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PHENOLIC COMPOUNDS IN TWO *SOLIDAGO* L. SPECIES FROM *IN VITRO* CULTURE

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Abstract: The regenerated *S. virgaurea* and *S. graminifolia*, obtained previously by micropropagation from shoot tips and the callus tissue of *S. virgaurea*, were analysed for phenolic compounds. Preliminary used 2D–TLC methods (fingerprinting) showed the presence of phenolic glucoside – leiocarposide (*S. virgaurea* only), C–glycosyflavones – schaftoside and isoschaftoside (*S. graminifolia*), flavonoids and caffeoylquinic acids, similar to the ones reported earlier for naturally growing plants with the exception of the callus tissue. The latter contained predominantly caffeoylquinic acids, which were isolated and identified by NMR as 5–O–caffeoylquinic and 3,5–di–O–caffeoylquinic acids. The content of leiocarposide (HPLC) and flavonoids (colorimetry) in *S. virgaurea* was determined.

Keywords: *Solidago* sp. from *in vitro* culture, *S. virgaurea*, *S. graminifolia*, flavonoids, leiocarposide, caffeoylquinic acids, 2D–TLC, NMR, HPLC.

In Poland, the genus *Solidago* L. (Goldenrod) is represented by four species: *Solidago canadensis* L., *S. gigantea* Ait. (= *S. serotina* Ait.), *S. graminifolia* (L.) Salisb. and *S. virgaurea* L. The latter two appear on a limited area only. The rest belong to a group of foreign plants mostly rampant in the Polish flora. Herba Solidaginis has been applied in the Middle Ages in diseases of urinary tract, nephrolithiasis and prostate, while flowers and leaves were used as a natural yellow dye. The plant was then forgotten, but found its place again in modern phytotherapy. *Solidago virgaurea*, less or more supplemented with *S. canadensis* and *S. gigantea*, is the component of many herbal mixtures and specifics (e.g.1,2). According to Pharmacopée Française goldenrod herb should be obtained from *S. virgaurea*, and such material (*Solidaginis virgaureae herba*) is inserted in the ESCOP publication (3).

The genus *Solidago* has not only became of interest due to its biological action. It contains numerous interesting secondary metabolites such as: flavonoids, triterpenoid saponosides, phenolic acids and glucosides, polisaccharides, diterpenes, tannins, essential oils and others. It is noteworthy that essential oils of four *Solidago* species native to Poland, have been shown recently to contain numerous constituents and found to possess biological activity (4,5). The urological activity is ascribed mainly to flavonoids and phenolic ester glucoside – leiocarposide (*S. virgaurea* only), compounds described in many publications reviewed in e.g. (1,6).

The four native species of the genus *Solidago* were obtained by micropropagation from shoot tips (6,7). They have been growing in the soil, blooming and fruiting there normally for some years, now.

Biotechnology, which can markedly improve production of plants and their tissues with biologically active metabolites, has been applied in the case of medicinal plants (8,9).

This paper presents results of research on the contents of some secondary metabolites of phenolic nature in the tissues produced *in vitro* and plants established in the soil of *S. virgaurea* and *S. graminifolia*.

EXPERIMENTAL

Plant material

The micropropagation of the *Solidago* species from shoot tips was carried out on a solidified MS medium supplemented with kin and IAA according

* Abbreviations: MS – Murashige and Skoog; kin – kinetin; IAA – indole–3 acetic acid; 2,4–D – 2,4–dichlorophenoxyacetic acid; BA – benzyloadenine; $CHCl_3$ – chloroform; MeOH – methanol; MeCOEt – methylethylketone; Ac_2CH_2– acetylacetone; AcOEt – ethyl acetate; HCOOH – formic acid; EtOH – ethanol; HOAc –acetic acid; n–BuOH–butyl alcohol; $AlCl_3$ – aluminium chloride

to (6,7). The acclimatised in the soil plants were harvested at the blooming stage and dried at room temperature. Callus tissue was originated from fragments of seedling, roots and cotyledons on MS medium with 2,4–D and BA, and plantlets – from shoot tips culture on MS medium containing kin and IAA (6). Callus tissues and plantlets were dried at the temperature of 60°C. The voucher specimen of the plants are deposited at our department.

Extraction and preliminary analyses

The material was extracted with MeOH and the extracts were fractionated into chloroform, butanol and water as previously described (10). Butanol fractions were subjected for phytochemical analysis which was performed by two – dimensional (2D–TLC) or one – dimensional (1D–TLC) thin–layer chromatography using the following systems: for *S. virgaurea* on polyamide 2D–TLC – I dimension: $CHCl_3$–MeOH–MeCOEt–Ac_2CH_2 (9:4:2:1) and II dimension: H_2O–EtOH–Ac_2CH_2 (4:2:1) (10); on silica gel F_{254} 1D–TLC, AcOEt – HCOOH – HOAc – H_2O (100:11:11:26) (12); for *S. graminifolia* on cellulose – I dimension: 1–BuOH – HOAc – H_2O (4:1:5), II dimension : 15% HOAc. The plates were viewed under ultraviolet light (λ = 365 nm) before and after spraying with 1% solution of $AlCl_3$. The pattern of spots was compared with those observed for naturally grown plants (10,17). The appropriate standard pure compounds were also chromatographed, like leiocarposide, schaftoside, isoschaftoside, which were isolated from naturally growing plants and identified by NMR analysis (6,10,17).

Isolation of caffeoylquinic acids

The n–BuOH fraction of callus tissue of *S. virgaurea*, was dissolved in MeOH and applied as a streak to polyamide 6 (Macherey–Nagel) home–made plates (layer thickness 0.5 mm), which were developed first with H_2O, and then, after drying, with MeOH. The broad band close to the start and showing blue fluorescence under UV_{365}, was eluted with 0.01% (v/v) ammonia in MeOH. The eluate was concentrated, redissolved in MeOH and analysed on silica gel plate (precoated, Merck) in AcOEt–HCOOH–H_2O (10:1:9) (11) against reference caffeoylquinic acids from *S. canadensis* (6). For detection, the plate was viewed under UV_{365} light before and after spraying with 0.1% solution of 2–aminoethanol diphenylborate (Naturstoffreagenz A, Roth) followed by 5% solution of polyethylene glycol. For isolation of individual compounds the eluate was applied on silica gel (60 PF_{254}, Merck) home–made plates (layer thickness 0.5 mm), which were developed in : AcOEt – HOAc–HCOOH–H_2O (50:2.5:0.5:10). The UV–visible bands at R_f 0.21 and 0.81 were immediately eluted with H_2O saturated AcOEt and concentrated . Two samples were further purified by successive column chromatography on small polyamide (Roth) columns eluted with 0.01% ammonia in MeOH and then on Sephadex LH–20 (Pharmacia) columns eluted with MeOH to give compounds **I**–3 mg and **II**–6 mg as amorphous, beige substances. Their ^1H (300 MHz) and ^1H–^1H–DQF–COSY (compound **I** only) and ^{13}C (75MHz) (compound **II** only) NMR spectra were recorded in CD_3OD using TMS as internal standard.

Quantitative analyses

Blooming herbs (20 cm long) of the species derived from *in vitro* cultures and growing in the soil, (garden of Department of Medicinal Plant, K. Marcinkowski University of Medicinal Sciences in Poznań) were collected for three subsequent years (1997 – 1999).

In *herba Solidaginis virgaureae* phenol glucoside – leiocarposide content was determined using the HPLC method as described previously (12). Powdered plants were extracted with methanol at 90°C, 1h. Extracts were filtered through membrane filter (0.45 µm) and subjected to RP – HPLC analysis. This determination was made on the La Chrom apparatus (Merck Hitachi) equipped with: an A pump of the L–7100 type and an injection valve with a sample loop of 20 µl capacity; a UV/Vis DAD detector; a Lichrospher 100 column RP–18e (5 µm), 250 x 4.6 mm (Merck). The conditions were: elution – isocratic with: methanol–water; detection – at λ = 216 nm; flow rate – 1 ml·min^{-1}; peak identification – by retention time (8.37 min) and UV/Vis spectrum of the studied substance; quantitative calculations by using the internal standard method (HSM programme). Fla-

Table 1. Quantitative analysis of leiocarposide and flavonoids in *S. virgaurea* L. from *in vitro* culture

Year of harvest	Leiocarposide	Flavonoids
	% in dry material*	
1997	0.24	1.09
1998	0.14	0.83
1999	0.21	1.23

* values are means of three measurements

Table 2. NMR data for compounds **I** and **II**, δ ppm (multiplicity, J in Hz).

Atom	Compounds		
	I	**II**	
	H	H	C
Quinic acid			
1			73.8
2ax	2.22–1.92 (m)	2.30 (brd, ~14)	39.7
2eq		2.20–2.00 (m)	
3	4.12 (dt, 3/~3)	5.40 (dt, ~3/3.9)	72.3*
4	3.69 (dd, 3.1/9.3)	3.93 (dd, 3.6/9.0)	72.0
5	5.36 (ddd, 4.5/~10/~10)	5.48 (brdd, 7.5/~9)	72.4*
6	2.22–1.92 (m)	2.20–2.00 (m)	37.1
7			179.0
Caffeoyl			
1'	7.04 (d, 2.1)	7.07/7.06 (each d, 2.1)	128.1/127.9
2'			116.5 /116.5
3'			146.85/146.81
4'	6.77 (d, 8.1)	6.77 (2H, d, 8.1)	149.6/149.5
5'	6.94 (dd, 2.1/8.1)	6.96 (2H dd, 2.1/8.1)	116.0/115.5
6'	7.60 (d, 15.9)	7.61/7.58 (each d, 15.9)	123.0/123.0
7'	6.28 (d, 15.9)	6.39/6.29 (each d, 15.9)	147.0/146.9
8'			115.2/115.1
9'			169.3/168.8

* interchangeable assignments
ax – axial, eq – equatorial

vonoid content was determined by the Christ–Müller method (13) and converted into hyperoside.

RESULTS AND DISCUSSION

Analyses, using chromatography 2D–TLC–fingerprinting showed that biosynthesis of leiocarposide, flavonoids and caffeoylquinic acids occurs as early as the stage of shoots formation in *S. virgaurea in vitro* cultures (6). The analysis of plantlets and plants of micropropagated *S. graminifolia* confirms the above findings in respect to flavonoids. This plant material was found to contain flavonoid C–glycosides – schaftoside and isoschaftoside, previously isolated from naturally growing plants (17).

The content of phenol glucoside – leiocarposide in *S. virgaurea in vitro* propagated plants was determined in samples collected in the three subsequent years (Table 1). The compound occurs only in this species and is an indicator of a proper herbal material (14). In these analyses, the average glucoside content was 0.18%. The content of flavonoid compounds converted into hyperoside was about 1.05%. It was reported (15) that naturally growing plants contained 0.2 – 1.0% of phenol glucosides and 1.5% of flavonoids, converted into quercetin. Other investigations showed, that the contents of leiocarposide ranged from 0.8% to < 0.1% or from 0.0 to 1.6% and the flavonoid glucosides from 0.4 to 2.4% in dependence on the growth area (16). According to (12) the leiocarposide content in dried raw material varied from 0.4 to 1.6% depending on the kind of sample different: cutting height, harvesting time, and natural state or cultivation origin. The present work showed the lowest levels of leiocarposide and flavonoids in 1998 year (Table 1).

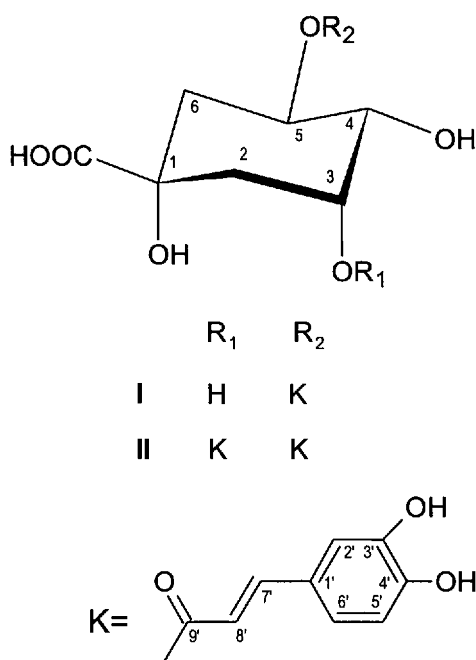

Figure 1. Structures of caffeoylquinic acids from *Solidago virgaurea* L. callus tissue.

The TLC and HPLC analyses of *S. virgaurea* L. callus extracts did not show the presence of leiocarposide, but only trace amounts of flavonoids. Instead, phenolic acids derivatives were predominant. They were isolated by two– stage preparative thin–layer chromatography. First separation, on a polyamide, using elution mode previously employed with the column chromatography technique (10), allowed the separation of the mixture of compounds from other constituents, while the second separation, done on silica gel, gave individual compounds.

Two compounds obtained, after clean–up by column chromatography on polyamide and Sephadex, were identified as 5–O–caffeoylquinic acid (=chlorogenic acid) (**I**) and as 3,5–di–O–caffeoylquinic acid (**II**) (Figure 1) on the basis of ^{1}H NMR spectra including ^{1}H–^{1}H–DQF–COSY for compound **I** (18) (Table 2). In the case of compound **II** ^{13}C NMR spectrum could also be obtained, which was in agreement with reported data (19). It should be mentioned, that ^{1}H NMR shifts and signals pattern, multiplicity, coupling constants for caffeoyl moieties and H–3, H–4 and H–5 of quinic acid moiety, obtained in the spectra measured at 300 MHz (Table 2) were satisfactory for reliable differentiation of isolated compounds from other possible caffeoylquinic acids isomers.

Previous investigations (6) showed the similar composition of caffeoylquinic acids, i.e. chlorogenic , 3,5–di–O–caffeoylquinic and 4,5–di–O–caffeoylquinic acids, in all four taxa of *Solidago* growing in natural conditions.

Significant quantitative differences of secondary metabolites (flavonoids and leiocarposide), found in the species of the *Solidago* genus (14,16), arise questions, if controlled soil cultivation would give better effects. A conclusion may be drawn, that due to continuous reduction in natural plant populations as well as to their intoxication with metals, a farm cultivation will be the basic source of medical herbs in future. Leiocarposide content in different parts of the herbs – flowers, leaves and stems – made the basis for selection of various chemotypes of *Solidago virgaurea* (14) . These findings show also that a most valuable material provides only the tips of blooming shoots, not having numerous stems increasing the biomass, which should lead to the selection of genotypes of optimal plant habit. Such genotypes should be clonally propagated and *in vitro* method is an alternative one. The described previously method of micropropagation (6,7) may be useful for the mass clonal propagation of valuable for planting material of *Herba Solidaginis virgaureae.*

The *in vitro* cultures may also be applied for the propagation of the *Solidago graminifolia* species rarely found in Poland, which is exceptional for its ability to synthesize flavonoid C–glycosides, not observed in other species of the *Solidago* genus so far.

Recently, *Solidago* species attracted considerable attention (20). Secondary metabolites characteristic for the species of the *Solidago* genus, as well as the wide spectrum of biological effects of the herbal material and drugs, ensure, that the plants will stay an important element of phytotherapy.

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