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Phenolics in Aerial Parts of Persian Clover *Trifolium resupinatum*

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The nutritional quality of Persian clover (*Trifolium resupinatum*), an important pasture crop, depends not only on a high protein content but also on the occurrence of animal health and welfare promoting phytochemicals. Nine phenolic constituents present in the aerial parts of this species were isolated and their structures confirmed by NMR and ESI-MS analyses. The compounds included two chlorogenic acids, four quercetin and two kaempferol glycosides, as well as the isoflavone formononetin-7-glucoside. The concentration of isoflavone was low, not exceeding 1.2 mg/g of dry matter. The concentration of flavonols ranged between 5.9 and 11.8 mg/g, depending on the sampling dates, with the highest concentration occurring in the first cut. A similar trend in the concentration was found for chlorogenic acids, which ranged from 2 mg/g in summer to 7.3 mg/g in spring.

Keywords: Persian clover, *Trifolium resupinatum*, flavonols, phenolic acids, composition.

The genus *Trifolium* (Papilionoidae-Trifollieae) includes about 250-300 species, which are distributed both in temperate and subtropical regions [1]. Out of this number, only a few species have gained economic significance as pasture crops. These include *Trifolium pratense* L., *T. repens* L., *T. resupinatum* L., *T. incarnatum* L., *T. hybridum* L., *T. pannonicum* Jacq., *T. subterraneum* L., *T. fragiferum* L. and *T. medium* L. Some of these species have been characterized for secondary metabolites, such as triterpene saponins [2-5], flavonoids [1,6], isoflavones [7,8] and cyanogenic glucosides [9,10].

The recent survey of seeds of 57 species of *Trifolium* showed a big diversity in secondary metabolite profiles [11]. Similar diversity can be also be found in aerial parts of *Trifolium* species [12], suggesting that some species can be recognized as important sources of natural products for food, feeding stuffs and cosmetic industries.

T. resupinatum (Persian clover, Reversed clover, Shaftal clover) has some importance as an annual pasture crop. The species originates from the Mediterranean and Middle East (Iran, Afganistan) region, where it has been cultivated for centuries. In temperate regions it has been cultivated since the beginning of the twentieth century, but problems exist with seed reproduction. Recent work on genetic selection has resulted in new varieties, which give the same yield of dry matter per hectare as the original Mediterranean populations, but also are able to produce satisfactory yield of seeds [13]. In spite of the wide geographical distribution and its usefulness as a pasture crop, little is known about the secondary metabolites of this species and their possible significance in animal nutrition [1]. Thus, the aim of the present work was to isolate and identify the major phenolic constituents of the green parts of this species.

In our previous work on phenolic content of 57 *Trifolium* species, three subspecies of *T. resupinatum* were classified into clusters high in phenolic acids (0.4-1.0% of dry matter), with some flavonoids, but free of cloveamids [12]. One of the subspecies contained low level of isoflavones.

Separation of *T. resupinatum* phenolics using liquid chromatography allowed us to distinguish a number of compounds, which were classified into three groups: two phenolic acids, six flavonols and one isoflavone. These were separated into individual components using low pressure preparative chromatography.

Two phenolic acids showed similar UV spectra and MS pseudomolecular ions at *m/z* 353 and fragmentations patterns characteristic for chlorogenic acids (caffeic and quinic acids). From NMR and MS data, compound **1** was shown to be 3-caffeoylquinic acid, known as chlorogenic acid, and compound **2** 4-caffeoylquinic acid, known as crypto-chlorogenic acid, with the second molecule being the dominant.

The six isolated flavonoids, based on their UV, MS and NMR spectra, were identified as quercetin and kaempferol derivatives, namely quercetin 3-*O*-β-D-glucopyranosyl-1→2-β-D-galactopyranoside (**3**), quercetin 3-*O*-β-D-glucopyranosyl-1→3-α-L-rhamnopyranosyl-1→6-β-D-galactopyranoside (**4**), kaempferol 3-*O*-β-D-glucopyranosyl-1→2-β-D-galactopyranoside (**5**), kaempferol 3-*O*-β-D-glucopyranosyl-1→3-α-L-rhamnopyranosyl -1→6-β-D-galactopyranoside (**6**), quercetin 3-O-β-D-glucopyranoside (**7**), quercetin 3-*O*- (6"-malonyl)-β-D-glucopyranoside (**8**), and formononetin 7-*O*-β-D-glucopyranoside (**9**). Compounds **3** and **5** are constituents of *Panax notoginseng* [14], while the triglycosides (**4**) and (**6**) occur in the leaves of tea (*Camellia sinensis)* [15]. Formononetin 7-*O*-β-D-glucopyranoside (**9**) is an isoflavone commonly occurring in plants belonging to the Leguminosae family [16,17].

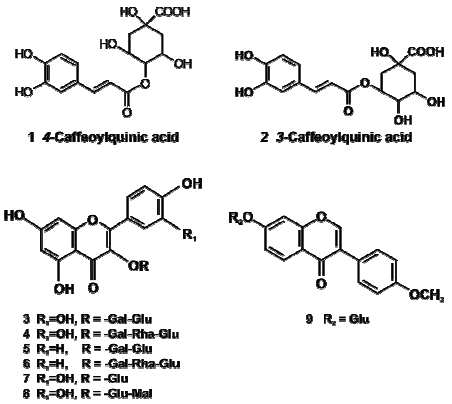

Figure 1: Chemical structures of phenolics isolated from *T. resupinatum* aerial parts.

Table 1: Concentration of phenolics in aerial parts of *T. resupinatum* var. Irain [three sampling dates (cuts) during one vegetation season].

Compounds	Cut 1* (mg/g)	Cut 2 (mg/g)	Cut 3 (mg/g)
1	0.54±0.08	0.48±0.09	0.35±0.05
2	6.74±0.43	3.24±0.59	1.68±0.58
3	0.75±0.07	0.27±0.03	0.24±0.03
4	0.89±0.07	0.36±0.05	0.54±0.06
5	0.72±0.09	0.35±0.05	0.20±0.02
6	1.80±0.20	1.59±0.23	1.56±0.14
7	5.18±0.41	2.55±0.28	2.20±0.24
8	2.53±0.35	1.10±0.13	1.13±0.18
9	1.18±0.08	0.52±0.05	0.58±0.08
Total	**20.33**	**10.46**	**8.48**

*Cut 1 – 6 June, Cut 2 – 4 July, Cut 3 -15 August 2007

The isolated compounds were used to develop an HPLC method for their determination in the plant material. These standard compounds were used both for the localization of phenolics in the HPLC profile, as well as for the preparation of standard curves for their determination. All nine isolated compounds were successfully separated by gradient elution (Figure 2).The concentration of isolated phenolics was measured in the aerial parts collected three times during the growing season. As shown in Table 1,

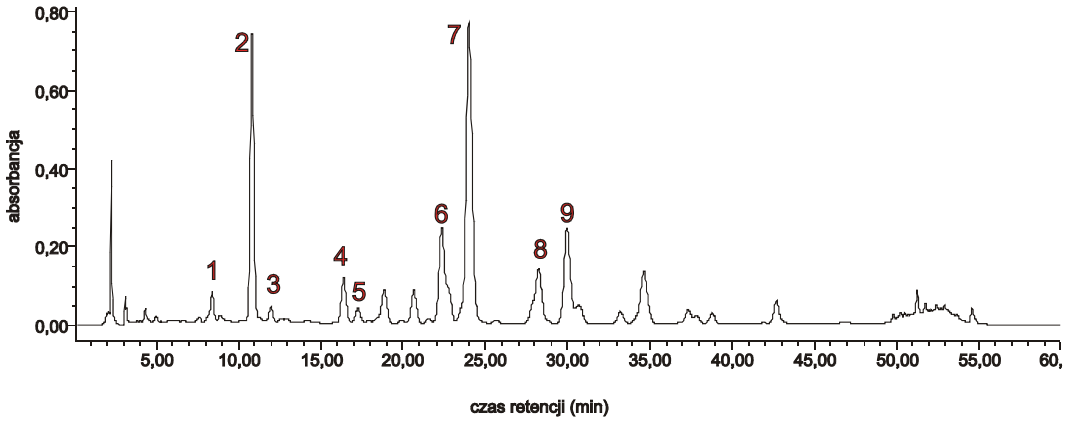

Figure 2: HPLC profile of *T. resupinatum* var. Ira phenolics.

the concentration of phenolics was highest in the first, spring collected material. The concentration of all three groups: chlorogenic acids, flavonols and isoflavone was double in spring samples as compared with the following two sampling dates. This remains in a good agreement with previous findings, showing successive decrease in the total flavonoid concentration in alfalfa aerial parts, during three growing seasons [18].

The overall concentration of phenolics in the aerial parts was quite high in a June sampled material and was about 2% of dry matter, dropping down by a half in the next two sampling dates.

The concentration of phenolics in the present research was very similar to the data obtained previously for three *T. resupinatum* subspecies [12]. The high flavonol concentration and thus their high antioxidant capacity, improves the nutritional value of this plant when used as feedstuff.

Experimental

Plant materials: Seeds of *T. resupinatum* L. var. Majus Boiss. were obtained from Genebank, Zentralinstitute fűr Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany (Herbarium Voucher TRIF 81/83). Seeds of *T. resupinatum* L. var. Ira were obtained from Bartazek Breeding Station, Poland. Seeds were planted in the field on small plots 2 x 2 m at IUNG Experiment Station. Plants of *T. resupinatum* var. Majus Boiss were collected at THE early flowering stage, freeze dried, powdered and used for isolation of phenolics.

Spectroscopic analysis: ESI-MS were recorded on a Mariner Biospectrometry workstation (PerSeptive Biosystems). NMR experiments were performed on a Bruker DRX-600 spectrometer equipped with a Bruker 5 mm TCI CryoProbe at 300 K. All 2D-NMR spectra were acquired in CD_3OD (99.95%, Sigma Aldrich) and standard pulse sequences and phase cycling were used for DQF-COSY, HSQC, HMBC spectra. The NMR data were processed on a Silicon Graphic Indigo2 Workstation using the Bruker XWIN-NMR software.

Extraction and separation of phenolics: Phenolics and saponins were extracted with 70% aqueous MeOH and purified according to a previously described procedure [19]. The purified phenolic fraction was loaded onto a preparative column (400 x 30 mm i.d., LiChroprep RP-18, 40-60 μm, Merck), which was washed with water and then with increasing concentrations of MeOH in water (linear gradient, Beckman Gradient Former) and 10 mL fractions were collected in a fraction collector. Fractions were analyzed by TLC (DC-Alufolien Cellulose, Merck, solvent 15% OHAc) and those showing similar profiles were combined and evaporated (34 fractions). The fractions were analyzed by HPLC (Waters with 996 PAD detector, 616 pump and Millenium software) and, based on HPLC profiles, isocratic systems were optimized for each fraction to purify individual compounds. The purification was performed on preparative column (400 x 20 mm i.d., LiChroprep RP-18, 25-40 μm, Merck) using an isocratic system (CH_3CN/1%H_3PO_4). Nine compounds were isolated:

3-caffeoylquinic acid (chlorogenic acid) (1)

UV/Vis λ_{max} (MeOH) nm: 323.
Rt min.: 8.2
^{1}H NMR (600 MHz, CD_3OD): 1.95 (1H, dd, *J* = 9.0, 14.0 Hz, H-6ax), 2.13 (2H, m, H-2eq, H-6eq), 2.20 (1H, dd, *J* = 4.0, 15.0 Hz, H-2ax), 3.63 (1H, dd, *J* = 3.0 and 9.0 Hz, H-4), 4.14 (1H, ddd, *J* = 3.0, 9.0, 9.0 Hz, H-5), 5.34 (1H, ddd, *J* = 3.0, 3.0, 4.0 Hz, H-3), 6.30 (1H, d, *J* = 16.0 Hz, H-8'), 6.76 (1H, d, *J* = 8.0 Hz, H-5'), 6.93 (1H, dd, *J* = 2.0, 8.0 Hz, H-6'), 7.04 (1H, d, *J* = 2.0 Hz, H-2'), 7.58 (1H, d, *J* = 16.0 Hz, H-7').
^{13}C NMR (150 MHz, CD_3OD): 36.7 (C-2), 41.5 (C-6), 68.3 (C-5), 73.0 (C-3), 74.8 (C-4), 75.4 (C-1), 115.1 (C-2'), 115.8 (C-8'), 116.4 (C-5'), 122.9 (C-6'), 127.9 (C-1'), 146.7 (C-3'), 146.8 (C-7'), 149.4 (C-4'), 169.0 (C-9'), 178.3 (C-7).
ESI-MS/MS: *m/z* 707 $[2M-H]^-$, 353 $[M-H]^-$, 179 [caffeic acid-H]$^-$, 191 [quinic acid-H]$^-$.

4-caffeoylquinic acid (crypto-chlorogenic acid) (2)

UV/Vis λ_{max} (MeOH) nm: 243, 328.
Rt min.: 10.9
^{1}H-NMR (600 MHz, CD_3OD): 2.00 (1H, dd, *J* = 9.0, 14.0 Hz, H-6ax), 2.06 (1H, ddd, *J* = 3.0, 14.0 Hz, H-2eq), 2.17 (1H, dd, *J* = 4.0 and 14.0 Hz, H-2ax), 2.20 (1H, ddd, *J* = 4.0, 14.0 Hz, H-6eq), 4.23 (1H, ddd, *J* = 4.0, 9.0, 9.0 Hz, H-5), 4.37 (1H, ddd, *J* = 3.0, 3.0, 4.0 Hz, H-3), 4.80 (1H, dd, *J* = 3.0, 9.0 Hz, H-4), 6.37 (1H, d, *J* = 16.0 Hz, H-8'), 6.78 (1H, d, *J* = 8.0 Hz, H-5'), 6.96 (1H, dd, *J* = 2.0, 8.0 Hz, H-6'), 7.06 (1H, d, *J* = 2.0 Hz, H-2'), 7.65 (1H, d, *J* = 16.0 Hz, H-7').
^{13}C NMR (150 MHz, CD_3OD): 38.4 (C-2), 42.7 (C-6), 65.6 (C-5), 69.6 (C-3), 76.6 (C-1), 79.3 (C-4),

115.1 (C-2'), 115.3 (C-8'), 116.5 (C-5'), 123.0 (C-6'), 127.8 (C-1'), 146.7 (C-3'), 147.1 (C-7'), 149.6 (C-4'), 168.9 (C-9'), 177.4 (C-7)
ESI-MS/MS: *m/z* 353 $[M-H]^-$, 335 $[M-H_2O-H]^-$, 179 [caffeic acid-H]$^-$, 161 [caffeoyl-H]$^-$, 135 [caffeoyl-CO_2-H]$^-$.

Determination of phenolics in plant material: Dried samples (200 mg) were extracted with 80% aqueous MeOH (20 mL, 20 min by boiling). Extracts were condensed to remove MeOH and loaded onto C18 Sep-Pak cartridges, preconditioned with water. Cartridges were washed first with water (10 mL) and phenolics were washed out with 40% aq. MeOH (10 mL), condensed and dissolved in 1 mL of MeOH for HPLC analyses. Separations were performed on a RP-18 column (4.6 x 250 mm; Eurospher 100, 10 μm, Säulentechnik, Germany) using a gradient system of A → 100%B (where A: 10% CH_3CN in1%H_3PO_4 and B: 40% CH_3CN in 1%H_3PO4) at a flow rate of 1mL/min for 50 min. All samples were analyzed 5 times.

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