

Phenolics from Cell Suspension Cultures of *Penstemon serrulatus*; Relation to Plant Organs

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By repeated selection of pigment portions of tissue the red callus induced from root seedlings of *Penstemon serrulatus* Menz. was chosen for suspension culture, which was maintained in Schenk and Hildebrandt medium supplemented with naphthaleneacetic acid (0.2 mg/l), 6-benzylaminopurine (2 mg/l) and sucrose (50 g/l). From the cultured cells eight phenolic compounds were isolated. They were identified as cyanidin 3-O-glucoside, delphinidin 3-O-glucoside, luteolin, luteolin 7-O-glucoside, norartocarpetin 7-O-glucoside, verbascoside, martynoside and leucosceptoside A. The kind of cell line, its age and light irradiation were important factors in flavonoid production, but production of phenylpropanoid glycosides was found to be unaffected by these factors. The phenolic composition found in the cell culture was compared with those in the flowers and leaves of original plants of *P. serrulatus*.

Introduction

The genus *Penstemon* Mitch. belongs to the Scrophulariaceae and comprises about 300 species widespread in the western part of North America. These plants are known to contain numerous different types of iridoids and phenylpropanoid glycosides (Junior, 1984, Gering and Wichtl, 1987, Gering-Ward and Junior, 1989, Teborg and Junior, 1989). Previous studies on *Penstemon serrulatus* tissue cultures led to the identification of several isovaleric acid ester iridoids, which have antiproliferative properties (Wysokińska *et al.*, 1992).

In continuation of our phytochemical investigations of the tissue culture of *P. serrulatus* we report now on the isolation and identification of phenolics (such as flavonoids and phenylpropanoid glycosides) from cell cultures of this plant. Biological activity of these compounds in humans and their importance in pathogen resistance have been demonstrated (Shoyama *et al.*, 1987, Grunberger *et al.*, 1988, Molonar *et al.*, 1989, Kim *et al.*, 1996). This paper also describes the results of comparative analysis of the phenolic compounds produced *in vitro* by cell suspension culture and *in vivo* by plant organs (flowers and leaves) of *P. serrulatus*.

Experimental

Plant material and culture method

The callus tissue used in this study was initiated from the roots of 2-week-old, aseptically germinated seedlings of *P. serrulatus*. The callus was cultured on Schenk and Hildebrandt (1972) (SH) medium supplemented with 0.2 mg/l naphthaleneacetic acid (NAA), 2 mg/l 6-benzylaminopurine (BAP) and sucrose (30 g/l), and solidified with 0.8% agar. For the selection of red pigment-producing callus, red portions of tissue were selected and transplanted to fresh medium. This procedure was repeated at several passages until a friable and callus culture red in appearance was obtained. The callus tissue was subcultured over 4 years at 35 day intervals. The suspension culture was initiated by transferring *ca* 1 g visually selected red callus into 50 ml SH liquid medium containing 0.2 mg/l NAA, 2 mg/l BAP and 50 g/l sucrose. The culture was maintained in 300 ml Erlenmeyer flasks on a rotary shaker at intervals of 14–28 days. Callus and suspension cultures were kept at 26±2°C under continuous light (40 µEm⁻²·s⁻¹). The cell suspension culture was maintained for one year before starting the experiments.

Sterile anthers from flower buds of original plants of *P. serrulatus* were also used as explants for callus culture as previously described (Wysokińska and Świątek, 1991). The calli were subcultured on SH agar medium with 3-indolebutyric acid (IBA) 0.5 mg/l and BAP 0.2 mg/l under the same

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conditions as above. They were yellowish-green in color. On the basis of visual evaluation it was found that the calli were unable to produce any red pigment. By transferring small pieces of tissue from the calli into SH liquid medium with IBA (0.5 mg/l) and BAP (0.2 mg/l) yellow colored cell line was then selected.

For experimental purposes, flowers and leaves of original *P. serrulatus* plants were collected at the Botanical Garden of Warsaw in July 1996. The voucher specimen of the plant is being kept in the Botany Department of the Medical University of Łódź.

Instruments

Mass spectrometer: Finnigan MAT. Molecular fragmentation of the samples was achieved by bombardment with cesium-ions. Matrix: glycerol. NMR: Bruker AM 300 (^1H :300MHz, ^{13}C :75.5MHz). ^1H - and ^{13}C - chemical shift are given relative to tetramethylsilane (TMS), δ -ppm, J -given in Hz. UV/VIS: Philips PU 8620.

Column chromatography: Bourzeix absorbent (20 g Polyclar AT, Serva, 20 g Kieselgel 60G for thin-layer chromatography and 70 g Kieselgel 60 for column chromatography, Merck, were mixed with boiling HCl; the packed column was washed with H_2O until the reaction for chloric ions disappeared); Sephadex LH – 20 (Pharmacia), Polyamide (Roth), Silica gel 60 (70–230 mesh, Merck).

Thin layer chromatography (TLC): Cellulose (plastikfolien, Merck), Silica gel 60 F_{254} (Merck).

Chromatography paper (PC): Whatman 1 Chr. (England).

Enzymes: β -glucosidase (Fluka AG), β -glucuronidase (Sigma).

PC and TLC systems: n-butanol:acetic acid:water 4:1:5, upper phase (BAW), 1% HCl (aq.), 60% HOAc (aq.), CHCl_3 -MeOH- H_2O (75:25:2 v/v). Detection: UV, 2% methanolic FeCl_3 solution, 5% methanolic AlCl_3 solution (for flavonoids); ceric sulphate in 65% H_2SO_4 (heated to 120°C for 15 min) and vanillin – H_2SO_4 (heated to 120°C for 2–3 min) to detect phenylpropanoid glycosides; aniline phthalide reagent (for sugars).

Extraction and isolation of anthocyanins

Approximately 5 g of fresh plant material (flowers or leaves) or 1 g (fresh wt.) cells (collected by filtration of the suspension cultures) were extracted with the mixture of MeOH-HOAc- H_2O (10:0.5:10 v/v) for 12h at 5°C. After filtration the extract was concentrated (to 2 ml) at 30°C, passed through Bourzeix absorbent (column length 15 cm

\times 1.5 cm) and eluted with H_2O , AcOEt, MeOH and 0.01% methanolic HCl successively. The last fraction containing pigment was concentrated and separated by preparative TLC (cellulose plates) using BAW as the developing solvent. The pigment bands were scraped off from the plates and then repassed through Bourzeix absorbent (column length 15 cm \times 1.5 cm). Elution of the column with 0.01% methanolic HCl gave two pigments **1** and **2** (for the cell suspension) or **1a** and **3a** (for the flowers).

Extraction and isolation of flavones and phenylpropanoid glycosides

The air-dried cells of the suspension (100 g) were extracted twice with boiling MeOH. Combined methanolic extracts were concentrated *in vacuo*. The residue (brown oil, 35 g) was dissolved in hot H_2O (150 ml). The aqueous solution was extracted with petrol until the extracts were colourless. The aqueous phase was chromatographed on polyamide (column length 78 cm \times 3.5 cm) using H_2O , a mixture H_2O -MeOH (1:1 v/v) and MeOH-AcOEt (1:1 v/v), as eluents. Fractions were monitored by TLC and those containing flavones and phenylpropanoid glycosides were combined and after concentration *in vacuo* passed through Sephadex (column length 80 cm \times 2.5 cm). Elution was performed with MeOH. Each of the methanol fractions was examined by TLC and combined into two main fractions containing flavones and phenylpropanoid glycosides, respectively.

The flavone fraction (3.2 g) was separated on Sephadex (column length 80 cm \times 2.5 cm) using MeOH to give: **4a** (5.2 mg) and a mixture of **4** and **5**. The mixture (82 mg) was separated by preparative TLC on cellulose using AcOEt-MeOH- H_2O (8:1:1 v/v) as solvent. Flavones (R_f 0.42 and R_f 0.53) were subsequently extracted from the absorbent using MeOH. This yielded 46 mg of **4** and 3.2 mg of **5**.

The leaves (5 g dry weight) were extracted and separated under the same conditions to give: **6** (6 mg), **7** (8.3 mg) and **4a** (2.5 mg). The analysis of flowers (5 g dry weight) was also carried out in the same way to give: **6** (4.5 mg), **7** (5 mg) and mixture of **4a** and **7a**. The mixture (15 mg) was separated by preparative TLC on cellulose using 60% HOAc to give: **4a** (2 mg) and **7a** (3.8 mg).

The phenylpropanoid glycoside fraction (16 g) was fractionated on a column (length 78 cm \times 3.5 cm) of silica gel (200 g) eluting CHCl_3 -MeOH- H_2O (75:25:2 v/v). The fractions were monitored by TLC and combined into three fractions, which

contained, **8** (300 mg), **9** (100 mg) and **10** (32 mg), respectively. Compounds **8**, **9**, **10** were further purified by silica gel column with the same solvent system as mentioned above.

Enzymatic hydrolysis

A mixture of isolated compound **4-7** (2–3 mg) and β -glucosidase (5 mg) for **4** and **5** or β -glucuronidase (0.15 ml) for **6** and **7** in H_2O (0.5 ml) was incubated at 36°C overnight. The solution was extracted with EtOAc. The organic layer was evaporated to dryness to give aglycones. The aqueous phase was evaporated *in vacuo*. The residue was dissolved in H_2O -MeOH (9:1v/v) and used for detection of sugars by PC.

Identification of compounds no 1-12

Cyanidin 3-O-glucoside (**1**)

FAB-MS positive mode (gly) m/z 287 [M-glucose]⁺, 449 [M]⁺

UV/VIS $\lambda_{max}^{0.01\% \text{ HCl-MeOH}}$ 528, shifted bathochromically with $AlCl_3$

λ_{max} 534; E_{440}/E_{max} 0.25

Delphinidin 3-O-glucoside (**2**)

FAB-MS positive mode (gly) m/z 303 [M-glucose]⁺, 465 [M]⁺

UV/VIS $\lambda_{max}^{0.01\% \text{ HCl-MeOH}}$ 532, shifted bathochromically with $AlCl_3$

λ_{max} 538; E_{440}/E_{max} 0.31

Pelargonidin (**3a**)

FAB-MS positive mode (gly) m/z 271 [aglycone]⁺

Norartocarpetin 7-O-glucoside (**5**)

¹H-NMR (CD_3OD , 300 MHz, δ -ppm, *J* given in Hz) 7.57 (1H, d, *J* = 2.2 Hz, H-3'), 7.53 (1H, dd, *J* = 2.2Hz, *J* = 8.6Hz, H-5'), 7.47 (1H, d, *J* = 8.6Hz, H-6'), 6.65 (1H, s, H-3), 6.41 (1H, d, *J* = 2.1Hz, H-8), 6.21 (1H, d, *J* = 2.1Hz, H-6), 5.06 (1H, d, *J* = 7.2Hz, glc. H-1'), 3.6–4.1 (6H, m, glc. H-2' – H-6') UV-VIS (λ_{nm}) MeOH: 324, 270, 236sh; NaOMe: 382, 296 sh, 268, 236sh; $AlCl_3$: 386, 350, 290sh, 278, 262sh; $AlCl_3/HCl$: 386, 346, 288sh, 280, 260sh; NaOAc: 324, 270, 232sh; NaOAc/ H_3BO_3 : 330, 280sh, 270, 240sh.

FAB-MS positive mode (gly) m/z 287 (M+H)⁺, 449.1 [(M+H)-glucose]⁺; negative mode (gly) m/z 284.5 (M-H)⁻, 446.8 [(M-H)-glucose]⁻.

Compounds **4**, **4a**, **6**, **7**, **7a** spectral data as reported by Mabry *et al.* (1970) and Klimek (1995).

Verbascoside (**8**) ¹H – and ¹³C-NMR as reported by Gering and Wichtl (1987).

Martynoside (**9**) ¹H – and ¹³C-NMR as reported by Teborg and Junior (1989).

Leucosceptoside A (**10**) ¹H – and ¹³C-NMR as reported by Miyase *et al.* (1982).

Results and Discussion

Cell suspension cultures

The calli of *P. serrulatus* were separated on the basis of color into two cell lines, which gave different phenolic composition. The pigment cell line of *P. serrulatus* was established from red callus. The callus was initiated from roots of seedlings by selection of small red-colored spots visible in the callus tissue. Four years after initiation the callus was transferred onto SH liquid medium supplemented with NAA (0.2 mg/l), BAP (2 mg/l) and sucrose (50 g/l) and red-cell suspension culture was obtained. We also established cell suspension culture derived from callus initiated from anther explants. The cell line showed a yellow-green color and accumulated phenylpropanoid glycosides (**8-10**), but it formed no anthocyanins and flavones. On the other hand, the red-cell line cultivated in continuous light produced at least three types of phenolic compounds, namely anthocyanins (**1**, **2**), flavones (**4**, **4a**, **5**) and phenylpropanoid glycosides (**8**, **9**, **10**). All the compounds were isolated and identified by their chromatographic (Rf) and spectral properties (UV-VIS, FAB-MS, ¹H – and ¹³C – NMR).

Identification of anthocyanins

Anthocyanins were extracted from the reddish cells of *P. serrulatus* in the presence of acidified methanol. The anthocyanins were studied by means of the positive FAB-MS. Signals at m/z 449 and 465 which corresponded to the quasi-molecular ions of cyanidin 3-O-glucosides (**1**) and delphinidin 3-O-glucoside (**2**), respectively, and the anthocyanin fragments resulting from the loss of glucosyl moiety at m/z 287 and m/z 303 were observed. The E_{440}/E_{vismax} ratios of both pigments (**1**=25% and **2**=31%) are characteristic of 3-substituted anthocyanins (Harborne, 1958). The intensity of FAB-MS peaks showed that the anthocyanin spectrum in *P. serrulatus* cells was dominated by cyanidin 3-O-glucoside; relative intensity peak of cyanidin aglycone was 11.07 compared to 5.32 of the delphinidin aglycone. The intensity of absorbance peaks also suggested that the biosynthesis

of anthocyanins was strongly activated between weeks 2 and 3 after transfer to fresh medium.

Although cell cultures of some plants, such as *Bupleurum falcatum* (Hiraoka *et al.*, 1986), *Ajuga reptans* (Callebaut *et al.*, 1990) and *Aralia cordata* (Sakamoto *et al.*, 1994) are known to produce anthocyanins in the dark, *P. serrulatus* belongs to the plants which require light for pigment biosynthesis. After the transfer of red-pigmented cell material to darkness the color faded during 3 weeks of subculture. The return of these cell cultures to light led to *de novo* synthesis of pigments. Matsumoto *et al.*, (1973) reported that the activity of phenylalanine ammonia-lyase (PAL), which is the key enzyme in anthocyanin biosynthesis, is enhanced as cells are exposed to light irradiation.

Identification of flavones and phenylpropanoid glycosides

We also studied the presence of flavonoids in the aqueous phase of methanolic extract of suspension cultures of *P. serrulatus* (red-cell line). The chemical analysis resulted in the isolation of three compounds belonging to flavone structural type. The main flavone was luteolin 7-O-glucoside (**4**). Aglycone, luteolin (**4a**) was also found in red cells. Moreover, norartocarpetin 7-O-glucoside (**5**), as the minor flavone, was isolated. This is the first record of the occurrence of norartocarpetin (5,7,2'-tetrahydroxyflavone) in the glucosyl form, although free norartocarpetin has been isolated previously from the roots of *Scutellaria baicalensis* (Tomimuri *et al.*, 1984) and *Flemingia stricta* (Rao *et al.*, 1982). The compound has also been found in plants from family Moraceae (Radhakrishnan *et al.*, 1965; Gerber, 1986) and its anti-tumor activity has been proven (Nishino *et al.*, 1986).

Further examination of aqueous phase of methanolic extract of *in vitro* cultured cells led to the isolation three phenylpropanoid glycosides (**8-10**). Among these, verbascoside (**8**) was the most abundant compound (70%), followed by martynoside (**9**) (23%) and leucosceptoside A (**10**) (7%). The last two compounds are distinguished from the verbascoside by the type of methyl unit esterified at the position 3''' (leucosceptoside A) or at the positions 3''' and 4 of martynoside (Fig. 1).

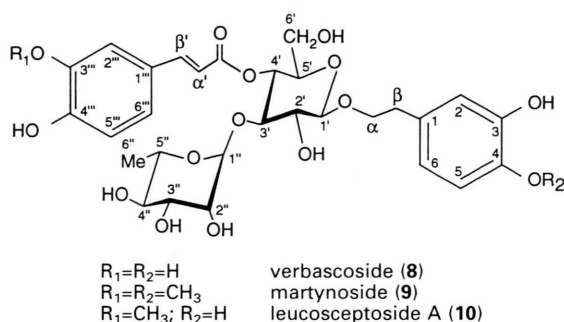


Fig. 1. Structures of phenylpropanoid glycosides from *Penstemon serrulatus* cell suspension cultures.

Effect of ageing on phenolic composition

All the data reported above were recovered from about 1-year-old cultures. The remarkable variations with respect to growth rate, color and phenolic pattern were observed with the increase of culture time. After 40 passages (2 years after initiation), the suspension culture turned yellowish-green and lost its capacity to be induced for anthocyanin and flavone formation. The growth rate of such „old“ suspension culture was much higher (2-weekly subcultures) than the suspension culture with red pigment production (4-weekly subcultures). It is possible that dilution of the original callus cells by the new cells that produce less pigment (but grow faster) caused a gradual loss of flavonoid synthesis. Instability of product formation in cell cultures is well documented in the literature, not only with respect to anthocyanins (Hirasuna *et al.*, 1991), but also to other secondary metabolites (Deus-Neumann and Zenk, 1984). On the other hand, the ability of our cell line of *P. serrulatus* to produce phenylpropanoid glycosides was maintained even after 3 years of subculturing. It was also observed that the original callus culture has continued to retain its red color and adequate growth rate and recently after 89 transfers (about 7 years after initiation) it formed both flavonoids (anthocyanins and flavones) and phenylpropanoid glycosides (data not presented). It is possible that the visual selection of pigment producing cells at callus subcultures can be used to stabilize flavonoid production under *in vitro* conditions. Thus, even after extended time, the new cell line with anthocyanin production can be easily derived from the red callus.

Table I. Phenolic constituents present in cell suspension culture and plant organs of *Penstemon serrulatus*.

Compound ^a	Phenolics (mg/g dry weight) from			
	Cultured cells		Original plant tissue	
	1-year-old	2-year-old	Flower	Leaf
Cyanidin 3-O-glucoside (1) (5,7,3',4'-tetrahydroxyflavylium 3-O-glucoside)	0.80	–	n.d.	–
Delphinidin 3-O-glucoside (2) (5,7,3',4',5'-pentahydroxyflavylium 3-O-glucoside)	0.40	–	n.d.	–
Cyanidin (1a) ^b (3,5,7,3',4'- pentahydroxyflavylium)	–	–	1.00	–
Pelargonidin (3a) ^b (3,5,7,4'- tetrahydroxyflavylium)	–	–	0.60	–
Luteolin 7-O-glucoside (4) (5,3',4'-trihydroxyflavone 7-O-glucoside)	0.46	–	–	–
Luteolin (4a) (5,7,3',4'-tetrahydroxyflavone)	0.05	–	0.40	0.50
Noratocarpetin-7-O-glucoside (5) (5,2',4'-trihydroxyflavone 7-O-glucoside)	0.03	–	–	–
Luteolin-7-O-glucuronide (6) (5,3',4'-trihydroxyflavone 7-O-glucuronide)	–	–	0.90	1.20
Apigenin-7-O-glucuronide (7) (5,4'-dihydroxyflavone 7-O-glucuronide)	–	–	1.00	1.70
Apigenin (7a) (5,7,4'-trihydroxyflavone)	–	–	0.80	–
Verbascoside (8)	3.00	3.00	1.80	3.50
Martynoside (9)	1.00	1.00	t	0.80
Leucosceptoside A (10)	0.32	0.32	–	0.50

Culture conditions: red-cell line, SH (Schenk and Hildebrandt, 1972) medium supplemented with naphthaleneacetic acid (NAA) 0.2 mg/l and 6-benzylaminopurine (BAP) 2 mg/l and sucrose 50 g/l.

Compounds were isolated from cells grown under light (compounds no **1**, **2**, **4**, **5** and **8–10**) or dark (compounds **8–10**).

^a Calculated on the basis of the amounts of the isolated compounds from the dried material.

^b only the aglycones were identified.

Symbol abbreviations: – not detectable;

t – traces;

n.d. – not determined.

Comparison between in vitro and in vivo phenolic compositions

Phenolics from cell cultures of *P. serrulatus* were compared with those of the flowers and leaves of the original plants by PC, TLC and spectral techniques (FAB-MS, ¹H-NMR). The results are shown in Table I. The anthocyanin profile of flowers of *P. serrulatus* contains two anthocyanidins, which were identified as cyanidin (**1a**) and pelargonidin (**3a**). In general, anthocyanidins are present in a conjugated form, bound to sugars. Chromatographic analysis (PC) suggested that *P. serrulatus* flower pigments might be diglucosides.

However, the nature of sugars and their position(s) in these pigments remain to be determined. None of the anthocyanins was found in the leaf extract. The anthocyanins have already been reported to occur in flowers of *Penstemon* genus but they were not known to occur in *P. serrulatus*. According to Scogin and Freeman (1987) the genus of *Penstemon* is characterized by the occurrence of three anthocyanidins i.e. cyanidin, delphinidin and pelargonidin. However, the co-occurrence of all three anthocyanidins or delphinidin plus pelargonidin was not observed. Furthermore, only three glycosidic types, namely 3-glucosides, 3,5-diglucosides and 3-arabinosides were detected among the

studied species of *Penstemon* (Scogin and Freeman, 1987).

Four flavones were isolated from flowers of *P. serrulatus*. They were identified as luteolin 7-O-glucuronide (**6**), apigenin 7-O-glucuronide (**7**) and their aglycones (**4a**, **7a**). The same flavones, except for apigenin (**7a**) were isolated from the leaf extract. The presence of glucuronic acid in compounds **6** and **7** was supported by results of FAB-MS and enzymatic hydrolysis. The signals at m/z 461 $[M-H]^-$ and m/z 445 $[M-H]^-$ for quasi-molecular ions of luteolin 7-O-glucuronide (**6**) and apigenin 7-O-glucuronide (**7**), respectively, were observed. The negative spectrum gave signals $[(M-H) -176]^-$ after loss of glucuronyl moiety at m/z 285 for luteolin (**4a**) and at m/z 269 for apigenin (**7a**). Glucuronides were not produced by *in vitro* grown cells of *P. serrulatus*. It is possible that in cell suspension due to unknown reasons the oxidation at the C-6 position of glucose is suppressed.

Generally, our study indicated, that both non-organized cells and differentiated plant organs are

characterized by the formation of phloroglucinol-based flavones. The flavones differ in the position and number of hydroxyl groups in the B-ring. Another distinguishing feature is a type of sugar substitution, which was, however, always linked to the C-7 hydroxyl group of aglycone. The phenylpropanoid glycoside profiles of the leaves and cell suspension of *P. serrulatus* showed a good correlation (Table I). The flowers contained no leucosceptoside A (**10**), but they produced verbascoside (**8**) (major compound) and martynoside (**9**), which was detected only in trace amounts.

This is the first study concerning isolation of phenolic compounds from *P. serrulatus*. The results indicate that *P. serrulatus* cell suspensions, irrespective of the origin, are a suitable candidate to produce phenylpropanoid glycosides. Further investigations are in progress to determine the factors which control phenolic production in *P. serrulatus* cell suspension cultures.

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