ANTIMICROBIAL ACTIVITY, ANTIOXIDANT POTENTIAL AND TOTAL PHENOLIC CONTENT OF TRANSGENIC ATCKX1 CENTAURY (Centaurium erythraea Rafn.) PLANTS GROWN IN VITRO

Milana Trifunović-Momčilov1*, Dijana Krstić-Milošević1, Snežana Trifunović2, Ana Ćirić1, Jasmina Glamočlija1, Sladana Jevremović1, Angelina Subotić1

1Institute for Biological Research “Siniša Stanković”, National Institute of Republic of Serbia, University of Belgrade, Bulevar despota Stefana 142, 11060 Belgrade, Serbia
2Faculty of Chemistry, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia

Abstract

Common centaury, Centaurium erythraea Rafn., represent the best known and the most investigated medicinal plant species of genus Centaurium. Centaury has been used for centuries in traditional medicine. Secondary metabolites such as bitter secoiridoid glucosides (gentiopicrin, swertiamarin and sweroside), xanthones (eustomin and demethyleustomin), and phenolic acids are the main constituents responsible for the therapeutic properties of centaury. Previous investigation showed that overexpression of the AtCKX genes in transgenic centaury plants did not result in a decrease of total cytokinin (CK) content, but in an altered CK profile leading to a decline of bioactive, the most important physiologically active group of CKs. The aim of this study was to investigate antibacterial and antifungal activity of transgenic centaury methanol extracts as well as pure secoiridoid and xanthone compounds on four Gram positive, four Gram negative bacteria and eight species of microfungi. All tested methanol extracts of control and transgenic AtCKX1 centaury shoots and roots showed better antibacterial activity, while pure compounds (gentiopicrin, swertiamarin, eustomin and demethyleustomin) showed better antifungal activity. The results obtained in this work suggest that centaury methanol extracts and pure compounds represent potential antimicrobials confirming the possibility of using these compounds in agronomy, veterinary, medicine or food industry.

Key words: antibacterial activity, antifungal activity, AtCKX genes, Centaurium erythraea Rafn., secondary metabolites

Received: January, 2019; Revised final: February, 2019; Accepted: March, 2019; Published in final edited form: September, 2019

1. Introduction

Microbes cause numerous and the most diverse infections in humans worldwide. The increasing in use of antibiotics and antifungal drugs has led to the development of multidrug-resistant pathogens (van de Sande-Brunisma et al., 2008). Thus, there is a need for discovering novel and effective tools for controlling these pathogenic microorganisms. More than one hundred secondary plant metabolites are being used as drugs. It is already known that medicinal plants produce a numerous physiologically active compounds used in the treatment of various chronic and infectious diseases (Dhama et al., 2015). It was discovered that plants producing terpenoids, alkaloids and polyphenols represent a very useful source of bioactive compounds with high antimicrobial activities (Silva and Fernandez, 2010).

Reactive oxygen species (ROS) including superoxide radical ($\text{O}_2^-$), hydroxyl radical (OH) and hydrogen peroxide ($\text{H}_2\text{O}_2$) represent an important mediators of cell injuries such as membrane damage, lipid peroxidation, carbohydrate damage, protein oxidation and fragmentation, mutagenesis and...
carcinogenesis (Valko et al., 2007). Thus, ROS are directly or indirectly involved in numerous chronic diseases including cancer and heart diseases. Antioxidants reduce the risk of chronic diseases and at the same time play a very important role as health protecting factors (Cui et al., 2004). Endogenous and exogenous antioxidants have an equally important role in the protection of the organism against oxidative stress. Most of the antioxidant compounds with a number of chemical properties have been detected in numerous agricultural and horticultural crops and medicinal plant species (Ksouri et al., 2009). Primary sources of naturally occurring antioxidants, such as vitamins C and E, carotenoids, phenolic acids etc., are fruits and vegetables (Prior and Cao, 2000). The antioxidant activity of plant extracts is primarily due to phenolic compounds. Structurally, phenols are molecules that have one or more hydroxyl substituents attached to the carbon atom of an aromatic ring (Bravo, 1998). The antioxidant activity is based on the redox properties which allow phenolic compounds to serve as reducing agents or hydrogen atom donors. Thus, natural antioxidants act as free-radical scavengers (Tosun et al., 2011).

Genus Centaurium (Gentianaceae) comprises about 50 species widespread in the northern hemisphere (Chevallier, 2000). In traditional medicine common centaury, Centaurium erythraea Rafn. (syn. C. umbellatum Gillib. and C. minus Moench., Gentiana centaurium L., Erythraea centaurium), has been used for centuries and represents a very important medicinal plant in the treatment of gastrointestinal tract diseases. Also, centaury represents the most investigated medicinal plant species of genus Centaurium. Because of the numerous biologically active pharmacological compounds this cosmopolitan plant species has been denoted as a plant with bitter digestive properties (Blumenthal et al., 1998; Hänsel et al., 1992), diuretic effect (Haloui, 2000), hepato-protective activity (Mroueh et al., 2004), antipyretic activity (Berkan et al., 1991), anti-inflammatory activity (Capasso et al., 1983; Hänsel et al., 1992; Newall et al., 1996) and antioxidant potential (Valenão et al., 2001; 2003). Phytochemical investigation of the genus Centaurium revealed the presence of bitter secoiridoid glucosides swertiamarin, gentiopicroin and sweroside (Jensen and Schripsema, 2002; Van der Sluis et al., 1983) and xanthones such as eustomin and demethyleustomin (Van der Sluis, 1985a, 1985b). Kumarasamy et al. (2003a, 2003b) described the bioactivity of centaury secoiridoid glycosides sixteen years ago.

Cytokinin oxidase/dehydrogenase (CKX, EC 1.5.99.12) is the only known catabolic enzyme that catalyzes specific cytokinin (CK) degradation in plant tissues (Mok and Mok, 2001; Schmülling et al., 2003). Genetic transformation of plants using specific AtCKX genes is very useful tool for investigation of numerous physiological processes controlled by CKs. Obtained AtCKX plants are expected to be with increased CKX activity and decreased endogenous CKs content, accordingly. An efficient protocol for A. tumefaciens-mediated transformation of centaury and production of stable transformants overexpressing AtCKX genes were previously described (Trifunović et al., 2013). Transgenic AtCKX centaury plants showed changed CK content and altered CK homeostasis which resulted in reduced level of bioactive CKs and, at the same time, increased contents of storage, inactive CK forms and/or CK nucleotides (Trifunović et al., 2015).

Notwithstanding that centaury is widely used in traditional medicine there are no many literature data considering biological effects of pure xanthones isolated from centaury. Numerous biological activities of centaury have mainly been ascribed to whole plant extracts. Therefore, the aim of this study was to investigate antibacterial and antifungal activity of non-transformed and transgenic centaury methanol extracts as well as pure secoiridoid and xanthone compounds on four Gram positive, four Gram negative bacteria and eight species of microfungi. Also, the purpose of this work was to investigate the antioxidant capacity and the total phenolic content of centaury, very important medicinal plant, and to evaluate the its potential antioxidants for medicinal and food purposes.

2. Material and methods

2.1. Plant material and culture conditions

Gene coding for Arabidopsis CK isoform, AtCKX1, was introduced into root explants of C. erythraea Rafn. as previously described (Trifunović et al., 2013). Four AtCKX1 centaury lines were selected for further analyses. All selected lines showed increased expression of AtCKX1 transgenes and at the same time increased level of CKX activity. Among all analysed transgenic centaury lines only one line, AtCKX1-29, produced increased content of xanthones compared to control (Trifunović et al., 2015). Control and only one transgenic centaury line, AtCKX1-29, were used in this study. Both centaury lines were cultured on solid, hormone free and half-strength MS medium (½MS, Murashige and Skoog, 1962) during four weeks. The used medium was supplemented with 3% sucrose and 100 mgL⁻¹ myo-inositol. All in vitro cultured plants were grown at 25 ± 2°C and a 16h/8h photoperiod (“Tesla” white fluorescent lamps, 65W, 4500K; light flux of 47 47 µmol·m⁻²·s⁻¹).

2.2. HPLC analyses

Extraction and quantification of secondary metabolites from control and transgenic AtCKX1-29 centaury line was carried out as follows. The air-dried and powered AtCKX transgenic and control C. erythraea 4-weeks-old shoots and roots were extracted with methanol for 48h at room temperature in the dark. The ratio between plant material and solvent was 1:20, w/v. Analyses of extracts were carried out on an Agilent series 1100 HPLC instrument with DAD detector and a reverse phase
Antimicrobial activity, antioxidant potential and total phenolic content of transgenic ATCKX1 centaury

Zorbax SB-C18 analytical column (150 x 4.6 mm, 5 μm). Mobile phase consisted of 1%, v/v solution of orthophosphoric acid in water (solvent A) and acetonitrile (solvent B). The flow rate was 1 mL min⁻¹. Injection volume of sample was 5 μl and the gradient elution was as follows: 98-90% A, 0-5 min, 90-85% A, 5-10 min, 85% A, 10-13 min, 85-70% A, 13-15 min, 70-10% A, 15-20 min, 10% A, 20-22 min, 10-0% A, 22-25 min. Detection wavelengths were set at 260 nm for secoiridoids and 320 nm for xanthones.

Xanthones, eustomin and demethyleustomin, were isolated from aerial parts of C. erythreae plants collected from nature. Their structures were confirmed by spectroscopic techniques: UV, 1D and 2D NMR spectroscopy and MS spectrometry. Secoirdoids, swertiamarin and gentiopicrin, were supplied by Cfm (Srbolek, Belgrade, Serbia) and ketoconazole (Zorkapharma, Šabac, Serbia), were used as positive controls (1–3500 μg/mL). All experiments were performed in duplicate and repeated three times.

2.3. Antibacterial and antifungal activity

Antibacterial and antifungal activities were determined using the microdilution method as described previously (Božunović et al., 2018). The antibacterial activity of investigated extracts was evaluated using eight bacterial strains: Escherichia coli (ATCC 35210), Pseudomonas aeruginosa (ATCC 27853), Salmonella typhimurium (ATCC 13311), Enterobacter cloacae (human isolate), Bacillus cereus (clinical isolate), Micrococcus flavus (ATCC 10240), Listeria monocyogenes (NCTC 7973) and Staphylococcus aureus (ATCC 6538). The antibacterial assay was carried out by a microdilution method (CLSI, 2009; Tsukatani et al., 2012). The centaury extracts (10 mg/mL) and pure compounds (1 mg/mL) were dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) and added in Tryptic Soy broth (TSB) medium (100 µl) with bacterial inoculum (1.0×10⁴ CFU per well). The results were presented as mg/g of dry weight (DW).

The antifungal activity of the extracts used in this study was evaluated using eight fungal species such as Aspergillus fumigatus (human isolate), Aspergillus versicolor (ATCC 11730), Aspergillus ochraceus (ATCC 12066), Aspergillus niger (ATCC 6275), Trichoderma viride (IAM 5061), Penicillium funiculosum (ATCC 36839), Penicillium ochrochloron (ATCC 9112) and Penicillium verrucosum var. cyclopium (food isolate). A modified microdilution technique was used (Espinel-Ingroff, 2001; Hanel and Raether, 1988). Antifungal results were expressed by MICs and minimum fungicidal concentrations (MFCs). A serial dilution technique using microtiter plates was used for determination of MICs. Investigated centaury extracts (10 mg/mL) and pure compounds (1 mg/mL) were dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v). Further, centaury extracts were added in broth Malt medium supplemented with inoculum. The microtiter plates were further incubated in a rotary shaker (160 rpm) at 28°C during 72h. Minimum inhibitory concentrations were determined as lowest concentrations without visible growth. Also, a serial dilution technique using microtiter plates was used for determination of MFCs. The serial subcultivation of tested compounds (2 µL) were dissolved in medium and inoculated for 72h. Microtiter plates containing 100 µL of broth per well were further incubated at 28°C during 72h. Minimum fungicidal concentrations, indicating 99.5% killing of the original inoculum, were determined as lowest concentrations with no visible growth. Solution of 5% DMSO was used as a negative control, commercial fungicides, bifonazole (Srbolek, Belgrade, Serbia) and ketoconazole (Zorkapharma, Šabac, Serbia), were used as positive controls (1–3500 μg/mL). All experiments were performed in duplicate and repeated three times.

2.4. DPPH radical scavenging activity

The DPPH assay is a rapid and simple method for measuring antioxidant capacity, based on the reduction of 1,1-diphenyl-2-picrylhydrazyl (DPPH), a stable free radical. The effect of centaury methanol extracts on DPPH free radicals was estimated according to the procedure described by Brand-Williams et al., 1995, with some modifications. Centaury extracts were dissolved in methanol (dilution series from 0.125 – 2 mg/mL) and mixed with methanol solution of DPPH 150 μM). The resulting mixture was shaken and incubated for 20 minutes at room temperature in the dark. Thereafter, absorbance (A) of the samples was measured spectrophotometrically at 517 nm. Radical scavenging activity was calculated by following formula: % Inhibition=([(A blank sample-A centaury extract)/A blank sample] x 100. The IC₅₀ values were calculated by linear regression of plots where the abscissa represented the concentration of tested samples and ordinate the average percent of inhibition activity from three separate tests.

2.5. Determination of total phenolic content

The level of total phenols in centaury methanol extracts was determined by using Folin–Ciocălteu reagent and external calibration with gallic acid. Determination of total phenolic content was carried out according to the procedure described by (Singleton et al., 1999) with slight modification. To 100 mL of each extract (5 mg/mL), 500 μL of Folin-Ciocălteu reagent (previously tenfold diluted with distilled water) was added and mixed. After 5 minutes, 400 μL of sodium carbonate (7.5 g/mL) was added, and the mixture was incubated at room temperature in the dark for 2 hours. The absorbance was measured spectrophotometrically at 760 nm. Results were expressed as milligrams of gallic acid equivalent per gram of dry weight of plant extract (mg GAE/ g DW).
Triplicate measurements were taken and mean values were calculated.

2.6. Statistical analysis

All statistical analyses were performed using StatGrafics software version 4.2 (STSC Inc. and Statistical Graphics Corporation, 1985–1989, USA). The data were subjected to analysis of variance (ANOVA) and comparisons between the mean values were made using the least significant difference (LSD) test calculated at a confidence level of \( p \leq 0.05 \).

3. Results and discussion

3.1. Secondary metabolite content

HPLC analysis of centaury methanolic extracts revealed that there were no differences in detected secondary metabolites from transgenic centaury line \( AtCKX1-29 \) grown \( \text{in vitro} \) and control centaury plants. The common peaks of secoiridoids and xanthones were detected in all tested extracts. Quantitative differences in secondary metabolites content from centaury shoots and roots were presented in Table 1. Increased xanthones content and decreased content of secoiridoids was determined in transgenic centaury line \( AtCKX1-29 \) in comparison to control centaury extracts. It was also noticed that in control centaury shoots grown \( \text{in vitro} \) secoiridoid swertiamarin was dominated (Table 1). However, in shoots and roots of transgenic line \( AtCKX1-29 \), production of swertiamarin significantly decreased and the content of this bitter glucoside reduced to only 1.34 mg/g DW in the shoots. Also very low content of gentiopicrin was detected (0.47 mg/g DW). On the contrary, the amounts of xanthones were higher in both transgenic shoots and roots compared to control extracts. The highest amount of eustomin (4.71 mg/g DW) and demethylleustomin (1.70 mg/g DW) was determined in the roots of transgenic line \( AtCKX1-29 \). Typical chromatographic profile of centaury methanol extract is shown in Fig. 1.

Swertiamarin was the most abundant secoiridoid in control centaury shoots grown \( \text{in vitro} \). On the other hand, the roots of control centaury contained gentiopicrin as the dominant secoiridoid. The results of this work are corresponding with previous reports only partially. Earlier investigations showed that swertiamarin was the dominant secoiridoid in centaury shoots and roots collected from the natural habitat but secoiridoidgentiopicrin was dominated in centaury shoot and roots grown \( \text{in vitro} \) while swertiamarin was detected only in traces (Janković et al., 1997; Piatczak et al., 2005; Van der Sluis, 1985a, 1985b).

In \( AtCKX1-29 \) transgenic centaury shoots and roots significantly lower content of swertiamarin and gentiopicrin was detected compared to control shoots and roots. Based on the content of centaury swertiamarin and gentiopicrin Piatczak et al. (2005) concluded that \( \text{in vitro} \) culture conditions influenced on reduced production of gentiopicrin. However, the results presented in this work indicated that \( \text{in vitro} \) culture conditions stimulated the production of gentiopicrin but only in centaury roots.

Table 1. Content of secoiridoids (swertiamarin and gentiopicrin) and xanthones (eustomin and demethylleustomin) in shoots and roots of 4-week-old control and \( AtCKX1-29 \) transgenic Centaurium erythraea plants grown \( \text{in vitro} \). Data represent mean ± standard error. Means marked with an asterisk are significantly different from the control according to the LSD test (\( p \leq 0.05 \))

<table>
<thead>
<tr>
<th>Secondary metabolites (mg/g DW)</th>
<th>Shoots</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>( AtCKX1-29 )</td>
</tr>
<tr>
<td>swertiamarin</td>
<td>64.77 ± 3.92</td>
<td>1.34 ± 0.11*</td>
</tr>
<tr>
<td>gentiopicrin</td>
<td>1.56 ± 0.19</td>
<td>0.47 ± 0.10*</td>
</tr>
<tr>
<td>eustomin</td>
<td>1.18 ± 0.12</td>
<td>1.48 ± 0.02</td>
</tr>
<tr>
<td>demethylleustomin</td>
<td>1.30 ± 0.15</td>
<td>1.17 ± 0.01</td>
</tr>
</tbody>
</table>

Fig. 1. HPLC profile of methanol extract of transgenic centaury roots (\( \lambda = 260 \) nm). Peaks: swertiamarin (1), gentiopicrin (2), eustomin (3), demethylleustomin (4)
Contrarily, Janković et al. (1997) showed increment of gentiopicrin content only in centaury shoots grown in vitro compared to the shoots from natural habitat. It is already known that genetic transformation could affect the production of centaury secondary metabolites. Transformed centaury roots obtained by inoculating using A. rhizogenes (strain A4M70GUS) accumulated only xanthones but no secoiridoids at all (Janković et al., 2002). There is also a literature which demonstrated that transformed centaury shoots inoculated by A. rhizogenes (strain LBA9402) produced two times higher amount of total secoiridoids compared to non-transformed shoots (Piatczak et al., 2006). In any case, it can be said that genetic transformation of centaury with AtCKX genes influenced the secondary metabolism of this plant species. Considering that control and transgenic centaury plants were grown in vitro, under the same temperature and light intensity conditions, it is possible that genetic transformation itself influenced on altered secondary metabolites content, especially on suppression of gentiopicrin production. In the metabolic pathway of iridoids, secoiridoid gentiopicrin originates from the swertiamarin. In AtCKX transgenic centaury shoots increased accumulation of gentiopicrin and at the same time reduced swertiamarin content was detected. Accumulation of secoiridoid gentiopicrin is apparently result of increased activity of the enzyme that converts swertiamarin to gentiopicrin. It was also noted that in roots of centaury transgenic line AtCKX-29 the production of eustomin and demethyleneustomin was stimulated. Although it is already known that in vitro culture could stimulate secondary metabolites production in response to abiotic stress, it can be also assumed that stress caused by genetic transformation itself additionally affected metabolic changes resulted in increased content of xanthones in AtCKX1 transgenic centaury roots.

3.2. Antibacterial and antifungal activity of centaury plants grown in vitro

Methanol extracts of control and AtCKX1-29 transgenic centaury plants grown in vitro as well as pure secoiridoids and xanthones showed antimicrobial activity against all tested bacteria. The results of antibacterial activity are presented in Table 2. Generally, all tested secondary metabolites (methanol extracts, pure secoiridoids and xanthones) showed MIC in the range of 0.004-0.030 mg/mL and MBC in the range of 0.005-0.040 mg/mL on all tested bacteria. Methanol extracts of all tested centaury shoots and roots showed antibacterial activity on all tested bacteria. MIC of centaury methanol extracts was in the range of 0.090-0.500 mg/mL, while MBC was in the range of 0.125-1.000 mg/mL. On the other hand, methanol extracts of control and AtCKX1-29 transgenic centaury roots showed low antibacterial activity (MIC/MBC were at 0.500/1.000 mg/mL) on M. flavus, E. coli and E. cloacae. Gentiopicrin also showed high antifungal potential against all tested mycotics except for P. funiculosum, A. fumigatus, A. ochraceus and P. ochrochloron. Pure compounds such as eustomin, demethyleneustomin, swertiamarin and gentiopicrin showed good antifungal effect and MIC values were 0.002-0.030 mg/mL and fungidal activity was 0.004-0.060 mg/mL. The majority of these compounds showed similarly high activity against all tested fungi. It was also noted that all analysed pure compounds were more effective than any methanol extracts against all fungi with the exception of control centaury root extract against P. funiculosum. The commercial antifungal agent, bifonazole, showed MIC at 0.100-0.200 mg/mL and MFC at 0.200-0.250 mg/mL. Ketoconazole showed fungistatic activity at 0.150-2.500 mg/mL and fungidical effect at 0.200-3.500 mg/mL. The methanol extracts of control centaury shoots and roots grown in vitro exhibited higher antifungal potential than mycotics against A. Versicolor and P. funiculosum. Contrarily, methanol extracts of AtCKX1 transgenic centaury shoots and transgenic roots showed lower activity than both mycotics except for P. funiculosum, where oil possessed inhibitory activity higher than mycotics. Xanthone eustomin exhibited higher antifungal potential than both mycotics (even 100 times higher).
Table 2. Antibacterial activity of the methanol extracts of centaury control shoots and roots cultured in vitro, transgenic Ackx1-29 shoots and roots cultured in vitro, xanthones (eustomin and demethyl/eustearmin) and secoiridoids (swertiamarin and gentiopicrocin). Mean values of MIC (minimum inhibitory concentration) and MBC (minimal bactericidal concentration) are presented in mg/mL ± SD. Different letters in each row indicate significant differences between the extracts (p≤ 0.05)

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Staphylococcus aureus</th>
<th>Bacillus cereus</th>
<th>Listeria monocytogenes</th>
<th>Micrococcus flavus</th>
<th>Pseudomonas aeruginosa</th>
<th>Escherichia coli</th>
<th>Salmonella typhimurium</th>
<th>Enterobacter cloacae</th>
</tr>
</thead>
<tbody>
<tr>
<td>control shoots in vitro</td>
<td>MIC 0.090±0.003a</td>
<td>0.090±0.005b</td>
<td>0.250±0.003c</td>
<td>0.250±0.010b</td>
<td>0.375±0.008b</td>
<td>0.187±0.004a</td>
<td>0.250±0.001a</td>
<td></td>
</tr>
<tr>
<td>transgenic shoots in vitro</td>
<td>MIC 0.125±0.008a</td>
<td>0.125±0.002c</td>
<td>0.500±0.02a</td>
<td>0.500±0.020b</td>
<td>0.500±0.007d</td>
<td>0.500±0.030a</td>
<td>0.250±0.020d</td>
<td>1.000±0.070b</td>
</tr>
<tr>
<td>control roots in vitro</td>
<td>MIC 0.187±0.003d</td>
<td>0.125±0.006e</td>
<td>0.375±0.120c</td>
<td>0.187±0.004d</td>
<td>0.375±0.008e</td>
<td>0.125±0.008e</td>
<td>0.250±0.020d</td>
<td>1.000±0.030c</td>
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<tr>
<td>transgenic roots in vitro</td>
<td>MIC 0.250±0.007e</td>
<td>0.250±0.010g</td>
<td>0.500±0.010e</td>
<td>0.250±0.002f</td>
<td>0.500±0.020e</td>
<td>0.500±0.020e</td>
<td>0.187±0.010f</td>
<td>1.000±0.020e</td>
</tr>
<tr>
<td>eustomin</td>
<td>MIC 0.090±0.003d</td>
<td>0.125±0.006e</td>
<td>0.375±0.008e</td>
<td>0.500±0.010e</td>
<td>0.375±0.008e</td>
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</tr>
<tr>
<td>demethyl/eustearmin</td>
<td>MIC 0.125±0.002e</td>
<td>0.250±0.010i</td>
<td>0.500±0.001a</td>
<td>1.000±0.050d</td>
<td>0.500±0.010f</td>
<td>1.000±0.070c</td>
<td>0.250±0.010f</td>
<td>1.000±0.10d</td>
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<tr>
<td>swertiamarin</td>
<td>MIC 0.010±0.001a</td>
<td>0.010±0.001a</td>
<td>0.010±0.001a</td>
<td>0.025±0.002a</td>
<td>0.020±0.003a</td>
<td>0.100±0.007a</td>
<td>0.025±0.002a</td>
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<tr>
<td>gentiopicrocin</td>
<td>MIC 0.000±0.001a</td>
<td>0.000±0.001a</td>
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<td>secoiridoids</td>
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<tr>
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<td>0.100±0.001e</td>
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<td>0.050±0.001e</td>
<td>0.050±0.002b</td>
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<tr>
<td>ampicillin</td>
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<td>0.300±0.007g</td>
<td>0.150±0.010d</td>
<td>0.200±0.010e</td>
<td>0.500±0.030f</td>
<td>0.200±0.002e</td>
<td>0.200±0.020e</td>
<td></td>
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There are numerous reports describing antiviral, antibacterial, antifungal and anti-inflammatory properties of plants (Fierascu et al., 2017; Kapoor et al., 2015; Namita and Mukesh, 2012; Pushpa et al., 2013; Saranraj and Sivasakthi, 2014; Silva and Fernandes, 2010; Tanase et al., 2018). Some of these observations helped in identifying active compounds responsible for specific activities and certainly helped in developing novel drugs for therapeutic use in humans. In the present study secondary metabolites characteristic for C. erythraea species (swertiamarin, gentiopicrocin, eustomin and demethyl/eustearmin) exhibited higher antibacterial activity on all tested bacteria than centaury methanol extracts and antibiotics. All the tested methanol extracts of centaury shoots and roots exhibited antibacterial activity. Pure swertiamarin, gentiopicrocin, eustomin and demethyl/eustearmin exhibited higher antibacterial activity on all tested bacteria than centaury methanol extracts. These compounds showed higher antibacterial activity even better than commercial antibiotics (streptomycin and ampicillin) used as positive control. It can be concluded that high antimicrobial activity could be ascribed to bitter secoiridoid glycosides as well as xanthones. These results are in accordance with previous findings considering the antibacterial activity of swertiamarin and gentiopicrocin (Siler et al., 2010, 2014). Beside antibacterial all tested methanol extracts of centaury shoots and roots as well as pure secoiridoids and xanthones showed antifungal effect. The majority of these compounds showed high activity against all tested fungi. It was also noted that all analysed pure secoiridoids and xanthones were more effective than any centaury methanol extracts against all fungi. Interestingly, xanthone eustomin exhibited higher antifungal potential, even 100 times higher, than both applied mycotics (ketoconazole and bifonazole).
Table 3. Antifungal activity of the methanol extracts of centaury control shoots and roots cultured in vitro, transgenic AtCKX1-29 shoots and roots cultured in vitro, xanthones (eustomin and demethylxanthones) and secoiridoids (swertiamarin and gentiopicrin).

Mean values of MIC (minimum inhibitory concentration) and MFC (minimum fungicidal concentration) are presented in mg/mL ± SD. Different letters in each row indicate significant differences between the extracts (p ≤ 0.05).

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Aspergillus fumigatus</th>
<th>Aspergillus niger</th>
<th>Trichoderma viride</th>
<th>Penicillium italicum</th>
<th>Penicillium ochrochloron</th>
<th>Penicillium verrucosum var. cyclopium</th>
</tr>
</thead>
<tbody>
<tr>
<td>control shoots in vitro</td>
<td>0.500±0.020</td>
<td>0.060±0.001a</td>
<td>0.125±0.008a</td>
<td>0.125±0.008a</td>
<td>0.250±0.020a</td>
<td>0.125±0.023a</td>
</tr>
<tr>
<td>MFC</td>
<td>1.000±0.070</td>
<td>0.125±0.008a</td>
<td>0.250±0.020a</td>
<td>1.125±0.040a</td>
<td>0.250±0.020a</td>
<td>0.500±0.020a</td>
</tr>
<tr>
<td>transgenic shoots in vitro</td>
<td>1.000±0.070a</td>
<td>0.500±0.030b</td>
<td>1.000±0.001b</td>
<td>0.250±0.007c</td>
<td>0.125±0.008a</td>
<td>0.500±0.020b</td>
</tr>
<tr>
<td>MFC</td>
<td>1.250±0.080a</td>
<td>1.000±0.100c</td>
<td>1.250±0.084d</td>
<td>0.500±0.007c</td>
<td>0.250±0.010a</td>
<td>1.000±0.070b</td>
</tr>
<tr>
<td>control roots in vitro</td>
<td>1.000±0.050b</td>
<td>0.500±0.018b</td>
<td>1.000±0.070a</td>
<td>0.500±0.020a</td>
<td>0.250±0.010a</td>
<td>1.125±0.080d</td>
</tr>
<tr>
<td>MFC</td>
<td>1.250±0.080</td>
<td>0.100±0.020f</td>
<td>1.250±0.050d</td>
<td>0.500±0.020a</td>
<td>0.015±0.002g</td>
<td>1.500±0.070d</td>
</tr>
<tr>
<td>transgenic roots in vitro</td>
<td>1.000±0.001c</td>
<td>0.500±0.050f</td>
<td>1.000±0.200f</td>
<td>0.500±0.020a</td>
<td>1.250±0.001f</td>
<td>1.000±0.001c</td>
</tr>
<tr>
<td>MFC</td>
<td>1.125±0.040f</td>
<td>1.000±0.050f</td>
<td>1.125±0.020f</td>
<td>1.000±0.070f</td>
<td>0.250±0.077a</td>
<td>1.125±0.020f</td>
</tr>
<tr>
<td>eustomin</td>
<td>0.015±0.002b</td>
<td>0.015±0.002b</td>
<td>0.015±0.001b</td>
<td>0.004±0.001b</td>
<td>0.003±0.007f</td>
<td>0.015±0.002f</td>
</tr>
<tr>
<td>MFC</td>
<td>0.060±0.003c</td>
<td>0.060±0.003c</td>
<td>0.060±0.003c</td>
<td>0.060±0.003c</td>
<td>0.060±0.001c</td>
<td>0.060±0.003f</td>
</tr>
<tr>
<td>swertiamarin</td>
<td>0.015±0.001b</td>
<td>0.015±0.001b</td>
<td>0.015±0.002b</td>
<td>0.015±0.002b</td>
<td>0.015±0.002b</td>
<td>0.030±0.001f</td>
</tr>
<tr>
<td>MFC</td>
<td>0.060±0.003c</td>
<td>0.060±0.003c</td>
<td>0.060±0.003c</td>
<td>0.060±0.003c</td>
<td>0.060±0.001c</td>
<td>0.060±0.003f</td>
</tr>
<tr>
<td>gentiopicrin</td>
<td>0.008±0.001c</td>
<td>0.002±0.002c</td>
<td>0.004±0.001c</td>
<td>0.002±0.001f</td>
<td>0.004±0.001f</td>
<td>0.004±0.001f</td>
</tr>
<tr>
<td>MFC</td>
<td>0.015±0.002b</td>
<td>0.004±0.001c</td>
<td>0.008±0.001c</td>
<td>0.008±0.001c</td>
<td>0.008±0.001c</td>
<td>0.008±0.001c</td>
</tr>
<tr>
<td>ketoconazole</td>
<td>0.200±0.070</td>
<td>0.200±0.010a</td>
<td>0.150±0.020a</td>
<td>0.200±0.007d</td>
<td>2.500±0.200f</td>
<td>0.200±0.020f</td>
</tr>
<tr>
<td>MFC</td>
<td>0.500±0.020c</td>
<td>0.500±0.005c</td>
<td>0.200±0.020d</td>
<td>0.500±0.001c</td>
<td>3.500±0.100c</td>
<td>0.500±0.030d</td>
</tr>
<tr>
<td>bifonazole</td>
<td>0.150±0.010d</td>
<td>0.100±0.020b</td>
<td>0.150±0.001b</td>
<td>0.150±0.007b</td>
<td>0.200±0.020c</td>
<td>0.200±0.030g</td>
</tr>
<tr>
<td>MFC</td>
<td>0.200±0.001b</td>
<td>0.200±0.001b</td>
<td>0.200±0.001b</td>
<td>0.200±0.010a</td>
<td>0.250±0.020d</td>
<td>0.200±0.020b</td>
</tr>
</tbody>
</table>

Although century secondary metabolites and their bioactivity have previously been investigated in details (Kumarsamy et al., 2003a, 2003b; Van der Sluis, 1985a) there are no literature data considering pure xanthones eustomin and demethylxanthones as potential antimicrobials. Thus, the results obtained in this work present the first report considering century xanthones as compounds with high antifungal activity.

3.3. DPPH radical scavenging activity and total phenolic content

The antioxidant activity of plant extracts is one of the most important factor in protection against oxidative damage. The potential antioxidant activity of century extracts was assessed on the basis of the scavenging activity for the stable DPPH radicals. The results showed in Table 4 indicated that the highest radical scavenging activity was observed in AtCKX1 transgenic century roots cultured in vitro where the concentration of methanol extracts lower than 1 mg/mL (IC₅₀ = 0.65 mg/mL) already inhibited 50% of the DPPH reference signal. Similarly, high antioxidant activity was also detected in control century roots (IC₅₀ = 1.10 mg/mL) and AtCKX1 transgenic century shoots (IC₅₀ = 1.81 mg/mL). On the other hand, control century shoots in vitro indicated the lowest antioxidant activity where IC₅₀ value was 3.12 mg/mL. It was also noted that century root extracts were more effective than shoot extracts, with activities two to five times higher for root extracts.

Based on the results presented in Table 4 total phenolic content was higher in the century roots compared to shoots. The highest level of total phenolic content was detected in AtCKX1 transgenic century roots grown in vitro (163.28 mg GA/g DW). Similar total phenolic level was determined in control century roots. In aerial parts of century control and transgenic shoots significantly lower total phenolic content revealed.
It can be concluded that all tested methanol extracts as in correlation with the highest total phenolic content. Generally, the antioxidant activities of phenolic compounds are mainly due to their ability to act as hydrogen donors, reducing agents and radical scavengers (Mai et al., 2009). It is known that there is a positive relationship between antioxidant activity potential and amount of phenolic compounds of the crude extracts of \textit{Camellia sinensis} (Mello and Quadros, 2014). Significantly higher phenolics content and antioxidant activity were also detected in \textit{Capsella bursa-pastoris} and \textit{Marrubium vulgare} (Neagu et al., 2019) as phenolic compounds, xanthones have already been described for their antioxidant properties (Pinto et al., 2005). Antioxidant properties of \textit{C. erythraea} have been already previously characterised (Valentão et al., 2001). In this study \textit{AtCKX1} transgenic centaury methanol extracts were analysed for the first time for their antioxidant activities.

The results showed that methanol extract of transgenic roots, line \textit{AtCKX1-29}, with highest phenolic content exhibited the highest capacity for the scavenging of the DPPH radicals. Considering that in roots of centaury transgenic line \textit{AtCKX1-29} detected stimulated production of eustomin and demethyleustomin, antioxidant properties of analysed methanol extracts could be assigned to elevated xanthone compounds.

### 4. Conclusions

The results of present investigation clearly indicate that the antibacterial and antifungal activity depend on plant material. All tested methanol extracts of control and transgenic \textit{AtCKX1} centaury shoots and roots showed better antibacterial activity, while pure secoiridoids (gentiopicrin and swertiamarin) and xanthones (eustomin and demethyleustomin) were more active against fungi.

Generally, transgenic \textit{AtCKX1} centaury shoots and roots showed higher antioxidant activity compared to the control shoots and roots. The highest antioxidant activity (IC$_{50}$ = 0.65 mg/mL) was determined in transgenic \textit{AtCKX1-29} roots which is in correlation with the highest total phenolic content. It can be concluded that all tested methanol extracts as well as pure secoiridoid and xanthone compounds represent potential bacterial and mold inhibitors confirming the possibility of using them in agronomy, veterinary, medicine and food industry. Thus, centaury plants with increased content of secondary plant metabolites, especially xanthones, could be of significant interest in the development of novel drugs.

### Acknowledgements

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant No. ON173015 and ON173032).

### Abbreviations

- HPLC – High pressure liquid chromatography
- MS – Murashige and Skoog medium
- NMR – Nuclear magnetic resonance spectroscopy
- UV – Ultraviolet–visible spectroscopy

### References


Mok D.W.S., Mok M.C., (2001), Cytokinin metabolism and action, Plant Physiology and Plant Molecular Biology, 52, 89-118.


