Acta Societatis Botanicorum Poloniae
Journal homepage: pbsociety.org.pl/journals/index.php/asbp
ORIGINAL RESEARCH PAPER Received: 2010.07.05 Accepted: 2011.01.31 Published electronically: 2011.08.19 Acta Soc Bot Pol 80(3):253-256 DOI: 10.5586/asbp.2011.019

Terpenoids from a multiple shoot culture of *Telekia speciosa*

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Abstract

Multiple shoots of *Telekia speciosa* were cultivated on MS medium containing 4.44 μM BAP and 0.54 μM NAA, solidified with agar. After eight weeks of culture the shoots were harvested and extracted with methanol. From the methanol extract one pseudoguaianolide – 2,3-dihydroaromaticin and three thymol derivatives: 8-hydroxy-9,10-diisobutyryloxythymol, 10-isobutyryloxy-8,9-epoxythymyl isobutyrate and 10-(2-methylbutyryloxy)-8,9-epoxythymyl isobutyrate were isolated as major secondary metabolites. Moreover, the shoots produced megastigmane and monoterpene glucosides, which were isolated for the first time from the species. The content of 2,3-dihydroaromaticin in the shoot culture was similar to that found in the intact plant, whereas yields of the three thymol derivatives were higher from multiple shoots than from the plants grown in the open field.

Keywords: megastigmane, monoterpene, multiple shoots, sesquiterpene lactone, *Telekia speciosa*

Introduction

Heartleaf oxeye [*Telekia speciosa* (Schreb.) Baumg., Asteraceae, tribe Inuleae, basionym – *Buphtalmum speciosum* Schreb.] is a perennial plant native to Southeastern Europe and Asia Minor. It is the only species belonging to the genus *Telekia* Baumg. [1], which, accordingly to recent molecular phylogenetic studies based on both cpDNA and ITS sequence analysis [2] is closely related to *Carpesium* spp. and a group of resiniferous taxa of *Inula* L., including *Inula helenium* – a well known medicinal plant. Likewise the roots of the resiniferous *Inula* species, roots of *T. speciosa* contain essential oil with eudesmane-type sesquiterpene lactones as major constituents. Except for sesquiterpene lactones of eudesmanolide and pseudoguaianolide type, the plant reportedly contains xanthanolides, terpenoid cyclopropenone derivatives, polyacetylene, thymol and nerol derivatives [3-6]. Data on medicinal uses of *T. speciosa* are sparse, however, biological activity of secondary metabolites produced by the plant have gained some interest. Except for widely investigated alantolactone and isoalantolactone, other sesquiterpene lactones – telekin and 2,3-dihydroaromaticin have also shown marked antiproliferative activity against human cancer cell lines in vitro [7,8]. Moreover, 2,3-dihydroaromaticin is a potent inhibitor of lipopolisaccharide induced nitric oxide synthesis and a moderate inhibitor of the transcription factor NF-κB activation [9,10]. Telekin affected activity of some antioxidant and drug-metabolizing enzymes in rat liver and kidney [11,12]. In a recently published report on antiproliferative and direct cytotoxic activity of extracts from *T. speciosa* an interference of these preparations with cell proliferation in vitro has been proven [13].

The aim of the present study was to achieve in vitro multiplication of *T. speciosa* shoots and to investigate secondary metabolism of the culture as a potential source of biologically active compounds.

Material and methods

Plant material

Seeds of *Telekia speciosa* (Schreb.) Baumg. were delivered by Botanical Garden in Nantes (France). Multiple shoots of the plant were collected from in vitro cultures maintained in our laboratory. As a reference material, aerial parts of *Telekia speciosa* (Schreb.) were harvested in July-August from plants grown in the Garden of Medicinal Plants, Institute of Pharmacology, Polish Academy of Sciences, Kraków, where a voucher specimen (10/08) was deposited. The plants were obtained by acclimatization of in vitro regenerated plantlets and were collected in the second year of their vegetation outside.

Multiple shoot culture

An in vitro shoot culture of *T. speciosa* was derived by inoculation of nodal explants excised from aseptic seedlings onto a solidified (0.8% agar) MS nutrient medium [14], containing 3% sucrose, 4.44 μM BAP and 0.54 μM NAA, with pH adjusted to 5.8, before autoclaving (20 min at 121°C). The culture was maintained in baby food jars (Sigma, USA) covered with Magenta B-caps (Sigma, USA), under continuous illumination (40 μmol $m^{-2}s^{-1}$), at 25 ± 3°C. Four explants per culture vessel were inoculated. After eight weeks of cultivation, the multiplied

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shoots were harvested, dried and used for phytochemical analysis. Some of the in vitro regenerated shoots were also used as a starting material in the next multiplication cycle or were rooted on hormone free MS medium with reduced concentration of macronutrients (1/2) and planted into the garden.

Isolation of terpenoids from multiple shoots

Dried and pulverized shoots (17 g) were exhaustively extracted with MeOH at room temperature. The extract was evaporated in vacuo providing an oily residue (6 g) which was subjected to column chromatography on silica gel (Merck, art. 7754) using hexane-EtOAc (up to 100% EtOAc) followed by EtOAc-MeOH (up to 10% MeOH) gradient solvent systems. Fractions eluted with hexane-EtOAc (9:1 v/v) were further separated by semipreparative RP-HPLC on a Delta-Pak C18 cartridge column (25 × 100 mm, particle size – 15 µm, Waters, Milford, MA) using MeOH-H_2O (13:7 v/v) as a solvent system (flow rate – 6 ml min^{-1}) to give 18-hydroxy-9,10-diisobutyryloxythymol (2.1 mg, Rt – 36 min; Fig. 1a), 10-isobutyryloxy-8,9-epoxythymyl isobutyrate (7.5 mg, Rt – 64 min; Fig. 1b) and 10-(2-methylbutyryloxy)-8,9-epoxythymyl isobutyrate (4.0 mg, Rt – 99 min; Fig. 1c). A Waters (Milford, MA, USA) model 600E solvent delivery system coupled to a Waters 2487 dual wavelength UV/VIS absorbance detector operating at 205 nm and 275 nm was employed. Fractions eluted from the conventional silica gel column by hexane-EtOAc (4:1 v/v) were further separated by preparative TLC on precoated silica gel plates (Merck, art. 5553) in a solvent system identical with that used in the column chromatography. The fractions yielded 30 mg of 2,3-dihydroaromaticin (Fig. 1d). From the fractions eluted with EtOAc, roseoside (1.5 mg; Fig. 1e) and 6β-hydroxypiperitol-3-O-β-D-glucopyranoside (1.5 mg; Fig. 1f) were isolated by means of semipreparative RP-HPLC, using MeOH-H_2O (2:3) as a solvent system (flow rate – 3 ml min^{-1}). Retention times of the compounds were 28 min and 59.5 min, respectively.

Structures of the isolated compounds were determined by comparison of their spectral data (1H NMR, 500.13 MHz or 300.18 MHz; UV) with those found in the literature [4,5,15-18] and those of the compounds isolated previously in our laboratory.

Quantification of thymol derivatives

Thymol derivatives were quantified by RP-HPLC as described elsewhere [19].

Quantification of 2,3-dihydroaromaticin

To the dry, pulverized plant material (100 mg) 50 µl of a freshly prepared santonin solution (0.5% in MeOH) were added as an internal standard. The plant tissue was subsequently extracted with chloroform (10 ml) at room temperature for 3 h on a gyratory shaker. The mixture was filtered and the residue was extracted once more with 5 ml of a fresh solvent. The extracts were combined and evaporated to dryness under reduced pressure. The dry residue was redissolved in 0.75 ml of MeCN, diluted with 0.25 ml of water, left to stand overnight at 4°C in the dark, and centrifuged (13000 rpm, 3 min) prior to HPLC analysis. Quantitative analysis of 2,3-dihydroaromaticin was performed by analytical RP-HPLC using an Agilent 1200 Series HPLC system (Agilent Technologies, USA) equipped with a Rheodyne manual sample injector, quaternary pump, degasser, column oven and a diode array detector. Chromatographic separations were carried out at 40°C, on a Purospher RP-18e column (3 × 125 mm, 5 µm particle size; Merck KGaA, Germany) as described by Grass et al. [20]. The sample injection volume was 5 µl. The amount of 2,3-dihydroaromaticin was estimated by comparing the peak area obtained for this compound (detection wavelength 225 nm) with the peak area obtained for the internal standard santonin.

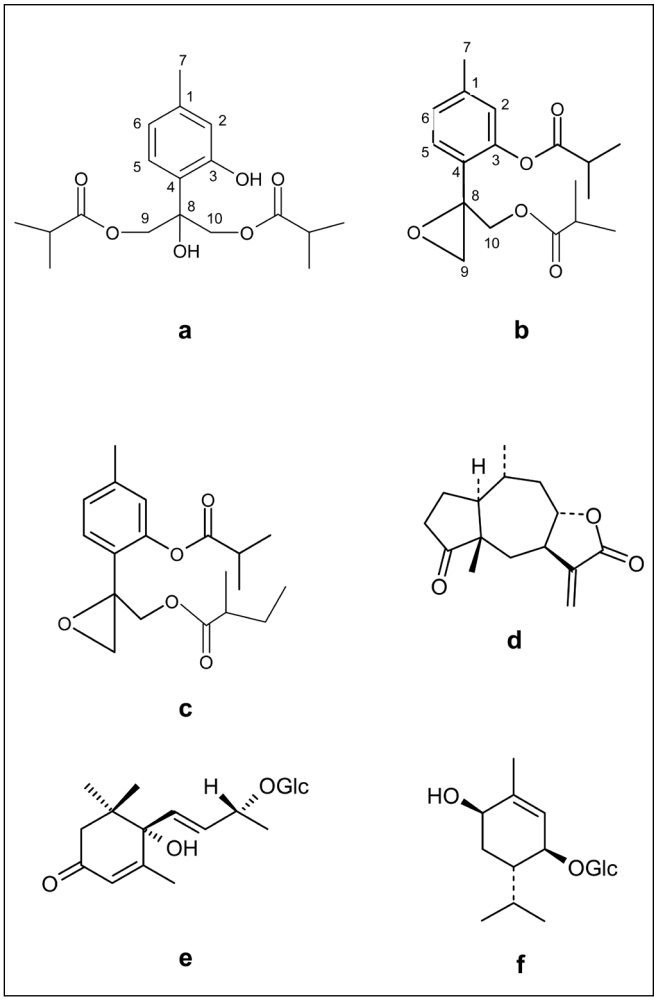

Fig. 1 Structures of terpenoids isolated from multiple shoot culture of *Telekia speciosa*: **a** 8-hydroxy-9,10-diisobutyryloxythymol; **b** 10-isobutyryloxy-8,9-epoxythymyl isobutyrate; **c** 10-(2-methylbutyryloxy)-8,9-epoxythymyl isobutyrate; **d** 2,3-dihydroaromaticin; **e** roseoside; **f** 6β-hydroxypiperitol-3-O-β-D-glucopyranoside.

Statistics

Results are presented as mean values (± SD) derived from three independent experiments.

Results and discussion

The shoot multiplication medium applied in our experiment was reportedly used for in vitro propagation of *Arnica montana* [21]. Multiplcation rates achieved for *T. speciosa* were similar to those for *A. montana*, after initial month of culture (4-5 shoots per explant, see Fig. 2). A growth index of the culture, calculated as a ratio of a final fresh weight of an explant with regenerated shoots (after eight week culture) to a fresh weight of inoculum, reached 60 (Tab. 1). Extension of the culture cycle to eight weeks caused raise of *T. speciosa* multiplication rate to 11. Moreover, the shoots

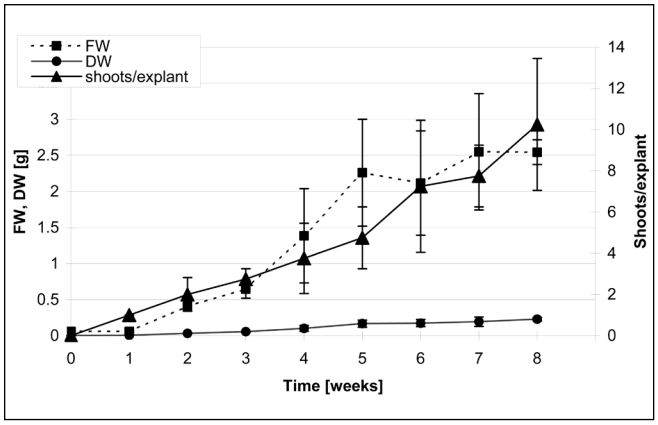

Fig. 2 Time course of biomass accumulation and axillary shoot proliferation in in vitro cultures of *Telekia speciosa* initiated from nodal explants. Bars represent standard deviation. FW – fresh weight; DW – dry weight.

harvested after eight week culture were longer and possessed well developed hirsute leaves. Six terpenoids were isolated from multiple shoots of *T. speciosa*. 10-Isobutyryloxy-8,9-epoxythymyl isobutyrate and 2,3-dihydroaromaticin were previously isolated from the plants of the species [4]. The remaining four compounds: 8-hydroxy-9,10-diisobutyryloxythymol, 10-(2-methylbutyryloxy)-8,9-epoxythymyl isobutyrate, roseoside, and 6β-hydroxypiperitol-3-O-β-D-glucopyranoside, to our knowledge, were found for the first time in the plant, though 10-isovaleryloxy-8,9-epoxythymyl isobutyrate – an isomer of 10-(2-methylbutyryloxy)-8,9-epoxythymyl isobutyrate and two thymol derivatives of unknown structure have been found recently in essential oil from aerial parts of *T. speciosa* [22].

Tab. 1 Axillary shoot proliferation from nodal explants of *Telekia speciosa* inoculated on solidified MS medium supplemented with 4.44 μM BA and 0.54 μM NAA, after eight week of culture (data derived from ten consecutive passages ± SD).

Number of explants	200
Mean fresh weight of an explant (g)	0.058 ± 0.016
Number of regenerated shoots (>1 cm long) per explant	10.9 ± 3.1
Mean fresh weight of an explant with regenerated shoots (g)	3.5 ± 1.3

Pseudoguaianolide 2,3-dihydroaromaticin was the only sesquiterpene lactone produced by the culture, whereas several sesquiterpene lactones were reported from the aerial parts of the intact plant [3-5]. Its content in multiple shoots, after 8 week culture was similar to that in leaves of the intact plant. Probably due to the stage of morphological development, the multiple shoots harvested after eight week of culture accumulated higher amount of 2,3-dihydroaromaticin than that found in shoots after 4 week culture (Tab. 2). Pseudoguaianolides were also produced by multiple shoots [23] and in vitro grown plantlets of *Arnica montana* [23,24]. Multiple shoots cultivated on a solidified medium and plantlets grown in a liquid medium as well as in vitro cultivated rooted plantlets on solidified media, accumulated the same set of sesquiterpene lactones: helenalin, 11α,13-dihydrohelenalin and their esters. The yields of compounds from multiple shoots and plantlets in liquid medium were, however, five to ten times lower than those found in rooted plantlets grown on solidified media. In vitro grown plantlets of *A. montana* produced higher amounts of pseudoguaianolides than the parent plants. The contents of helenalin and its derivatives were particularily affected. The most abundant compound – helenalin methacrylate, constituted 0.32% of the dry weight of in vitro grown plantlets. The content (0.27% dry weight) of 2,3-dihydroaromaticin in multiple shoots of *T. speciosa* seems to be encouraging to undertake attempts of the culture conditions optimization.

Tab. 2 Contents of 2,3-dihydroaromaticin (as% dry weight ± SD) in *T. speciosa* multiple shoots and leaves of the intact plant.

Plant material	2,3-Dihydroaromaticin
Multiple shoots after four week culture	0.05 ± 0.01
Multiple shoots after eight week culture	0.26 ± 0.01
Leaves of the plant	0.22 ± 0.01

Thymol derivatives are common constituents of Inuleae plants. The contents of 8-hydroxy-9,10-diisobutyryloxythymol and the other thymol derivatives in the multiple shoots were higher than those in the intact plant (Tab. 3). This result is in agreement with our previous observation that juvenile tissues of *Inula* spp. accumulated higher amounts of the monoterpenoids than the mature plants do [19]. However, the yields of thymol derivatives from *T. speciosa* multiple shoots were much lower than those from root cultures of *Inula* spp. [19] and hairy roots of *Arnica montana* [25]. Thus, multiple shoot culture of *T. speciosa* seems to be of low interest as a thymol derivative producer.

The monoterpene glycoside – 6β-hydroxypiperitol-3-O-β-D-glucopyranoside and roseoside – (6S,7E,9R)-6,9-dihydroxymegastigma-4,7-dien-3-one are constituents of different Asteraceae plants, representatives of several tribes (e.g.: Cichorieae – [26,27]; Anthemideae – [28]; Eupatorieae – [16]), but to our best knowledge their presence in *Telekia* and *Inula* spp. has not been reported yet.

In conclusion: multiple shoots of *T. speciosa* which accumulate terpenoids of interesting biological activities, produce 2,3-dihydroaromaticin in amount similar to that found in the leaves of the intact plant. Taking into consideration the

Tab. 3 Contents of thymol derivatives (as % dry weight ± SD) in *T. speciosa* multiple shoots and leaves of the intact plant.

Plant material	a	b	c
Multiple shoots after eight week culture	0.015 ± 0.003	0.051 ± 0.007	0.023 ± 0.001
Leaves of the plant	0.010 ± 0.001	0.010 ± 0.001	0.017 ± 0.002

a 8-Hydroxy-9,10-diisobutyryloxy-thymol. **b** 10-Isobutyryloxy-8,9-epoxythymyl isobutyrate. **c** 10-(2-Methylbutyryloxy)-8,9-epoxythymyl isobutyrate.

antiproliferative activity of the compound against the cancer cell lines in vitro the culture is worth further studies on the optimization of the production.

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