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# Young herbaceous legumes – a natural reserve of bioactive compounds and antioxidants for healthy food and supplements

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## Summary

Young plants of clover (*Trifolium pratense* L. and *T. medium* L.), medick (*Medicago sativa* L. and *M. lupulina* L.), sainfoin (*Onobrychis viciifolia* Scop.) and milkvetch (*Astragalus glycyphyllos* L. and *A. cicer* L.), were investigated for total contents of phenolics, flavonoids, isoflavones, condensed tannins and triterpene saponins as well as their extracts for antiradical and ferrous ion chelating activity. The impact of two sample drying methods on the aforementioned characters was compared. The phytochemical concentrations were higher in the freeze-dried legumes; however, antioxidant activities were generally higher of oven-dried samples. Both the composition of health promoting phytochemicals and antioxidant properties were strongly species-dependent. Among the species tested, *Trifolium* spp. were most abundant in isoflavones, *Medicago* spp. – in saponins and *O. viciifolia* – in tannins. Plants of *T. medium* and *O. viciifolia* were rich in TPC. The extracts of *T. pratense, O. viciifolia* and *A. cicer* possessed significant antiradical activity; the extracts from *Astragalus* spp. proved to be promising chelators of ferrous ion. We concluded that young perennial legumes could be considered as potential candidates for the development of nutraceuticals and functional food ingredients to accommodate the need for a particular bioactive component or property.

**Key words:** perennial Fabaceae; phenolic compounds; saponins; antioxidant properties; drying methods

## Introduction

Nowadays, the live question is not only to eat for survival, but to be aware of what we eat and know that the food will provide the opportunity to enjoy the quality of life for longer (Muzquiz et al., 2012). The increasing occurrence of non-infectious diseases such as cancer or cardiovascular diseases might be caused by nutritional and lifestyle habits. Legumes play an important role in the traditional diets of many regions of the world and are used both in staple and functional foods (Prati et al., 2007). Though forage legumes are not common for human consumption, there is evidence that mankind in all ages and in various countries has been using young plants, leaves, or flowers of various perennial legumes in food and phytotherapy (Butler, 1995; Redžić, 2010). According to Barrett (1990), humans have been using leaves of total 88 genera with 290 species in Fabaceae, including 63 genera and 205 species in the sub-family Faboideae as vegetables: raw, steamed, boiled, fried or cooked mixed in with other foods. At present, leaves, flowers and seeds of alfalfa (*M. sativa* L.) and red clover (*T. pratense* L.) are sold as bulk powdered herb, capsules, and extracts in health food stores or different online shops. Potential health benefit of legume consumption is associated with the presence of different phenolic compounds like isoflavones, coumestans, tannins and other phytochemicals, for instance saponins, in their plant materials (Prati et al., 2007; Muzquiz et al., 2012). The diversity and complexity of the phytochemical composition of plants of Fabaceae family may explain their polyvalent pharmacological activity. Proanthocyanidins (condensed tannins) constitute an important group of natural polyphenols. They occur naturally in many plants, including legumes. Many of their biological effects of nutritional interest derive from antibacterial and antioxidative properties providing protection against radical mediated injury and cardiovascular disease (Cos et al., 2004). Other health-promoting components specific to legumes are isoflavones: they have come into focus of interest due to several reports about their positive effects on human health, in particular prevention of hormone-dependent cancers, cardiovascular diseases, osteoporosis, adverse menopausal manifestations and age-related cognitive decline (Pilšáková et al., 2010). Saponins are exceptional plant metabolites because of their invaluable pharmaceutical properties (Cheok et al., 2014). Clinical studies have suggested that saponins affect the immune system in ways that help to protect the human body against cancers, and also lower cholesterol levels, blood lipids, cancer risks, and blood glucose response (Shi et al., 2004).

There are many biological activities associated with the total phenolics and individual groups of metabolites, preeminently their antioxidant and anticarcinogenic properties (Campos-Vega et al., 2010). Numerous findings emphasize that dietary antioxidants are useful radioprotectors and play an important role in preventing many human diseases (cancer, atherosclerosis, diabetes and others) (Fang et al., 2002). Therefore, there is an increasing trend towards finding alternative sources of valuable phytochemicals due to their diverse potentialities in food industry and pharmaceutical applications (Barreira et al., 2016). To our knowledge, no comprehensive studies have been done on phytochemicals combined with antioxidant activities of perennial legumes of branching stage. Isoflavones have been quantitatively studied only in a limited number of species of these legumes before.

Sample preparation and drying methods are important factors to preserve stability of natural bioactive compounds and their antioxidant properties. A review of the existing researches, done by Abascal et al. (2005) indicates that freeze-drying has unanticipated and significant effects on the constituent profiles of medicinal plants. Many authors argue that freeze-drying proved to preserve more the quality of the plant materials, including antioxidant activity, phenolics and other bioactive substances (Pinela et al., 2011; Orphanides

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et al., 2013). However, conflicting evidence has been found by Que et al. (2008) who suggest that hot air-dried pumpkin flour exhibited higher reducing power, free radical scavenging and metal chelating activities than freeze-dried flour. Esparza-Martínez et al. (2016) reported that drying at high temperatures shows higher antioxidant activity than at low temperatures. Little information exists on the influence of drying methods on the concentration of phytochemicals and antioxidant properties of plant material of perennial legume species.

Thus, the aims of this study were to quantify bioactive compounds in 7 species of perennial legumes from the sub-family Faboideae, cut at branching stage, and to assess *in vitro* antioxidant activity of plant extracts as well as to evaluate the effects of two drying methods on the phytochemical profile and properties.

## Materials and methods

### Plant material

The 7 species chosen for the study represent legumes from the following genera: red and zigzag clovers, lucerne, black medick, sainfoin, liquorice and cicer milkvetches (Tab. 1).

The germplasm collection was established in a field trial in 2014 in the Central Lowland of Lithuania (55°23′49″N; 23°51′40″E), at the Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry. The soil of the experimental site is *Endocalcari-Epihypogleyic Cambisol*. The seeds of the entries were sown in a single row (2.5 m long with 0.5 m spacing between plants) in 4 replications. No herbicides were applied in the collection nursery.

The plant material represents whole aerial parts collected at the plant branching stage. The samples were washed thoroughly with tap water, rinsed with distilled water and blotted on filter paper. Then each of the samples was divided into two subsamples. One subsample was pre-dried in an oven at 105 °C for 15 minutes to rapidly stop metabolism and then oven-dried at 65±5 °C for 24 hours. The other subsample was freeze-dried. Sublimation /lyophilisation was performed in a Sublimator 3×4×5 (ZIRBUS Technology GmbH, Germany), the condenser temperature was -85 °C, and the vacuum was $2 \times 10^{-6}$ mPa, the samples were frozen at -40 °C in a laboratory freezer, and then left in a freeze-drier for 72 hours. Both oven- and freeze-dried samples were ground to pass a 1 mm screen.

### Reagents and chemicals

Folin-Ciocalteu phenol reagent (2N), gallic acid monohydrate (≥98.0%), sodium carbonate (anhydrous, 99.5-100%), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid monosodium salt (ferrozine, 97%), methanol (≥99.9%), acetone (≥99.8%), hexane (≥97.0%), acetic acid (≥99.7%), sulphuric acid (95-98%), vanillin (4-hydroxy-3-methoxybenzaldehyde, ≥97%), catechin hydrate (≥98%), biochanin A (≥98%), daidzein (≥98%), formononetin (≥99%), genistein (≥98%), oleanolic acid (≥97%) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). A stable DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical (95%) and iron(II) chloride (anhydrous, 99.5%) were supplied by Alfa Aesar GmbH & Co KG (Karlsruhe, Germany). Acetic acid (100%), aluminium chloride hexahydrate (≥95%), hexamethylene tetramine (≥99%) and rutin trihydrate (≥95%) were obtained from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). LC-MS grade methanol, acetic acid and formic acid were obtained from Fluka (Sigma-Aldrich). Ethanol 96.3% (v/v) was purchased from Stumbras AB (Kaunas, Lithuania). Double-deionized water with conductivity lower than 18.2 MΩ was purified by using a Milli-Q Direct 8 water purification system (Millipore, Bedford, MA, USA).

### Preparation of extracts

The assays for total phenolics, flavonoids and antioxidant properties revealed that the best results in the recovery of the bioactive properties were achieved when extraction was performed with 70% (v/v) aqueous ethanol through ultrasonic agitation at 50 °C for a 15 min period of sonication (data not shown). About 0.25 g (precision ±0.0001 g) of plant material was sonicated with 25 mL of the 70% (v/v) aqueous ethanol for 15 min using an ultrasonic bath Elmasonic S40H (Elma Schmidbauer GmbH, Germany). The suspension was filtered and supernatant was adjusted to 25 mL. Two replicate extractions were performed for each plant sample.

Extraction procedure for condensed tannins was carried out as follows: 500 mg of plant sample was extracted with 5 mL acetone/water mixture (70/30, v/v) containing 0.5% (m/v) of ascorbic acid by vortexing for 1 h. Ascorbic acid was added to prevent tannin oxidation during the extraction. Then the sample was centrifuged at 3000 × g for 15 min and resulting supernatant was filtered through 0.20 µm nylon filter. Three mL of hexane was added to 1 mL of fine solution to remove chlorophyll. The aqueous layer was separated and taken for spectrophotometric analysis.

Both acid hydrolysis and extraction of isoflavones were performed in a single step. The representative amount of sample (250 g) was extracted with 10 mL of methanol/water (8:2, v/v) containing 2 M HCl using sonication for 30 min at room temperature before being hydrolysed at 80-85 °C for 1.5 h. The extracts were filtered through a 0.2 µm nylon syringe filter and analyzed.

For the hydrolysis and extraction of triterpene saponins, 0.100 g of plant material was treated with 10 mL of 2 M HCl in methanol/water (1:1 v/v) under reflux for 8 h. Methanol was removed under vacuum and the aglycones were extracted with ethyl acetate (2 × 5 mL). The combined organic phase was evaporated to dryness, the residue dissolved in 5 mL of methanol then filtered through 0.2 µm nylon syringe filter and analyzed.

**Tab. 1:** List of germplasm collection of the perennial species from the sub-family Faboideae (Fabaceae) studied.

| Species (tribe[a]) | Entry No[b] | Cultivar (cv.) or wild ecotype (WE) | Entry notation | Origin[c] |
|---|---|---|---|---|
| Red clover, *T. pratense* (Trifolieae) | 31 | cv. Sadūnai | *Tpr* | Lithuania |
| Zigzag clover, *T. medium* (Trifolieae) | 2148 | WE | *Tme* | Lithuania |
| Lucerne, *M. sativa* (Trifolieae) | 2097 | cv. Malvina | *Msa* | Lithuania |
| Black medick, *M. lupulina* (Trifolieae) | 10 | cv. Arka | *Mlu* | Lithuania |
| Sainfoin, *O. viciifolia* (Hedysareae) | 28 | cv. Meduviai | *Ovi* | Lithuania |
| Liquorice milkvetch, *A. glycyphyllos* (Galegeae) | 13 | WE | *Agl* | Latvia |
| Cicer milkvetch, *A. cicer* (Galegeae) | 71 | WE | *Aci* | Lithuania |

[a] Tribes are indicated according to Lewis et al. (2005); [b] Entry No in Catalogue of Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry; [c] Country where seeds were collected.

**Total phenolics**

Total phenolic contents (TPC) in the plant extracts were determined spectrophotometrically by the Folin-Ciocalteu method using gallic acid as a reference. One mL of standard solutions of gallic acid at different concentrations or appropriately diluted extract was assayed with 5 mL 0.2 N Folin-Ciocalteu reagent and, after 5 min, 4 mL of sodium carbonate (7.5%, w/v) solution was added to the mixture and shaken. After a 60 min period of incubation at room temperature, the absorbance was determined at 765 nm using a UV/VIS spectrophotometer Spectronic Genesys 2 (Spectronic Instruments, USA). Quantification was done on the basis of the standard curve of gallic acid (solution of gallic acid in 96% (v/v) ethanol, 11-350 μg $mL^{-1}$). The concentration of TPC was expressed in mg of gallic acid equivalents (GAE) g of dry mass (mg GAE $g^{-1}$ DM).

**Total flavonoids**

Analysis of total content of flavonoids (TFC) in extracts was performed by a colorimetric assay, based on complexation with Al(III). One mL aliquot of 70% (v/v) ethanolic plant extract (10 g $L^{-1}$) was added to a 25 mL volumetric flask containing 10 mL of 96% (v/v) ethanol. Then, 0.5 mL of 33% acetic acid, 1.5 mL 10% $AlCl_3$ and 2 mL 5% hexamethylenetetraamine solutions were pipetted into the flask and made up with distilled water. The absorbance was read at 407 nm after 30 min at 20 °C versus the prepared blank. Blank samples were prepared from 1 mL of plant extract, 10 mL of 96% (v/v) ethanol and 0.5 mL of 33% acetic acid and diluted to 25 mL with distilled water. The absorbance of a reference solution, which was prepared by using 1 mL of rutin solution instead of plant extract, was measured simultaneously. Standard rutin solution was prepared by dissolving 0.05 g of rutin in 100 mL of 96% ethanol. Samples were analyzed in three replications. TFC was expressed as milligrams of rutin equivalents (RE) per g of dry mass (mg RE $g^{-1}$ DM).

**Condensed tannins**

Condensed tannins (CT) were performed using vanillin assay. 10 μL of aqueous sample was incubated with 2 mL of 1.8 M sulphuric acid solution in methanol, 2 mL of 10 g $L^{-1}$ vanillin solution in methanol and 990 μL pure methanol for 5 minutes. The absorbance was measured at 500 nm using a PG Instrument UV-Vis spectrophotometer T60 (Oasis Scientific Inc., USA). A solution of (+)-catechin in methanol (1 g $L^{-1}$) was used as the standard. The CT concentration was expressed as mg of catechin equivalents (CE) per g of dry mass (mg CE $g^{-1}$ DM). The limit of CT quantification (LOQ) was 3.2 mg CE $g^{-1}$ DM.

**Total triterpene saponins**

Saponin aglycones were analyzed on a 1290 Infinity UPLC system equipped with a 6410 triple quadrupole mass spectrometer (Agilent Technologies, USA). The atmospheric pressure chemical ionization source was operated in the negative ion mode and MS data acquisition was performed in the selected ion monitoring mode. Data were acquired and processed using the MassHunter software (Agilent). The Acquity UPLC BEH Shield C18 (2.1 × 100 mm, 1.7 μm) column (Waters) was employed for the separations. The mobile phase was composed of (A) water and (B) methanol both containing 0.25% (v/v) formic acid. The gradient elution program was as follows: 0-15 min, 40-100% B linear; 15-17 min, 100-40% B linear; 17-22 min, 40% B isocratic. The column temperature was maintained at 30 °C, the mobile phase flow rate was 0.25 mL $min^{-1}$, and the injection volume was 5 μL. The total amount of saponin aglycones was measured using internal calibration with oleanolic acid. The limit of quantification was 0.25 mg $g^{-1}$ DM.

**Quantification of isoflavones**

The quantification of the four isoflavones (daidzein, genistein, and their 4'-methylated derivatives, formononetin and biochanin A) was performed by ultra-performance liquid chromatography (UPLC) using a Waters Acquity UPLC system (Waters, Milford, MA) equipped with diode array detector (DAD). Data were collected and managed using the HyStar 3.2 software (Bruker). Analytes in the extracts were identified according to our recently published procedure (Lemežienė et al., 2015; Taujenis et al., 2015) and by comparing the retention times with those of the corresponding standards. Quantification was performed by external calibration and results were expressed in mg per 1 g of the dry material (mg $g^{-1}$ DM). The limits of quantification defined as the concentration resulting in a signal of ten times the noise level were 0.15 mg $L^{-1}$ (0.006 mg $g^{-1}$ DM) for biochanin A and formononetin, 0.20 mg $L^{-1}$ (0.008 mg $g^{-1}$ DM) for genistein, and 0.25 mg $L^{-1}$ (0.010 mg $g^{-1}$ DM) for daidzein. The sum of four isoflavones was presented in the current study.

**DPPH radical-scavenging activity**

The ability to scavenge the stable free 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was determined spectrophotometrically. The solution of DPPH in 96% (v/v) ethanol ($6 \times 10^{-5}$ M) was prepared daily before measurements. To initiate the antioxidant reaction an aliquot of 50 μL of plant extracts (10 g $L^{-1}$), i.e. an aliquot containing bioactive compounds from $0.5 \times 10^{-3}$ g of plant material was transferred into a test tube containing 2 ml of DPPH solution (containing 0.12 μmol of pure DPPH). The mixture was incubated for 30 min (until the reaction reached the steady state plateau) in the dark at room temperature. The decrease in absorbance due to the scavenging of DPPH was monitored with a spectrophotometer at 515 nm. The absorption of a blank sample containing the same amount of 70% (v/v) ethanol and DPPH solution was determined before each analysis. Radical scavenging capacity of plant extracts was calculated as a percentage of DPPH inhibition as follows:

$$DPPH(\%) = [(A_c - A_s) \times 100] / A_c,$$

where $A_c$ is the absorption of blank sample and $A_s$ is the absorption of the solution with the analyzed extract (t = 30 min). Finally the results were recalculated as μmoles of DPPH free radicals scavenged by extract from 1 g of plant material (on a DM basis):

$$DPPH(\mu mol\ g^{-1}) = (a \times DPPH\%) / (m \times 100),$$

where a is μmoles of pure DPPH in the aliquot, and m – plant material mass (g) in the extract volume used for the test.

**$Fe^{2+}$ chelating efficacy**

The ferrous ion-chelating (FIC) potential of legume extracts was monitored spectrophotometrically by measuring ferrous iron-ferrozine complex formation by its absorbance at 562. An aliquot of 50 μL of 2 mM $FeCl_2$ solution (containing 0.1 μmol $Fe^{2+}$) was added to a 1 mL of 70% (v/v) ethanolic plant extract (10 g $L^{-1}$). One mL of extract contained $Fe^{2+}$-chelating compounds from 0.01 g of plant material. After 5 min, the reaction was initiated by the addition of 0.2 mL 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was thereafter measured at 562 nm. One mL of 70% (v/v) ethanol was used instead of the sample for the control. FIC capacity was calculated as amount of $Fe^{2+}$ μmoles bound by chelating agents in extract from 1 g of plant material (on a DM basis):

$$FIC(\mu mol\ g^{-1}) = (a \times FIC\%) / (m \times 100),$$

where a is $Fe^{2+}$ μmoles in the aliquot of $FeCl_2$ solution, m – plant material mass (g) equivalent to the volume of extract used for the test. FIC % is a percentage calculated by using the formula:

$$FIC(\%) = [(A_c - A_s) \times 100] / A_c,$$

where $A_c$ is the absorbance of the reaction mixture with blank and $A_s$ is the absorbance of the reaction mixture with the plant extract.

All bioactive compounds and antioxidant properties were analyzed in triplicate.

**Statistical analysis**

Statistical analysis of the results was performed by using software Statistica 7.0 for Windows (StatSoft Inc., USA). T-test for dependent samples was performed to determine the significance of the drying method on the concentration of bioactive compounds and properties tested for the individual legume entry. One-factor repeated-measures ANOVA followed by Duncan's test was carried out to test for simple main differences among legume entries separately for the drying method. Significance of differences was defined at the 1% level ($p < 0.01$).

## Results

**Effects of drying method on bioactive compound concentrations and antioxidant activity**

Means of bioactive characters for all species dried by a different method and probability values of T-test revealed that concentrations of bioactive compounds and properties of plant extracts responded differently to sample drying method subject to both entry and bioactive character (Tab. 2). Total mean values of freeze-dried and oven-dried samples for total phenolic and flavonoid contents were very close; however, according to the probability values, the drying method significantly affected the concentration of total phenolics for *T. pratensis* and *A. glycyphyllos*, and total flavonoid content for *M. sativa*, *A. glycyphyllos* and *A. cicer.*

All four individual isoflavones quantified as well as their sum were tightly reliant on the drying method for *T. medium*. Generally, almost all legume species statistically significantly differed in the sum of isoflavones except for *M. lupulina* and *A. glycyphyllos*. The impact of the sample drying method as a factor was statistically significant on the concentrations of individual isoflavones for one or two species only. The mean values of condensed tannins, triterpene saponins and isoflavones (individual and sum) for freeze-dried samples were variably higher than those for oven-dried samples. At the same time means of antioxidant activities clearly showed an opposite direction: extracts of oven-dried legumes on average displayed higher potential as free radical scavengers and ferrous ion chelators than those of freeze-dried ones. Both DPPH radical scavenging and ferrous ion chelating capacity (FIC) of extracts of *T. pratensis* and *M. lupulina* were under the significant influence of the drying method.

**Bioactive compounds**

The amount of total phenolic, flavonoid contents (TPC and TFC, respectively) and capacity of their ethanolic extracts to scavenge free radicals as well as to chelate ferrous ions varied depending on the legume species (Tab. 3, Fig. 2) for both sample sets freeze-dried and oven-dried. In plant materials of different perennial legumes of branching stage, TPC ranged from 7.94 to 40.9 mg GAE $g^{-1}$ for freeze-dried samples and from 7.68 to 42.0 mg GAE $g^{-1}$ for oven-dried samples (Tab. 3). The highest TPC values (>40 mg GAE $g^{-1}$) were observed in the plants of *T. medium* and *O. viciifolia*. *M. sativa* and *M. lupulina* were the poorest among the legumes tested for TPC. The TFC varied in the similar ranges both in oven-dried and freeze-dried sample sets (3.69 - 41.9 and 3.42 - 36.9 mg RE $g^{-1}$, respectively). The results clearly indicated that the richest source of total flavonoids was *O. viciifolia* containing TFC more than twice above the mean for the investigated plants (15 mg RE $g^{-1}$, Tab. 2).

Plant material of *T. medium* also had a noticeably higher content of total flavonoids than the mean and significantly higher than *T. pratense, Medicago* spp. and *Astragalus* spp. had (Tab. 3). Plants of *A. cicer* were the least rich in TFC than other species. Generally, significant differences both in TPC and TFC were observed between species of the same genera (*Trifolium*, *Medicago* and *Astragalus*), except for TPC in *Medicago* species.

Quantifiable concentrations of proanthocyanidins (CT) were determined in sainfoin (11.2 and 13.3 mg CE $g^{-1}$ for oven- and freeze-dried plants respectively), in other legume species, vanillin assay showed only CT traces (<LOQ). *M. sativa and M. lupulina* were the only two species of perennial legumes which exhibited the quantifiable concentrations of triterpene saponins (7.71 and 7.88 mg $g^{-1}$ for oven-dried and 9.02 and 8.59 mg $g^{-1}$ for freeze-dried samples, respectively). Using UPLC-MS (spectra not shown) in the other plant species studied there were found also a few compounds attributable to saponins (two in clover entries and one in sainfoin and milkvetch species); however, the total concentrations of these

**Tab. 2:** Significance of the drying method effect on bioactive characters (separately for each legume entry) and mean values of phytochemical concentration and bioactivity for oven- and freeze-dried samples.

| Bioactive character | Probability values | | | | | | | Mean[a] for | |
|---|---|---|---|---|---|---|---|---|---|
| | *Tpr* | *Tme* | *Msa* | *Mlu* | *Ovi* | *Agl* | *Aci* | OD[b] | FD[c] |
| Total phenolic content | **0.036**[d] | 0.562 | 0.380 | 0.065 | 0.929 | **0.001** | 0.094 | 23.74 | 23.69 |
| Total flavonoid content | 0.184 | 0.687 | **0.0003** | 0.689 | 0.230 | **0.005** | **0.021** | 15.12 | 15.07 |
| Formononetin | 0.055 | **0.032** | 0.413 | 0.075 | 0.081 | 0.240 | **0.002** | 3.36 | 3.69 |
| Biochanin A | 0.155 | **0.007** | **0.0003** | 0.057 | 0.057 | 0.253 | 0.395 | 3.01 | 3.65 |
| Daidzein | **0.048** | **0.004** | - | - | - | - | - | 0.113 | 0.146 |
| Genistein | 0.197 | **0.004** | - | - | - | 0.423 | - | 1.04 | 1.26 |
| Sum of isoflavones | **0.010** | **0.009** | **0.028** | 0.077 | **0.020** | 0.477 | **0.046** | 11.2 | 13.3 |
| Condensed tannins | - | - | - | - | 0.283 | - | - | 6.85 | 7.92 |
| Triterpene saponins | - | - | 0.313 | 0.559 | - | - | - | 7.79 | 8.81 |
| DPPH | **0.008** | 0.283 | 0.675 | **0.007** | 0.116 | 0.120 | **0.037** | 72.2 | 59.5 |
| FIC | **0.00001** | **0.0004** | 0.622 | **0.002** | 0.192 | **0.015** | 0.839 | 7.32 | 6.77 |

[a] Units of mean values for character – in Material and methods.
[b] Oven-dried.
[c] Freeze-dried.
[d] Probability levels of differences in bold are statistically significant at $P < 0.05$.

**Tab. 3:** Contents of total phenolic (TPC), total flavonoid (TFC), condensed tannin (CT), triterpene saponin (TS) and sum of isoflavones in the oven- and freeze-dried perennial legumes. Values are means ± SD.

| Bioactive character | *Tpr* | *Tme* | *Msa* | *Mlu* | *Ovi* | *Agl* | *Aci* |
|---|---|---|---|---|---|---|---|
| | Oven-dried | | | | | | |
| TPC (mg GAE $g^{-1}$) | 37.1±0.29d | 42.0±1.88e | 7.68±0.98a | 8.10±0.07a | 40.9±1.91e | 13.2±0.97b | 17.2±0.93c |
| TFC (mg RE $g^{-1}$) | 12.4±0.68c | 22.5±1.70d | 9.50±0.51b | 5.28±0.44a | 41.9±0.45e | 10.6±0.20bc | 3.69±0.07a |
| CT (mg CE $g^{-1}$) | <LOQ | <LOQ | <LOQ | <LOQ | 11.2±1.42 | <LOQ | <LOQ |
| TS (mg $g^{-1}$) | <LOQ | <LOQ | 7.71±0.687 | 7.88±0.908 | <LOQ | <LOQ | <LOQ |
| Sum of isoflavones (mg $g^{-1}$) | 18.3±0.43b | 28.5±0.46c | 0.258±0.014a | 0.212±0.005a | 0.283±0.005a | 0.203±0.013a | 0.223±0.016a |
| | Freeze-dried | | | | | | |
| TPC (mg GAE $g^{-1}$) | 31.9±0.13d | 40.9±0.30e | 7.94±0.17a | 8.27±0.04a | 40.8±0.06e | 17.1±0.03b | 18.9±0.02c |
| TFC (mg RE $g^{-1}$) | 11.0±0.68bc | 22.8±1.02d | 12.7±0.45c | 5.90±0.05ab | 36.9±2.24e | 12.8±0.08c | 3.42±0.14a |
| CT (mg CE $g^{-1}$) | <LOQ | <LOQ | <LOQ | <LOQ | 13.3±1.11 | <LOQ | <LOQ |
| TS (mg $g^{-1}$) | <LOQ | <LOQ | 9.02±1.014 | 8.59±0.944 | <LOQ | <LOQ | <LOQ |
| Sum of isoflavones (mg $g^{-1}$) | 19.8±0.19b | 34.5±0.82c | 0.208±0.007a | 0.233±0.015a | 0.256±0.004a | 0.211±0.011a | 0.256±0.005a |

Mean values of variable followed by the same letter are not significantly different among species (in the row) at the $P < 0.01$ level by Duncan's multiple range test computed separately for differently dried sample set.

compounds were lower LOQ. Differences between *Medicago* species in total TS content were not significant. Although the impact of drying methods on the TS and CT levels was not statistically significant, concentrations of the metabolites indicated the advantage of the freeze-drying method to retain these bioactive compounds.

*T. medium* and *T. pratense* of the branching stage were characterised by isoflavone abundance. According to the total amount of four isoflavones averaged for both drying sets, the species ranked as follows: *T. medium* >> *T. pratense* >> *O. viciifolia* ≥ *A. cicer* = *M. sativa* = *M. lupulina* ≥ *A. glycyphyllos*. The rank for non-clover species slightly differed between the oven- and freeze-dried sample sets. Generally, freeze-drying proved to be the method of plant material preparation, preserving a higher isoflavone concentration than oven-drying, except for *O. viciifolia* and *M. sativa*. Moreover, qualitative UPLC-DAD chromatograms of non-hydrolysed extracts of red clover showed that drying conditions affected the composition of isoflavones and their conjugates (Fig. 1). Freeze-dried samples had higher levels of formononetin and biochanin A aglycones and lower levels of their glucoside malonates than oven-dried samples.

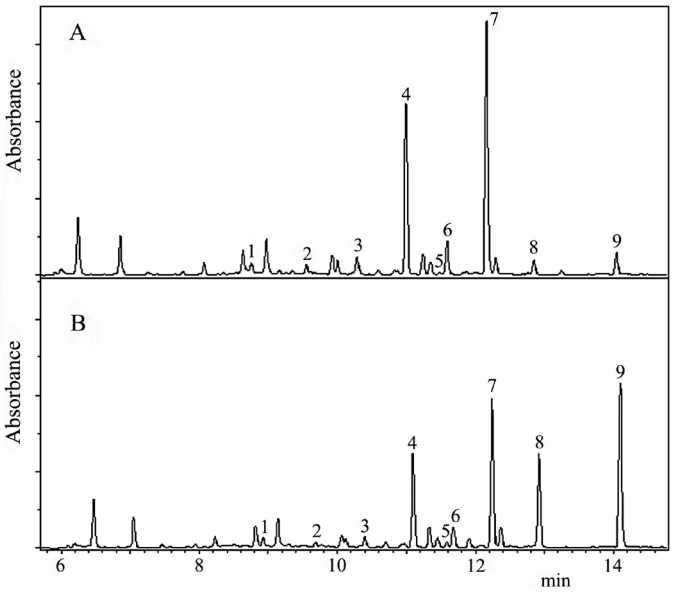


**Fig. 1:** UPLC-DAD profiles of isoflavones and their conjugates of non-hydrolysed extracts of differently dried red clover of branching stage. A – oven-dried; B – freeze-dried. Peaks: 1 – daidzein glucoside malonate; 2 – genistein glucoside malonate; 3 – formononetin glucoside; 4 – formononetin glucoside malonate; 5 – genistein; 6 – biochanin A glucoside; 7 – biochanin A glucoside malonate; 8 – formononetin; 9 – biochanin A.

**Antioxidant properties**

The results of DPPH quenching showed the presence of radical scavenging compounds in all investigated legume species. Comparison of antiradical activity of ethanolic extracts obtained from different legumes revealed that their radical scavenging capacities were highly variable (one gram of plant material eliminated from 12.98 to 174.88 μmol DPPH) (Fig. 2A). The extracts of *T. pratense* and *A. cicer* exhibited the highest antiradical activity (102.47 and 136.73 μmol $g^{-1}$, respectively for freeze-dried samples and 174.88 and 129.00 μmol $g^{-1}$, respectively for oven-dried samples). Despite the relatively high phenolic content in *T. medium* and moderate level in *A. glycyphyllos* (Tab. 3), fairly low activity against DPPH was distinctive for the extracts of the above-mentioned species (36.91 and 17.58 μmol $g^{-1}$, on average for oven- and freeze-dried samples of respective legume). Extracts of *M. sativa* possessed the weakest scavenging properties among the analyzed plants. Overall, the DPPH radical scavenging assay showed higher antioxidant activity of most extracts prepared from the legume samples which were oven-dried at 65 °C combined with pre-drying at 105 °C compared with freeze-dried samples.

Ferrous ion chelating (FIC) assay exhibited that extracts from raw materials of all the investigated species were able to capture $Fe^{2+}$ ions (Fig. 2B). It was determined that FIC values in extracts strongly differed subject to species of legume plants tested and ranged from 3.61 to 9.30 μmol of bound $Fe^{2+}$ per chelating agents in g of freeze-dried plant materials and from 5.22 to 9.29 μmol $g^{-1}$ of oven-dried ones (Fig. 2B). According to the average FIC potential of the oven- and freeze-dried samples, the species fell in the following rank: *A. cicer* (9.29 and 9.30 μmol $g^{-1}$) = *A. glycyphyllos* (8.50 and 9.19 μmol $g^{-1}$) > *M. sativa* (7.17 and 7.26 μmol $g^{-1}$) > *T. medium* (6.66 and 5.17 μmol $g^{-1}$) > *M. lupulina* (5.22 and 6.19 μmol $g^{-1}$) ≥ *O. viciifolia* (5.29 and 4.97 μmol $g^{-1}$) ≥ *T. pratense* (6.09 and 3.61 μmol $g^{-1}$).

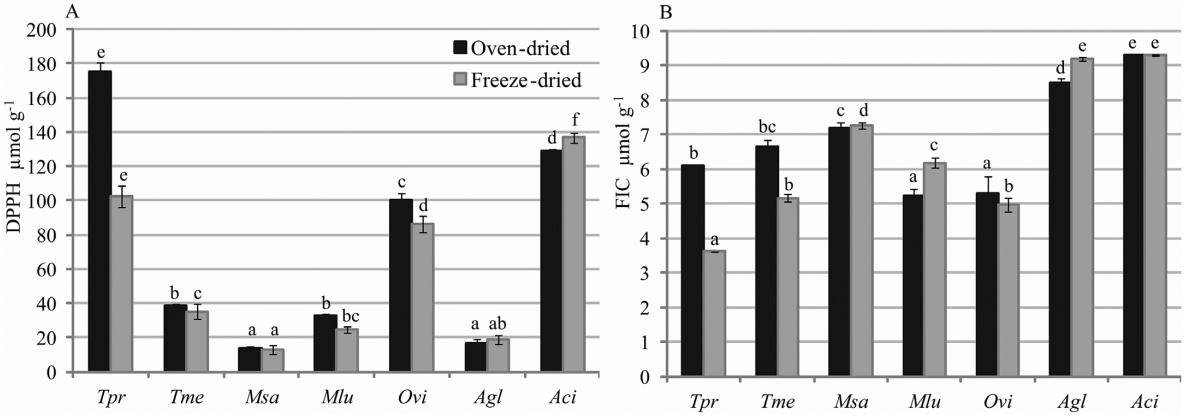


**Fig. 2:** Mean of DPPH radical scavenging (A) and ferrous ion chelating activities (B) of ethanolic extracts from the perennial legumes. Mean values (columns of the identical color) labeled by the same letter for bioactive character are not significantly different among species at the 0.01 level by Duncan's multiple range test computed separately for differently dried sample set; error bars indicate ± SD.

## Discussion

### Effect of drying method on antioxidant activity and bioactive substances

Disagreement on the effect of drying method on both antioxidant activity and bioactive substances has been found in literature (Que et al., 2008; Pinela et al., 2011; Orphanides et al., 2013; Esparza-Martínez et al., 2016). The chosen legume species have not been studied before or studied only fragmentary for the aspect of drying method influence on phytochemical profile and antioxidant properties of plant material. The results on this issue obtained in our study are in line with those of Sang et al. (2014) who revealed that the differences between total phenolic content and antioxidant activity of the differently dried Malaysian forage legume leaves were genera-specific. However, we observed higher differences among species in bioactivity response to drying conditions. The antioxidant activity of plant material of *Trifolium* species showed a stronger response to drying method than other legume species. This may be related to the transformation of the phytochemicals, both investigated and not investigated in the current study, including distinctive compounds for clovers – isoflavones. Antioxidant and biological activities of isoflavones have been known to depend on the concentrations and structures of isoflavones and their derivatives (Andres et al., 2015). In regard to these compounds, Swinny and Ryan (2005) revealed that oven-drying promotes decarboxylation of the malonyl glucosides to the acetyl glucosides, However, our results did not show such oven-drying effect on decarboxylation of the malonyl glucosides. This contradiction could be related to different oven-drying conditions, used by Swinny and Ryan (2005) (100 °C) and in the current study (65 °C in combination with short sample pre-drying at 105 °C). Yuan et al. (2009) disclosed that the decarboxylation of glycosides malonates occurred at a very low rate for the first period of 20 min when the soybeans were heated at 110 °C, and an inconsiderable change in isoflavone profile was observed while the temperature was lower (90 °C). Even small alteration in the drying conditions may affect the composition and properties of the plant materials. For instance, Swinny and Ryan (2005) found that immediate freeze-drying of red clover samples after being collected inhibited the conversion of the isoflavone glycosides to the aglycones; whereas Tsao et al. (2006) demonstrated that freeze-dried samples contained mainly the aglycones of isoflavones, when samples were kept at -5 °C for a few days before freeze-drying. In our study, samples were put in a sublimator within a few hours after being washed and blotted up. Such delayed procedure probably led to higher levels of aglycones through partial hydrolysis of conjugates, than in oven-dried samples (Fig. 1). Short and prompt sample pre-drying at 105 °C used before oven-drying in mild conditions (65 °C) as a factor retaining antioxidant properties of plant material should not be dismissed. In summary, sample preparation could be one of the causes of variation in the biological activity of isoflavones as well as of other phytochemicals, therefore the choice of drying conditions is important for particular plant use in food and phytotherapy. Our results highlighted the need for additional studies on this matter.

### Phytochemical composition and antioxidant properties

To our knowledge, this is the first inter- and intra-generic phytochemical characterisation of the plant materials of young perennial legumes. The findings of our study revealed that the concentrations of isoflavones, triterpene saponins, and condensed tannins in perennial legumes and antioxidant properties are taxon-dependent features. Although *Trifolium* and *Medicago* are classified in the same tribe, the quantitative phytochemical compositions of plant materials as well as the antioxidant activities of their ethanolic extracts differed radically between the species of the two genera. Under the tested set of features, the differences between the species of the same genus were smaller, but obvious.

Scanty information was found in the literature on the question concerning both bioactive substances and properties of the legume species studied. With regard to red clover, our results on TPC and DPPH radical scavenging are in agreement with those obtained by Khorasani Esmaeili et al. (2015) when phenolics extraction with methanol from *in vivo* grown red clover plants was used. However, TFC in our study was lower than that in the work mentioned above. Ince et al. (2012) confirmed also that sainfoin is a good source of phenolics, and its methanolic extracts exhibited high DPPH radical scavenging activity. In the recent study of Karimi et al. (2013), both TPC content and DPPH inhibition percentage of *M. sativa* leaf extract were found to be much higher, compared with the findings presented here; total flavonoids concentration matched that observed in our work. Admittedly, it is not appropriate to directly compare the results of our study with the scarce literature data owing to the differences in the plant genotype, maturity, plant parts, pedoclimatic dissimilarity, peculiarities in assay conditions, units of data expression, etc. In addition, no specific quantitative data have been found on bioactive properties in other species involved in the current study.

One of the most substantial attributes of sainfoin – the CT content in our study was higher than that reported by Theodoridou et al. (2011) (3.66-6.40 g $kg^{-1}$ DM); though, according to Azuhnwi et al.

(2011), the concentration of CT in 15 Swiss accessions of sainfoin was considerably higher and varied within a wide range of 47-80 g $kg^{-1}$ DM. Summarized data of numerous studies showed that CT in most legumes including *T. pratense* and *M. sativa* are located in generative plant organs, like flowers, seeds or seed coats (Piluzza et al., 2014). Furthermore, Piluzza et al. (2014) noticed that plant tannin content is often difficult to compare between laboratories because of the different methods or standards used for quantification.

Among the legume entries discussed in this paper only isoflavones in red clover have been quantified fairly in detail (Tsao et al., 2006; Oleszek, 2007; Lemežienė et al., 2015). The concentration of total isoflavones we found in red clover (19.6 mg $g^{-1}$) agrees with the range reported by the mentioned authors, except for the results published by Lemežienė et al. (2015). Such mismatch has resulted from the dissimilarities in plant maturity. The total mean content of isoflavones in different extracts from *M. sativa* (2.29-8.39 mg $kg^{-1}$) that was observed by Rodrigues et al. (2014) proved to be noticeably lower than we found (0.209 mg $g^{-1}$, Tab. 2). Reliable data on isoflavone quantification in other species of plants tested in our work are not available currently, only sporadic descriptive information can be found in the literature.

Generally, the concentration of triterpene saponins in our study was in agreement with the report of Tava et al. (1999), who found that total saponin concentration in *M. sativa* varied from 2.3 to 10 mg $g^{-1}$ DM depending on the cultivar and harvesting time. However, regarding the saponin concentration in black medick, Górski et al. (1984) found 2.5-3-fold higher values in cv. Renata (21-25 mg $g^{-1}$) than we determined in cv. Arka (7.98 mg $g^{-1}$). In contrast to the low concentrations of TPC, TFC and inhibiting activity against DPPH, the extracts from two species of the genus *Medicag*o were found to possess moderate activity in FIC assay. This may be related to the presence of genus-specific bioactive compounds – saponins. Joshi et al. (2013) revealed that saponins in all concentrations exhibited high FIC values.

Extracts of *A. glycyphyllos* exhibited the highest ferrous ion chelation potential. The high FIC capacity of the extracts from *Astragalus* species suggests that they contain higher amounts of ligands to compete with ferrozine and their extracts could prevent the generation of chemically reactive molecules containing oxygen, also known as reactive oxygen species (ROS). Currently, synthetic compounds are used in the chelation therapy and they have certain side-effects (Sudan et al., 2014). Therefore, chelation of metal ions by natural phytochemicals from Fabaceae plants, particularly from *Astragalus* species can prove to be of therapeutic importance.

Generally, it is recognized that among important forage crops there are accessions, including species studied in the current work, with relatively high bioactive and antioxidant properties, which have the potential to be used for the development of new functional, medicinal food and nutraceuticals (Kroyer, 2004; Prati et al., 2007; Ionkova, 2008; Ince et al., 2012; Karimi et al., 2013). The bioactive characteristics (substances and antioxidant properties) observed in legumes in our studies showed that each of the investigated species exhibited distinct and exceptional value. Perennial legumes are not common for human consumption; however, there is potential to include young legume plants as functional food ingredients to accommodate the need for a particular bioactive component/property or use them in nutraceuticals and supplements production. At this point, the present work is an initial phase for the forethought of further aspects of investigation on studying species including preclinical and epidemiological studies.

## Conclusions

Bioactive compounds and properties of legumes respond differently to sample drying method: freeze-drying retained higher concentration of phytochemicals, and antioxidant activity in most cases was higher of the oven-dried samples. Plants of the seven Fabaceae species of branching stage contain a considerable amount of bioactive compounds, and their extracts exhibit significant antioxidant activity; however, all properties vary significantly among the investigated species. Plant materials of *T. medium* and *O. viciifolia* are abundant in total phenolics. The extracts of *T. pratense, O. viciifolia* and *A. cicer* possess significant antiradical activity; the extracts from *Astragalus* species prove to be promising chelators of ferrous ion. *O. viciifolia* contains the highest concentrations of total phenolics, total flavonoids and condensed tannins among the species tested. Perennial legumes, particularly *Trifolium*, are important sources of isoflavones. *Medicago* species contain saponins. Hence, all these findings proved that perennial legumes of branching stage merit to be considered as a potential source of valuable bioactive compounds for medicinal application and for functional components in foods which may increase the intake of potentially health-promoting phytochemicals.

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