Journal of Ethnopharmacology 176 (2015) 297–304

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Ethnopharmacological uses of *Sempervivum tectorum* L. in southern Serbia: Scientific confirmation for the use against *otitis* linked bacteria

Dejan Stojković [a,*], Lillian Barros [b], Jovana Petrović [a], Jasmina Glamoclija [a], Celestino Santos-Buelga [c], Isabel C.F.R. Ferreira [b], Marina Soković [a,*]

[a] Institute for Biological Research "Siniša Stanković", Department of Plant Physiology, University of Belgrade, Bulevar Despota Stefana 142, 11000 Belgrade, Serbia
[b] Mountain Research Centre (CIMO), School of Agriculture, Polytechnic Institute of Bragança, Campus de Santa Apolónia, 1172, 5301-855 Bragança, Portugal
[c] Grupo de Investigación en Polifenoles (GIP-USAL), Faculty of Pharmacy, University of Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain

ARTICLE INFO

Article history:
Received 7 May 2015
Received in revised form
4 November 2015
Accepted 5 November 2015
Available online 10 November 2015

Keywords:
Ethnopharmacological survey
Sempervivum tectorum L.
Otitis
Phenolic compounds
Organic acids
Antimicrobial activity

ABSTRACT

Ethnopharmacological relevance: *Sempervivum tectorum* L. (Crassulaceae), known as houseleek, is used in traditional medicine in the treatment of ear inflammation. It can be spread as a pack on wounds, sores, burns, and abscesses and also on painful areas attacked by gout as a refrigerant and astringent. Drinking tea prepared from leaves of *S. tectorum* is recommended for ulcer treatment. The present study was designed to investigate ethopharmacological use of *S. tectorum* in the southern Serbia and to further scientifically justify and confirm effectiveness of the leaf juice used in ethnomedicine for ear inflammation, against *otitis* linked bacteria.

Material and methods: Ethnopharmacological survey on the use of *S. tectorum* in southern Serbia was performed using semi structured questionnaires via a face-to-face interview. Chemical composition of the leaf juice regarding phenolic compounds and organic acids was analyzed. Antimicrobial activity was tested on bacteria isolated from ear swabs of the patients suffering from the ear pain (*otitis*). Anti-quorum-sensing activities of the juice were further investigated on *Pseudomonas aeruginosa*.

Results: Ethnopharmacological survey revealed the use of *S. tectorum* in southern Serbia for the treatment of ear pain, warts, cancer, stomachache, ulcer and high blood sugar level with the highest fidelity level (FL) for the ear pain. The phenolic composition of the *S. tectorum* leaf juice consisted of flavonol glycosides, with kaempferol-3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside as the majority compound. Organic acids composition revealed malic acid as the most dominant one. Antimicrobial and anti-quorum-sensing activities of the juice showed to be promising.

Conclusion: Ethnopharmacological use of *S. tectorum* juice for treating ear pain is justified, since the juice possessed antimicrobial activity towards clinical isolates of bacteria linked to *otitis*.

1. Introduction

Traditional medicines are continuously increasing in their status, and show that there are some features, which are unique to them, contributing to both efficacy and safety (Nanjan, 2010). In recent years, special attention has been given to alternative natural bio-remedies to cure different diseases (Savikin et al., 2013) because of their less or no side effects, high efficacy and low cost.

Sempervivum tectorum L. (Crassulaceae), known as houseleek, is an evergreen plant with perennial root, crowned with imbricated fleshy leaves, which are smooth on both sides and ciliated at the margin, with the stem rising from the center of the tuft of leaves and terminated with a cymose corymb flowers (Muselin et al., 2014). Its use in the treatment of ear inflammation in Serbian folk medicine has been reported (Savikin et al., 2013), having also an antinociceptive activity (Alberti et al., 2012). Fresh juice from squeezed leaves of *S. tectorum* is used as a folk medicine almost exclusively for external purposes. It can be spread as a pack on wounds, sores, burns, and abscesses and also on painful areas attacked by gout as a refrigerant and astringent. Drinking tea prepared from leaves of *S. tectorum* is recommended for ulcer treatment (Bremness, 1996).

Otitis is a general term used to describe inflammation or infection of the ears. *Otitis* is classified as *otitis interna*, *otitis media* and *otitis externa*, depending on it affects inner ear, middle ear and outer ear and canals, respectively (NIDOCD, 2006). *Otitis media* is linked to inflammation of the middle ear, which often

* Corresponding authors. Fax: +351-273-325405.
E-mail addresses: mris@ibiss.bg.ac.rs (D. Stojković), iferreira@ipb.pt (I.C.F.R. Ferreira).

http://dx.doi.org/10.1016/j.jep.2015.11.014
0378-8741/

begins when infections that cause sore throats, colds, or other respiratory or breathing problems spread to the middle ear (Berman et al., 1997). Acute *otitis media* is a bacterial infection of the mucosal line ear (Culpepper and Froom, 1997). Chronic suppurative *otitis media* is the condition when the ear drum has been perforated. In an acute attack of *otitis media* the infection remains patent and becomes chronic upon secondary invaders: *Staphylococcus aureus* and *P. aeruginosa* (Tierney et al., 2005). *Otitis externa* may be caused by bacteria and fungi, particularly *P. aeruginosa*, *S. aureus*, *Candida albicans* and some *Aspergillus* species (Karma et al., 1978).

Quorum sensing (QS) is an intercellular signaling system in which bacteria communicate and regulate gene expression by releasing small compounds called autoinducers in the environment. Due to its role in various regulatory processes it can serve as an important target. Knowledge about quorum sensing is resulting in identification of new targets for therapeutics against *P. aeruginosa* infection (Petrović et al., 2014; Glamočlija et al., 2015).

The aim of this study was first to investigate folk use of *S. tectorum* in Vranje area, southern Serbia. After the data about the use in folk medicine were obtained, chemical characterization of the squeezed *S. tectorum* juice was analyzed by HPLC–DAD–ESI/MS. Since the fidelity level revealed the most frequent use of *S. tectorum* for ear inflammation, bacteria were isolated from several patients suffering with *otitis*. Antimicrobial activity of *S. tectorum* juice was tested against the isolated bacteria. Furthermore, influence of the juice was tested against certain quorum-sensing-regulated functions in *Pseudomonas aeruginosa* clinical isolate.

2. Material and methods

2.1. Ethnopharmacological investigation

2.1.1. Study area

Vranje is administrative, health care, educational and cultural center of Pcinja district, located in southern Serbia. The City of Vranje covers the space of 860 km^2. Vranje is the economic, political and cultural center of Pčinja District consisting of Bosilegrad, Bujanovac, Vladičin Han, Preševo, Surdulica, Trgovište and Vranje municipalities. The city is located in the southwest of Vranje valley, on the left bank of the South Morava River (Fig. 1). All the participants interviewed were living in the municipality of Vranje or surrounding villages.

2.1.2. Ethnopharmacological survey

The survey was performed using semi structured questionnaires via a face-to-face interview and circulating these questionnaires among cross section of people above 20 years of age; 212 filled up reports were collected visiting the Vranje area in southern Serbia. A questionary in Serbian language was prepared about the use of *S. tectorum* L. (Crassulaceae) by the local people in Vranje area. All the respondents were aware of the present investigation and have signed the informed consent. The survey was conducted during two months. The survey covered different age groups of both the sexes, whose gender, age, educational background, professional status and knowledge on the use of *S. tectorum* were also documented. Each participant was interviewed separately to generate data on diseases, regarding the treatment through medicinal plant *S. tectorum*. The record of questionnaires used included the following information: (a) the local name,

Fig. 1. Study area-location of the city of Vranje on the map of Serbia and Europe.

(b) part of the plant used, (c) method of preparation, (d) mode of application, and (e) ethnomedicinal uses.

Fidelity level (FL) was applied for diseases or ailments that were reported (Sarma and Devi, 2015). It is a ratio of informants claiming the use of a plant species for a particular purpose (Np) and number of informants using the plant to treat any disease (*N*). It was calculated by the expression:

$$FL(\%) = Np/N \times 100$$

2.2. Chemical characterization

2.2.1. Sample preparation

S. tectorum L. (Crassulaceae) (local name: čuvarkuća; english name: houseleek) was a cultivated species, collected during the summer of 2014. A voucher specimen was deposited at the Herbarium of the Institute for Biological Research "Siniša Stanković" (University of Belgrade, Serbia), under the code ST-14-DSSD. Fresh leaves were harvested and gently pressed to obtain juice. Then, the juice was filtered through Whatman No. 4 paper and further kept at −20 °C prior to lyophilisation. The sample was lyophilized (LH Leybold, Lyovac GT2, Frenkendorf) in order to obtain crude mass of juice that was used for further experiments.

2.2.2. Phenolic compounds extraction and analysis

Phenolic extraction was performed using the squeezed and lyophilized *S. tectorum* juice (0.5 g) stirring with 30 mL of methanol:water (80:20, *v*/*v*) at 25 °C and 150 rpm for 1 h, and filtered through Whatman No. 4 paper. The residue was then extracted with one additional 30 mL portion of the hydromethanolic mixture. The combined extracts were evaporated at 35 °C under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) to remove the methanol. For purification, the extract solution was deposited onto a C-18 SepPak® Vac 3cc cartridge (Phenomenex), previously activated with methanol followed by water; sugars and more polar substances were removed by passing through 10 mL of water and the phenolic extract was further eluted with 5 mL of methanol:water (80:20, *v*/*v*). The extract was concentrated under vacuum, lyophilized, re-dissolved in 1 mL of 20% aqueous methanol and filtered through a 0.22-μm disposable LC filter disk for HPLC analysis.

Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA) as previously described by the authors (Barros et al., 2013a). Double online detection was carried out in a DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were identified by comparing their retention time, UV–vis and mass spectra with those obtained from standard compounds, when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of other compound from the same phenolic group: kaempferol 3-*O*-glucoside ($y=236.33x+70.006$; $R^2=1$); kaempferol 3-*O*-rutinoside ($y=182.94x+96.644$; $R^2=1$) and quercetin-3-*O*-rutinoside ($y=280.87x+373.73$; $R^2=0.999$). The results were expressed in μg per g of methanolic extract.

2.2.3. Organic acids extraction and analysis

Organic acids were determined using ultra-fast liquid chromatography coupled to a photodiode array detector (UFLC–PDA). Samples (~0.5 g) were extracted by stirring with 10 mL of meta-phosphoric acid (25 °C at 150 rpm) for 25 min and subsequently filtered through Whatman No. 4 paper. Before analysis, the sample was filtered through 0.2 μm nylon filters (Barros et al., 2013b). The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan). Separation was achieved on a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C_{18} column (5 μm, 250 mm × 4.6 mm i.d.) thermostatted at 35 °C. The elution was performed with sulfuric acid (3.6 mM) using a flow rate of 0.8 mL/min. Detection was carried out in a PDA, using 215 and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 and 245 nm with calibration curves obtained from commercial standards of each compound: ascorbic acid ($y=1E+08x+751815$; $R^2=0.999$); citric ($y=1E+06x+16276$; $R^2=1$); fumaric acid ($y=148083x+96092$; $R^2=0.999$); malic acid ($y=863548x+55571$; $R^2=1$); oxalic acid ($y=9E+06x+377946$; $R^2=0.998$); succinic acid ($y=603298x+4994.1$; $R^2=1$). The results were expressed in mg per g of dry weight (dw).

2.3. Antimicrobial activity

2.3.1. Microorganisms

Bacteria used in this study were collected and cultured from the ear swab of the patients suffering from *otitis*. All samples were collected using standard microbiological techniques in the Microbiological laboratory of the Institute of Public Health, Health Center Vranje. Bacteria were isolated and identified using standardized microbiological techniques. Isolated clinical bacteria from ear swabs were: *Proteus mirabilis* (1 isolate), *P. aeruginosa* (2 isolates) and *S. aureus* (1 isolate). *S. aureus* (ATCC 6538) and *P. aeruginosa* (ATCC 27853) were used for the comparison of activities with clinical isolates.

2.3.2. Antibacterial activity

The antibacterial assay was carried out by a microdilution method (CLSI, 2009). The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0×10^5 CFU/mL. *S. tectorum* juice was dissolved in 5% DMSO solution containing 0.1% Tween 80 (*v*/*v*) (10 mg/mL) and added in Tryptic Soy broth (TSB) medium (100 μL) with bacterial inoculum (1.0×10^4 CFU per well). The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (Minimum Inhibitory Concentration; MIC). The MICs obtained from the susceptibility testing of various bacteria to tested juice were determined also by a colorimetric microbial viability assay based on reduction of an INT color (*p*-iodonitrotetrazolium violet [*syn*, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride]; Sigma) and compared with positive control for each bacterial strain (Tsukatani et al., 2012). The MBCs (Minimum Bactericidal Concentration) were determined by serial sub-cultivation of 2 μL into microtitre plates containing 100 μL of broth per well and further incubation for 24 h. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at a wavelength of 655 nm by Microplate manager 4.0 (Bio-Rad Laboratories) and compared with a blank (broth medium plus diluted lyophilized juice) and the positive control. Streptomycin (Sigma P 7794) and Ampicillin (Panfarma, Belgrade, Serbia) were used as positive controls (1 mg/mL in sterile physiological saline). Five percent DMSO was used as a negative control.

2.4. Antiquorum (AQ) sensing activity

P. aeruginosa used in this study was a clinical isolate. Bacteria were routinely grown in Luria-Bertani (LB) medium (1% *w*/*v* NaCl,

1% *w*/*v* Tryptone, 0.5% *w*/*v* yeast extract) with shaking (220 rpm) and cultured at 37 °C.

Filter paper discs were impregnated with tested juice solutions (subMIC; 0.30 mg/disc, 0.15 mg/disc, 0.07 mg/disc), streptomycin and ampicillin (subMIC; 0.5 MIC, 0.25 MIC and 0.125 MIC; 0.1, 0.05, 0.025 mg/disc for streptomycin and 0.4, 0.2 and 0.1 mg/disc for ampicilin) to determine whether they have antiquorum activity against bacteria and impair bacterial growth. To do this, filter paper (filter paper 4 mm; Whatman) was used. Discs were soaked in the indicated solutions, then dried at room temperature (3 h, protected from light), and aseptically placed onto the plates prior to bacterial inoculation. After incubation, it was recorded whether the inhibition or antiquorum zones were obtained (Petrović et al., 2014).

2.4.1. Twitching and flagella motility

After growth in the presence or absence of juice, the cells of *P. aeruginosa* (clinical isolate) were washed twice with sterile PBS and resuspended in PBS at 1×10^8 cfu/mL (OD of 0.1 at 660 nm). Briefly, cells were transferred into a nutrient agar plate with a sterile toothpick and incubated overnight at 37 °C. Plates were then removed from the incubator and kept at room temperature for two more days. Colony edges and the zone of motility were measured with a light microscope (O'May and Tufenkji, 2011; Petrović et al., 2014). Fifty microlitres of tested juice (sub MIC; 0.5 MIC, 0.25 MIC and 0.125 MIC) was mixed into 10 mL of molten MH medium and poured immediately over the surface of a solidified LBA plate as an overlay. The plate was point inoculated with an overnight culture of *P. aeruginosa* (clinical isolate) once the overlaid agar had solidified and incubated at 37 °C for 3 days. The twitching motility was evaluated by measuring the area of the colony on swimming plates (Glamočlija et al., 2015).

2.4.2. Influence of S. tectorum on pyocyanin production in P. aeruginosa

Overnight culture of *P. aeruginosa* PA01 was diluted to OD_{600} 0.2. Then, tested juice (250 µL dissolved as 0.5 MIC, 0.25 MIC and 0.125 MIC) was added to *P. aeruginosa* (4.75 mL) and incubated at 37 °C for 24 h. The treated culture was extracted with chloroform (3 mL), followed by mixing the chloroform layer with 0.2 M HCl (1 mL). Absorbance of the extracted organic layer was measured using the UV–visible spectrophotometer (UV1601, Shidmazu, Kyoto, Japan) at 520 nm (Petrović et al., 2014).

3. Results and discussion

3.1. Ethnopharmacological uses of S. tectorum in Vranje area

Although the literature published previously (Savikin et al., 2013) revealed the use of *S. tectorum* in traditional medicine in some parts of Serbia (only for ear pain relief), ethnopharmacological investigation on the use of *S. tectorum* in the southern Serbia was not previously reported. Table 1 covers demographical information of the informants (gender, age cohort, education) from the Vranje municipality. Ninety-nine informants were females (70.71%) and 41 were males (29.29%). The informants aged between 50 and 80 were observed to have the highest participation rate (39.29%), followed by the group aged between 20 and 30 years with participation rate of 25%. The majority of educational status of the 140 informants was reported to be secondary education (63.57% of the total informants), followed by the informants with high educational status (26.43%). Table 1 also reports the ethnopharmacological use of *S. tectorum*. The ailments claimed to be treated by informants with *S. tectorum* included: ear pain, warts, cancer, stomachache, ulcer and high blood sugar level. Fidelity level (FL) revealed the most frequent use of *S. tectorum* in the treatment of ear pain (a total of 93 citations with FL of 66.43%). All of the informants claimed the use of directly squeezed *S. tectorum* juice in ears for ear pain relief. With FL of 32.14% skin warts are on the second place that could be treated with directly squeezed *S. tectorum* juice. Cancer with FL of 25.71% was on the third place for treatment with *S. tectorum*. The forms of folk remedies, in which *S. tectorum* is used for this ailment, mostly included combination of *S. tectorum* leaves with honey and lemon juice. Although with low FL, stomachache and ulcer are claimed to be treated with *S. tectorum* leaves prepared in the form of tea.

3.2. Chemical characterization of S. tectorum juice

The phenolic profile of *S. tectorum* leaves juice, recorded at 370 nm is shown in Fig. 2; peak characteristics, tentative identities and quantification are presented in Table 2. In global, eight phenolic compounds were detected being all flavonol derivatives, most of them derived from kaempferol possessing different patterns of sugar substitution (Table 2). The presence of kaempferol derivatives as major phenolic compounds in *S. tectorum* leaves was also reported by Abram and Donko (1999) and Alberti et al. (2008, 2012).

Peaks **1** and **3** were identified as quercetin derivatives owing to the product ion observed at *m/z* 301. MS^2 fragments of peak **1** revealed the alternative loss of deoxyhexosyl (*m/z* at 609; −146 u)

Table 1
Data obtained from ethnopharmacological survey about *Sempervivum tectorum* ethnomedicinal uses.

Gender		Age group		Education	
Male	41 (29.29%)	20–30	35 (25.00%)	No formal	2 (1.43%)
Female	99 (70.71%)	30–40	22 (15.71%)	Primary	12 (8.57%)
		40–50	28 (20.00%)	Secondary	89 (63.57%)
Total informants	140	50–80	55 (39.29%)	High	37 (26.43%)

Ethnopharmacological use	Citation for particular disease	Fidelity level (%)
Ear pain	93	66.43
Warts	45	32.14
Cancer	36	25.71
Stomachache	23	16.43
Ulcer	11	7.85
High blood sugar level	5	3.57

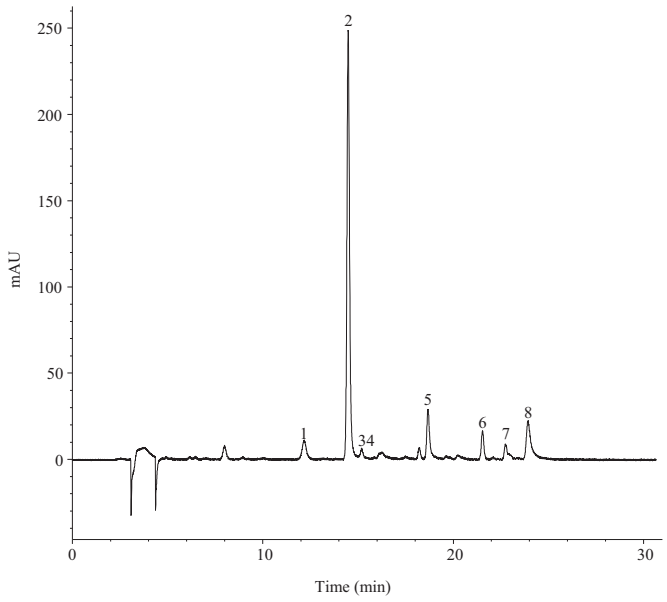

Fig. 2. HPLC chromatogram of the phenolic compounds in *S. tectorum* leaf juice recorded at 370 nm.

and deoxyhexosyl-hexoside (*m/z* at 447; −308 u) residues, indicating location of each residue on different positions of the aglycone, so that the compound was tentatively identified as quercetin-*O*-(deoxyhexosyl-hexoside)-*O*-deoxyhexoside. To the best of our knowledge this compound was not previously described in *S. tectorum* leaves. Peak **3**, with a pseudomolecular ion $[M–H]^-$ at *m/z* 609, released fragments at *m/z* 463 (−146 u; loss of deoxyhexosyl residue) and 301 (−162 u; loss of hexosyl residue). The presence of a compound with similar characteristics had already been observed in leaf juice samples of *S. tectorum* from Hungary by Alberti et al. (2008, 2012) and tentatively identified as rutin (i.e., quercetin-3-*O*-rutinoside). However, comparison with a rutin standard showed that the compound detected in our samples showed different chromatographic retention, so that it was just identified as a quercetin-*O*-deoxyhexosyl-hexoside.

Peaks **2** and **4–8** were identified as kaempferol glycosides based on their UV spectra and the production of an MS^2 fragment at *m/z* 285. Among them, only peak **8** was positively identified as kaempferol-3-*O*-glucoside according to its retention, mass and UV–vis characteristics by comparison with a commercial standard. A kaempferol hexoside was also reported in *S. tectorum* leaves by Alberti et al. (2008, 2012), although its precise identity was not given.

Mass characteristics of the majority peak **2** ($[M–H]^-$ at *m/z* 739) indicated that it corresponds to a kaempferol derivative bearing two deoxyhexosyl and one hexosyl residues. The observation of MS^2 fragments at *m/z* 593 (−146 u) and 431 (−308 u) indicated the alternative loss of deoxyhexose and deoxyhexosyl-hexose moieties, respectively, pointing to each of those residues are located at a different position onto the aglycone. A compound with the same characteristics was also reported by Alberti et al. (2008, 2012) as the most abundant flavonoid in leaf juice samples of *S. tectorum* from Hungary and positively identified as kaempferol-3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside by NMR (Alberti et al., 2012). Thus, that identity was also assumed for the compound detected herein.

Peaks **4** ($[M–H]^-$ at *m/z* 709), **5** ($[M–H]^-$ at *m/z* 593) and **7** ($[M–H]^-$ at *m/z* 577) showed similar fragmentation pattern as peak 2. In all cases, the loss of a deoxyhexosyl residue (146 u) was observed, yielding fragment ions at *m/z* 563 (peak 4), 447 (peak 5) and 431 (peak 7), together with another common fragment at *m/z* 431 from the respective losses of deoxyhexosyl-pentose (278 u), hexose (162 u) and deoxyhexose (146 u) moieties. As for compound 2, the alternative loss of two glycosyl residues pointed to they are located on different positions of the aglycone, so that these peaks were tentatively assigned as kaempferol-*O*-deoxyhexosyl-pentoside-*O*-deoxyhexoside (**4**) and kaempferol-*O*-deoxyhexoside-*O*-hexoside (**5**) and kaempferol-*O*-deoxyhexoside-*O*-deoxyhexoside (**7**). The pseudomolecular ion of peak **6** ($[M–H]^-$ at *m/z* 871) indicated that it corresponded to a kaempferol derivative bearing two deoxyhexoses, one pentose and one hexose. In this case, the observation of an MS^2 fragment ion at *m/z* 725 from the loss of a deoxyhexosyl group also pointed to that sugar was located at a different position in relation to the other glycosyl residues, which are lost together as revealed by the production of another fragment ion at *m/z* 285 (kaempferol). Thus, despite a fragment at *m/z* 431 from the alternative loss of those glycosyl residues was not observed, a tentative identity as kaempferol-*O*-deoxyhexosyl-pentosyl-hexoside-*O*-deoxyhexoside could be assumed for the compound.

From the mass spectra no information can be obtained about the nature and position of substitution of the sugar moieties, although, by analogy with peak **2**, the positions 3 and 7 could be suspected for the location of the glycosyl substituents, and the hexosyl and and deoxyhexosyl residues might correspond to glucose and rhamnose. Compounds with similar mass characteristics as peaks **4–7** were also found in leaf juices of *S. tectorum* samples by Alberti et al. (2012), although no identity of the type of sugars and substitution location was offered, either.

Furthermore, organic acids composition of the sample was investigated and the results are presented in Table 3. Malic acid was

Table 2
Retention time (R_t), wavelengths of maximum absorption (λ_{max}), mass spectral data, relative abundances of fragment ions, tentative identification and quantification of the phenolic compounds in *Sempervivum tectorum* leaf juice (mean ± SD).

Peak	R_t (min)	λ_{max} (nm)	Molecular ion $[M–H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (μg/g)
1	12.2	354	755	609(17),447(33),301 (17)	Quercetin-*O*-(deoxyhexosyl-hexoside)-*O*-deoxyhexoside	tr
2	14.5	348	739	593(69),431(15),285(9)	Kaempferol-3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside	1383 ± 5
3	15.2	350	609	463(100),301(33)	Quercetin-*O*-deoxyhesoyl-hexoside.	tr
4	18.2	348	709	563(42),431(12),285(8)	Kaempferol-*O*-deoxyhexosyl-pentoside-*O*-deoxyhexoside	30 ± 3
5	18.7	350	593	447(33),431(22),285 (15)	Kaempferol-*O*-deoxyhexoside-*O*-hexoside	180 ± 5
6	21.5	344	871	725(23),285(5)	Kaempferol-*O*-deoxyhexosyl-pentosyl-hexoside-*O*-deoxyhexoside	91 ± 1
7	22.7	348	577	431(33),285(48)	Kaempferol-*O*-deoxyhexoside-*O*-deoxyhexoside	55 ± 1
8	23.9	348	447	285(100)	Kaempferol-3-*O*-glucoside	195 ± 5
					Total flavonols	1934 ± 20

tr- Traces.

Table 3
Composition of organic acids in *S. tectorum* leaf juice (mean ± SD).

Organic acids	mg/g dw
Oxalic acid	0.90 ± 0.01
Malic acid	138 ± 4
Ascorbic acid	0.33 ± 0.02
Citric acid	63 ± 2
Succinic acid	42 ± 1
Fumaric acid	0.19 ± 0.01
Total organic acids	202 ± 2

dw – Dry weight.

found as the major organic acid present in the sample with 138 mg/g dw, followed by citric (63 mg/g dw) and succinic (42 mg/g dw) acids. Oxalic, ascorbic and fumaric acids were also identified (Table 3) in lower amounts (less than 1 mg/g dw). The concentration of the total organic acids was 202 mg/g dw. Previous data have shown that juice squeezed from *S. tectorum* contained citric (up to 20 mg/mL) and malic (up to 14 mg/mL) acids, as also ascorbic acid (205 mg/kg of fresh leaves), all determined by RP-HPLC (Sentjurc et al., 2003). Our results are in agreement with previous findings stating that organic acids in *Crassulaceae* family can be represented with more than 20% of dw (Pucher, 1942).

3.3. Antimicrobial activity of S. tectorum juice

In order to scientifically confirm the effectiveness of *S. tectorum* traditional use, we have further evaluated its antimicrobial and anti-quorum activities. Since the highest FL was reported for the use of *S. tectorum* juice in ear pain (*otitis*) issues, bacteria isolated from patients suffering *otitis* were used in antimicrobial tests. Although ear inflammation and pain might have different causes, the most frequently linked to *otitis* are bacterial infections (Rashid et al., 2014). Furthermore, previously reported antinociceptive activity (Alberti et al., 2012) might play a significant role in pain relief. The leading cause of *otitis* – bacteria isolated from ear swabs and their susceptibility to *S. tectorum* juice were not previously investigated. Table 4 presents the antimicrobial activity of *S. tectorum* juice on isolated and reference bacteria strains: *P. mirabilis*, *S. aureus* and *P. aeruginosa*. It is evident that the juice tested possessed antimicrobial activity towards pathogenic bacteria. Reference strain of *P. aeruginosa* (ATCC 27853) was the most susceptible to the effect of *S. tectorum* juice, with MIC of 0.153 mg/mL and MBC 0.290 mg/mL, followed by the two clinical isolates of *P. aeruginosa* that showed the same sensitivity to the tested juice with MIC of 0.290 mg/mL and MBC of 0.552 mg/mL. The most resistant strain was reference strain *S. aureus* (ATCC 6538) with equal range of values for MIC and MBC (1.990 mg/mL). The activity of *S. tectorum* juice against the assayed bacteria increased in order: *S. aureus* < *P. mirabilis* < *P. aeruginosa*. Since *P. mirabilis* and *P. aeruginosa* are Gram negative bacteria and *S. aureus* a Gram positive strain, the difference in MICs/MBCs might be due to the different structure of the bacterial cell wall. All of the assayed strains were more susceptible to the effects of standard antibacterial drugs like streptomycin and ampicillin (Table 4). The higher values of MICs and MBCs on the tested strains might be attributed to the possible resistance of the isolated strains, but further studies are necessary to confirm these findings. The antimicrobial activity of the tested sample could be linked to the organic acids and flavonol derivatives present in *S. tectorum*. A previous study conducted by Akroum et al. (2009) has described the *in vitro* antibacterial effects of kaempferol glucosides against bacteria including *S. aureus* and *P. aeruginosa*. The antimicrobial activity of malic acid is well documented against *B. subtilis* and *E. coli* with MIC values of 2 mg/mL (Gao et al., 2012). The same study showed the activity of citric and succinic acids with MICs of 1.667–6.667 mg/mL against tested bacteria.

Inhibition of the growth of selected food spoilage-related microorganisms in the presence of 5% addition of homogenized fresh leaves of *S. tectorum* L. in the culture media was reported previously (Abram and Donko, 1999). That study showed that the growth of *S. aureus* (94.5%) and *Bacillus cereus* (92.1%) was the most strongly inhibited, while the growth of *Geotrichum* sp. (60.0%) and *Enterococcus faecalis* (57.5%) was moderately inhibited, and that of *Escherichia coli* (9.3%) and *Proteus morgani* (5.1%) slightly inhibited (Abram and Donko, 1999).

Table 4
Antimicrobial activity of *Sempervivum tectorum* leaf juice and positive controls against bacteria causing ear infection (mg/mL).

Bacteria	*S. tectorum* (MIC/MBC)	Streptomycin (MIC/MBC)	Ampicillin (MIC/MBC)
Proteus mirabilis[a]	0.552 ± 0.003	0.150 ± 0.003	0.150 ± 0.003
	1.049 ± 0.015	0.300 ± 0.00	0.300 ± 0.00
Staphylococcus aureus[a]	1.049 ± 0.007	0.250 ± 0.020	0.100 ± 0.007
	1.990 ± 0.010	0.500 ± 0.007	0.150 ± 0.050
Staphylococcus aureus	1.990 ± 0.008	0.250 ± 0.030	0.100 ± 0.007
	1.990 ± 0.007	0.500 ± 0.007	0.150 ± 0.070
Pseudomonas aeruginosa[a]	0.290 ± 0.007	0.100 ± 0.00	0.200 ± 0.01
	0.552 ± 0.011	0.100 ± 0.00	0.200 ± 0.01
Pseudomonas aeruginosa[a]	0.290 ± 0.005	0.100 ± 0.00	0.200 ± 0.01
	0.552 ± 0.009	0.100 ± 0.00	0.200 ± 0.01
Pseudomonas aeruginosa	0.153 ± 0.015	0.050 ± 0.00	0.100 ± 0.00
	0.290 ± 0.015	0.100 ± 0.00	0.200 ± 0.01

[a] Clinically isolated bacteria.

3.4. Anti-QS activities of S. tectorum juice on P. aeruginosa

The flask incubation assay was used to quantify quorum sensing inhibitory activity of the *S. tectorum* sample. The tested juice demonstrated a concentration-dependent pyocyanin inhibitory activity and showed reduction of the pigment at all the assayed concentrations (0.5 MIC, 0.25 MIC and 0.125 MIC) (Table 5).

In addition to QS, the initiation of biofilm formation by *P. aeruginosa* depends on two cell-associated structures; the flagellum and type IV pili. The flagellum is responsible for swimming motility while the type IV pili are responsible for twitching motility. Both types of motility are important in the initial stages of biofilm formation by *P. aeruginosa* (Henrichsen, 1972; O'Toole and Kolter, 1998). Therefore, determination of the juice influence on both motilities was investigated. The motile *P. aeruginosa* strain was used as standard (control) for motility on swimming plates. In the absence of the juice, the colonies of *P. aeruginosa* were flat with a rough appearance displaying irregular colony edges (Fig. 3D) and a hazy zone surrounding the colony. After 2 days of incubation at

Table 5
Anti-quorum activitities of *Sempervivum tectorum* leaf juice against *Pseudomonas aeruginosa*.

S. tectorum		0.125 MIC	0.25 MIC	0.5 MIC	Control
Anti-quorum activity	Colony diameter (mm)	8.33 ± 0.58	7.67 ± 0.58	6.33 ± 0.58	21.67 ± 3.06
	Length of flagella shape (μm)	176 ± 48	152 ± 32	–	184 ± 40
	Pyocianin reduction (%)	12.31	25.42	77.23	–

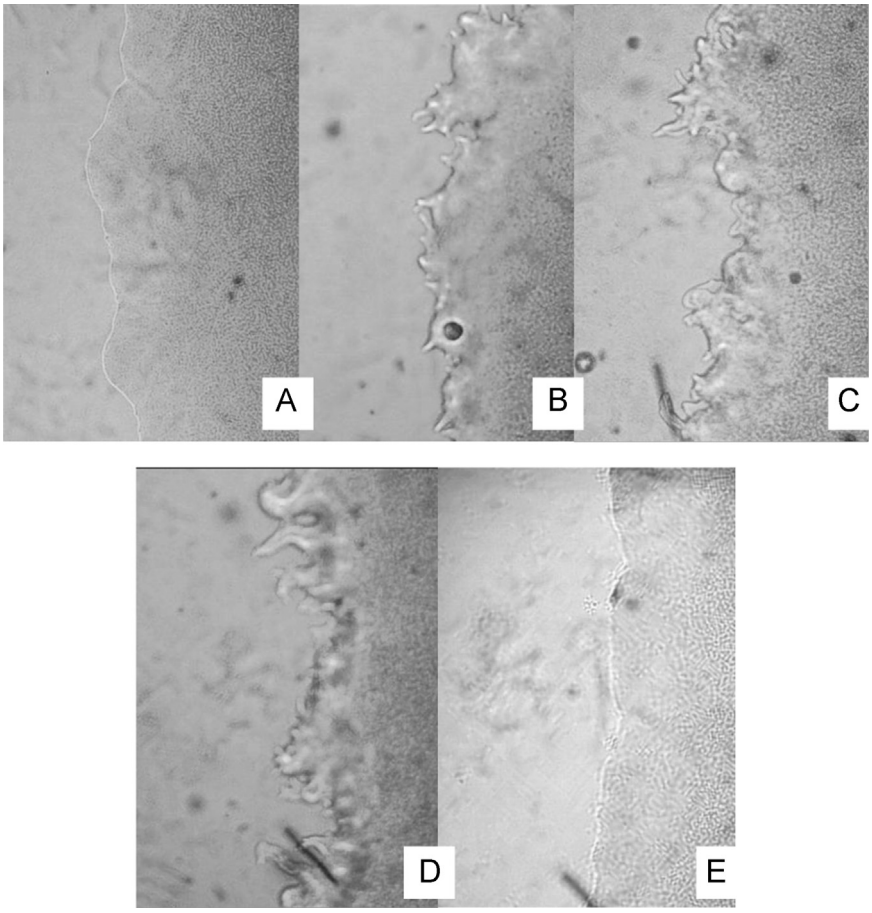

Fig. 3. Light microscopy of colony edges of *Pseudomonas aeruginosa* in twitching motility plates, grown in the presence or absence of *Sempervivum tectorum* juice. Colonies from the bacteria grown with *S. tectorum* juice at 0.5 MIC (A), 0.25 MIC (B) and 0.125 MIC (C). The colonies of *P. aeruginosa* produced a flat, widely spread, irregularly shaped colony in the absence of juice (D). *P. aeruginosa* colony with presence of Streptomycin without flagella shape (E). Magnification: (A–D)x200.

ambient temperature, colony expansion occurred very rapidly due to twitching motility (Table 5). Bacteria that were grown in the presence of the *S. tectorum* juice were incapable of producing such a twitching zone and had almost round, smooth, regular colony edges (Fig. 3A–C); the flagella were reduced completely at 0.5 MIC (Table 5 and Fig. 3A), while the samples treated with 0.25 MIC and 0.125 MIC showed reduced flagella shapes both in size and number (Table 5 and Figs. 3B and 3C), and the diameter of the colony swimming zones was also reduced (Table 5 and Fig. 3A–C). Streptomycin reduced the flagella completely (Fig. 3E).

4. Conclusions

Ethnopharmacological survey revealed the use of *S. tectorum* in southern Serbia for the treatment of ear pain, warts, cancer, stomachache, ulcer and high blood sugar level, with the highest fidelity level for the ear pain. The phenolic composition of *S. tectorum* leaf juice was characterised by the presence of flavonol glycosides, with kaempferol-3-*O*-rhamnosyl-glucoside-7-*O*-rhamnoside (1.383 mg/g dw) as the majority derivative. Organic acids quantification revealed malic acid as the most dominant one (138 mg/g dw). The moderate antimicrobial activity, tested on bacteria linked to *otitis*, highlighted and confirmed the reason for effectiveness of *S. tectorum* in ear pain relief. Furthermore, quorum sensing functions in *P. aeruginosa* were effectively regulated with leaf juice of *S. tectorum*. The observed antimicrobial activity could be linked mostly to the organic acids and probably to flavonol derivatives presented in the sample. More extensive studies are necessary to confirm these findings in ear pain relief, by formulating and standardizing *S. tectorum* pharmaceutical preparation and further *in vivo* confirmation should be explored on ear pain relief caused by bacteria linked to *otitis*.

Acknowledgments

The authors are thankful to Slobodan Davidovic for providing the sample of *S. tectorum* during the hectic interpersonal relations and to Danijela Stanisavljevic for helping during the interview process. The authors are grateful to the Ministry of Education, Science and Technological Development of Serbia for financial support (Grant no. 173032) and to the Foundation for Science and Technology (FCT, Portugal) for financial support to the research centre CIMO (PEst-OE/AGR/UI0690/2014).

References

Abram, V., Donko, M., 1999. Tentative identification of polyphenols in *Sempervivum tectorum* and assessment of the antimicrobial activity of *Sempervivum* L. J. Agric. Food Chem. 47, 485–489.

Akroum, S., Bendjeddou, D., Satta, D., Lalaoui, K., 2009. Antibacterial activity and acute toxicity effect of flavonoids extracted from *Mentha longifolia*. Am.–Eurasian J. Sci. Res. 4, 93–96.

Alberti, A., Blazics, B., Kery, A., 2008. Evaluation of *Sempervivum tectorum* L. flavonoids by LC and LC–MS. Chromatographia 68, S107–S111.

Alberti, A., Beni, S., Lacko, E., Riba, P., Al-Khrasani, M., Kery, A., 2012. Characterization of phenolic compounds and antinociceptive activity of *Sempervivum tectorum* L. leaf juice. J. Pharm. Biomed. Anal. 70, 143–150.

Barros, L., Pereira, E., Calhelha, R.C., Dueñas, M., Carvalho, A.M., Santos-Buelga, C., Ferreira, I.C.F.R., 2013a. Bioactivity and chemical characterization in hydrophilic and lipophilic compounds of *Chenopodium ambrosioides* L. J. Funct. Foods 5, 1732–1740.

Barros, L., Pereira, C., Ferreira, I.C.F.R., 2013b. Optimized analysis of organic acids in edible mushrooms from Portugal by ultra fast liquid chromatography and photodiode array detection. Food Anal. Methods 6, 309–316.

Berman, S., Byrns, P.J., Bondy, J., Smith, P.J., Lezotte, D., 1997. Otitis media-related antibiotic prescribing patterns, outcomes and expenditures in a pediatric medicaid population. Pediatrics 100, 585–592.

Bremness, L., 1996. Velika knjiga o zeliscih. In: Mladinska Knjiga: Ljubljana, Slovenija, (Translated from The Complete Book of Herbs; Dorling Kindersley Limited: London, U.K., 1988), p. 128.

CLSI – Clinical and Laboratory Standards Institute, 2009. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. In: Approved Standard, 8th ed. CLSI Publication M07-A8. Clinical and Laboratory Standards Institute, Wayne, PA.

Culpepper, L., Froom, J., 1997. Routine antimicrobial treatment of acute otitis media: is it necessary? J. Am. Med. Assoc. 278, 1643–1645.

Gao, Z., Shao, J., Sun, H., Zhong, W., Zhuang, W., Zhang, Z., 2012. Evaluation of different kinds of organic acids and their antibacterial activity in Japanese Apricot fruits. Afr. J. Agric. Res. 7 (35), 4911–4918.

Glamočlija, J., Ćirić, A., Nikolić, M., Fernandes, Â., Barros, L., Calhelha, R.C., Ferreira, I.C.F.R., Soković, M., Van Griensven, L.J.L.D., 2015. Chemical characterization and biological activity of Chaga (*Inonotus obliquus*), a medicinal "mushroom". J. Ethnopharmacol. 162, 232–332.

Henrichsen, J., 1972. Bacterial surface translocation: a survey and a classification. Bacteriol. Rev. 36, 478–503.

Karma, P., Jokipii, L., Ojala, K., Jokipii, A.M., 1978. Bacteriology of the chronically discharging middle ear. Acta Otolaryngol. 86, 110–114.

Muselin, F., Trif, A., Stana, L.G., Romeo, C.T., Gravila, C., Macinic, I., Dumitrescu, E., 2014. Protective effects of aqueous extract of *Sempervivum tectorum* L. (Crassulaceae) on aluminium-induced oxidative stress in rat blood. Trop. J. Pharm. Res. 13, 179–184.

Nanjan, M.J., 2010. Quality control and standardisation of traditional medicine/herbal drugs. In: Proceedings of an International Conference on Challenging and Emerging Dimensions in Medicinal/Herbal Plants and their Products.

NIDOCD, 2006. National Institute on Deafness and Other Communication Disorder, USA. pp. 65–69.

O'May, C., Tufenkji, N., 2011. The swarming motility of *Pseudomonas aeruginosa* is blocked by cranberry proanthocyanidins and other tannin-containing materials. Appl. Environ. Microbiol. 77, 3061–3067.

O'Toole, G.A., Kolter, R., 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. 30, 295–304.

Petrović, J., Glamočlija, J., Stojković, D., Nikolić, M., Ćirić, A., Fernandes, A., Ferreira, I.C.F.R., Soković, M., 2014. Bioactive composition, antimicrobial activities and the influence of *Agrocybe aegerita* (Brig.) Sing on certain quorum-sensing-regulated functions and biofilm formation by *Pseudomonas aeruginosa*. Food Funct. 5, 3296–3303.

Pucher, G.W., 1942. The organic acids of the leaves of *Bryophylum calycinum*. J. Biol. Chem. 145, 511–523.

Rashid, H., Zeb, M., Jamal, Q., Waqar, M., Farooqi, B.J., Majid, A., 2014. Frequency and antimicrobial susceptibility pattern of *Pseudomonas aeruginosa* in ear swabs. World Appl. Sci. J. 30, 812–817.

Sarma, R., Devi, R., 2015. Ethnopharmacological survey of *Garcinia pedunculata* Roxb. fruit in six different districts of Assam, India. Int. J. Pharm. Sci. Invent. 4, 20–28.

Savikin, K., Zdunić, G., Menković, N., Živković, J., Ćujić, N., Tereščenko, M., Bigović, D., 2013. Ethnobotanical study on traditional use of medicinal plants in South-Western Serbia, Zlatibor district. J. Ethnopharmacol. 146 (3), 803–810.

Sentjurc, M., Nemec, M., Connor, H.D., Abram, V., 2003. Antioxidant activity of *Sempervivum tectorum* and its components. J. Agric. Food Chem. 51, 2766–2771.

Tierney Jr., L.M., McPhee, S.J., Papadakis, M.A., 2005. Current Medical Diagnosis and Treatment, 44th ed McGraw-Hill, New York, NY.

Tsukatani, T., Suenaga, H., Shiga, M., Noguchi, K., Ishiyama, M., Ezoe, T., Matsumoto, K., 2012. Comparison of the WST-8 colorimetric method and the CLSI broth microdilution method for susceptibility testing against drug-resistant bacteria. J. Microbiol. Methods 90, 160–166.