering antimicrobial effects of those compounds, only isomontanolide, isolated from the roots of *L. siler* was reported to reduce bacterial growth (MIC 128 μg/ml) of fast-growing mycobacteria.[28] A trilobolide, guaianolide lactone closely related to thapsigargin, that was isolated from *L. siler* and *L. archangelica*, belongs to potent chemiotherapeutics in therapy of slow proliferative carcinomas, and its potential application in resistant microorganisms was also reported.[12] Our results may point out the potential of these slovanolides as antimicrobial agents that might be further considered for antimicrobial food preservation.

Conclusions

Sesquiterpene lactones, principal components of extracts of *L. zernyi* and *L. ochridanum* underground parts, as well as laserpitine, the most abundant compound in extract of *L. latifolium* underground parts, showed promising antimicrobial properties in tested food spoilage bacteria and fungi, and in the tests of inhibition of biofilm formation. Effect of all tested plant metabolites was higher than fluconazole, a reference compound. Among the tested compounds, guaianolide lactone isomontanolide showed highest effect. However, further tests aiming the possible mode of action are required, in order to

explain the efficiency of these compounds. Further studies should clarify the possible safe doses that might be applied in actual food systems in order to prolong shelf life of food products and keep the product safe from microbial contamination.

Competing interests

The authors declare no competing financial interest.

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Table 1: Activity of extracts of the underground parts of three *Laserpitium* species and a reference compound streptomycin, against food spoilage microbes in microdilution test. MIC – minimum inhibitory concentration, MBC – minimum bactericidal concentration in mg/ml.

	L. latifolium		*L. zernyi*		*L. ochridanum*		Streptomycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
L. monocytogenes	1.25	2.50	5	7.50	2.50	5.00	0.05	0.10
S. aureus	1.25	1.25	1.25	2.50	1.25	1.25	0.05	0.10
E. coli	1.25	1.25	1.25	2.50	1.25	2.50	0.20	0.40
P. aeruginosa	1.25	2.5	5.00	7.50	2.50	5.00	0.05	0.10
S. typhimurium	1.25	1.25	1.25	2.50	1.25	1.25	0.10	0.20

Table 2: Activity of extracts of the underground parts of three *Laserpitium* species and a reference compound ketoconazole against fungal strains in microdilution test. MIC – minimum inhibitory concentration, MFC – minimum fungicidal concentration in mg/ml.

	L. latifolium		*L. zernyi*		*L. ochridanum*		Ketoconazole	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
A. fumigatus	1.25	2.50	1.25	2.50	2.50	5.00	0.20	0.50
A. niger	1.25	2.50	2.50	5.00	2.50	5.00	0.20	0.50
C. albicans	1.25	2.50	7.50	10.00	5.00	7.50	-	-
C. krusei	1.25	2.50	7.50	10.00	5.00	7.50	-	-
P. funiculosum	1.25	2.50	1.25	2.50	2.50	5.00	1.00	1.00

403 **Table 3.**Activity of laserpitine (LAS), tarolide (TAR), isomontanolide (ISM), montanolide (MON), and a reference compound
404 streptomycin against food spoilage microbes in microdilution test. MIC – minimum inhibitory concentration, MBC – minimum
405 bactericidal concentration in mg/ml.

	LAS		TAR		ISM		MON		Streptomycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
L. monocytogenes	0.20	0.30	0.20	0.30	0.10	0.30	0.20	0.30	0.05	0.10
S. aureus	0.10	0.20	0.10	0.20	0.10	0.20	0.10	0.20	0.05	0.10
E. coli	0.10	0.20	0.20	0.30	0.20	0.30	0.10	0.20	0.20	0.40
P. aeruginosa	0.20	0.30	0.20	0.30	0.20	0.30	0.20	0.30	0.05	0.10
S. typhimurium	0.10	0.20	0.20	0.30	0.10	0.20	0.10	0.20	0.10	0.20

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414 **Table 4.**Activity of laserpitine (LAS), tarolide (TAR), isomontanolide (ISM), montanolide (MON), and a reference compound
415 ketoconazole against fungal strains in microdilution test. MIC – minimum inhibitory concentration, MFC – minimum fungicidal
416 concentration in mg/ml.

	LAS		TAR		ISM		MON		Ketoconazole	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
A. fumigatus	0.20	0.20	0.50	0.60	0.05	0.20	0.10	0.20	0.20	0.50
A. niger	0.10	0.20	0.50	0.60	0.10	0.20	0.10	0.20	0.20	0.50
C. albicans	0.20	0.30	0.40	0.50	0.05	0.10	0.20	0.30	-	-
C. krusei	0.20	0.30	0.40	0.50	0.20	0.30	0.20	0.30	-	-
P. funiculosum	0.10	0.20	0.50	0.60	0.05	0.20	0.10	0.20	1.00	1.00

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Table 5. Activity of extracts of the underground parts of three *Laserpitium* species and a reference compound fluconazole against biofilm (plaque) formation of two *Candida* strains. MIC – minimum inhibitory concentration, MFC – minimum bactericidal concentration in mg/ml.

	L. latifolium		*L. zernyi*		*L. ochridanum*		Fluconazole	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans	6.3	12.5	15.0	15.0	10.0	25.0	8.0	9.0
C. krusei	6.3	12.5	37.5	37.5	10.0	25.0	2.0	3.0

Table 6. Activity of laserpitine (LAS), tarolide (TAR) isomontanolide (ISM), montanolide (MON), and a reference compound fluconazole against biofilm (plaque) formation of two *Candida* strains. MIC – minimum inhibitory concentration, MFC – minimum fungicidal concentration in mg/ml.

	LAS		TAR		ISM		MON		Fluconazole	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans	0.40	1.00	1.00	1.00	0.25	0.50	0.40	1.00	8.00	9.00
C. krusei	0.40	1.00	1.00	1.00	0.25	0.50	0.40	1.00	2.00	3.00

Figure 1. HPLC chromatograms of methanol-soluble parts of extracts (1 mg/ml) of underground parts of three *Laserpitium* species (225 nm) in the HPLC conditions optimised for a terpenoid separation. a) Chromatogram of the extract of *L. latifolium*; b) Chromatogram of the extract of *L. zernyi*; c) Chromatogram of the extract of *L. ochridanum*. Constituents are marked as follows: 1-Laserpitin, 2-Isomontanolide, 3-Montanolide and 4-Tarolide.

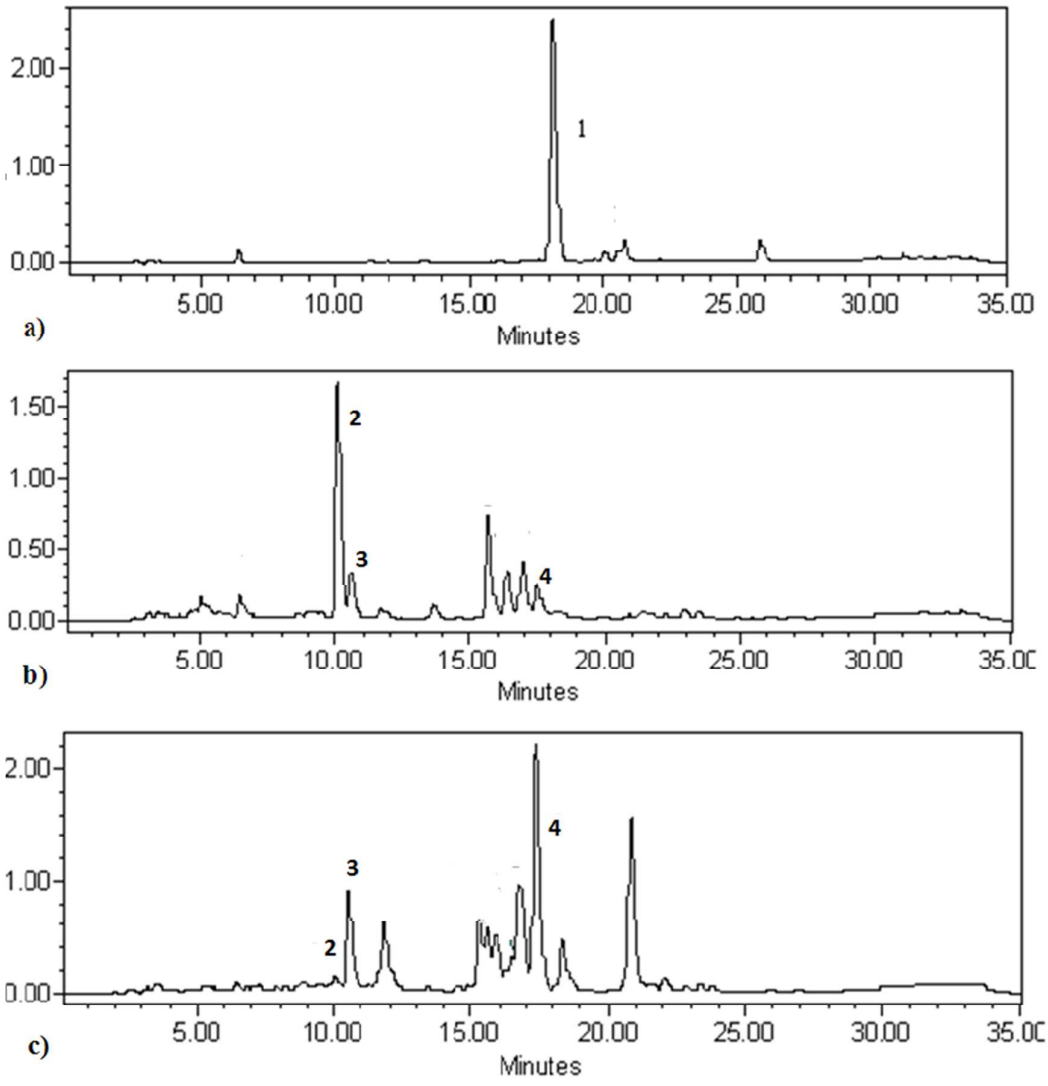

Figure 2. Structures of laserpitine, isomontanolide, montanolide and tarolide.