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Triterpene saponins and flavonoids in the seeds of *Trifolium* species

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Abstract

Seeds of 57 species of the genus *Trifolium* have been studied for the occurrence and concentration of soyasapogenol B glycosides and flavonoids. It was shown that all tested species contained soyasaponin I and in some species astragaloside VIII and/or 22-*O*-glucoside and 22-*O*-diglucoside of soyasaponin I were also present. Total concentration of saponins ranged from trace amounts up to 10 mg/g^{DM}. It was suggested that soyasapogenol B glycosides could be recognized as chemotaxonomic character of Fabaceae family. All but three tested species contained flavonoids. The majority of species contained quercetin as a sole flavonoid or in the mixture with a number of unidentified flavonoid components. Concentration of quercetine in some species was at the level of about 3 mg/g^{DM}. This high quercetin concentration and soyasaponin occurrence makes the seeds of some *Trifolium* species a potential source of health beneficial phytochemicals, to be used in human nutrition.

Keywords: Genus *Trifolium*; Seeds; Soyasaponins; Quercetin

1. Introduction

The genus *Trifolium* (Papilionoidae–Trifolieae) includes about 250–300 species distributed in temperate and subtropical regions of both hemispheres (Bisby et al., 1994). Some of them including *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. are used as pasture crops. Due to the economic significance the chemistry of some of these species has been well recognized. Triterpene saponins (aglycones) have been reported in *T. pratense* roots (Oleszek and Jurzysta, 1986), *T. repens* aerial parts (Sakamoto et al., 1992), seeds of *T. incarnatum* (Jurzysta et al., 1989) and seeds of *T. resupinatum* (Simonet et al., 1999).

Similarly, flavonoid compounds have been researched only in a few species including *T. repens*, *T. pratense, T. medium. T. subterraneum* and *T. incarnatum* (Bisby et al., 1994). Cyanogenic glycosides have been extensively studies in *T. repens* (Butler, 1965; Stochmal and Oleszek, 1997).

The goal of the present paper was to analyse saponin and flavonoid composition and concentration in the seeds of 57 *Trifolium* species.

2. Results and discussion

Extraction of seeds of 57 *Trifolium* species with 70% MeOH followed by solid phase extraction/fractionation on C18 micro-columns afforded in each case several fractions. They were first analyzed by TLC using Silica gel plates for saponins and cellulose for phenolics. Washing micro-columns with water removed carbohydrates, which usually make up quite large portion of the matrix. Further washing with 40% MeOH was expected to remove phenolics (flavonoid glycosides and phenolic acids), while saponins should still retain on the micro-column (Oleszek, 1988). Indeed TLC of 40% MeOH fraction did not contain saponins but also analysis for phenolics showed only in some cases trace amounts of flavonoids. In consequence this fraction was not further analysed either by TLC or by HPLC.

Fraction obtained by washing micro-columns with 70% MeOH contained saponins as indicated by TLC, but some yellow spots were also observed. Close examination of these fractions with HPLC and PAD

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detector confirmed that the saponin fraction also contained compounds showing UV absorption maxima characteristic for flavonoids. The retention time of overwhelming flavonoid compound and its absorption spectrum was identical to the values obtained for quercetin standard. Thus, when 70% MeOH fraction was analyzed by HPLC, integrating chromatograms at 210 nm it was possible to quantify saponins, while the same run integrated at 350 nm provided chromatogram showing only flavonoid components. The presence of flavonoids in 70% MeOH fraction indicated they were quite hydrophobic, presumably being aglycones or monoglycosides. From the number of available flavonoid standards (quercetin, kaempferol, apigenin, luteolin, and their glucosides) tested by co-chromatography with the extract, only quercetin was identified and separately determined. The concentration of the other remaining flavonoids was measured by calibrating chromatogram at 350 nm and calculating the total flavonoid peak area and concentration as quercetin equivalent.

2.1. Saponins

TLC of saponin fractions on Silica gel showed after spraying with Liebermann-Burchard reagent that all samples contained saponins. Closer analysis with appropriate standards allowed identification of four structurally different glycosides. These included soyasaponin I (S1), astragaloside VIII (S2), 22-*O*-glucoside (S3) and 22-*O*-diglucoside (S4) of soyasaponin I. Soyasaponin I was present in all tested species and was found in a trace amount (detected by TLC but not determined by HPLC) only in *T. curvisepalum, T. desvauxii, T. fragiferum* ssp. *bonanni, T. heldreichianum* and *T. pallidum*. The concentration in the other remaining species ranged from 0.6 mg/g^{DM} for *T. resupinatum* var. *minus* to 5.57 mg/g^{DM} for *T. arvense* and 7.16 mg/g^{DM} for *T. phleoides* (Table 1). Astragaloside VIII that differs from soyasaponin I just with the inner sugar component (xylose in astragaloside and galactose in soyasaponin I) was found only in a half of the studied species and its concentration was generally quite low. Even lower occurrence frequency was found for 22-*O*-glucoside of soyasaponin I (21 species) and its concentration was relatively low (highest concentration 1.4 mg/g^{DM} found for *T. clypeatum*). In contrary 22-*O*-diglucoside of soyasaponin I was the second most frequently occurring saponin and its concentration was similar to the range of concentrations of soyasaponin I, with the highest amount found in *T. apertum* (5.02 mg/g^{DM}) and *T. cherleri* (8.74 mg/g^{DM}). Total concentration of saponins in tested species varied from about 0.1% up to 1%^{DM}.

Composition of four saponins did not seem to be a chemotaxonomic character. For some species, for which subspecies were available, saponin composition was different even inside the species. From two subspecies of *T. fragiferum*, the ssp. *bonanni* contained all four saponins, while in ssp. *fragiferum* only soyasaponin I was registered. Two subspecies of *T. medium* differed in saponin composition; they both contained saponins S1 and S4 and differed only in S2 and S3. Similarly one of the four subspecies of *T. pratense* (ssp. *sativum* Mart.) contained all four glycosides, while in three other species saponin S3 was not present. Only in the case of *T. resupinatum* all four glycosides were found in three tested subspecies.

The soyasaponin I, or rather its aglycone soyasapogenol B, seems to be a taxonomic marker for Trifolieae. In the present work, soyasaponin I was found in the seeds of all tested *Trifolium* species. Previous reports showed that soyasapogenol B was also present in seeds of 36 *Medicago* species (Jurzysta et al., 1992). It occurred in all but two species, *M. hybrida* and *M. doliata*. But even in case of these two species presence of soyasapogenol B could not be excluded as this was overlapped by extremely high concentration of hederagenin. Soyasapogenol B glycosides were also reported in a number of other leguminous species with some indication that DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one) conjugates may be useful as chemotaxonomical markers (Okubo and Yoshiki, 1996). This suggests that soyasapogenol B can also be a marker for the Fabaceae.

Its physiological function is not known, but there were some reports indicating that soyasapogenol B can play signaling function. The work of Lynn (1985) documented that root exudates from *Lespedeza cuneata* (Dumont) G. Don contained soyasapogenol B and E, and these aglycones were capable of inducing differentiation of the haustorium in parasitic angiosperm, *Agalinis purpurea*. Wide occurrence in Fabaceae, the localization of high concentration of soyasapogenol B glycosides in physilogically active tissues and in the root hairs (Tsurumi et al., 1992) where rhizobia inducing flavonoids are also present in increased concentration, may suggest that also soyasapogenol B glycosides or the aglycone itself participate in legume-rhizobia colonization processes (Oleszek et al., 1999).

2.2. Flavonoids

All but three species, *T. cernuum*, *T. scabrum* and *T. subterraneum* contained detectable levels of flavonoids (Table 1). The only flavonoid identified with available standards was quercetin. In eight species (*T. carmeli, T. hirtum, T. isodon, T. leucanthum, T. miegeanum, T. ochroleucon, T. squarrosum* and *T. tomentosum*) this was the only flavonoid present, while in five species (*T. cherleri, T. dubium, T. fragiferum* ssp. *bonanni, T. michelianum* and *T. stellatum*) other than quercetin flavonoids were present.

Table 1
The presence and concentration of saponins and flavonoids in the seeds of *Trifolium* species

Species	Concentration (mg/g dry matter)						
	S1	S2	S3	S4	Total saponin	Q	Other flavonoids
T. alexandrinum	1.62	+	–	0.70	2.32	0.59	0.29
T. alpestre	2.29	–	+	+	2.29	0.27	0.38
T. ambiguum	1.82	–	–	–	1.82	1.37	0.17
T. angustifolium	1.16	0.32	–	–	1.48	1.85	0.11
T. apertum	4.63	–	–	5.02	9.65	1.09	0.43
T. arvense	5.57	–	–	2.03	7.60	2.03	0.64
T. bocconei	3.88	–	–	–	3.88	2.21	0.46
T. campestre	4.09	–	–	1.79	5.88	0.08	0.17
T. carmeli	2.19	–	–	4.35	6.54	0.60	–
T. cernuum	1.42	–	–	–	1.42	–	–
T. cherleri	2.00	+	+	8.74	10.74	–	1.70
T. clypeatum	1.40	1.23	1.40	3.82	7.85	0.19	0.04
T. curvisepalum	+	2.48	0.79	–	3.27	1.71	0.19
T. desvauxii	+	–	+	1.86	1.86	2.53	0.48
T. dubium	3.52	–	–	–	3.52	–	0.11
T. echinatum	2.06	–	–	–	2.06	0.22	0.32
T. fragiferum ssp. *bonanni*	+	+	0.93	+	0.93	–	0.09
T. fragiferum ssp. *fragiferum*	1.63	–	–	–	1.63	1.57	0.30
T. glomeratum	1.84	+	+	2.34	4.18	1.75	2.52
T. heldreichianum	+	2.08	–	–	2.08	0.55	0.79
T. hirtum	1.28	–	–	+	1.28	0.04	–
T. hybridum	3.29	+	–	1.10	4.39	2.07	0.96
T. incarnatum	4.23	–	–	1.47	5.70	0.90	0.11
T. isodon	2.10	1.04	–	2.85	5.99	0.22	–
T. isthmocarpum	1.81	1.76	–	–	3.57	0.20	0.81
T. lappaceum	4.76	1.88	+	–	6.64	1.14	0.35
T. leucanthum	5.50	–	–	1.04	6.54	0.63	–
T. ligusticum	3.52	+	–	+	3.52	0.91	0.19
T. medium var. *medium*	1.02	–	+	1.93	2.95	1.39	0.59
T. medium var. *sarosiense*	1.26	2.30	–	1.46	5.02	0.99	0.16
T. michelianum	1.49	+	+	2.26	3.75	–	0.09
T. michelianum ssp. *balansae*	1.91	–	–	–	1.91	2.80	1.22
T. miegeanum	1.56	0.76	–	3.20	5.52	0.42	–
T. montanum	2.58	–	–	–	2.58	0.83	0.70
T. nigrescens	3.20	1.00	–	0.78	4.98	0.85	0.42
T. occidentale	3.56	–	–	+	3.56	1.49	0.24
T. ochroleucon	1.37	–	1.08	2.54	4.99	0.33	–
T. pallidum	+	–	–	1.42	1.42	3.07	0.42
T. pannonicum	1.20	+	+	2.53	3.73	0.41	0.20
T. phleoides	7.16	+	–	1.42	8.58	3.17	4.99
T. pratense	1.92	+	–	3.99	5.91	0.97	0.44
T. pratense ssp. *expansum*	1.73	+	–	2.86	4.59	0.83	0.45
T. pratense ssp. *sativum*	3.37	0.81	–	1.69	5.87	1.17	0.62
T. pratense ssp. *sativum* Alef.	5.22	0.88	1.21	1.14	8.45	0.89	0.20
T. repens	1.12	0.86	+	1.18	3.16	1.16	0.68
T. resupinatum var. *majus*	2.84	+	0.69	0.95	4.48	1.03	0.15
T. resupinatum var. *minus*	0.66	+	0.43	+	1.09	0.44	0.17
T. resupinatum var. *resupinatum*	2.39	+	+	+	2.39	0.47	0.08
T. rubens	2.52	–	+	0.89	3.41	1.55	0.28
T. scabrum	2.85	–	+	0.94	3.79	–	–
T. spumosum	2.10	–	+	–	2.10	2.05	1.35
T. squarrosum	1.61	1.04	–	2.36	5.01	0.27	–
T. stellatum	4.79	–	0.41	1.23	6.43	–	0.08
T. striatum	5.24	–	–	2.63	7.87	0.05	0.18
T. subterraneum ssp. *subter*.	3.46	1.75	–	1.02	6.23	–	–
T. tomentosum	1.86	–	–	–	1.86	1.73	–
T. xerocephalum	3.82	–	–	0.88	4.70	2.71	0.92

+ under the limit of HPLC detection (<0.05 mg/g), the presence registered only by TLC.

Table 2
List of species examined of genus *Trifolium*

Species (subspecies, variety)	Origin	Herbarium voucher
Trifolium alexandrinum Jusl.	India	TRIF 30/79
Trifolium alpestre L.	Unknown	TRIF 201/93
Trifolium ambiguum M. Bieb.	Unknown	TRIF 178/75
Trifolium angustifolium L.	France	TRIF 139/78
Trifolium apertum Bobr.	Unknown	TRIF 44/83
Trifolium arvense L.	Unknown	TRIF 40/78
Trifolium bocconei Savi	Portugal	TRIF 81/79
Trifolium campestre Schreb.	Sweden	TRIF 99/91
Trifolium carmeli Boiss.	Israel	TRIF 100/81
Trifolium cernuum Brot.	Portugal	TRIF 52/96
Trifolium cherleri Jusl.	Unknown	TRIF 74/96
Trifolium clypeatum L.	Israel	TRIF 129/96
Trifolium curvisepalum Tackh.	Unknown	TRIF 53/94
Trifolium desvauxii et Blume	USA	TRIF 143/82
Trifolium dubium Sibth.	Germany	TRIF 103/79
Trifolium echinatum M. Bieb. subsp. *supinu* (Savi) Aschers. et Graebn.	Romania	TRIF 104/95
Trifolium fragiferum L. subsp. *bonanni* (Presl) Soj.	Hungary	TRIF 208/79
Trifolium fragiferum L. subsp. *fragiferum*	Unknown	TRIF 37/83
Trifolium glomeratum L.	Marocco	TRIF 107/81
Trifolium heldreichianum Hausskn.	Unknown	TRIF 149/92
Trifolium hirtum All.	Unknown	TRIF 213/78
Trifolium hybridum L.	Unknown	TRIF 6/82
Trifolium incarnatum L.	Czech Rep.	TRIF 82/83
Trifolium isodon Murb.	Denmark	TRIF 133/79
Trifolium isthmocarpum Brot.	Portugal	TRIF 77/91
Trifolium lappaceum L.	Uknown	TRIF 55/83
Trifolium leucanthum M. Bieb.	Israel	TRIF 131/77
Trifolium ligusticum Balb.	Portugal	TRIF 113/83
Trifolium medium Grufb. var. *medium*	Unknown	TRIF 35/83
Trifolium medium Grufb. var. *sarosiense* (Hazsl)	Unknown	TRIF 179/83
Trifolium michelianum Savi s l.	Unknown	TRIF 79/81
Trifolium michelianum Savi s. l. subsp. *balansae* (Boiss.) Thell	Bulgaria	TRIF 145/76
Trifolium miegeanum Maire	Portugal	TRIF 116/79
Trifolium montanum L.	Unknown	TRIF 147/95
Trifolium nigrescens Viv. subsp. *nigrescens*	Portugal	TRIF 117/96
Trifolium occidentale D.E. Coombe	France	TRIF 255/91
Trifolium ochroleucon Huds.	Slovakia	TRIF 173/96
Trifolium pallidum Waldst. et Kit.	Italy	TRIF 253/95
Trifolium pannonicum Jacq.	Unknown	TRIF 8/91
Trifolium phleoides Pourr.	Unknown	TRIF 132/94
Trifolium pratense L.	Turkey	TRIF 186/75
Trifolium pratense L. subsp. *expansum* (Waldst. et Kit) Simk)	Turkey	TRIF 188/83
Trifolium pratense L. subsp. *sativum* (Schreb.) Schübl. et Mart.	Unknown	TRIF 151/78
Trifolium pratense L. subsp. *sativum* (Schreb.) Schübl. et Mart. f. *albiflorum* Alef.	Unknown	TRIF 171/79
Trifolium repens L.	Unknown	TRIF 15/79
Trifolium resupinatum L. var. *majus* Boiss.	Iran	TRIF 61/83
Trifolium resupinatum L. var. *minus* Boiss.	Unknown	TRIF 57/83
Trifolium resupinatum L. var. *resupinatum*	Unknown	TRIF 43/80
Trifolium rubens L.	Unknown	TRIF 32/82
Trifolium scabrum L.	Portugal	TRIF 120/79
Trifolium spumosum L.	Unknown	TRIF 67/83
Trifolium squarrosum L.	Unknown	TRIF 122/79
Trifolium stellatum L.	Croatia	TRIF 215/94
Trifolium striatum L.	France	TRIF 70/96
Trifolium subterraneum L. subsp. *subterraneum*	USA	TRIF 259/91
Trifolium tomentosum L.	Portugal	TRIF 218/79
Trifolium xerocephalum Fenzl.	Unknown	TRIF 80/75

The concentration of quercetin ranged between trace amounts (0.05 mg/g in *T. striatum*) to around 3 mg/g in some species. Highest concentration was found in *T. desvauxii* (2.53 mg/g), *T. michelianum* ssp. *balansae* (2.80 mg/g), *T. xerocephalum* (2.71 mg/g), *T. pallidum* (3.07 mg/g) and *T. phleoides* (3.17 mg/g). This high concentration of quercetin can be advantageous for seeds due to its high antioxidant and radical scavenging properties. As shown in numeral studies quercetin is one of the most potent antioxidant and radical scavenging

compound from the group of flavonoids (Burda and Oleszek, 2001).

Flavonoids play an important role in human nutrition as health promoting natural chemicals (Paré, 2000; Rice-Evans, 2000). The most important sources of flavonoids in human diet include tea, onion and apples, and average daily intake was estimated to be about 30 mg/day (Rice-Evans, 2000). All these three sources possess quercetin and kaempferol glucosides, bioavailability of which is much higher than widely occurring quercetin rutinoside. Health promoting activities are shown also by some saponins, including soyasapogenol glycosides (Rao and Gurfinkel, 2000). Concentration of these glycosides in seeds of *Trifolium* species is similar to the concentration in other leguminous plants. High concentration of quercetin and presence of soyasapogenol B glycosides make seeds of some *Trifolium* species, a promising plant material to be used in human nutrition as nutraceuticals or food additives.

3. Experimental

Seed of authenticated material of 57 species of *Trifolium* were obtained from Genebank, Zentralinstitut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany (Table 2).

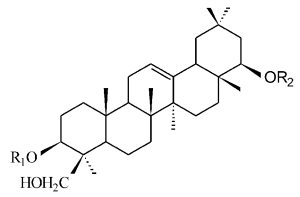
3.1. Extraction and SPE purification

Finely powdered seeds (200 mg) were extracted under reflux for 1 h with 70% MeOH (10 ml). The extracts were concentrated until MeOH was nearly removed and loaded onto C18 cartridges (Waters, Poland) preconditioned with water. Cartridges were washed with water (10 ml) and then successively with 40% MeOH and MeOH (5 ml each). Methanolic fractions were evaporated to the dryness and redissolved in 1 ml of MeOH and used for analyses.

3.2. TLC

Thin layer chromatography of saponins was performed on Silica gel (Kieselgel 60 F_{254}, Merck) and reversed phase C18 (DC-Alufolien RP-18, F_{254}, Merck). Plates were developed in EtOAc–OHAc–H_2O (7:2:2) and MeOH–H_2O (6:4) for Silica gel and RP-18, respectively. Plates were dried, sprayed with Liebermann–Burchard reagent and heated at 105 °C for visualization.

Saponin standards (Fig. 1) of 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-galactopyranosyl(1→2)-β-D-glucuronopyranoside]soyasapogenol B (soyasaponin I, S1), 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-xylopyranosyl(1→2)-β-D-glucuronopyranoside] soyasapogenol B (astragaloside VIII, S2), 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-galactopyranosyl(1→2)-β-D-glucuronopyranosyl]-22-*O*-[glucopyranoside]soyasapogenol B (S3), 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-galactopyranosyl(1→2)-β-D-glucuronopyranosyl]-22-*O*-[glucopyranosyl(1→2)glucopyranoside]soyasapogenol B (S4) (Simonet et al., 1999) were run together with plant extracts on the same plate and R_f values were used for confirmation of identity.



S1 (Soyasaponin I)	R_1= -GlcAp←Galp←Rhap	R_2=H
S2 (Astragaloside VIII)	R_1= -GlcAp←Xylp←Rhap	R_2=H
S3	R_1= -GlcAp←Galp←Rhap	R_2=-Glcp
S4	R_1= -GlcAp←Galp←Rhap	R_2=-Glcp←Glcp

Fig. 1.

Flavonoids were chromatographed on cellulose (DC Alufolien, Merck) using 15 and/or 50% OHAc. Chromatograms after drying the solvent were observed under UV (254 nm). Flavonoid standards were run in parallel with plant samples.

3.3. HPLC

High performance liquid chromatography of saponins and flavonoids was performed on Waters apparatus equipped with 996 PAD detector, 616 pump and Millenium software. Separations were performed on RP-18 (4.6×250 mm; Eurospher 100, 10 μm, Säulentechnik, Germany) using gradient system of H_3PO_4→90% AcN at flow rate 1 ml/min during 50 min. Chromatograms were integrated at 210 nm for saponins and 350 nm for flavonoids. Peaks were identified on the basis of R_t for saponins and on R_t and PAD absorption spectra for flavonoids in relation to appropriate standards. Quantification was based on one point peak area of appropriate saponin and flavonoid standards.

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