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Adventitious root cultures of *Castilleja tenuiflora* Benth. as a source of phenylethanoid glycosides

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ABSTRACT

Castilleja tenuiflora is a highly valued medicinal plant that grows in pine–oak woods in Mexico. In this study, we identified for the first time verbascoside and isoverbascoside as the major phenylethanoid glycosides (PhGs) in *C. tenuiflora*. These compounds have proven biological activities, including anti-inflammatory, antioxidant, and cytotoxic activities, which may be related to the traditional uses of this plant. We developed a reverse-phase high-performance liquid chromatography (RP-HPLC) procedure to analyze PhGs, and determined their concentrations in various different tissues of wild plants. Verbascoside accumulated mainly in roots and inflorescences (9.23 and 7.88 mg g^{-1} dry biomass, respectively), while isoverbascoside accumulated mainly in the roots (7.13 mg g^{-1} dry biomass). To provide an alternative source of material for production of bioactive compounds, we established *in vitro* adventitious root cultures in which roots were grown in B5 medium containing either 10 μM indole 3-acetic acid (IAA) or 10 μM α-naphthaleneacetic acid (NAA). The greatest dry biomass yield (30 g L^{-1}) was achieved at 30 days after transfer of roots into IAA-containing medium. The highest specific yields of PhGs were also obtained using this auxin; the maximum level of verbascoside was 14.62 mg g^{-1} dry root biomass (438.6 mg L^{-1}) at 30 days after root transfer, and the maximum yield of isoverbascoside was 37.32 mg g^{-1} dry root biomass (522.48 mg L^{-1}) at 23 days after root transfer. Adventitious root cultures of *C. tenuiflora* are a promising system for further studies on scale-up and phenylethanoid glycosides biosynthesis.

1. Introduction

The genus *Castilleja* comprises more than 220 herbaceous annual or perennial species, which are mainly distributed in the Americas. Some of these species are rare and/or endemic (Egger, 2008; Nesom, 1992; Tank et al., 2009). This genus was recently reclassified from the family Scrophulariaceae into the family Orobanchaceae on the basis of molecular phylogenetic analyses and because of its hemiparisitic characteristics (Tank et al., 2009; Thorne, 2000; Wesselingh and van Groenendael, 2005). Most *Castilleja* species are highly ornamental and a few have medicinal uses. *Castilleja tenuiflora* Benth., known as "Cola de borrego" in Spanish, "Atzoyatl" in Nahuatl, or "Indian paintbrush" in English, is distributed in mountainous areas of the Southern United States and Mexico (Holmgren, 1976). It has been used in Mexican folk medicine since the 16th century to treat conditions associated with cancer, respiratory illnesses, and gastrointestinal disorders (Alonso-Castro et al., 2011; Béjar et al., 2000). Teas, decoctions, or infusions are prepared from various organs of wild-harvested plants. For instance, preparations from flowers and leaves are used to relieve coughs whereas root preparations are used to treat colic (Béjar et al., 2000). To date, there has been little research conducted on any aspect of this species. Recently, we showed that methanolic extracts from wild plants and from *in vitro* organ cultures have free-radical scavenging properties and reducing power (Trejo-Tapia et al., 2011).

C. tenuiflora accumulates iridoid glycosides, such as aucubin and geniposidic acid, in its aerial parts (Jimenez et al., 1995) and roots (Trejo-Tapia et al., 2011). Such iridoids have antitumor (Gálvez et al., 2005; Hung et al., 2008) and cytotoxic activities (Nguyen et al., 2005), which may be associated with the traditional uses of this plant. As well as iridoid glycosides, the phenylethanoid glycosides (PhGs) verbascoside and isoverbascoside have also been detected in the genus *Castilleja* (Gardner et al., 1987; Pettit et al., 1990; Stermitz et al., 1991). PhGs are water-soluble compounds characterized by a dihydroxyphenethyl β-D-glucopyranoside, a phenylpropenoic acid (cinnamic, *p*-coumaric, caffeic, and ferulic

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acids) as an ester, and a monosaccharide residue (apiose, rhamnose, glucose, and xylose between others) (Kawada et al., 2002; Sinaphet et al., 2006). These compounds have restricted occurrence among plants in the order Lamiales, which includes the families Scrophulariaceae and Orobanchaceae (Scogin, 1992). Verbascoside is also known as acteoside, kusaginin, and orobanchin whereas its isomer, isoverbascoside, is also known as isoacteoside (Saimaru and Orihara, 2009; Scogin, 1992). PhGs are natural compounds, absorbable by human intestinal cells (Cardinali et al., 2010, 2011) and have a wide spectrum of biological activities (*in vitro* and *in vivo*) (Dembitsky, 2005). For instance, verbascoside effectively scavenges free radicals and inhibits cholinesterases (Georgiev et al., 2011a; Kahraman et al., 2010; Martin et al., 2009; Shindo et al., 2008). It is a potent anti-inflammatory agent because it inhibits the accumulation of pro-inflammatory molecules such as nitric oxide and cytokines along with the expression of the cyclooxygenases COX-1 and COX-2 (Gyurkovska et al., 2011). Verbascoside also presents vasorelaxant effects in isolated rat thoracic aorta (Yoshikawa et al., 2006), antibacterial activity against *Staphylococcus aureus* by affecting protein synthesis (Avila et al., 1999) and anti-hyperalgesic activity (Isacchi et al., 2011). Isoverbascoside presents strong free radical scavenging activity (Arthur et al., 2011; Kim et al., 2009; Martin et al., 2009), inhibits cell proliferation and reversed malignant phenotypic characteristics of human gastric cancer cell line MGC803 (Chen et al., 2002). Both PhGs show *in vitro* activity against respiratory syncytial virus (Kernan et al., 1998) and herpes simplex virus HSV-1 and HSV-2 (Martins et al., 2009); they exhibit *in vivo* hepatoprotective effects (Morikawa et al., 2010), and are cytotoxic against leukemia cells (Pettit et al., 1990) and murine melanoma B16F10 (Nagao et al., 2001). Compounds with properties for various purposes such as PhGs are being intensively sought to treat pathological processes such as inflammation or chronic diseases (e.g. rheumatoid arthritis and Alzheimer) (Georgiev et al., 2011a; Gyurkovska et al., 2011) being attractive for the food and pharmaceutical industries (Cardinali et al., 2011; Georgiev et al., 2010; Stancheva et al., 2011).

Plant cell cultures are an alternative and effective source for production of valuable phytochemicals such as PhGs (Georgiev et al., 2011b; Stancheva et al., 2011). They are also useful for propagation and germplasm conservation, and thus represent a way to protect plant biodiversity (Lubbe and Verpoorte, 2011; Sarasan et al., 2011). As well as being an alternative source of bioactive compounds, *in vitro* cultures of *C. tenuiflora* may also be useful for research on the chemistry and biochemistry of this species. As a part of our research on the conservation and sustainable management of medicinal plant species, we have focused on several aspects of *C. tenuiflora* biotechnology, including the establishment of *in vitro* cultures and analyses of its chemical constitution and biological activities (Martínez-Bonfil et al., 2011; Salcedo-Morales et al., 2009; Trejo-Tapia et al., 2011). Here, we report for the first time the identification of the PhGs verbascoside (**1**) and isoverbascoside (**2**) in wild plants of *C. tenuiflora*, and show that the highest concentrations of both compounds are in the root. In addition, we established adventitious root cultures and analyzed their ability to produce PhGs.

2. Materials and methods

2.1. Plant material

C. tenuiflora plants were collected at the flowering stage (approx. 50 cm in height) from a wild population in Amecameca, State of Mexico, Mexico (3500 m a.s.l.) in November 2008. Plants were separated into roots, stems, leaves, and inflorescences, and then dried at room temperature in the dark for 2 weeks. Dry material was milled in an electric grinder to a particle size of <4 mm (Cutting mills, Pulvex plastic, Model 95). Species identification was confirmed by the HUMO Herbarium of Universidad Autónoma del Estado de Morelos, Mexico (UAEM), where a voucher specimen has been deposited (HUMO13234).

2.2. Preparation of extracts

Dried, ground material of organs from wild *C. tenuiflora* plants (roots, stems, leaves, and inflorescences; 2 g each) was extracted with methanol (40 mL) in a water bath at 60 °C for 30 min. The methanolic extract was filtered and evaporated to dryness in a rotary evaporator (Büchi-490; Büchi, Switzerland) under low pressure at 50 °C. All extracts were stored at 18 °C in the dark until analysis. Both of the phenylethanoid glycosides were obtained from root extracts (160 g dried root material in 3 L methanol at 50 °C).

2.3. Isolation and identification of PhGs

The methanolic extract from roots of wild plants (7.2 g) was suspended in 600 mL chloroform to obtain a less polar fraction (**F1**, 1 g, 13.88%) and a precipitate (**F2**, 6.2 g, 86.12%). The solid fraction was partitioned adding 600 mL acetone to yield a soluble fraction (**F3**, 1.6 g, 22.2%) and a new precipitate (**F4**, 4.6 g, 63.88%). The acetone fraction was separated into 40 fractions using an open chromatography column (40 cm height × 4 cm internal diameter) containing 40 g silica gel 60 and a chloroform/acetone/methanol gradient ascendant polarity system in which the proportion of methanol was gradually increased to 100%. The 50 mL fractions were grouped based on the similarity of spots obtained by thin layer chromatography (TLC). The fractions 27 and 28 were combined (0.26 g) and further separated by column chromatography over silica RP18 and eluted with a solvent gradient consisting of water (5% trifluoroacetic acid, TFA) and acetonitrile. The initial ratio of solvents in this system was 80:20 water + TFA:acetonitrile. The proportion acetonitrile was slowly increased and 13 sub-fractions were collected. Sub-fraction **sF3** (95 mg) contained a white precipitate, which was washed with acetone to yield verbascoside **1** (10 mg). In the same way, isoverbascoside **2** was purified from fractions 36 + 37 (0.017 g) from the first chromatographic separation. The isolated verbascoside and isoverbascoside were identified by ^{1}H (500 and 300 MHz, respectively) and ^{13}C NMR (125 and 75 MHz, respectively) data obtained using a Varian Innova spectrometer and comparisons of the resulting spectra with reported data (Andary et al., 1982; Li et al., 2005). The assignments were confirmed from ^{1}H–^{1}H COSY, ^{1}H–^{1}H NOESY, ^{1}H–^{13}C HSQC, and ^{1}H–^{13}C HMBC spectral analyses.

2.4. Thin layer chromatography

For TLC, aliquots of fractions were spotted onto silica gel 60 F_{254} aluminium plates (Merck) and eluted with a $CHCl_3$/MeOH (7:3) solvent system. The plates were examined by UV fluorescence (365 nm) and then were sprayed with ethanol, 0.5% *p*-anisaldehyde, and 10% H_2SO_4, and then heated at 105 °C for 1–2 min (Wagner et al., 1983).

2.5. Quantification of PhGs by HPLC

We developed a method for analysis of PhGs by HPLC using a Waters 2695 separation module HPLC system equipped with a Waters 996 photodiode array detector and Empower Pro software (Waters Corporation, USA). Compounds were separated on a LiChrospher 100 RP-18 column (4 mm × 250 mm, 5 μm) (Merck, Darmstadt, Germany) connected to a guard column. The mobile phase consisted of water (pH 3.5, containing 5% TFA) (solvent