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Bioreactor Cultivation of *Zeltnera beyrichii* (Torr. & A. Gray) Mans.: A Novel Source of Biologically Active Compounds

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Abstract: With regard to world's increasing demand for biologically active compounds, a novel source of xanthones and secoiridoid glycosides has been studied. *Zeltnera beyrichii* (Torr. & A. Gray) Mans., an insufficiently acknowledged North American medicinal plant species, may be considered a pharmacological substitute for commercial *C. erythraea* Rafn, since it accumulates in aerial parts nearly the same amount of secoiridoid glycosides: swertiamarin, gentiopicrin, and sweroside (13.76, 7.56, and 0.17 mmol per 100 g dry weight, respectively) in plants grown under greenhouse condition, and a considerable amount of xanthones: decussatin and eustomin. Additionally, *Z. beyrichii* produced as much biomass during cultivation in RITA® temporary immersion bioreactors as greenhouse-grown plants, in a third of the time. Plants grown in bioreactors contained moderate levels of total phenolics and total flavonoids, and possessed modest antioxidant activity and antimicrobial potential against eight bacterial and eight fungal species. Therefore, this species may be highly recommended for cultivation either in natural environment, or in bioreactors under *in vitro* conditions, for producing compounds of interest of modern pharmacology and food industry.

Keywords: *Zelnera beyrichii;* temporary immersion bioreactors; secoiridoid glycosides; xanthones; antioxidant activity; antimicrobial activity.

1. Introduction

An increasing demand for plant-derived biologically active compounds bear conceptions about alternative ways to derive them from underutilized plant species, improved technology of plant cultivation and the extraction of compounds of interest. Secoiridoid glycosides (SGs) and xanthones are for a long time known for their antimicrobial [1-7], hepatoprotective [8], gastroprotective [9], sedative [10,11], and even antitumor activity [12]. Primary commercial sources of these compounds are a few genera from the *Gentianaceae* family: *Gentiana*, *Swertia*, and *Centaurium* and from the latter, *Centaurium erythraea* Rafn is extensively utilized in food industry as a natural source for food and beverage embittering in category N2, which could be added to food stuffs in small quantities

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(0.0002%-0.0008%, [13]), as well as in pharmacology as "Centaurii herba" drug [14]. Some other species from the genus *Centaurium* also contain SGs and xanthones in considerable amounts [6,15-18]. Until recently, this genus was comprised of about 50 species [19]. However, after detailed floral descriptive and morphometric analysis, Mansion [20] has proposed division of polyphyletic genus *Centaurium* into four genera: *Cenataurium* Hill (predominantly European species), *Schenkia* Griseb. (Mediterranean basin, Australia, and Hawaii), *Zeltnera* Mans. (North and Central America), and *Gyrandra* Griseb. (Central America).

Genus *Zeltnera* contains 25 annual or biannual herbaceous species [20], including *Z. beyrichii* (Torr. & A. Gray) Mans. This species, also known as "mountain pink" or "quinine weed", grows on calcareous, rocky soils in Texas and Oklahoma. Plants are bushy, robust, up to 30 cm high and quite decorative with numerous pink flowers. No literature data regarding identification and yield of secondary metabolite compounds in *Zeltnera* species has been published. Therefore, this study was aimed at identification of the main secondary metabolites in *Z. beyrichii*, with particular attention to SGs and xanthones; its secondary metabolite profile is then compared to that of commercial species *Centaurium erythraea.* Another objective of this study was to evaluate antioxidant and antimicrobial potential of *Z. beyrichii* extract.

The potential of *in vitro* grown plants to produce various secondary metabolites has been studied for more than 30 years [21,22]. There are several publications describing the production of secondary metabolites in plants from the *Gentianaceae* family grown under *in vitro* conditions [17,23-26]. Temporary immersion systems are proposed as a possible way to obtain a higher growth rate of biomass of various species and increased accumulation levels of secondary metabolites in relation to regularly grown *in vitro* cultures [27,28]. According to Teisson and Alvard [29] culture vessels induced by temporary immersion are distinguished by: a better supply of nutrient elements by renewed direct contact with the medium, low disruption of gas exchanges between the plant and the atmosphere by short immersion periods, and complete renewal of the atmosphere inside the vessel at regular intervals. Thus, bioreactor-growing plants can be an alternative source of bioactive compounds in species whose field cultivation is troublesome and do not provide much success. The final goal of this research is to propound the most worthwhile biotechnological method for cultivation of this species to obtain large scale production of biomass and compounds of interest for the pharmaceutical purposes.

2. Materials and Methods

2.1. Plant Material and Experimental Postulation

Seeds of *Zeltnera beyrichii* were kindly provided by Kew Royal Botanic Gardens, Edinburg, UK, while those of *Centaurium erythraea* were collected by the authors in 2007 near Laznica, Serbia. After surface sterilization with 20% bleach, seeds were sown on solid MS nutrient medium [30] under aseptic conditions. The medium also contained 30 g L^{-1} sucrose (Suc), 7 g L^{-1} agar and 0.1 g L^{-1} *myo*-inositol. The pH value of the medium was adjusted to 5.8 prior to autoclaving. After germination, plants were grown *in vitro* in 760 mL glass jars on the same medium composition (100 mL per jar) until enough shoots for the experimentation were obtained. Some seedlings were also transferred to the greenhouse and, after acclimatization were grown on basal compost mixture for 6 months.

Five shoots per jar of both species, 2-3 cm long, were placed on solid ½MS medium (half-strength salts) supplemented with 30 g L^{-1} Suc, 7 g L^{-1} agar and 0.1 g L^{-1} *myo*-inositol, and were grown for 3 months under *in vitro* conditions, with 16 h light/ 8 h dark cycle at 25±2°C and a relative air humidity of 60-70%. White fluorescent tubes provided a photon flux rate of 32.5 μmol m^{-2} s^{-1} at the level of plant cultures. Shoots were, consequently, detached from the roots and air-dried until constant dry matter was reached. The experiment was set up in triplicate.

Shoots of *Z. beyrichii* were also cultivated in RITA® temporary immersion bioreactor (TIB) vessels (VITROPIC, Saint-Mathieu-de-Tréviers, France) as proposed by Teisson and Alvard [29],

each containing 300 mL of liquid ½MS culture medium supplemented with carbohydrate source: Suc (30 g L^{-1}), glucose (Glu) + fructose (Fru) (15+15 g L^{-1}), or Fru alone (30 g L^{-1}). TIB cultures were established with immersion frequency of four 15 min flooding cycles during 24 h. The flow rate of inlet air was 0.001 m^3 h^{-1} for each RITA® vessel. Each bioreactor was inoculated with 20 shoot explants, 2-3 cm long, and there were three vessels per each treatment. The cultures were grown for two months under the same growing conditions as stated above and, after harvesting, separation of aerial parts from roots and desiccation were performed.

2.2. Secondary Metabolite Analysis

2.2.1. Sample Preparation

Samples were prepared either from greenhouse-grown plants (Figure 1a), *in vitro*-grown plants (Figure 1b), or the plants cultured in TIB vessels (Figure 1c). They were made in triplicate by pooling either the aerial parts or roots belonging to plants of the same accession. Each sample contained 200 mg of powdered plant material, which was extracted with 10 mL of 99.8% methanol (AppliChem, Cheshire, CT), overnight. All samples were filtered through cellulose filters with 0.2 µm pore size (Agilent Technologies, Santa Clara, CA) and stored at 4°C until use.

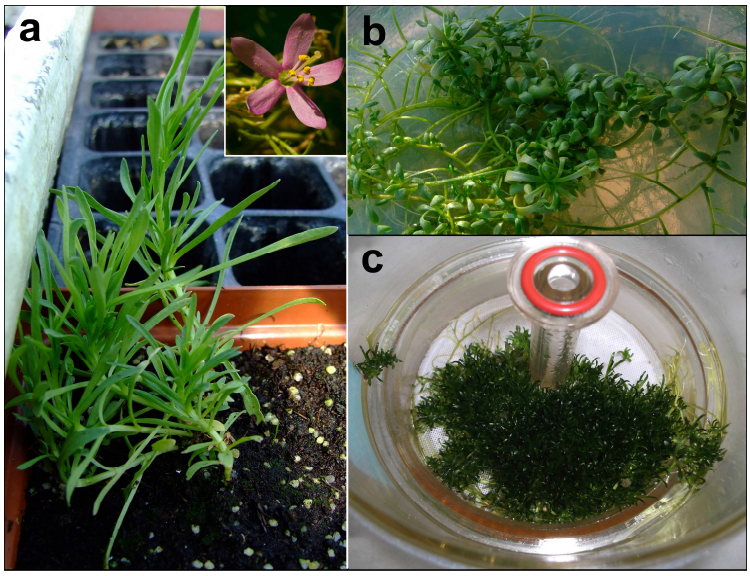

Figure 1. Plants of *Zeltnera beyrichii* grown in a) greenhouse, b) *in vitro* on solid ½MS medium, and c) in RITA® temporary immersion bioreactors with liquid ½MS medium.

2.2.2. UHPLC-PDA MS/MS Analyses of Secoiridoid Glycosides and Xanthones

Separation, determination and quantification of compounds of interest in each sample were performed using Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Bremen, Germany) equipped with a diode array detector (DAD) and connected to a triple-quadrupole mass spectrometer. Elution was performed at 30°C on Hypersil gold C18 column (50 × 2.1 mm) with 1.9 µm particle size (Thermo Fisher Scientific, USA). The mobile phase consisted of (A) water + 0.1% formic acid, and (B) acetonitrile (all: MS grade, Fisher Scientific, Leics, UK), which were applied in the following gradient elution: 5-20% B in first 6 min, 6-10 min 20–40% B, 10-15 min from 40% to 50% B, 15-17 min 50–60% B, 17-21 min 60–95% B, 21-23 min 95% B, 23-24 min from 95% to 5% B, and 24-30 min 5% B. The flow rate was set to 0.4 mL min^{-1} and the detection wavelength to 260 and 320 nm, respectively. The injection volume was 10 µL. All analyses were performed in triplicate.

Standard solutions were prepared by dissolving 1 mg of gentiopicrin (**GP**) (>90% purity, Roth, Germany), swertiamarin (**SM**), and sweroside (**SW**) (both 98% purity, Oskar Tropitzsch, Germany) in 1 mL methanol. Further calibration levels were prepared by diluting the stock with methanol. Linear regression analyses of calibration curves of these compounds revealed an excellent linearity with a correlation coefficient of r = 0.999, $P < 0.001$ for each compound. Total amount of compounds **SM**, **GP** and **SW** in both aerial parts and roots of plants grown in the greenhouse, *in vitro* on solid ½MS medium, or in TIB vessels, was evaluated by the calculation of peak areas and is expressed as mmol per 100 g of DW.

Qualitative analysis of each sample was performed on a TSQ Quantum Access Max triple-quadrupole mass spectrometer (Thermo Fisher Scientific, Basel, Switzerland), equipped with heated electrospray ionization (HESI) source. Vaporizer temperature was kept at 350°C and ion source settings were set as follows: spray voltage 4000 V, sheet gas pressure 50 AU, ion sweep gas pressure 0 AU and auxiliary gas pressure at 20 AU, capillary temperature at 300°C, skimmer offset 0 V. Mass spectrometry data were acquired in a positive mode. Multiple mass spectrometric scanning modes, including full scanning (FS), product ion scanning (PIS), selected reaction monitoring (SRM), and neutral loss scanning (NL) were conducted. Collision-induced fragmentation experiments were performed using argon as the collision gas, and collision energy was set to 30 eV. SRM experiment was performed by using two fragments for each compound, which were previously defined as dominant in PIS experiments. SRM is a non-scanning technique which increases specificity of detection of known molecules, and is usually used for quantitative analysis. Since standard compounds of decussatin and eustomin were not available, mangiferin (Sigma-Aldrich, Germany) was used as reference xanthone compound in MS and MS/MS analyses.

2.3. Determination of Total Phenolic and Total Flavonoid Content

Total phenolics and total flavonoids were quantified in methanol extract of aerial parts of *Z. beyrichii* grown in TIB vessels on fructose medium. Folin-Ciocalteau assay described by Singleton and Rossi [31] was used for total phenolics estimation, with minor modifications. Plant extract (50 µL) was mixed with 475 µL of the 5% sodium carbonate solution. The mixture was left for 3-5 min and then 475 µL of the 50% Folin-Ciocalteau reagent was added. The solution was well mixed and allowed to stand at room temperature in darkness for 1 h. Absorbance of the resultant solution was measured at 724 nm. The gallic acid standard curve was used for determination of total phenolics content in the sample. Result is expressed as mmol of gallic acid equivalents per g of dry weight (mmol GAE g^{-1} DW).

Total flavonoid content of the sample was determined according to Karadeniz et al. [32], with some modifications. 50 µL of plant extract and 600 µL distilled water were well shaken and mixed with 40 µL of 5% KNO_2. The mixture was allowed to stand at room temperature for 6 min and subsequently 70 µL of 26% $AlCl_3$ solution was added. After 5 min at room temperature, and the addition of 240 µL of 1M NaOH to the mixture, the solution was well mixed. The absorbance was measured at 510 nm. The total flavonoids content in the extract was calculated from the standard curve based on rutin (Sigma-Aldrich, Germany) and is expressed as mmol of rutin equivalents per g dry weight (mmol RE g^{-1} DW). All measurements were performed in triplicate.

2.4. Antioxidant Activity

Methanol extract of aerial parts of *Z. beyrichii* grown in TIB vessels on fructose medium was used in the evaluation of antiradical and antioxidant activity.

2.4.1. ABTS Radical Scavenging Activity

The ABTS radical cation decolorization assay was carried out as previously described by Re et al. [33], with some modifications. $ABTS^{\bullet+}$ was generated by oxidation of 7 mM ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate), Sigma-Aldrich, Germany) with 2.45 mM solution of potassium persulphate (final concentration) and subsequent incubation at room temperature in dark for 16 h. The $ABTS^{\bullet+}$ solution was then diluted with 80% ethanol and equilibrated at 30°C until an absorbance of 0.700±0.020 AU at 734 nm was reached. Reaction mixture was prepared by mixing 970 μL of the $ABTS^{\bullet+}$ solution and 30 μL of plant extract. The mixture was incubated for 10 min at room temperature, and absorbance at 734 nm was subsequently measured using UV-visible spectrophotometer (Agilent 8453, Agilent Technologies, Waldbronn, Germany). Methanol solutions of Trolox (Hoffman-La Roche, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma-Aldrich, Germany) were used for the calibration curve construction and the result are expressed as mmol Trolox equivalents scavenging activity per g of dry weight (mmol TEAC g^{-1} DW). The measurement was performed with three independent extracts.

2.4.2. DPPH Radical Scavenging Activity

DPPH assay was performed as previously described by Brand-Williams et al. [34], with slight modifications. The reaction mixture (1 mL) consisted of 30 μL of sample and 100 μM DPPH solution (1,1-diphenyl-2-picrylhydrazil, Sigma-Aldrich, Germany) in methanol. After 30 min of the incubation at room temperature the absorbance was measured at 517 nm. Trolox was used as the reference compound to create the standard curve. The measurement was run with three independent samples. The result is expressed as mmol TEAC g^{-1} DW.

2.4.3. Ferric Reducing/Antioxidant Power (FRAP)

The ferric reducing/antioxidant power (FRAP) assay was carried out according to the procedure described by Benzie and Strain [35], with some modifications. Working FRAP reagent contained 300 mM Na-acetate buffer (pH=3.6), 20 mM ferric chloride and 10 mM Fe^{3+}-TPTZ (ferric-2,4,6-tri(2-pyridil)-1,3,5-triazine, Sigma-Aldrich, Germany) at 10:1:1 ratio. The sample was prepared by mixing 950 μL freshly prepared FRAP reagent with 50 μL of extract. Absorbance reading was taken after 4 min at 593 nm, along with the absorbance reading of the reagent blank of pure methanol. The measurement was run with three independent samples. Methanol solutions of Trolox were used for the calibration curve construction and the result is expressed as mmol TEAC g^{-1} DW.

2.5. Evaluation of Antimicrobial Activity

For the evaluation of antimicrobial activity, methanol extract of aerial parts of *Z. beyrichii* grown in TIB vessels on fructose medium was used. Methanol was removed at rotary evaporator (R-210, Büchi, Switzerland) at 40°C. The dry extract was re-dissolved in sterilized distilled water containing 0.02% Tween 80 at a concentration of 100 mg mL^{-1}, and stored at -20°C until use. Bacterial and fungal cultures were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Serbia.

2.5.1. Antibacterial Assays

Four Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Salmonella enteritidis* (ATCC 13076) and four Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Listeria monocytogenes* (NCTC 7973), *Bacillus cereus* (human isolate) and *Micrococcus flavus* (ATCC 10240) were used to evaluate

the antibacterial activity of *Z. beyrichii* methanol extract. The minimum inhibitory (MIC) and minimum bactericidal concentration (MBC) were determined by the microdilution method [36]. Briefly, fresh overnight cultures of bacteria were adjusted to a concentration of 1×10^5 CFU mL^{-1}, which was confirmed spectrophotometrically. Dilutions of inocula were cultured on solid medium to check their validity and verify the absence of contamination. Dilutions of extract were carried out over the wells containing 100 μL of TSB (Tripticase Soy Broth, Merck, Germany) and afterwards, 10 μL of inoculum was added to each well. The microplates were incubated for 24 h at 37°C. The lowest concentration that produced a significant inhibition of the growth of the bacteria in comparison with the positive control was identified as the MIC. The minimum inhibitory concentrations (MICs) obtained from the susceptibility testing of various bacteria to tested extracts were determined also by a colorimetric microbial viability assay based on reduction of a INT (p-iodonitrotetrazolium violet, Sigma-Aldrich, Germany) color and compared with positive control for each bacterial strain [37,38]. The MIC of the samples was detected after the addition of 40 μL of INT in concentration of 0.2 mg mL^{-1}, and incubation at 37°C for 30 min. MBC was determined by serial sub-cultivation of 10 μL of suspension into microplates containing 100 μL of TSB. The lowest concentration showing no visible growth after sub-culturing was recorded as the MBC. Standard drugs Streptomycin (Sigma-Aldrich, Germany) and Ampicillin (Panfarma, Belgrade, Serbia) were used as positive controls.

2.5.2. Antifungal Assays

Eight fungal species were used: *Aspergillus ochraceus* (ATCC 12066), *A. fumigatus* (human isolate), *A. niger* (ATCC 6275), *A. versicolor* (ATCC 11730), *Fusarium sporotrichoides* (IMT 496), *Penicillium funiculosum* (ATCC 6275), *P. ochrochloron* (ATCC 9112), and *Fulvia fulvum* (plant isolate). In order to investigate the antifungal activity of methanol extract, 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 spore suspensions were adjusted with sterile saline to a concentration of approximately 1×10^5 in a final volume of 100 μl per well. The inocula were stored at 4°C for further use. Dilutions of extract were carried out over the wells containing 100 μL of malt broth (MB) and afterwards, 10 μL of inoculum with fungal spores was added to each well. The microplates were incubated for 72 h at 28°C. The lowest concentration without visible growth was defined as MIC. The fungicidal concentrations (MFCs) were determined by serial sub-cultivation of 10 μL into microplates containing 100 μL of MB per well and further incubation at the same conditions. The lowest concentration with no visible growth was defined as MFC indicating 99.5% mortality of the original inoculum. Commercial fungicides ketoconazole (Zorkafarma, Šabac, Serbia) and bifonazole (Srbolek, Beograd, Serbia) were used as positive controls.

2.6. Statistical Analyses

Statistical analyses for SGs concentrations and biomass production data were performed using STATGRAPHICS Centurion XV software (version 15.1.02 StatPoint, Inc. 1982–2006, USA). The data were subjected to the analysis of variance and comparison between the mean values of accessions were made by the least significant difference (LSD) test calculated at $P \leq 0.05$ confidence level.

3. Results and Discussion

3.1. Identification of Secoiridoid Glycosides and Xanthones

UHPLC/+HESI-MS TIC (total ion count) chromatogram and UHPLC/DAD chromatogram of methanol extract of *Z. beyrichii* grown in TIB vessels is presented in Figure 2.

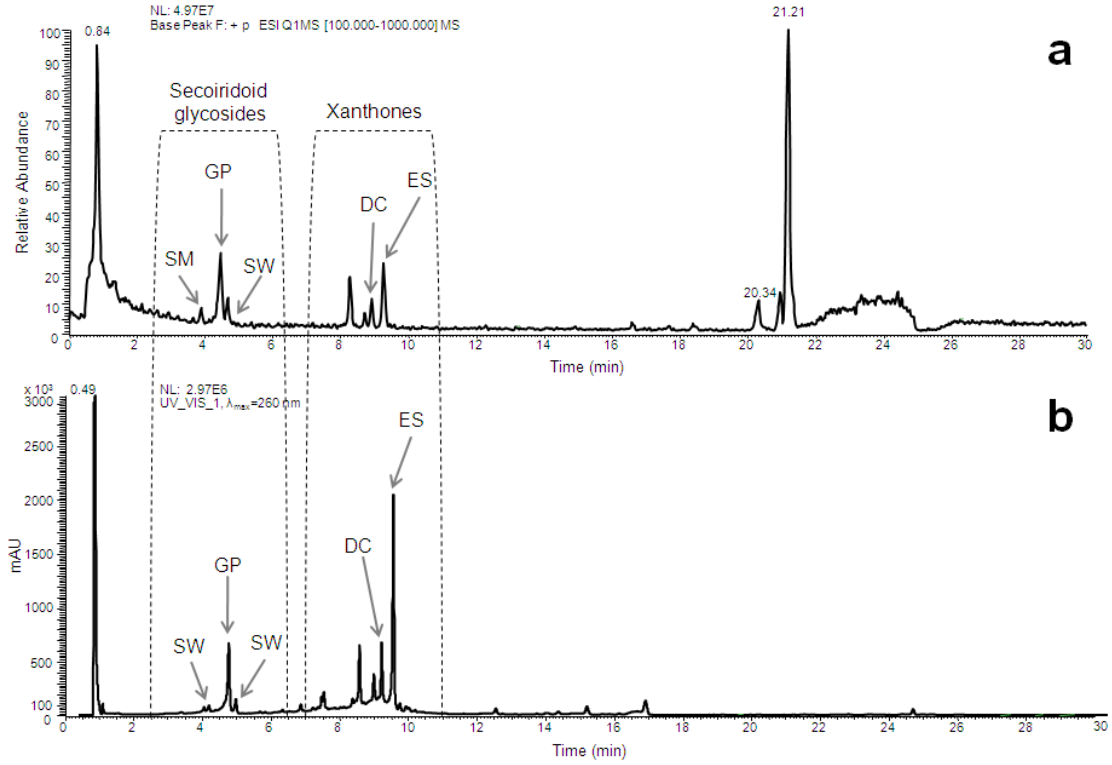

Figure 2. UHPLC/+HESI-MS TIC (total ion count) chromatogram (a) and UHPLC/DAD chromatogram (b) of *Zeltnera beyrichii* methanol extracts. Secoiridoid glycosides swertiamarin (**SM**), gentiopicrin (**GP**), and sweroside (**SW**) are identified, as well as xanthones decussatin (**DC**) and eustomin (**ES**).

Initially, full scan UHPLC/HESI-MS spectra were acquired in both positive and negative ion mode showing series of peaks between *m/z* 100 and 1000. UHPLC/+HESI-MS total ion chromatogram (TIC) showed several distinct peaks at 3.91, 4.48, and 4.67 min (Figure 3) assigned to secoiridoid glycosides: **SM** ($C_{16}H_{22}O_{10}$) with *m/z* $[M+H]^+$ at 375, **GP** ($C_{16}H_{20}O_9$) with *m/z* $[M+H]^+$ at 357, and **SW** ($C_{16}H_{22}O_9$) showing *m/z* $[M+H]^+$ of 359 which is in correlation with the standards (Figure 2a). UHPLC/DAD analysis of *Z. beyrichii* methanol extracts confirmed the presence of **SM**, **GP**, and **SW** (Figure 2b). UHPLC/+HESI-MS/MS data and the major product ions $+MS^2$ of these compounds are also presented in Table 1. Dominant $+MS^2$ fragments of **SM** were at *m/z* 147 and 178, for **GP** at *m/z* 121 and 177 and for **SW** at *m/z* 127 and 179 (Figure 3), and selected ions were utilized in SRM experiment for the accurate identification and quantification of these compounds. Although SGs are widely distributed throughout the *Gentianaceae* family [15,16], this is the first report on this group of secondary metabolites in the genus *Zeltnera*.

Two other dominant compounds (Figure 2) were identified as xanthones based on their characteristic UV spectra, as well as their MS and MS/MS spectra (Table 1). UHPLC/+HESI-MS total ion chromatogram (TIC) showed peaks at 8.96 and 9.31 min (Figure 3). In general, these compounds along with mangiferin, which was used as reference xanthone compound, were monitored and characterized using multiple mass spectrometric scanning modes, and they were subjected to the same MS fragmentation pathways. In the positive mode, the dominant product ions $[M + H]^+$ of those xanthones were different, although they undergo a similar fragmentation pattern. UHPLC/+HESI-MS and UHPLC/+HESI-MS/MS analyses of mangiferin gave a molecular ion *m/z* $[M + H]^+$ of 423 and major $+MS^2$ fragments at *m/z* 303 $[M + H - 120]^+$ and 273 $[M + H - 150]^+$. MS/MS analysis of the first unknown xanthone compound gave a molecular ion *m/z* $[M + H]^+$ of 303 and major $+MS^2$ fragments at *m/z* 245 $[M + H - 58]^+$ and 270 $[M + H - 33]^+$. MS/MS analysis of the second compound gave a molecular ion *m/z* $[M + H]^+$ of 363 while dominant $+MS^2$ fragments were at *m/z* 333 $[M + H - 30]^+$ and 305 $[M + H - 58]^+$ (Figure 3).

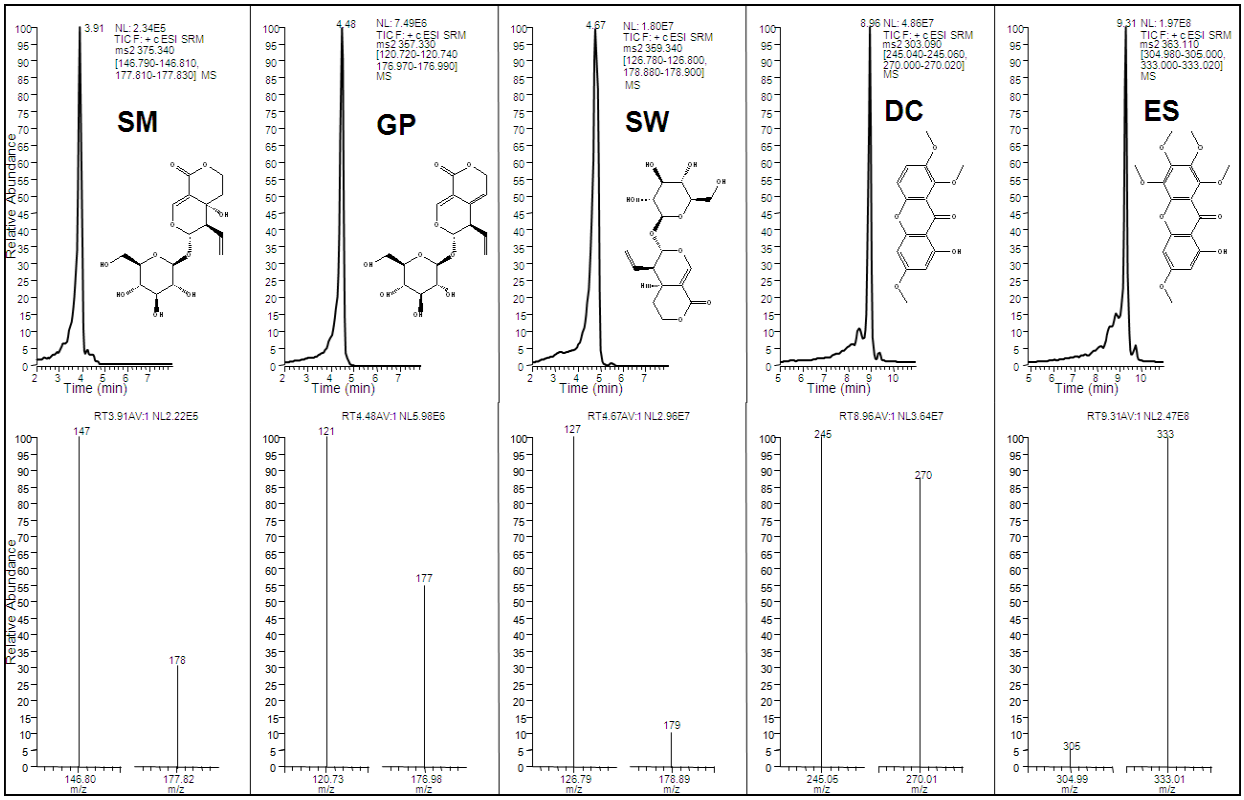

Figure 3. UHPLC/+HESI-MS/MS chromatograms of *Zeltnera beyrichii* methanol extracts representing SRM experiment: upper row of chromatograms represents TIC chromatograms of methanol extracts, while the lower row of chromatograms presents $+MS^2$ spectrums of the predefined main fragments of swertiamarin (**SM**), gentiopicrin (**GP**), sweroside (**SW**), decussatin (**DC**), and eustomin (**ES**).

According to these results and comparison with the literature data of known compounds in the genus *Centaurium*, we putatively identified these compounds as decussatin (8-hydroxy-1,2,6-trimethoxyxanthen-9-one) and eustomin (1-hydroxy-3,5,6,7,8-pentamethoxyxanthone). Tetraoxygenated and hexaoxygenated xanthones are widely distributed in species of the *Gentianaceae* family [16,39].

Decussatin (**DC**) has been reported for tribes *Chironieae*, *Gentianeae*, *Potaliae* [39,40], and *Helieae* [41,42]. Hexa-oxygenated xanthone eustomin (**ES**) has been reported only for the tribe *Chironieae* (*Centaurium*, *Chironia* and *Eustoma* [43,44]), and could therefore be recommended as a good chemotaxonomic marker [45]. This is the first report for **DC** and **ES**, and generally xanthones in the genus *Zeltnera*. In the end of the past century, xanthones have emerged as a class of compounds possessing a broad spectrum of biological and pharmacological activities such as: cytotoxic, antitumor, anti-inflammatory, and antifungal [39,46-48]. These facts make *Zeltnera beyrichii* an interesting object for further studies, and the course of our future work will be the identification and quantification of xanthone compounds in this species.

Table 1. UHPLC/+HESI-MS/MS characterization of components of *Z. beyrichii* methanol extracts using different mass spectrometric scanning modes and DAD data.

				MS data [*m/z*]			DAD data	
Peak label	**Assignment**	**Molecular formula**	**Molecular weight**	**Rt (min)**	**$[M+H]^+$**	**MS^2 fragments (*m/z*)**	**Rt (min)**	**λ_{max} (nm)**
SM	Swertiamarin[S,R]	$C_{16}H_{22}O_{10}$	374.12	3.89	375.34	**178**, **147**, 121	3.81	240
GP	Gentiopicrin[S,R]	$C_{16}H_{20}O_9$	356.11	4.43	357.33	**177**, 162, 159, 149, 146, 144, 131, **121**	4.38	210, 250, 270
SW	Sweroside[S,R]	$C_{16}H_{22}O_9$	358.34	4.63	359.34	197, **179**, 151, 137, 125, **127**, 123, 111	4.59	250
DC	Decussatin[R]	$C_{16}H_{14}O_6$	302.28	8.96	303.65	288, 287, 273, **270**, 269, 259, **245**, 241, 240, 227, 213	8.84	240, 280, 310
ES	Eustomin[R]	$C_{18}H_{18}O_8$	362.33	9.31	363.14	348, **333**, 330, 315, **305**, 287, 272, 261	9.18	250, 310, 370
/	Mangiferin[NP,S,R]	$C_{19}H_{18}O_{11}$	422.09	4.32	423.35	405, 387, 369, 351, 339, 327, **303**, 299, 285, **273**	4.22	260, 320, 360

S-compared with standard
R-compared with literature data
NP- not predicted
Bold numbers designate masses used for SRM (selected reaction monitoring) experiments

3.2. Production of Secoiridoid Glycosides of Zeltnera beyrichii in Comparison to Centaurium erythraea

Accumulation of total secoiridoid glycosides in *Z. beyrichii* aerial parts and roots is shown to be slightly lower in comparison to commercial species *C. erythraea,* grown under the same experimental conditions, both in greenhouse or *in vitro* (Figure 4). The most significant differences between the two species grown in greenhouse arose both in the **GP** content in aerial parts (with *Z. beyrichii* being more productive), and in **SM** content in aerial parts (where *C. erythraea* showed its dominance). Nevertheless, the results undoubtedly candidate *Z. beyrichii* as an eligible pharmacological substitute for *C. erythraea,* at least with respect to bioproduction of secoiridoid glycosides.

The aerial parts of *in vitro* grown *Z. beyrichii* and *C. erythraea* plants both accumulated slightly less total SGs per DW than greenhouse grown plants, with a significant difference in **SM**

content, but also both produced significantly more **SW** (Figure 4). Furhermore, the concentrations of **GP**, a dominant compound in roots, were significantly higher in greenhouse grown plants for both species. This could be due to the nature of *in vitro* conditions, which enforce cultured plants to use alternative carbon sources, such as Suc, Glu and/or Fru, thus becoming partially heterotrophic, which alters the primary and secondary metabolism. A significant decrease in secoiridoid glycosides' production, in relation to greenhouse or nature-grown plants, was observed earlier [23,49], which is the case for some other secondary metabolites (e.g. [28]). However, it should be noticed that greenhouse grown plants took 6 months to produce approximately equal biomass as *in vitro* grown plants did in only 3 months (Figure 5).

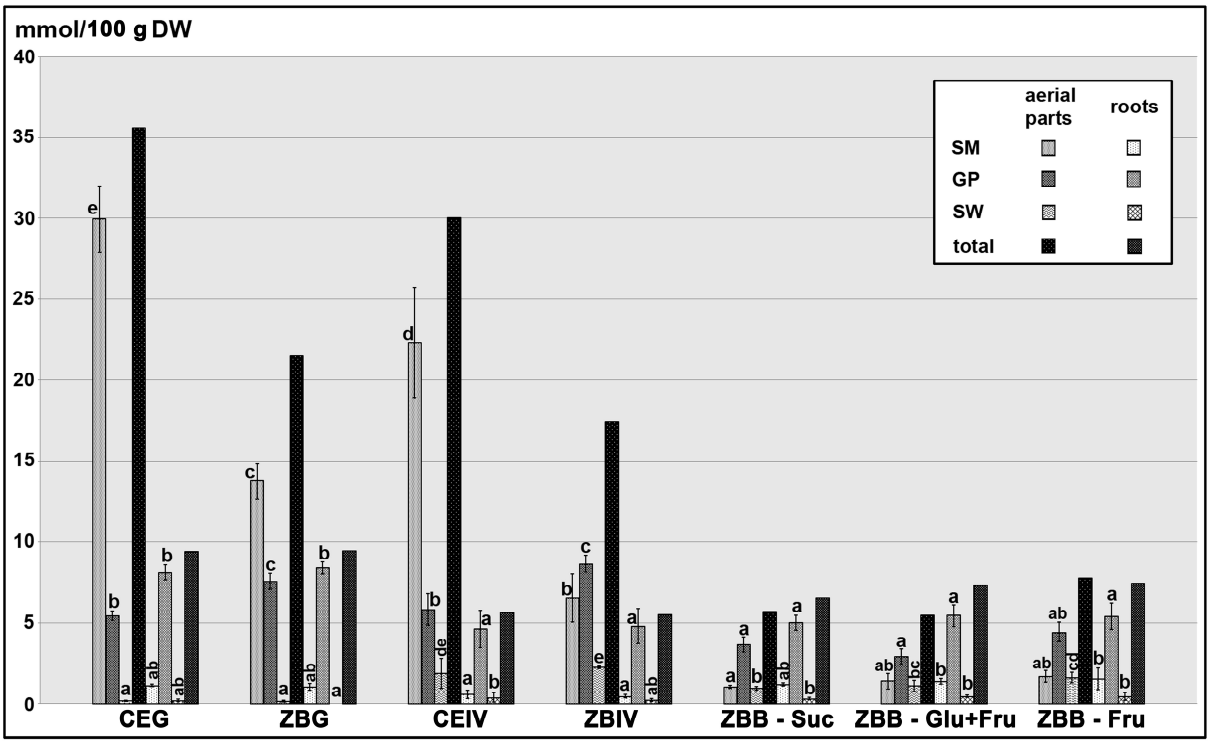

Figure 4. Secoiridoid glycoside accumulation (mmol per 100 g DW) in *Zeltnera beyrichii* aerial part and roots, cultivated under different growing conditions. Comparison with commercial species *Centaurium erythraea* is also given. CEG and ZBG: greenhouse grown *C. erythraea* and *Z. beyrichii*, respectively; CEIV and ZBIV: *in vitro* grown *C. erythraea* and *Z. beyrichii*, respectively; ZBB – Suc, ZBB – Glu+Fru, and ZBB – Fru: *Z. beyrichii* plants grown in RITA® temporary immersion bioreactors in medium supplemented with 30 g L^{-1} sucrose, 15 g L^{-1} glucose + 15 g L^{-1} fructose, or 30 g L^{-1} fructose, respectively. Letters above the bars indicate if the values are statistically different within each parameter and independent for each plant part, at the $P \leq 0.05$ level according to the LSD test.

3.3. Production of Secoiridoid Glycosides in Z. beyrichii in vitro

There were no significant differences either in quantitative content of the three SGs (with exception of **SW** content in aerial parts) among plants grown on media with different carbon source in TIB vessels (Figure 4), nor in their biomass production (Figure 5). However, it is noticeable that plants grown on medium supplemented with Fru produced the highest yield of total SGs in relation to other bioreactor experiments, both in aerial parts and roots. It is also worth to mention that plants grown for only two months in TIBs produced greater biomass in relation to standard *in vitro* treatment (Figure 5). Their roots generally produced significantly more **SM** and therefore total SGs. In word of total SGs accumulation per DW, green parts of TIB-grown plants still contained about twice lower amounts than

in vitro grown plants and nearly three times lower amounts than that grown in greenhouse. Nevertheless, this deficiency is compensated with high biomass production in TIBs, as DW per plant for these two months-old plants nearly fit with the values of 6 months-old greenhouse plants (Figure 5). In bioreactor culture of *Panax ginseng* Asaka et al. [50] remarked a similar phenomenon: biomass production in bioreactors was a few times higher than in static culture, but secondary metabolite production was decreased and therefore, they suggested a decrease in growth conditions. Our results imply that a specific spectrum of bioactive compounds could be achieved by choosing appropriate cultivation conditions.

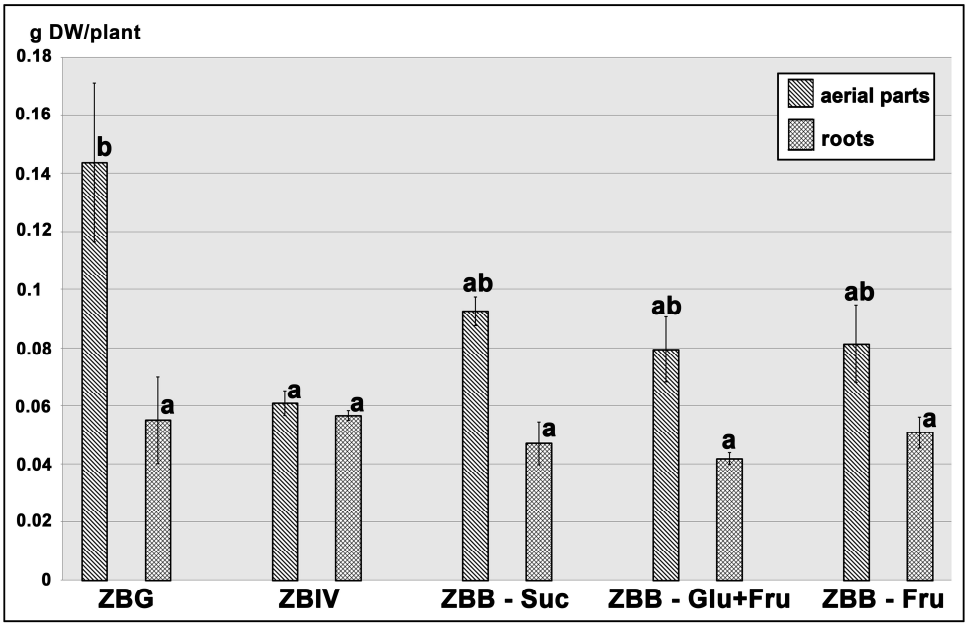

Figure 5. Biomass production (g DW per plant) of *Zeltnera beyrichii* grown under various growing conditions. ZBG and ZBIV: greenhouse and *in vitro* grown *Z. beyrichii*, respectively; ZBB – Suc, ZBB – Glu+Fru, and ZBB – Fru: *Z. beyrichii* plants grown in RITA® temporary immersion bioreactors in medium supplemented with 30 g L^{-1} sucrose, 15 g L^{-1} glucose + 15 g L^{-1} fructose, or 30 g L^{-1} fructose, respectively. Letters above the bars indicate if the values are statistically different within each parameter and independent for each plant part, at the $P \leq 0.05$ level according to the LSD test.

3.4. Antioxidant Potential

Z. beyrichii was analyzed for the first time for the antioxidant activities. Three methods were used as they differ in terms of their assay principles, giving the possibility to take into account various modes of action of antioxidant compounds. The methanol extracts of this species, grown in TIBs exhibited scavenging capacities against both $ABTS^{\bullet+}$ (0.903±0.014 mmol TEAC per g DW) and $DPPH^{\bullet}$ (0.359±0.038 mmol TAEC per g DW) radicals, and ferric reducing capacity in FRAP assay (2.204±0.088 mmol TEAC per g DW). In relation to other species from *Gentianaceae* family, methanol extract of *Z. beyrichii* showed slightly lower (*Gentiana kurro,* [51]) or similar (*Enicostema littorale,* [52]) potential for $DPPH^{\bullet}$ or $ABTS^{\bullet+}$ radical scavenging. Antioxidant activity of *Z. beyrichii* extract should be ascribed to phenolics, whose concentration was 15.46±0.06 mmol GAE per g DW, while the concentration of total flavonoids was 1.284±0.031 mmol RE per g DW. Xanthones and flavonoids, which belong to phenolics, are likely a significant component of *Z. beyrichii* methanol extracts and therefore strongly participate in its antioxidant potential. SGs have been previously reported to possess a low affinity to scavenge $DPPH^{\bullet}$ or $ABTS^{\bullet+}$ radical, and it may be presumed that glycosylation of secoiridoid glycosides suspends their antioxidant potential.

3.5. Antimicrobial Activity

	Z. beyrichii		**Streptomycin**		**Ampicillin**	
Bacteria	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Staphylococcus aureus	2.5	5	0.05	0.1	0.1	0.15
Bacillus cereus	1.25	2.5	0.5	0.5	0.1	0.15
Listeria monocytogenes	1.25	2.5	0.05	0.1	0.15	0.3
Micrococcus flavus	10	20	0.1	0.1	0.1	0.15
Pseudomonas aeruginosa	20	30	0.05	0.1	0.3	0.5
Escherichia coli	10	20	0.2	0.4	0.15	0.2
Salmonella typhimurium	5	10	0.1	0.2	0.1	0.2
Salmonella enteritidis	5	10	0.1	0.4	0.1	0.2

	Z. beyrichii		**Ketoconazole**		**Bifonazole**	
Fungi	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
Fusarium sporotrichoides	10	30	0.01	0.025	0.15	0.2
Aspergillus ochraceus	30	40	0.025	0.05	0.1	0.1
Fulvia fulvum	10	30	0.025	0.025	0.1	0.1
Aspergillus versicolor	10	20	0.025	0.1	0.1	0.1
Penicillium ochrochloron	10	20	0.1	0.25	0.1	0.1
Penicillium funiculosum	20	40	0.025	0.05	0.15	0.2
Aspergillus niger	30	50	0.025	0.05	0.1	0.2
Aspergillus fumigatus	30	50	0.025	0.05	0.1	0.2

Table 2. Antibacterial and antifungal activity of methanol extract of *Zeltnera beyrichii* shoots grown in RITA® temporary immersion bioreactors. MIC – minimum inhibitory concentration; MBC – minimum bactericidal concentration; MFC – minimum fungicidal concentration.

The results of antibacterial activity of methanol extract of *Z. beyrichii* are presented in Table 2. The extract inhibits the growth of bacteria in range of 1.25-30 mg mL^{-1}. The MIC values ranged from 1.25-20 mg mL^{-1}, while MBCs were 2.5-30 mg mL^{-1}. Among the tested bacteria, *B. cereus* and *L. monocytogenes* proved to be the most sensitive species with MICs of 1.25 and MBCs 2.5 mg mL^{-1}, while the most resistant was *P. aerogunosa* with MIC 20 mg mL^{-1} and MBC of 30 mg mL^{-1}. Generally, *Z. beyrichii* extract showed considerably inferior antimicrobial activity in comparison to antibiotics Streptomycin and Ampicillin.

Antifungal activity of methanol extract of *Z. beyrichii* is also presented in Table 2. Fungi, in general, appear to be more resistant than bacteria. The extract showed the moderate antifungal activity with MIC and MFC values ranging from 10-30 to 20-50 mg mL^{-1}, respectively. As observed, the extract possessed the highest activity against *A. versicolor* and *P. ochrochloron* with MIC 10 and MFC 20 mg mL^{-1}, respectively, in contrast to *A. niger* and *A. fumigatus* which proved to be the most resistant species with MIC 30 and MFC 50 mg mL^{-1}. The commercial antimycotics Bifonazole and Ketoconazole showed much stronger antifungal activity than the extract.

A quite modest antimicrobial action of *Z. beyrichii* extract, in relation to some *Centaurium* species [6], caused by reduced amount of biologically active compounds, especially phenolics, might be due to the origin of the extract, since the secondary metabolites' profile of plants grown in TIB

vessels significantly differ from that grown in nature or in greenhouse (Figure 4). According to that, additional antimicrobial assays with the extracts of plants from the nature or greenhouse should be performed.

In conclusion, there are many reasons to consider a commercial cultivation of this important but underutilized plant species in large-scale bioreactors for food industry (biomass production) and pharmaceutical purposes (production of bioactive secondary compounds). One of such species is *Zeltnera beyrichii*, barely investigated North American plant, with a huge potential for future extensive utilization for production of essential bioactive compounds, such as xanthones and secoiridoid glycosides. Although many challenges still exist for the commercial implementation of this alternative production system, it opens a new opportunity for pharmaceutical and industrial applications. The course of our further work is optimization of growth conditions and elicitation mechanisms in general, to achieve increased production of targeted phytochemicals.

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References

[1] W. G. van der Sluis and R. P. Labadie (1981). Secoiridoids and xanthones in the genus *Centaurium*, *Planta Med.* **41**, 221-231.

[2] W. G. van der Sluis and R. P. Labadie (1981). Fungitoxic activity of the secoiridoid glucoside gentiopicrin (gentiopicroside), *Planta Med.* **42**, 139-140.

[3] S. Rodriguez, J. L. Wolfender, E. Hakizamungu and K. Hostettmann (1995). An antifungal naphthoquinone, xanthones and secoiridoids from *Swertia calycina*, *Planta Med.* **61**, 362-364.

[4] Y. Kumarasamy, L. Nahar, P. J. Cox, M. Jaspars and S. D. Sarker (2003). Bioactivity of secoiridoid glycosides from *Centaurium erythraea*, *Phytomed.* **10**, 344-347.

[5] Y. Kumarasamy, L. Nahar and S. D. Sarker (2003). Bioactivity of gentiopicroside from the aerial parts of *Centaurium erythraea*, *Fitoterapia.* **74**, 151-154.

[6] B. Šiler, D. Mišić, J. Nestorović, T. Banjanac, J. Glamočlija, M. Soković and A. Ćirić (2010). Antibacterial and antifungal screening of *Centaurium pulchellum* crude extracts and main secoiridoid compounds, *Nat. Prod. Commun.* **5**, 1525-1530.

[7] S. A. Ross, A. El-Shanawany, G. A. Mohamed, A. M. Nafady, S. R. Ibrahim and M. M. Radwan (2011). Antifungal activity of xanthones from *Centaurium spicatum (Gentianaceae)*, *Planta Med*. **77**, P 43.

[8] Y. Kondo, F. Takano and H. Hojo (1994). Suppression of chemically and immunologically induced hepatic injuries by gentiopicroside in mice, *Planta Med.* **60**, 414-416.

[9] Y. Niiho, T. Yamazaki, Y. Nakajima, T. Yamamoto, H. Ando, Y. Hirai, K. Toriizuka and Y. Ida (2006). Gastroprotective effects of bitter principles isolated from gentian root and *Swertia* herb on experimentally-induced gastric lesions in rats, *J. Nat. Med.* **60**, 82-88.

[10] S. K. Bhattacharya, P. K. S. P. Reddy, S. Ghosal, A. K. Singh and P. V. Sharma (1976). Chemical constituents of Gentianaceae XIX: CNS depressant effects of swertiamarin, *J. Pharm. Sci.* **66**, 1547-1549.

[11] A. Rojas, M. Bah, J. I. Rojas and D. M. Gutiérrez (2000). Smooth muscle relaxing activity of gentiopicroside isolated from *Gentiana spathacea*, Planta Med. **66**, 765-767.

[12] K. Ishiguro, M. Yamaki, S. Takagi, Y. Ikeda, K. Kawakami, K. Ito and T. Nose (1998). Studies on iridoid-related compounds. V. Antitumor activity of iridoid derivatives-periodate oxidation products, *J. Pharmacobio-Dyn.* **11**, 131–136.

[13] C.A. Newall, L.A. Anderson and J.D. Phillipson (1996). Herbal Medicines. A Guide For Health-Care Professionals. The Pharmaceutical Press, London, UK. p.67.

[14] European Medicines Agency (EMA) (2009). Assesment report of *Centaurium erythraea* Rafn s.l. including *C. majus* (H. et L.) Zeltner and *C. suffruticosum* (Griseb.) Ronn. herba, for development of a community herbal monograph, Doc. Ref.: EMA/HMPC/105535/2008.

[15] W. G. van der Sluis (1985). Chemotaxonomical investigations of the genera *Blackstonia* and *Centaurium (Gentianaceae), Plant Syst. Evol.* **149**, 253-286.

[16] S.R. Jensen and J. Schripsema (2002). Chemotaxonomy and pharmacology of Gentianaceae, In: Gentianaceae – Systematics and Natural History, *eds*: L. Struwe and V.A. Albert, Cambridge University Press, Cambridge, UK., pp.573–631.

[17] D. Krstić, T. Janković, K. Šavikin-Fodulović, N. Menković and D. Grubišić (2003). Secoiridoids and xanthones in the shoots and roots of *Centaurium pulchellum* cultured *in vitro*, *In Vitro Cell Dev. Bio. Plant* **39**, 203-207.

[18] D. Mišić, B. Šiler, B. Filipović, Z. Popović, S. Živković, T. Cvetić and A. Mijović (2009). Rapid *in vitro* selection of salt-tolerant genotypes of the potentially medicinal plant *Centaurium maritimum* (L.) Fritsch., *Arch. Biol. Sci.* **61**, 57-69.

[19] L. Struwe, J.W. Kadereit, J. Klackenberg, S. Nilsson, M. Thiv, K.B. von Hagen, V.A. Albert (2002). Systematics, character evolution, and biogeography of Gentianaceae, including a new tribal and subtribal classification. In: Gentianaceae – Systematics and Natural History, *eds*: L. Struwe and V.A. Albert, Cambridge University Press, Cambridge, UK., pp.21–309.

[20] G. Mansion (2004). A new classification of the polyphyletic genus *Centaurium* Hill (*Chironiinae, Gentianaceae*): description of the New World endemic *Zeltnera,* and reinstatement of *Gyrandra* Griseb. and *Schenkia* Griseb., *Taxon* **53**, 719-740.

[21] J. E. Staba (1985). Milestones in plant tissue culture systems for the production of secondary products, *J. Nat. Prod.* **48**, 203-209.

[22] H. Dörnenburg and D. Knorr (1995). Strategies for the improvement of secondary metabolite production in plant cell cultures, *Enzyme Microb. Technol.* **17**, 674-684.

[23] A. Sabovljević, N. Rosić, T. Janković and D. Grubišić (2006). Secoiridoid content of *Blackstonia perfoliata in vivo* and *in vitro*, *In Vitro Cell Dev. Bio. Plant.* **42**, 427-431.

[24] B. Vinterhalter, T. Janković, K. Šavikin, R. Nikolić and D. Vinterhalter (2008). Propagation and xanthone content of *Gentianella austriaca* shoot cultures, *Plant Cell Tissue Organ Cult.* **94**, 329-335.

[25] S. Hayta, I. H. Akgun, M. Ganzera, E. Bedir and A. Gurel (2011). Shoot proliferation and HPLC-determination of iridoid glycosides in clones of *Gentiana cruciata* L., *Plant Cell, Tissue Organ Cult.* **107**, 175-180.

[26] B. Šiler, S. Avramov, T. Banjanac, J. Cvetković, J. Nestorović Živković, A. Patenković and D. Mišić (2012). Secoiridoid glycosides as a marker system in chemical variability estimation and chemotype assignment of *Centaurium erythraea* Rafn from the Balkan Peninsula, *Ind. Crops. Prod.* **40**, 336-344.

[27] E. Gontier, S. Piutti, A. Gravot, S. Milesi, A. Grabner, B. Massot, K. Lievre, M. Tran, J.L. Goergen and F. Bourgaud (2005). Development and validation of an efficient low cost bioreactor for furanocoumarin production with *Ruta graveolens* shoot cultures, In: Liquid Culture Systems for in vitro Plant Propagation, *eds*: A.K. Hvoslef-Eide and W. Preil, Springer, Dordrecht, The Netherlands, pp.509–524.

[28] D. Wilken, E. Jiménez González, A. Hohe, M. Jordan, R. Gomez Kosky, G. Schmeda Hirschmann and A. Gerth (2005). Comparison of secondary plant metabolite production in cell suspension, callus culture and temporary immersion system. In: Liquid Culture Systems for in vitro Plant Propagation, *eds*: A.K. Hvoslef-Eide and W. Preil, Springer, Dordrecht, The Netherlands, pp.525–537.

[29] C. Teisson and D. Alvard (1999). In vitro production of potato microtubers in liquid medium using temporary immersion, *Potato Res.* **42**, 499-504.

[30] T. Murashige and F. Skoog (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures, *Physiol. Plant.* **15**, 473-497.

[31] V. L. Singleton and J. A. Jr. Rossi (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, *Am. J. Enol. Vitic.* **16**, 144-158.

[32] F. Karadeniz, H. S. Burdulu, N. Koca and Y. Soyer (2005). Antioxidant activity of selected fruits and vegetables grown in Turkey, *Turk. J. Agric. For.* **29**, 297-303.

[33] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans (1999). Antioxidant activity applying an improved ABTS radical cation decolorisation assay, *Free Radicals Biol. Med.* **26**, 1231-1237.

[34] W. Brand-Williams, M. E. Cuvelier and C. Berset (1995). Use of free radical method to evaluate antioxidant activity, *Lebensm.-Wiss. Technol*. **28**, 25-30.
[35] I. F. F. Benzie and J. J. Strain (1996). The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": The FRAP assay, *Anal. Biochem*. **239**, 70-76.
[36] R. K. Daouk, S. M. Dagher and E. J. Sattout (1995). Antifungal activity of the essential oil of *Origanum syriacum* L., *J. Food Prot.* **58**, 1147-1149.
[37] Clinical and Laboratory Standards Institute (2009). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard, 8th ed. CLSI.
[38] T. Tsukatani, H. Suenaga, M. Shiga, K. Noguchi, M. Ishiyama, T. Ezoe and K. Matsumoto (2012). Comparison of the WST-8 colorimetric method and the CLSI broth microdilution method for susceptibility testing against drug-resistant bacteria, *J. Microb. Met.* **90**, 160-166.
[39] V. Peres, T. J. Nagem and F. F. de Oliveira (2000). Tetraoxygenated naturally occurring xanthones, *Phytochem.* **55**, 683-710.
[40] J. You, C. Ding, F. Zhu, X. Sun, Y. Li and Y. Suo (2007). Mass spectrometric identification of multihydroxy phenolic compounds in Tibetan herbal medicines, *Chromatographia* ***6***, 545-563.
[41] G. H. Stout, B. J. Reid *and G. D.* Breck *(*1969). Xanthones of the *Gentianaceae.* IV. Xanthones of *Macrocarpaea glabra*, *Phytochem.* ***8***, 2417-*2419.*
[42] A. M. Pohlit, E. V. M. dos Santos, T. C. M. da Silva, S.K.R. de Morais, S. M. Nunomura and L. Struwe (2012). A rare secoiridoid monoterpene and a xanthone from *Tachia grandiflora* Maguire & Weaver., *Biochem. Syst. Ecol.* **44**, 267-269.
[43] V. Peres and T. J. Nagem (1997). Naturally occurring pentaoxygenated, hexaoxigenated and dimeric xanthones: a literature survey, *Quím. Nova* **10**, 388-397.
[44] L. M. M. Vieira and A. Kijjoa (2005). Naturally-occurring xanthones: Recent developments, *Curr. Med. Chem.* **12**, 2413-2446.
[45] S. Mészáros (1994). Evolutionary significance of xanthones in *Gentianaceae*: A reappraisal, *Biochem. Syst. Ecol.* **22**, 85-94.
[46] H. Minami, M. Kinoshita, Y. Fukuyama, M. Kodama, T. Yoshizawaa, M. Sugiuraa, K. Nakagawaa and H. Tagoa (1994). Antioxidant xanthones from *Garcinia subelliptica*, *Phytochem.* **36**, 501-506.
[47] J. Asano, K. Chiba, M. Tada and T. Yoshii (1996). Cytotoxic xanthones from *Garcinia hanburyi*, *Phytochemistry*. **41**, 815-820.
[48] M. Iinuma, H. Tosa, T. Tanaka, F. Asai, Y. Kobayashi, R. Shimano and K. I. Miyauchi (1996). Antibacterial activity of xanthones from guttiferaeous plants against methicillin-resistant *Staphylococcus aureus*, *J. Pharm. Pharmacol.* **48**, 861-865.
[49] T. Janković, D. Krstić, K. Šavikin-Fodulović, N. Menković and D. Grubišić (1997). Comparative investigation of secoiridoid compounds of *Centaurium erythraea* grown in nature and cultured *in vitro*, *Pharm. Pharmacol. Lett.* **7**, 30-32.
[50] I. Asaka, I. Ii, M. Hirotani, Y. Asada and T. Furuya (1993). Production of ginsenoside saponins by culturing ginseng (*Panax ginseng*) embryogenic tissues in bioreactors, *Biotech. Lett.* **15**, 1259-1264.
[51] S. Surveswaran, Y. Z. Cai, H. Corke and M. Sun (2007). Systematic evaluation of natural phenolic antioxidants from 133 Indian medicinal plants, *Food Chem*. **102**, 938-953.
[52] R. Sathishkumar, P. T. V. Lakshmi and A. Annamalai (2010). Comparative analyses of non enzymatic and enzymatic antioxidants of *Enicostema littorale* blume, *Int. J. Pharma. Bio. Sci.* **V1(2)**, 1-16.