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Studies on the accumulation of phenolic acids and flavonoids in different *in vitro* culture systems of *Schisandra chinensis* (Turcz.) Baill. using a DAD-HPLC method

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ABSTRACT

Different *in vitro* culture systems of the East-Asian origin medicinal plant species – *Schisandra chinensis*, were tested in order to investigate their potential for the accumulation of two groups of phenolic compounds. *In vitro* cultures were maintained on the Murashige and Skoog (MS) medium supplemented with 3 mg/l BA and 1 mg/l NAA in an agar system (30- and 60-day growth cycles), and also in two different liquid systems: stationary and agitated. Stationary liquid cultures were grown in batch (30- and 60-day growth cycles) and fed-batch modes. Of the twenty compounds, seven free phenolic acids and of the eleven compounds, five flavonoids were quantified in methanolic extracts from lyophilized biomass and in the growth media using the RP-HPLC-DAD method. For comparison purposes, phytochemical analyses of leaf and fruit extracts from the parent plant were also conducted. The estimated compounds were not detected in the growth media. The highest total amounts of phenolic acids (71.48 mg/100 g DW) and flavonoids (29.36 mg/100 g DW) were found in extracts from the biomass of agar cultures harvested after 30 days of cultivation. The main metabolites in all the tested systems were: protocatechuic acid (max. 35.69 mg/100 g DW), chlorogenic acid (max. 13.05 mg/100 g DW), and quercitrin (max. 27.43 mg/100 g DW).

1. Introduction

Schisandra chinensis (Turcz.) Baill. – the Chinese magnolia vine (Schisandraceae), is a medicinal plant species whose value in the official phytotherapy worldwide has been increasing from year to year. The fruit of *S. chinensis* (*Schisandrae chinensis fructus*) is indicated as a pharmaceutical raw material by the United States Pharmacopeia (United States Pharmacopeia National Formulary, 1999), the International Pharmacopoeia edited by WHO (World Health Organization, 2007), as well as by the European Pharmacopoeia (European Pharmacopoeia 8.0, 2013; European Pharmacopoeia 9.1, 2017). The Schisandra fruit has long been recognized in the countries of the Far East (The Japanese Pharmacopoeia, 2006; The Korean Pharmacopoeia, 2002; The Pharmacopoeia of the People's Republic of China, 2005). Its origins date back to and are firmly rooted in the Traditional Chinese Medicine (TCM).

The numerous valuable biological activities of *S. chinensis* fruit extracts, such as antiviral, antiphlogistic, hepatoregenerative, adaptogenic, immunostimulant, antitumor and antioxidant properties, are the result of rich chemical composition (Szopa et al., 2016a). The main role is played by specific dibenzocyclooctadiene lignans known as "schisandra lignans" (Opletal et al., 2004; Szopa et al., 2016a). Nevertheless, the important role is attributed to some other ingredients such as: essential oils, triterpenoids, polysaccharides, vitamins, bioelements, phytosterols and organic acids (Hancke et al., 1999; Szopa et al., 2016a). Recent phytochemical studies have documented the presence of some phenolic acids and flavonoids in fruit extracts (Mocan et al., 2016a, 2016b, 2014; Szopa and Ekiert, 2012). Analyses of *S. chinensis* fruit extracts by our team have confirmed the presence of chlorogenic, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, salicylic and syringic acids (Szopa and Ekiert, 2012). Other authors have additionally proved the presence of gentisic acid and flavonoids: hyperoside, isoquercitrin, rutin and quercetin (Mocan et al., 2014).

The occurrence of phenolic compounds together with specific dibenzocyclooctadiene lignans and other compounds is associated

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with the common initial steps of their biosynthesis – the shikimic acid pathway. Phenolic acids and flavonoids are extensively studied plant secondary metabolites because of their important biological properties. They play an important role not only in therapy, but also as components of foodstuffs, food supplements, and cosmetics products (Piazzon et al., 2012). The principal property of polyphenols is their strong antioxidant activity. Moreover, phenolic acids, both benzoic and cinnamic acid derivatives, and especially depsides, show other experimentally proven properties. Among the phenolic acids that have been detected in *S. chinensis* fruit dominate derivatives of benzoic acid. These compounds show a very wide spectrum of biological properties. For example, gallic acid (2,5-dihydroxybenzoic acid) exhibits antiseptic, anti-inflammatory, hypoglycemic and anti-mutagenic activities (Khadem and Marles, 2010). *p*-Hydroxybenzoic (4-hydroxybenzoic acid) acid shows antimicrobial and estrogenic activities (Chong et al., 2009; Pugazhendhi et al., 2005). Protocatechuic acid (3,4-dihydroxybenzoic acid) has antibacterial, antifungal, antiviral, anti-inflammatory, antiulcer, antiatherosclerotic and anticancer properties (Kakkar and Bais, 2014; Kampa et al., 2004; Kedzierska et al., 2012; Khadem and Marles, 2010). Salicylic acid (2-hydroxybenzoic acid) possess anti-inflammatory, antiseptic, antifungal and keratolytic properties (Lin and Nakatsui, 1998). Syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid) shows antibacterial, hepatoprotective, antidiabetic and anticancer activities (Itoh et al., 2010; Krolicka et al., 2014).

Flavonoids constitute a polyphenolic subgroup of plant secondary metabolites that demonstrate equally significant biological actions. Both flavonoid aglycones and glycosides, apart from strengthening and sealing blood vessels and stimulating metabolism, also exhibit spasmolytic, anti-inflammatory, anti-allergic, antifungal, diuretic, hepatoprotective, and even anticancer properties (Kumar and Pandey, 2013; Procházková et al., 2011).

The capacity of plant cultures *in vitro* to accumulate phenolic acids and flavonoids, and the dynamics of their accumulation during growth cycles, could be a scientific issue of interest in plant biotechnology. There have been examples of successful production of some compounds from this group of metabolites, for instance, of rosmarinic acid in cultures *in vitro* of many Lamiaceae and Boraginaceae species (Ekiert et al., 2013), rosmarinic and chlorogenic acids in cell and organ cultures of *Eryngium planum* (Kikowska et al., 2012), ellagic acid in shoot cultures of *Rubus chamaemorus* (Thiem and Krawczyk, 2003), protocatechuic acid in shoot cultures of *Ruta graveolens* (Ekiert et al., 2009), and *p*-coumaric acid in shoot-differentiating callus cultures of *Ruta graveolens* ssp. *divaricata* (Ekiert et al., 2014). Furthermore, considerable amounts of flavonoids have been obtained in cultures *in vitro* of plant species such as *Astragalus missouriensis* (Ionkova, 2009), *Cyclopia genistoides* (Kokotkiewicz et al., 2014), *Hyoscyamus muticus* (Biondi et al., 2002), or *Dionaea muscipula* and *Drosera capensis* (Krolicka et al., 2008).

Our previous studies on the accumulation of phenolic acids in the biomass of *S. chinensis* agar cultures maintained *in vitro* in Magenta vessels on a few variants of the Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), supplemented with plant growth regulators: BA (6-benzyladenine) and NAA (1-naphthaleneacetic acid) at concentrations from 0.1 to 3.0 mg/l, had allowed us to obtain interesting results on the accumulation of phenolic acids and to nominate the best "production" medium for these compounds (MS medium with 3 mg/l BA and 1 mg/l NAA) (Szopa and Ekiert, 2012). In the current study, we tested different *in vitro* culture systems of *S. chinensis* for the production of two groups of secondary metabolites: phenolic acids and flavonoids. The experimental cultures were maintained on the same "productive" MS medium with 3 mg/l BA and 1 mg/l NAA, nominated by us earlier, in an agar system and also in two different liquid systems: stationary and agitated. All of these types of cultures were grown in batch mode (30 and 60 days). Stationary liquid cultures were additionally grown in fed-batch mode (60 days, including supplementation with fresh medium on day 30). Moreover, the dynamics of the accumulation of phenolic acids and flavonoids were studied in the agitated cultures. Phenolic acids and flavonoids were also estimated in fruit and leaf extracts of the parent plant for comparative purposes. Qualitative and quantitative profiles of the compounds estimated in the samples of extracts were obtained by the DAD-HPLC method (Ellnain-Wojtaszek and Zgorka, 1999; Sułkowska-Ziaja et al., 2016).

2. Materials and methods

2.1. Experimental cultures in vitro

Shoot-differentiating callus cultures of *Schisandra chinensis* were initiated and maintained as reported previously (Szopa et al., 2016b; Szopa and Ekiert, 2011). Experimental agar shoot-differentiating callus cultures, and shoot stationary liquid and agitated cultures (four series) were maintained on the MS medium (Murashige and Skoog, 1962) supplemented with 3.0 mg/l of cytokinin (BA) and 1.0 mg/l of auxin (NAA). The cultures were maintained under white fluorescent light (lamp 36 W, Philips; light intensity 88 ± 8 μmol m^{-2} s^{-1}), at a temp. of 25 ± 2 °C.

2.2. In vitro culture systems tested

2.2.1. Agar cultures

Agar shoot-differentiating callus cultures were cultivated in Magenta™ vessels (77 mm × 77 mm × 97 mm, Sigma-Aldrich). For cultivation, 3.0 g of inoculum was introduced into each vessel containing 25 ml of an agar-solidified medium. Cultures were collected after 30 and 60 days of batch-mode cultivation.

2.2.2. Stationary liquid cultures

Stationary liquid shoot cultures were maintained in Magenta™ vessels (77 mm × 77 mm × 97 mm) fitted with stainless steel mesh placed 13 mm above the bottom. For cultivation, 3.0 g of biomass was introduced into each vessel containing 100 ml of a liquid medium. Cultures of this type were grown in batch mode (samples were harvested after 30 and 60 days), and additionally in fed-batch mode (60 days, including supplementation with 40 ml of fresh medium on day 30).

2.2.3. Agitated cultures

Agitated shoot cultures were cultivated in 250 ml Erlenmeyer flasks containing 100 ml of a liquid medium. The amount of inoculum was 3 g. The biomass was agitated on a rotary shaker (120 rpm, 25.4 mm orbit, INNOVA 2300, Eppendorf). The cultures were grown in batch mode; the biomass was harvested every 10 days of cultivation for up to 60 days.

2.3. Plant material

The plant material was harvested in Poland in 2014, and was analysed for comparison purposes. It comprised the fruit and leaves of plants growing under natural conditions (Rogów Arboretum – Warsaw University of Life Sciences, Forest Experimental Station in Rogów, Poland).

2.4. HPLC-DAD analyses

Lyophilized, pulverized biomass (0.5 g of DW per sample) was subjected to extraction with methanol (50 ml) under a reflux condenser for 2 h in order to analyse it for free phenolic acids and

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