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Characterisation of red clover (*Trifolium pratense* L.) isoflavones under different extraction conditions

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KEY CONTRIBUTION

Compared to the whole red clover plant (*Trifolium pratense* L.), leaves are richer in isoflavones. Four isoflavones have been identified and quantified: daidzein, genistein, formononetin and biochanin A. The most dominant isoflavones are formononetin and biochanin A. The highest isoflavone content was obtained with 2 M HCl after evaporation to dryness.

ABSTRACT

Red clover (*Trifolium pratense* L.) is a widely used forage crop rich in isoflavones, bioactive compounds with diverse health-protective effects. In legumes, isoflavones occur in various chemical forms, but their health benefits are most pronounced in their free forms—aglycones. Although different extraction parametres (temperature, pH, sample-to-solvent ratio) can influence the efficiency of isoflavone extraction, this study specifically examined the effect of different HCl concentrations (2M, 4M, and 6M), with and without evaporation of the extract to dryness, on the hydrolysis of glycoside isoflavones into aglycones. The leaves and the whole plant of red clover were used as samples. High-Performance Liquid Chromatography (HPLC) was used for the identification and quantification of the isoflavones. The results revealed significant variations in isoflavone content among different plant parts. Red clover leaves had the highest total isoflavone content, with formononetin (3.739 mg/g DM) and biochanin A (2.484 mg/g DM) as the dominant compounds, whereas whole-plant extracts contained a higher content of daidzein (0.440 mg/g DM) and genistein (0.170 mg/g DM) compared to the leaves. Regarding the extraction method, the highest total isoflavone concentration (6.556 mg/g DM) was obtained from leaf extracts that were evaporated to dryness after hydrolysis with 2M HCl.

Introduction

Red clover (*Trifolium pratense* L.) is a species from the Leguminosae family and the *Trifolium* genus, which includes over 240 species (Dluhošová et al., 2018). As one of the most important forage species in grassland agroecosystems, red clover is a short-lived perennial legume, typically persisting for two to three years. It is highly adaptable to a wide range of climatic conditions, soil types, fertility levels, usage patterns, and management practices (Tucak et al., 2019). Although it is commonly used as animal feed, it has recently become very popular in the food, pharmaceutical and cosmetics industries as a valuable source of bioactive compounds (Petrauskas et al., 2023). The primary group of bioactive compounds found in red clover are isoflavones—secondary plant metabolites with mild estrogenic effects in mammals due to their structural similarity to 17β-estradiol, which classifies them as phytoestrogens (Daems et al., 2016; Mikulić et al., 2024). Therefore, the majority of the studies on the biological properties of *T. pratense* focused on its phytoestrogenic activity. Numerous clinical studies have demonstrated that isoflavones may contribute positively to human health and nutrition by reducing the risk of cardiovascular disease, alleviating menopausal symptoms, and helping to prevent certain types of cancer, cardiovascular disorders, and osteoporosis (Lipovac et al., 2012; Kolodziejczyk-Czepas, 2016). The amount of isoflavones varies depending on the part of the plant. In red clover, the highest concentrations of isoflavones are typically found in the leaves, followed by the stems, petioles, and flowers (Lemežienė et al., 2015; Butkutė et al., 2017). Besides parts of the plant, isoflavone contents also vary depending on the flowering stages of the plant. In the early stages of maturation, isoflavone concentrations are highest, while as the plant continues to grow, isoflavone concentrations tend to decrease (Tsao et al., 2006; Sivesind and Seguin, 2005). Gikas et al. (2008) analysed clover samples at different growth stages and found the highest proportion of isoflavones in the vegetative stage of plant development. Furthermore, the isoflavone content in red clover varies depending on origin, cultivar, genetic factors, and other environmental influences (Küçükboyaci et al., 2013).

More than 40 different isoflavones have been identified in red clover, with formononetin, biochanin A, daidzein, and genistein being the most dominant (Kumar et al., 2018; Tava et al., 2019; Tucak et al., 2019; Mikulić et al., 2024). In most studies on isoflavone extraction from red clover, the majority of the extracted compounds are glycosides, which are highly polar molecules. Because of their polarity, they have difficulty passing through the intestinal epithelium, resulting in lower bioavailability (Cosentino et al., 2019). Glycosides are broken down into aglycones with the help of intestinal bacteria in the digestive system, which improves their bioavailability (Beck et al., 2005). Given the better bioavailability of aglycone forms and their positive impact on health, they were the focus of this study. Typical extraction protocols first extract glycosides using an organic solvent, followed by acid hydrolysis to convert them into aglycones. Although different extraction parametres (temperature, pH, sample to solvent ratio) can influence the efficiency of isoflavone extraction, this study specifically examined the effect of different HCl concentrations (2M, 4M, and 6M), with and without extract evaporation to dryness, on the hydrolysis of glycoside isoflavones into aglycones. The study aimed to identify and quantify the proportions of the isoflavones biochanin, genistein, formononetin, and daidzein in hydrolysed red clover extracts, and to describe the impact of the hydrolysis extent on the obtained results.

Materials and methods

Sample preparation

Red clover (*Trifolium pratense* L.) samples were collected at the beginning of July 2023. The average leaf and whole plant samples (including leaves, stems, and flowers) were randomly collected from the central part of the experimental plot. The collected samples were first air-dried for one month, followed by oven-drying at 40

°C for two hours. The dried samples were then stored at −80 °C, lyophilised, and ground into a fine powder using an oscillating mill immediately before extraction.

Extraction

The extraction of isoflavones using acid hydrolysis was performed according to the partially modified procedure of Ramos et al. (2008). For extraction, 2 M, 4 M, and 6 M HCl were used, each with and without evaporation to dryness, as follows: 2 M without evaporation (mth1), 2 M with evaporation to dryness (mth2), 4 M without evaporation (mth3), 4 M with evaporation (mth4), 6 M without evaporation (mth5), and 6 M with evaporation to dryness (mth6). The homogenised red clover samples (0.05 g) were extracted with 4 mL of the respective HCl solution and incubated in a water bath at 100 °C for 15 min, with occasional vortexing. After cooling, the extracts were filtered, and the residue was washed with5 mL of MeOH. The purified samples were transferred and filtered into a volumetric flask and diluted to a final volume of 10 mL with distilled H_2O, then centrifuged at 4000 rpm for 10 min. The obtained extracts were stored at -21 °C until HPLC analysis (High-Performance Liquid Chromatography). Each sample was prepared in duplicate. Prior to HPLC analysis, all extracts were filtered through a 0.45 µm pore-size membrane filter.

HPLC analysis of isoflavones

Isoflavone separation was achieved using a PerkinElmer series, 200 HPLC system equipped with a quaternary pump, degasser, autosampler, and diode array detector (DAD) using a Phenomenex C18 column (150 × 4.6 mm, 5 µm). The mobile phase consisted of solvent A (water with 0,1% trifluoracetic acid) and solvent B (acetonitrile with 0,1% trifluoracetic acid). The following gradient programme was applied: 0-2 min 75% A and 25% B; 2–5 min from 25 to 35% B; 5–10 min from 35 to 50% B; followed by a post-run time of 3 min at 25% B. The flow rate was 1 mL/min, and the detection wavelength was 254 nm. The injection volume was 10 µL, and the analysis was performed at a constant temperature of 25 °C. Isoflavones were identified based on the retention time and UV spectra of corresponding standards—daidzein, genistein, formononetin, and biochanin A. The standards were dissolved in 80% MeOH. For quantification, five-point calibration curves were constructed for each compound with correlation coefficients of $r^2 \geq 0.999$. The isoflavone content was expressed in mg per g of dry weight (DW). All isoflavone standards (daidzein, genistein, formononetin) had a purity of ≥ 98%, whereas biochanin A, had a purity of 95%. Standards were purchased from Sigma-Aldrich, St. Louis, MO, USA. All other chemicals were of analytical reagent grade.

Statistical analysis

Statistical analyses were performed using the Statistica programme (version 14.2.0.18). Isoflavone concentrations were expressed as average values from two HPLC analyses of duplicate samples. Statistical differences among isoflavones extracted by six different methods were analysed using analysis of variance followed by a Fisher´s Least Significant Difference (LSD) post hoc test at $p < 0.05$.

Results and discussion

The present study demonstrated that both the plant part and extraction treatment significantly influenced the concentration and composition of isoflavones in *Trifolium pratense* L. Fig. 1 showed that leaf extract hydrolysed with 2 M HCl and evaporated to dryness yielded the highest total isoflavone content (6.556 mg/g DM), dominated by formononetin (3.739 mg/g DM) and biochanin A(2.484 mg/g DM). In contrast, the whole plant (Fig. 2) extract contained lower total isoflavone levels but exhibited a relatively higher proportion of daidzein (0.440 mg/g DM) and genistein (0.170 mg/g DM). These results highlight both tissue-specific isoflavone

accumulation and the critical role of hydrolysis conditions in maximising aglycone yield, which is in accordance with previous findings. Isoflavones are synthesised via the phenylpropanoid pathway, and their biosynthetic enzymes are more active in photosynthetically active tissues such as leaves (Klejdus et al., 2001; Lemežienė et al., 2015). The predominance of formononetin and biochanin A over daidzein and genistein is also consistent with the typical red clover isoflavone profile, where methoxylated derivatives are the major constituents (Mazur and Adlercreutz, 1998). The relatively higher concentration of daidzein in the whole-plant extract may reflect its localisation in non-foliar tissues or a differential rate of hydrolysis and conversion from its glycosidic precursors. Previous studies have shown that daidzein and genistein occur in lower abundance but can be enriched in stems or roots (Lemežienė et al., 2015). These differences may also arise from metabolic interconversion during extraction or degradation under acidic conditions (Klejdus et al., 2003). Acid hydrolysis is a key step in converting isoflavone glycosides to their aglycone forms, which are more biologically active and readily quantifiable by HPLC (Pilšáková et al., 2010). Hydrolysis with 2 M HCl followed by evaporation yielded the highest total isoflavone content, suggesting efficient glycosidic bond cleavage with minimal aglycone degradation. Increasing the acid concentration beyond 2 M may promote unwanted degradation or rearrangement of the phenolic compounds, thereby reducing total recoveries (Wang and Murphy, 1994; Lee et al., 2004). Evaporation to dryness likely enhanced aglycone concentration and facilitated the removal of volatile solvents and residual moisture that could otherwise interfere with chromatographic detection. Nevertheless, careful control of drying conditions is essential to prevent thermal degradation, as isoflavones are known to be thermolabile (Klejdus et al., 2003). The optimised protocol in our study demonstrated a balance between efficient hydrolysis and compound stability, producing higher yields than non-evaporated or over-hydrolysed extracts. The total isoflavone content obtained in this study (6.556 mg/g DM) corresponds to the lower to mid-range of reported values for red clover. Reported isoflavone contents in red clover leaves range from 5–12 mg/g DM (Lemežienė et al., 2015; Tucak et al., 2019) to approximately 15 mg/g DM when methanol extraction and enzymatic hydrolysis are applied. Differences between studies often arise from environmental factors (light intensity, temperature, soil nitrogen) and genetic variability among cultivars (Mikulić et al., 2024). Formononetin and biochanin A were identified as the predominant compounds, accounting for over 90% of total isoflavones, while daidzein and genistein were present at lower levels (Fig. 3, Fig. 4). These findings are consistent with previous studies that have identified red clover leaves as the main site of isoflavone biosynthesis and accumulation. Although daidzein and genistein are less abundant, they remain pharmacologically important due to their conversion to equol and *p*-ethylphenol metabolites in mammals (Pilšáková et al. 2010; Tava et al., 2019; Tava et al., 2015).

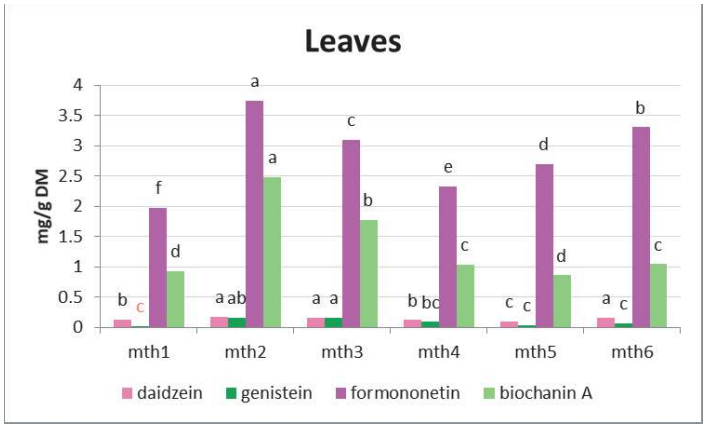

Figure 1 Average values of isoflavones in leaves obtained by mth1—2 M without evaporation; mth2—2 M with evaporation to dryness; mth3—4 M without evaporation; mth4—4 M with evaporation to dryness; mth5—6 M without evaporation; mth6—6 M with evaporation to dryness. Different letters indicate significant differences among the isoflavones extracted by six methods at $p < 0.05$ according to the LSD test

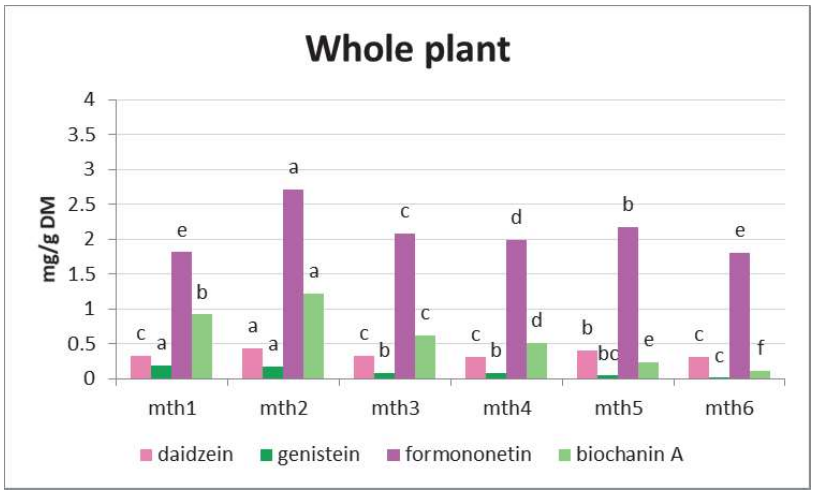

Figure 2 Average values of isoflavones in the whole plant obtained by mth1—2 M without evaporation; mth2—2 M with evaporation to dryness; mth3—4 M without evaporation; mth4—4 M with evaporation to dryness; mth5—6 M without evaporation; mth6—6 M with evaporation to dryness. Different letters indicate significant differences among the isoflavones extracted by the six methods at $p < 0.05$ according to the LSD test

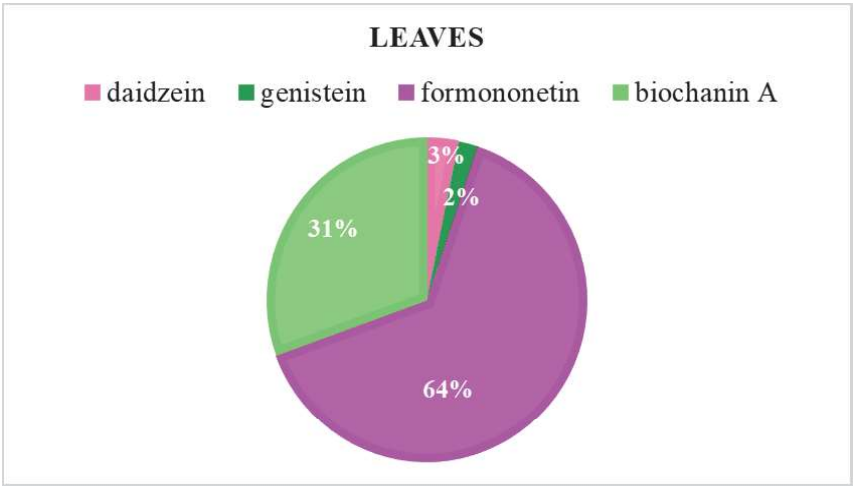

Figure 3 Proportion (%) of formononetin, biochanin A, daidzein and genistein of leaves

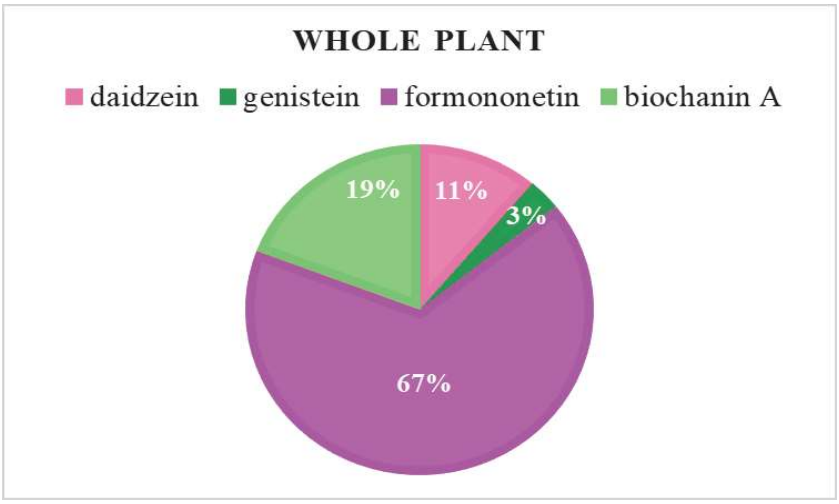

Figure 4 Proportion (%) of formononetin, biochanin A, daidzein and genistein of the whole plant

Conclusion

This study confirms that both the plant part and hydrolysis treatment significantly influence the yield and profile of isoflavones in *Trifolium pratense* L. The highest total isoflavone content was obtained from leaf extracts hydrolysed with 2 M HCl and evaporated to dryness, demonstrating that moderate acid hydrolysis efficiently converts glycoside forms into bioactive aglycones. Formononetin and biochanin A were identified as the predominant compounds, accounting for over 90% of the total isoflavones. The developed extraction and hydrolysis method offers an effective and reproducible approach for both research and industrial applications. Future investigations should address the effects of genotype, growth stage, and environmental conditions on isoflavone composition, and evaluate the use of enzymatic or eco-friendly hydrolysis methods to improve sustainability.

Author Contributions: M.K.B.: formal analysis, data analysis, and writing-original manuscript, M.K.: supervision, review and editing.; K.S.: data analysis, visualisation; D.H.: conceptualisation and review. All authors read and approved the final manuscript.

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***Conflicts of Interest*:** The authors declare no conflict of interest.

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