Industrial Crops & Products 111 (2018) 705–721

Contents lists available at ScienceDirect

Industrial Crops & Products

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

In vitro and *in vivo* transformations of *Centaurium erythraea* secoiridoid glucosides alternate their antioxidant and antimicrobial capacity

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ARTICLE INFO

Keywords:
Centaurium erythraea
Metabolomics
Secoiridoid glucosides
Aglycones
Antioxidant activity
Antimicrobial activity

ABSTRACT

The present study was principally aimed at ascertaining the differences in metabolomics profiles and biological activities between non-hydrolyzed (ME) and hydrolyzed methanol extract (HME) of *Centaurium erythraea* Rafn. *UHPLC–MS/MS Orbitrap* analysis showed that the enzymatic hydrolysis of the extract caused changes in β-D-glycoside/aglycone ratio of both flavonoid and secoiridoid compounds. *UHPLC/DAD/+HESI−qqqMS* characterization and/or quantification of secoiridoid glucosides (SGs) and their aglycones in both ME and HME revealed gentiopicral and erythrocentaurin as the major aglycones, the same metabolic products which appear after the hydrolysis of pure swertiamarin, the dominant secoiridoid glucoside of *C. erythraea*. SGs played an antioxidant role only in ABTS assay, whilst the remarkable antioxidant potential of *C. erythraea* methanol extract is ascribed chiefly to phenolics detected in it. Interestingly, antioxidant activities of swertiamarin and sweroside recorded in ABTS assay increased after the compounds have been hydrolyzed, which highlighted their possible antioxidant role during ingestion. Strong antimicrobial activities of ME and HME against a vast array of pathogens, which exceed the effects of the reference antibiotics and antimycotics, largely depended on the amount of secoiridoids in either of the glycosylation forms. Extracts and pure secoiridoids were especially effective against most of the tested *Penicillium* species. On the other hand, *P. funiculosum* has evolved an efficient mechanism of detoxification of sub-lethal concentrations of secoiridoid glucosides, involving their biotransformation and complete digestion. The presented findings will contribute to clarify the fate and role of the SGs after *C. erythraea* ingestion within *in vivo* systems, and to further promote this remarkable plant as a food preservation additive with significant health benefits.

1. Introduction

Centaurium erythraea Rafn (*Gentianaceae*), commonly known as "centaury", is a biennial herbaceous plant species widely distributed in Europe from Sweden to Mediterranean basin, and east to Asia, while naturalized in America and Australia (Marhold, 2011; U.S. National Plant Germplasm System, 2017). It is one of the most widely used bitter herbs, medicinally important due to the production of bioactive secondary metabolites. Pharmacological effects of centaury include anti-inflammatory and anti-pyretic (Berkan et al., 1991), hypoglycemic (Stefkov et al., 2014), antioxidant (Đorđević et al., 2017; Sefi et al., 2011; Šiler et al., 2014), antimicrobial (Šiler et al., 2014), hepatoprotective (Mroueh et al., 2004), gastroprotective (Tuluce et al., 2011), and many others, recently reviewed in Šiler and Mišić (2016). Many previous attempts have been conducted with the aim to introduce this plant into cultivation (Pataczek et al., 2017; Radušienė, 1995; Uzundzhalieva et al., 2013), and thus overcome the problem of its decreasing natural populations as the consequence of overexploitation and degradation of biological resources. This species is considered endangered in many European countries (e.g. Colling, 2005; Niklfeld and Schratt-Ehrendorfer, 1999; Węglarz et al., 2009). New concepts to alternatively propagate this plant and produce its bioactive compounds have been implemented, including cell and hairy root culture systems of *C. erythraea*, as well as of related species (e.g. Mišić et al., 2013; Piatczak et al., 2006; Subotić et al., 2009). Although some attempts to scale-up production of secoiridoids in bioreactors have been made (Mišić et al., 2013; Piatczak et al., 2006; Radović et al., 2013), a better understanding of elicitation mechanisms is still needed prior

Abbreviations: ME, methanol extract; HME, hydrolyzed methanol extract; SWM, swertiamarin; HSWM, hydrolyzed swertiamarin; SW, sweroside; HSW, hydrolyzed sweroside; GP, gentiopicrin; HGP, hydrolyzed gentiopicrin

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https://doi.org/10.1016/j.indcrop.2017.11.040
Received 30 June 2017; Received in revised form 20 November 2017; Accepted 21 November 2017

commercial production of these bioactive compounds on industrial scale.

Secoiridoid glucosides and phenolics (xanthones, flavonoids and phenolic acids) are reported to be the most abundant secondary metabolites in aerial parts of *C. erythraea* (Aberham et al., 2011; Banjanac et al., 2017). Among the secoiridoid glucosides (SGs) swertiamarin (SWM), gentiopicrin (GP) and sweroside (SW) are most commonly found. SGs are monoterpene compounds, with the basic hydrocarbon skeleton composed of two isoprene units (C10) conjugated with glucose. Their biosynthesis starts with geraniol, which is further through the series of oxidation, reduction, glycosylation, cyclization, methylation steps and a sequence of intermediates (8-hydroxygeraniol, 8-oxogeraniol, nepetalactol, iridotrial, 7-deoxyloganetic acid, 7-deoxyloganic acid, loganic acid, and loganin) converted to secologanin, the first secoiridoid in the pathway. Genes involved in this part of the biosynthetic pathway has been studied in *Catharanthus roseus* (Miettinen et al., 2014; O'Connor, 2012; Salim et al., 2014), *Gentiana macrophylla* (Hua et al., 2014), *Rauwolfia serpentine* (Ikeda et al., 1991), *Olea europea* (Alagna et al., 2016), and *Lonicera japonica* (Yamamoto et al., 2000). Biosynthetic branch leading from secologanin to SW, SWM, and GP is still unresolved, as well as are the steps involved in the catabolism of these compounds.

Iridoid glycosides, including secoiridoid glucosides, act as prodrugs, and their activities are induced when the compounds are activated by enzymes or by acid hydrolysis (Zeng et al., 2014). The β-glucosidases catalyze hydrolysis of the bond between iridoid and sugar moiety, thus converting less reactive glucosides into highly reactive aglycones (Pankoke et al., 2013). Some pharmacokinetic studies demonstrated that aglycone metabolites of some glucosides were more readily absorbed and more bioavailable in plasma; these glucosides are, after ingestion, metabolized in the gastrointestinal tract by the intestinal flora and/or endogenous β-glucosidases (Otieno and Shah, 2007; Setchell et al., 2001). Secoiridoid aglycones are relatively rarely identified within the *Gentianaceae* family (Parra et al., 1985; Zeng et al., 2014), probably due to high instability and limitations of the analytical methods applied. Although aglycones have not been previously prepared using either enzymatic or acid hydrolysis, biotransformation of SWM, GP, and SW by microorganisms (El-Sedawy et al., 1990; Jun et al., 2008; Zeng et al., 2014) has resulted in the production of several metabolites, including erythrocentaurin and gentiopicral, the common products of SWM and GP hydrolysis.

The study was basically aimed at ascertaining the differences in metabolomics profiles between non-hydrolyzed and hydrolyzed extract of *Centaurium erythraea.* Hydrolytic degradation of glycosylated compounds is a basic chemical reaction which is taking place after plant ingestion, and aglycones may differ from the corresponding glycosides in biological activity. Therefore, our next aim was to find if the non-hydrolyzed and hydrolyzed extracts demonstrate differences in antioxidant and antimicrobial activities and to figure out which of the profiled compounds may be mostly responsible for such extract's behavior. We were also interested to discover the fate of the secoiridoid compounds that some microorganisms readily digest.

2. Material and methods

2.1. Plant material and in vitro culture conditions

Centaurium erythraea Rafn seeds were collected in 2014 in Beočin (Serbia) and further stored in the seed collection at the Institute for Biological Research "Siniša Stanković", University of Belgrade. Seeds were surface sterilized in 20% commercial bleach solution for 10 min, rinsed 5 times with sterilized deionized water and transferred onto Petri dishes containing half-strength MS medium (Murashige and Skoog, 1962) adjusted to pH 5.8 and supplemented with 20 g l^{-1} sucrose and 7 g l^{-1} agar. Seeds were kept in growth chamber under long-day conditions (16 h light/8 h dark regime) and temperature of 25 ± 2 °C. Seedlings were aseptically transferred into 350 ml glass jars closed with polycarbonate caps, each containing 70 ml of the same medium, and cultivated for three months.

2.2. Preparation and in vitro enzymatic hydrolysis of C. erythraea methanol extract

C. erythraea aerial parts of three month-old plants were grounded with mortar and pestle using liquid nitrogen (LN). Around 100 mg was extracted with 1 ml 99.8% methanol (AppliChem, Cheshire, CT, USA) by sonication for 10 min at room temperature. Following centrifugation at 10,000*g* for 10 min, supernatant was filtered through 0.2 μm cellulose filters (Agilent Technologies, Santa Clara USA). Methanol was removed at 30–45 °C in a Vacuum Rotor Evaporator (Eppendorf Concentrator 5301, Germany). Subsequently, 6 mg of crude extract was dissolved in either 8 ml of 0.5 M citric phosphate buffer (pH = 5.5) or the same buffer supplemented with 0.1 mg ml^{-1} β-glucosidase purified from almond (SigmaAldrich, CAS 9001-22-3). Reaction mixture was incubated for 16 h at 35 °C and subsequently lyophilized.

For antioxidative assays, samples were diluted in 2 ml 99.8% methanol and sonicated in an ultrasonic bath for 15 min. Following centrifugation at 10,000*g* for 10 min, supernatants were filtered through 0.2 μm cellulose filters and stored at 4 °C until use.

To investigate antimicrobial activity of both untreated and β-glucosidase treated *C. erythraea* methanol extract, lyophilized samples were diluted in 5% DMSO to obtain concentration of 0.5 mg ml^{-1} DMSO.

2.3. In vitro enzymatic hydrolysis of secoiridoid glucosides

SW and SWM standards (1.5 mg each) were dissolved in 1 ml of 0.5 M citric phosphate buffer pH = 5.5, or in the same buffer containing 0.1 mg ml^{-1} almond β-glucosidase. Reaction mixture was incubated for 16 h at 35 °C and subsequently lyophilized. Residues were further dissolved in 3 ml of methanol, sonicated for 10 min and centrifuged for 10 min at 10,000*g*. Supernatants were filtered through 0.2 μm cellulose filters and used for *UHPLC/DAD/qqqMS* analysis and for antioxidant assays.

To examine antimicrobial activity of β-glucosidase, treated and non-treated SW and SWM standards were lyophilized and diluted in 5% DMSO to obtain concentrations of 0.5 mg ml^{-1} DMSO.

2.4. UHPLC/Orbitrap-MS/MS qualitative analysis

Chromatographic separation was performed using an ultrahigh-performance liquid chromatography (UHPLC) system consisting of a quaternary Accela 600 pump and Accela autosampler (ThermoFisher Scientific, Bremen, Germany). It was coupled to a mass spectrometry detector which combines linear ion trap and Orbitrap hybrid mass analyzer (LTQ OrbiTrap MS). Ion source was equipped with heated electrospray ionization probe (HESI-II, ThermoFisher Scientific, Bremen, Germany). A Syncronis C18 column (100 × 2.1 mm, 1.7 μm particle size, ThermoFisher Scientific, Bremen, Germany) at 40 °C was used for peak separation. Flow rate was 0.300 ml min^{-1} and the mobile phase was consisted of (A) water + 0.1% acetic acid and (B) acetonitrile. The injection volume was 5 μl and the linear gradient program was as previously described (Banjanac et al., 2017).

The mass spectrometer was operated in the negative ionization mode. Parameters of the ion source and MS detection settings were as reported in Banjanac et al. (2017). The ions of interest were isolated in the ion trap with an isolation width of 3 ppm and activated with 35% collision energy levels. Full scan analysis was employed to detect the monoisotopic mass of unknown compounds, while the fragmentation pathway was obtained by MS^4 and confirmed using Mass Frontier 6.0 software (ThermoFisher Scientific, Bremen, Germany). The molecule editor program, ChemDraw (version 12), was used to calculate accurate