

Article

Red Clover Isoflavones as Effective Longevity Agents for Anti-Aging and Regenerative Skin Applications

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Abstract

Red clover (*Trifolium pratense* L.) is a rich source of isoflavones with documented antioxidant and skin-protective properties, yet substantial differences in phytochemical composition exist among cultivars. In this study, fourteen *T. pratense* cultivars were compared with respect to formononetin and biochanin A contents (Milena, Pasięka, Pyza, Milvus, Nemaro, Maro, Larus, Hammon, Vesna, Fregata, Carbo, Forelia, Osimia, and Elanus), and the relationship between isoflavone profiles and skin-related biological activity was evaluated. High-performance liquid chromatography revealed pronounced cultivar-dependent variability with formononetin and biochanin A contents ranging from 1.60 to 7.80 mg/g DW and from 0.69 to 6.44 mg/g DW, respectively. The observed variability was further visualized by principal component analysis. The cultivar with the highest total isoflavone content—Hammon, was selected for biological assessment. Its extract exhibited antioxidant (DPPH IC₅₀ = 0.619 mg/mL; FRAP IC_{0.5} = 0.302 mg/mL) and enzyme inhibitory activities (elastase IC₅₀ = 0.602 mg/mL, hyaluronidase IC₅₀ = 22.44 mg/mL), and it significantly enhanced fibroblast migration in an in vitro scratch assay, indicating anti-aging and regenerative potential. These results demonstrate that red clover cultivars differ significantly in their suitability as sources of bioactive isoflavones and highlight the importance of cultivar selection for the development of standardized plant-derived anti-aging ingredients. However, it is worth emphasizing that isoflavones derived from red clover are a valuable group of active compounds with significant potential for topical application as anti-aging and regenerative agents, warranting further formulation development and in vivo validation.

Keywords: *Trifolium pratense* L.; isoflavones; anti-aging; skin regeneration; longevity



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1. Introduction

Isoflavones are bioactive polyphenolic compounds with considerable pharmacological importance due to their antioxidant, anti-inflammatory, estrogen-like, and enzyme-inhibitory properties [1]. In addition to their potential use in preventing chronic diseases, including through inhibition of metabolic enzymes such as α -glucosidase [2], and alleviating menopausal symptoms, recent studies have demonstrated their beneficial effects on

skin properties through modulation of oxidative stress, collagen synthesis, and extracellular matrix remodeling [3–5].

Trifolium pratense L. is a valuable source of isoflavones, as it is rich in the O-methylated derivatives formononetin and biochanin A [6]. Compared with soy isoflavones, these compounds exhibit higher lipophilicity, greater metabolic stability, and distinct estrogen receptor affinities, which translate into a specific biological activity profile relevant to skin physiology [7]. In the context of skin aging, progressive oxidative stress, chronic low-grade inflammation, and extracellular matrix degradation are the major factors responsible for the loss of tissue elasticity, impaired regeneration, and delayed wound healing [8]. Both formononetin and biochanin A have been reported to protect fibroblasts against oxidative stress, modulate inflammatory signaling, and influence ECM (Extracellular Matrix) remodeling, supporting their anti-aging and regenerative potential [9,10], which makes *Trifolium pratense* a particularly attractive plant source for anti-aging and regenerative dermatological applications. In addition to isoflavones, red clover is known to contain other classes of specialized metabolites, including flavonoids (e.g., flavonols and flavones) and phenolic acids [6]. Although these compounds may also contribute to antioxidant and skin-related activities [11], isoflavones represent characteristic marker constituents of *Trifolium pratense* and are widely used for its chemotaxonomic identification and standardization. Their specific estrogen-like and redox-modulating properties make them particularly relevant to skin aging and regeneration.

However, the biological performance of red clover extracts is highly inconsistent, largely due to pronounced variability in their phytochemical composition. The content of formononetin and biochanin A strongly depends on the genetic background of the plant, and different *T. pratense* cultivars can differ several-fold in their isoflavone levels [12]. In addition, extraction conditions critically determine the recovery of these compounds from plant material [13]. Despite this, most biological studies on red clover do not control for cultivar identity or extraction efficiency, which limits the reproducibility and translational relevance of the results.

Beyond antioxidant effects, red clover isoflavones are of particular interest for anti-aging and regenerative applications because of their ability to modulate enzymes involved in ECM degradation [3]. Elastase contributes to loss of skin elasticity and delayed wound healing, while hyaluronidase affects tissue hydration and cell migration [14,15]. Inhibition of these enzymes is a recognized strategy in skin aging and regeneration, yet the extent to which red clover extracts exert such effects depends on their precise isoflavone profile [16]. Likewise, fibroblast migration is a key cellular endpoint of skin regeneration, and isoflavones have been shown to stimulate this process via estrogen-dependent and redox-sensitive pathways [9,17].

In this study, we aimed to identify *T. pratense* cultivars with the highest potential for anti-aging and regenerative skin applications by linking their phytochemical profiles with relevant biological activities. A Box–Behnken design was used to establish an extraction process that reliably reflects the isoflavone content of red clover, and this method was applied to fourteen cultivars to reveal genotype-dependent differences in formononetin and biochanin A levels. The cultivar with the highest isoflavone abundance was subsequently selected to evaluate how this chemical profile translates into antioxidant activity, modulation of extracellular-matrix-degrading enzymes, and stimulation of fibroblast migration in a scratch assay. In this way, the study connects cultivar chemistry with functional properties that are directly relevant to skin aging and regeneration.

2. Results

2.1. Optimization of Isoflavone Extraction

The Box–Behnken design enabled the statistical evaluation of the influence of ethanol concentration, extraction temperature, and extraction time on the yield of total isoflavones (the sum of formononetin and biochanin A) from *Trifolium pratense* L. leaves. The analysis of variance (ANOVA) confirmed the adequacy of the model ($R^2 = 0.983$, adjusted $R^2 = 0.965$), indicating a strong correlation between the predicted and experimental data. The Pareto chart (Figure 1) revealed that ethanol concentration had the most significant positive effect on the extraction efficiency ($p < 0.01$), followed by extraction time and temperature.

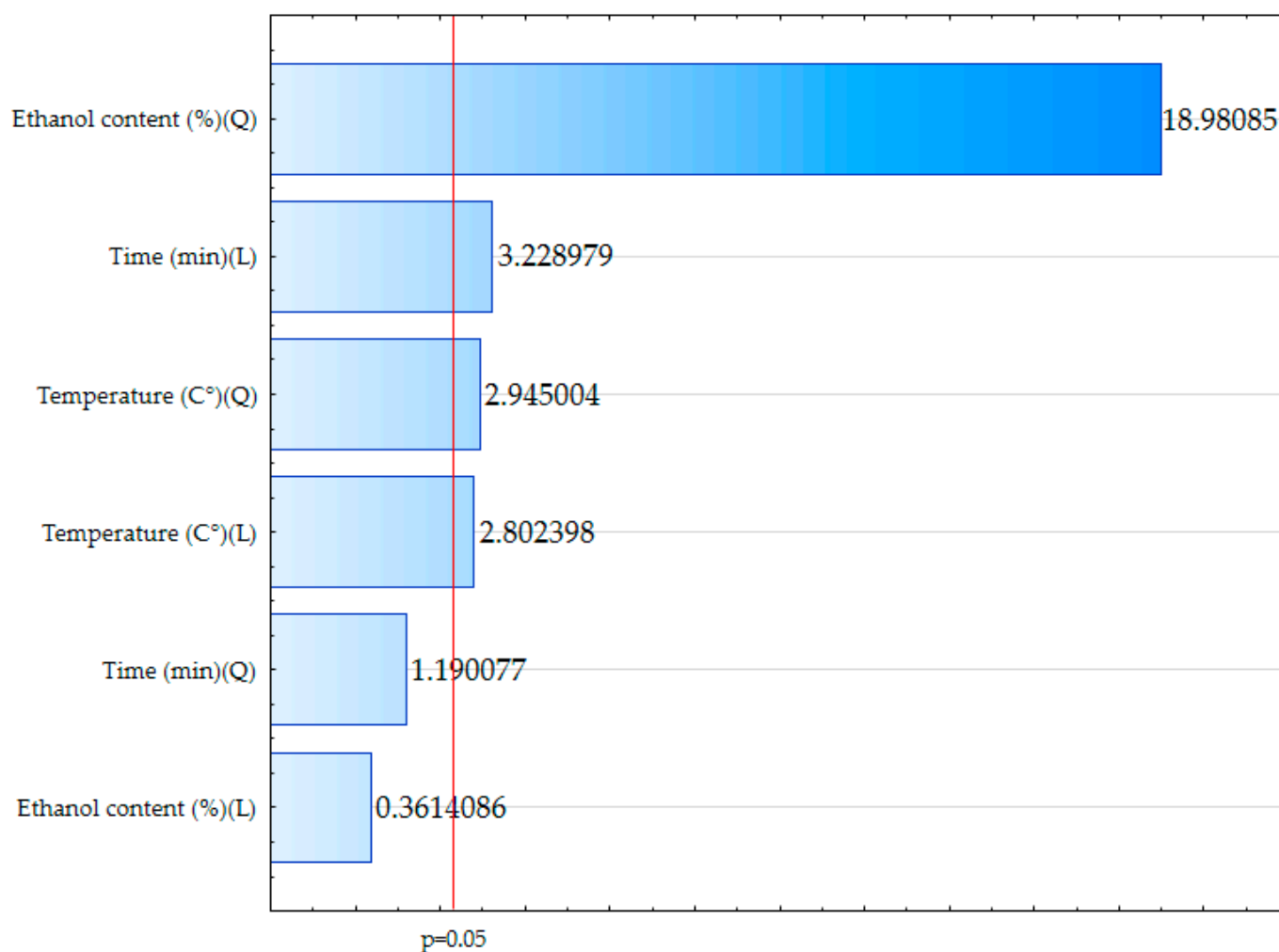


Figure 1. Pareto chart of standardized effects on total isoflavone content (sum of formononetin and biochanin A). L and Q denote linear and quadratic effects of the independent variables, respectively.

The response surface plots (Figure 2) demonstrated that moderate ethanol levels combined with longer extraction times enhanced the recovery of isoflavones.

The optimal extraction parameters were determined as 50.3% ethanol, 54.7 °C, and 82 min, yielding the maximum total isoflavone content. Under these conditions, the experimental values closely matched those predicted by the model, confirming its reliability.

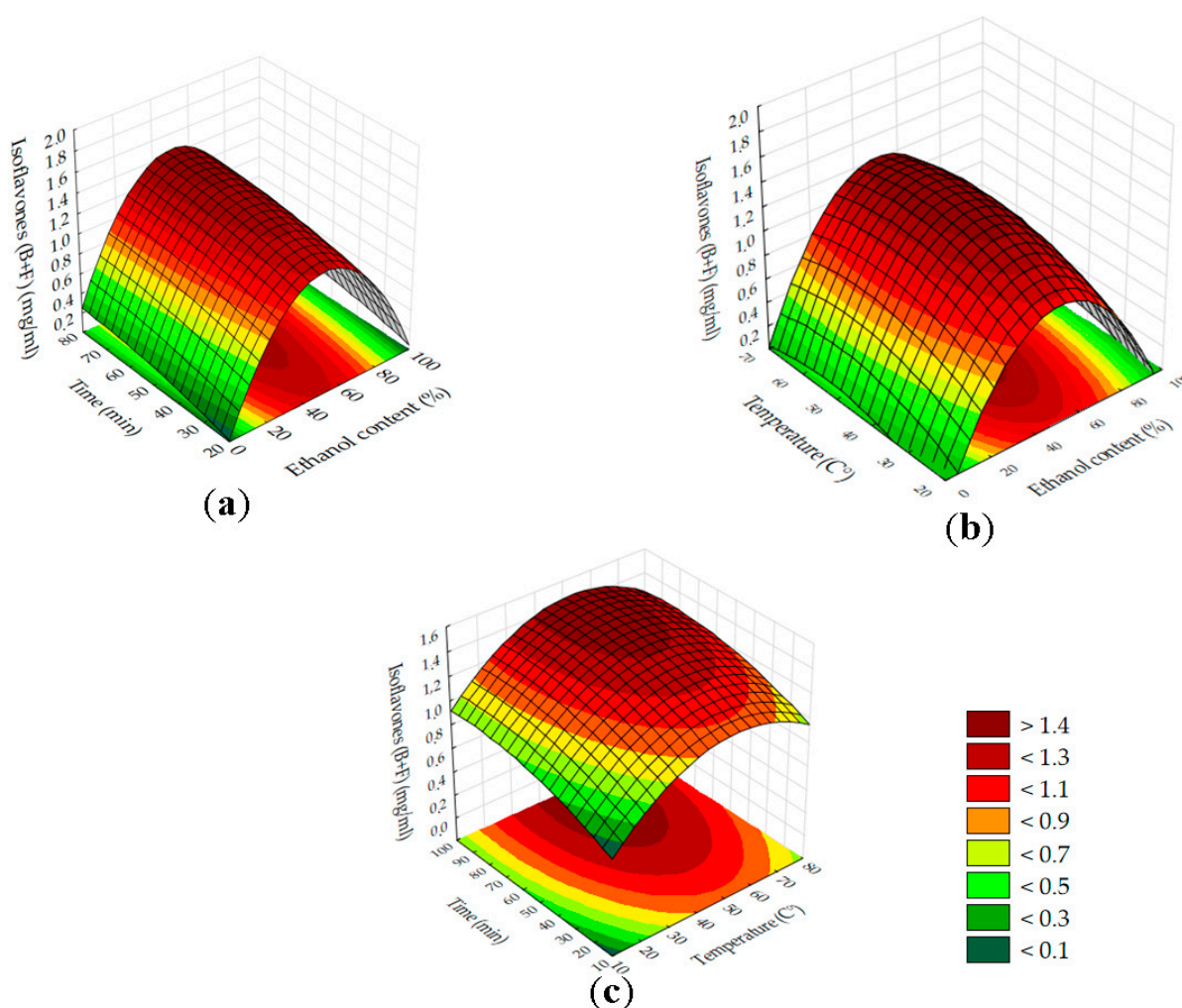


Figure 2. Response surface plots illustrating the effects of extraction parameters on total isoflavone content (sum of formononetin and biochanin A): (a) interaction between ethanol concentration and extraction temperature, (b) interaction between ethanol concentration and extraction time, and (c) interaction between extraction temperature and extraction time.

2.2. Cultivar Comparison

The optimized extraction procedure was applied to fourteen *Trifolium pratense* L. cultivars to evaluate inter-cultivar variability in isoflavone content. The analysis revealed pronounced differences in the concentrations of the two major phytoestrogens, formononetin and biochanin A (Table 1).

The content of formononetin ranged from 1.60 to 7.80 mg/g DW, while biochanin A levels varied between 0.69 and 6.44 mg/g DW (Table 1).

Among the fourteen *T. pratense* cultivars analyzed, those with the lowest isoflavone content, such as Milvus and Forelia, were primarily bred for forage performance and reduced estrogenic activity, traits that coincide with their comparatively limited capacity to accumulate formononetin and biochanin A. In contrast, cultivars with the highest isoflavone levels, including Hammon, Carbo and Larus, are characterized by agronomic features such as strong persistence, stress tolerance and extended regrowth, which may be associated with enhanced secondary metabolism and the biosynthesis of phenolic compounds.

Such variation reflects both genetic diversity among cultivars and potential environmental influences during plant growth. Cultivars exhibiting the highest yields can be considered potential candidates for future phytopharmaceutical or nutraceutical formulations that aim to provide standardized isoflavone-rich extracts. The cultivar Hammon,

characterized by the highest total isoflavone content under the applied extraction conditions, was selected for subsequent studies.

Table 1. Content of formononetin and biochanin A in *Trifolium pratense* L. cultivars determined by HPLC analysis (mg/g DW, mean $\pm$ SD, $n = 3$). Data were statistically evaluated using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$). Values with different letters in the same column differ significantly.

Cultivar	Formononetin (mg/g) DW $\pm$ SD	Biochanin A (mg/g) DW $\pm$ SD
Milena	2.875 $\pm$ 0.073 ^b	1.807 $\pm$ 0.067 ^c
Pasieka	3.204 $\pm$ 0.422 ^{bc}	2.806 $\pm$ 0.425 ^d
Pyza	3.133 $\pm$ 0.029 ^{bc}	1.733 $\pm$ 0.010 ^c
Milvus	1.650 $\pm$ 0.044 ^a	0.689 $\pm$ 0.036 ^a
Nemaro	5.940 $\pm$ 0.548 ^e	3.258 $\pm$ 0.011 ^e
Maro	4.059 $\pm$ 0.415 ^{cd}	1.746 $\pm$ 0.023 ^c
Larus	6.555 $\pm$ 0.059 ^{ef}	3.361 $\pm$ 0.139 ^e
Hammon	7.802 $\pm$ 0.428 ^f	6.441 $\pm$ 0.084 ^f
Vesna	3.045 $\pm$ 0.073 ^{bc}	1.136 $\pm$ 0.095 ^b
Fregata	2.649 $\pm$ 0.043 ^{ab}	1.812 $\pm$ 0.136 ^c
Carbo	6.906 $\pm$ 0.994 ^{ef}	3.443 $\pm$ 0.122 ^e
Forelia	1.600 $\pm$ 0.044 ^a	2.670 $\pm$ 0.032 ^d
Osimia	4.503 $\pm$ 0.055 ^d	1.738 $\pm$ 0.061 ^c
Elanus	2.718 $\pm$ 0.137 ^{ab}	1.567 $\pm$ 0.051 ^c

2.3. Principal Component Analysis (PCA)

Principal component analysis (PCA) was used to evaluate differences among *T. pratense* cultivars based on their formononetin and biochanin A content. The PCA biplot showed a clear separation of cultivars along the first principal component (PC1), which represented overall isoflavone abundance (Figure 3). Both formononetin and biochanin A loaded strongly and in the same direction on PC1, indicating a strong positive correlation and a common biosynthetic control of their accumulation.

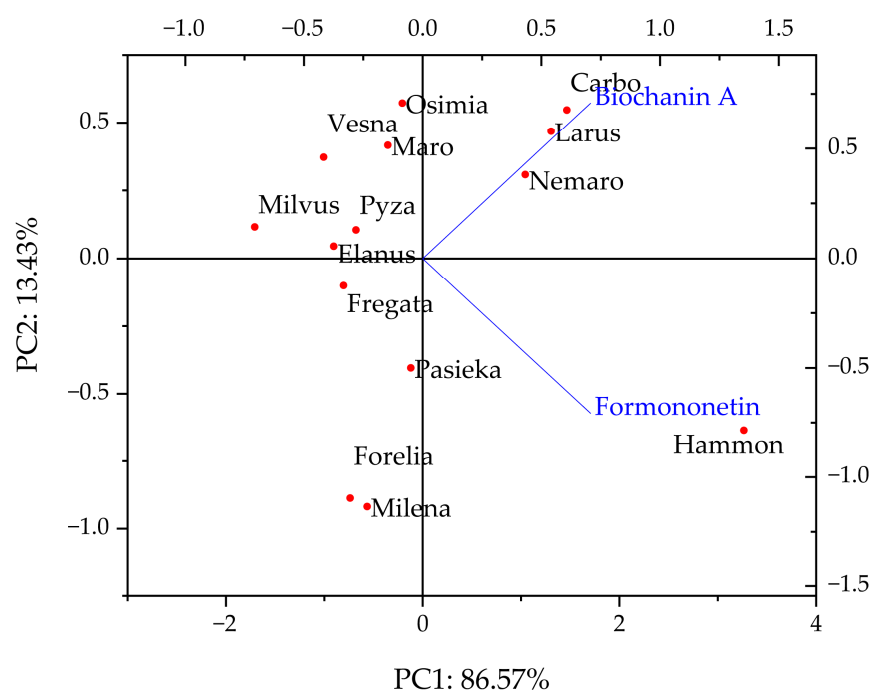


Figure 3. PCA biplot of *Trifolium pratense* L. cultivars based on formononetin and biochanin A content.

The second principal component (PC2) reflected differences in isoflavone composition, distinguishing cultivars with relatively higher biochanin A from those dominated by formononetin. Although PC2 explained a smaller proportion of the variance, it allowed discrimination between cultivars with similar total isoflavone levels but different compositional profiles.

2.4. Antioxidant and Enzyme Inhibition Activity of Red Clover Extract

The biological activity of the selected red clover extract was assessed using antioxidant and enzyme inhibition assays to characterize its functional profile. As shown in Table 2, the extract exhibited moderate antioxidant capacity in both radical scavenging and reducing power tests, indicating the presence of redox-active constituents. In enzyme inhibition assays, the extract showed a differentiated activity profile. Inhibition of elastase was comparable to that of the reference compound, whereas the inhibitory effect toward hyaluronidase was less pronounced. This pattern suggests a selective interaction of extract constituents with enzymes involved in extracellular matrix remodeling. The results confirm that the optimized red clover extract possesses measurable antioxidant and enzyme inhibitory activities, supporting its further use in formulation studies aimed at improving solubility and bioavailability.

Table 2. Antioxidant and enzyme inhibitory activities of the selected red clover extract expressed as IC (mg/mL, mean $\pm$ SD). Reference compounds were used as positive controls.

Assay	RCE IC _{50/0.5} (mg/mL)	Reference Compound IC _{50/0.5} (mg/mL)
DPPH	0.619 $\pm$ 0.028	0.075 $\pm$ 0.002 (Trolox)
FRAP	0.302 $\pm$ 0.004	0.013 $\pm$ 0.006 (Trolox)
Elastase inhibition	0.602 $\pm$ 0.041	0.723 $\pm$ 0.011 (Quercetin)
Hyaluronidase inhibition	22.443 $\pm$ 6.13	14.60 $\pm$ 2.776 (Quercetin)

2.5. Fibroblast Migration (Scratch Assay)

The regenerative potential of the optimized *T. pratense* extract was evaluated using an in vitro scratch assay. Treatment with the red clover extract (RC) significantly increased fibroblast migration compared with the untreated control. The percentage of wound closure rose from approximately 70–75% in control cultures to about 85–90% in extract-treated cells (** $p < 0.01$), indicating accelerated gap closure (Figure 4).

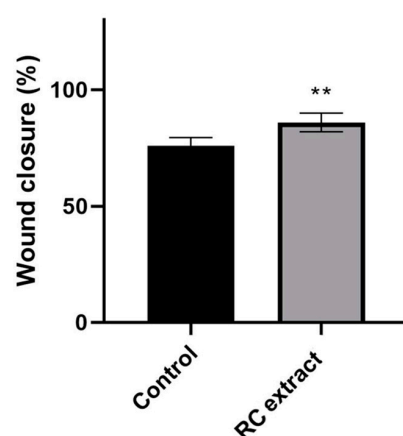


Figure 4. Wound-healing properties of RC 0 extract observed on human normal skin fibroblasts (Hs27 cells) after 24 h incubation. The extract was tested at the concentration of 10 μ g/mL. Results were statistically analyzed by a one-way ANOVA with a post hoc Tukey's test. Statistical significance was designated as: "***" when $p < 0.01$ (vs. control cells).

Images of fibroblasts (Figure 5) showing scratch closure in untreated control cultures and in cultures treated with the optimized *T. pratense* extract (RC). Images were taken immediately after scratching (0 h) and after the incubation period. Treatment with the red clover extract resulted in a visibly higher degree of gap closure compared with the control, indicating enhanced fibroblast migration.

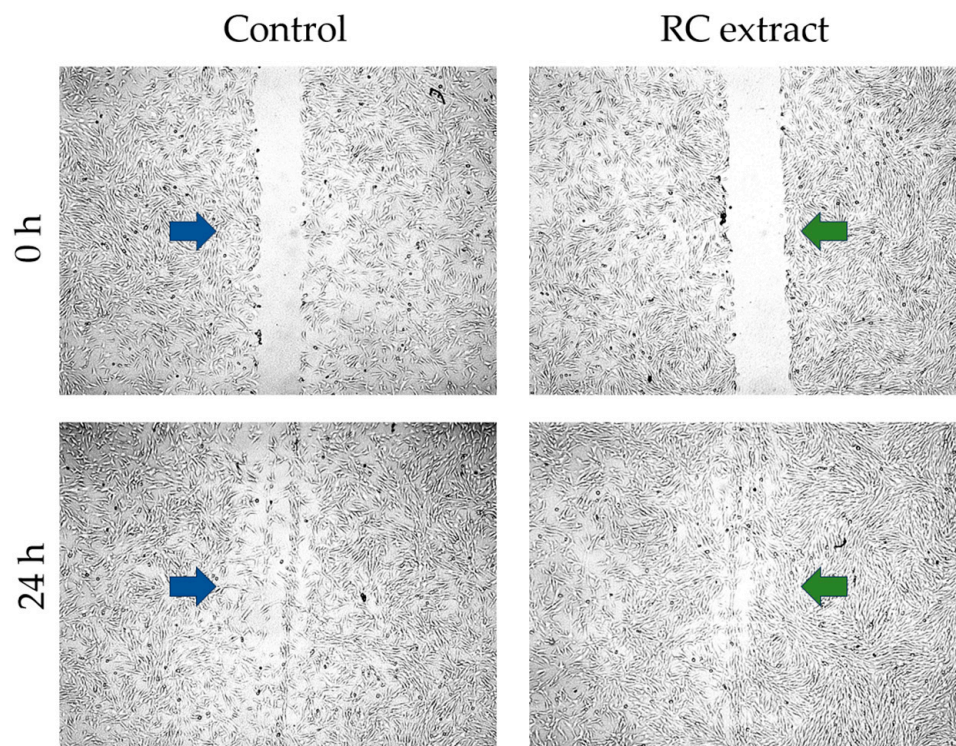


Figure 5. Images of effect of red clover extract on fibroblast migration in the scratch assay.

3. Materials and Methods

3.1. Experimental Material

Plant material for the study consisted of fourteen red clover (*Trifolium pratense* L.) cultivars. The aerial parts of the plants were collected at a comparable developmental stage, dried under controlled conditions (30° C, 15% RH), and stored until extraction. The plant material was obtained from experimental fields belonging to the Central Research Centre for Cultivar Testing in Słupia Wielka, near Poznań. The cultivars included diploid varieties (Milena, Pasięka, Pyza, Milvus, Nemaro, Maro) and tetraploid varieties (Larus, Hammon, Vesna, Fregata, Carbo, Forelia, Osmia, Elanus). Detailed agronomic characteristics of all cultivars are provided in Appendix A.

3.2. Optimization of Isoflavone Extraction

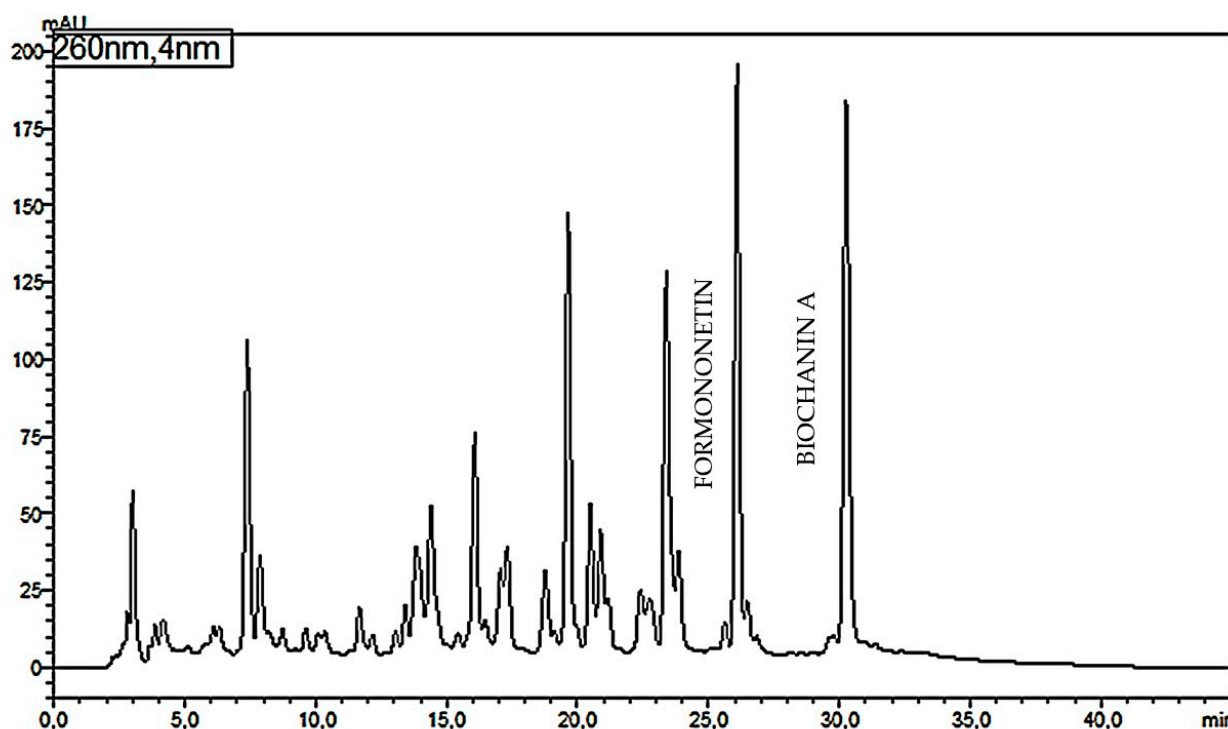
The extraction process of isoflavones from *Trifolium pratense* L. was optimized using a Box–Behnken experimental design (BBD) implemented in Statistica 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA). Three independent variables were selected based on preliminary screening: ethanol concentration (X_1 , 0–100%), extraction temperature (X_2 , 20–70 °C), and extraction time (X_3 , 15–90 min). The main isoflavones content, expressed as the sum of biochanin A and formononetin concentrations, was used as the response variable (Y).

Each extraction was performed with a constant solvent-to-solid ratio of 20 mL g^{−1} using ultrasonic bath (Ulsonix, Zielona Góra, Poland) under the conditions defined by the experimental design (Table 3).

Table 3. Box–Behnken experimental design for optimization of isoflavone extraction from *Trifolium pratense* L.

Sample Number	Ethanol Content (%)	Temperature (C°)	Time (min)
1	0	20	52.5
2	100	20	52.5
3	0	70	52.5
4	100	70	52.5
5	0	45	15
6	100	45	15
7	0	45	90
8	100	45	90
9	50	20	15
10	50	70	15
11	50	20	90
12	50	70	90
13	50	45	52.5
14	50	45	52.5
15	50	45	52.5

After extraction, the samples were centrifuged (10 min, 5000 rpm) and then filtered through a 0.45 µm PTFE membrane. Quantitative analysis of the main isoflavones, formononetin and biochanin A, was performed by HPLC (Figure 6) in accordance with the previously developed and validated method by Gościński et al. [18]. Briefly, the analysis was carried out using an HPLC–PDA system (HPLC–DAD, LC-2050C, Shimadzu Corp., Kyoto, Japan) equipped with a LiChrospher RP-18 column (250 mm × 4.6 mm, 5 µm). The mobile phase consisted of acetic acid/methanol/water (1:10:89, *v/v/v*; solvent A) and 1% acetic acid in methanol (solvent B), applied in gradient mode, with compound identification based on retention times and UV spectra of reference standards.

**Figure 6.** Representative HPLC–DAD chromatogram of *Trifolium pratense* L. extract obtained from the Hammon cultivar, recorded at 260 nm, showing the separation and identification of the main isoflavones: formononetin and biochanin A.

The experimental data were fitted to a second-order polynomial model, which describes the relationships between the variables and the response. The adequacy of the model was assessed based on the determination coefficient (R^2), the adjusted R^2 . Pareto charts and response surface plots were generated using Statistica 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA) to visualize the influence and interactions of the independent variables on the total isoflavone yield. After determining the optimal extraction parameters, the procedure was applied to fourteen cultivars of *T. pratense* to evaluate inter-cultivar variability in isoflavone content under the optimized conditions. Based on the obtained results, the cultivar exhibiting the highest total isoflavone content (sum of formononetin and biochanin A) was selected for further studies.

3.3. Preparation of the Lyophilized Extract

The extract from the *T. pratense* cultivar exhibiting the highest isoflavone content was again obtained under the optimized extraction conditions. The extract was subsequently lyophilized using a freeze-dryer (Telstar, Eden Prairie, MN, USA) at $-85\text{ }^{\circ}\text{C}$ and 0.2 MPa for 5 days to obtain a dry powder suitable for further formulation. The lyophilizate was stored in tightly sealed containers at $8\text{ }^{\circ}\text{C}$ until analysis.

3.4. In Vitro Biological Activities of Red Clover Extract

3.4.1. 2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging Assay

The antioxidant activity of red clover extract was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, as described in a previously published method with minor modifications [19]. The DPPH assay is based on the reduction in the stable DPPH radical by antioxidant compounds, resulting in a decrease in absorbance proportional to radical scavenging activity. Briefly, a methanolic solution of DPPH (0.1 mM) was prepared freshly. Aliquots of the extract at different concentrations were mixed with the DPPH solution and incubated in the dark at room temperature for 30 min. The decrease in absorbance was measured at 517 nm using a microplate reader (Multiskan GO 1510, Thermo Fisher Scientific, Vantaa, Finland). Methanol was used as a blank, and DPPH solution without the extract served as the control. The radical scavenging activity was expressed as a percentage inhibition. The DPPH radical scavenging activity was expressed as IC_{50} , defined as the extract concentration (mg/mL) required to inhibit 50% of the DPPH radical. Trolox was used as a positive control.

3.4.2. Ferric Reducing Antioxidant Power Assay

The ferric reducing antioxidant power (FRAP) of the extract was determined using the FRAP assay [19]. The FRAP assay measures the ability of antioxidants to reduce ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions, forming a colored complex with TPTZ that is quantified spectrophotometrically. The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution in a ratio of 10:1:1 (*v/v/v*). The extract was mixed with the FRAP reagent and incubated at $37\text{ }^{\circ}\text{C}$ for 30 min. Absorbance was measured at 593 nm. Antioxidant activity was expressed as the extract concentration (mg/mL) required to reach an absorbance value of 0.5 at 593 nm, calculated from the concentration–absorbance curve. Trolox was used as a positive control.

3.4.3. Hyaluronidase Inhibition Assay

The hyaluronidase inhibitory activity was determined using a method as previously described [20]. The hyaluronidase inhibition assay is based on the turbidimetric determination of undigested hyaluronic acid, allowing for the evaluation of enzyme inhibition by the tested extract. Briefly, the reaction mixtures consisted of 25 μL of hyaluronidase solution

(30 U/mL in acetate buffer, pH 7.0), 25 µL of acetate buffer (50 mM, pH 7.0, containing 77 mM NaCl and 1 mg/mL bovine serum albumin), 15 µL of acetate buffer (pH 4.5), and 10 µL of extract. The mixtures were incubated at 37 °C for 10 min, followed by the addition of a hyaluronic acid solution (0.3 mg/mL in acetate buffer, pH 4.5), and further incubation at 37 °C for 45 min. Undigested hyaluronic acid was precipitated by adding 200 µL of 2.5% CTAB in 2% NaOH. After standing at room temperature for 10 min, turbidity was measured as absorbance at 600 nm using a microplate reader (Multiskan GO 1510, Thermo Fisher Scientific, Vantaa, Finland). The inhibition of hyaluronidase activity was expressed as a percentage relative to the control. Quercetin was used as a positive control.

3.4.4. Elastase Inhibition Assay

The inhibitory activity of red clover extract against elastase was evaluated using a spectrophotometric method based on the hydrolysis of N-succinyl-Ala-Ala-Ala-p-nitroanilide (Suc-Ala₃-pNA) as the substrate [21]. Elastase inhibitory activity is determined by measuring the reduction in hydrolysis of a chromogenic substrate, reflecting the ability of the extract to inhibit elastase activity. The assay was performed in 100 mM Tris buffer (pH 8.0, 25 °C). The reaction mixture contained the extract solution, Tris buffer, and elastase solution (0.5 U/mL). After pre-incubation at 25 °C for 20 min, the substrate solution (4.4 mM Suc-Ala₃-pNA) was added, and the mixture was incubated at 37 °C for an additional 20 min. The release of p-nitroaniline was monitored by measuring absorbance at 410 nm using a microplate reader (Multiskan GO 1510, Thermo Fisher Scientific, Vantaa, Finland). Appropriate blanks and controls without the extract were included. Elastase inhibitory activity was expressed as a percentage inhibition relative to the control, Quercetin was used as a positive control.

3.5. Scratch Wound Healing Assay

The methodology applied was described in detail in a previous publication [22]. The scratch wound healing assay evaluates fibroblast migration by monitoring the closure of a cell-free gap created in a confluent cell monolayer over time. Hs27 cell line was purchased from the ATCC (Manassas, VA, USA) and maintained in DMEM–high glucose medium supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL). The cells were cultured in a humidified atmosphere at 5% CO₂ and 37 °C. On the day of experiment, cells were collected from monolayers with trypsin/EDTA and seeded onto a 6-well plate at a concentration of 1×10^5 cells/ml. When the confluency of cells reached ~90%, the vertical linear scratch was done with a sterile pipette tip. Cells were washed with PBS, and the fresh medium (for control group) or 10 µg/ml solutions of RC samples (directly dissolved in medium) were added to the respective wells. Images of the scratch were taken at 0 h and 24 h using Olympus CKX53 microscope coupled with XM10 digital camera (Olympus, Tokyo, Japan). Scratch area at the beginning of the experiment (0 h) was considered 100%. The open wound area was measured with the ImageJ software version 1.54g (NIH, Maryland, USA). Wound closure (in %) was calculated using the following formula:

$$\text{Closed wound area(\%)} = \frac{\text{open wound area at 0 h} - \text{open wound area at 24 h}}{\text{open wound area at 0 h}} \times 100\%$$

Results were expressed as the mean percentage of wound closure $\pm$ SD.

3.6. Statistical Analysis

Multivariate analysis of cultivar-dependent differences in isoflavone content was performed by principal component analysis (PCA) using OriginPro 2018 (OriginLab Corporation, Northampton, MA, USA). The PCA model was built using formononetin and biochanin

A concentrations as variables, which were standardized prior to analysis. Quantitative results were analyzed using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$) using Statistica software (version 13.3, TIBCO Software Inc., Palo Alto, CA, USA).

4. Discussion

These findings are consistent with our results, confirming that systematic optimization using a response surface or Box–Behnken design allows for the efficient recovery of biochanin A and formononetin under controlled ethanolic extraction conditions, resulting in extracts with high isoflavone content and strong biological potential. Although the specific extraction parameters differed slightly among studies, the optimal conditions obtained in our Box–Behnken design fell within a similar range, confirming that medium ethanol concentration and moderate temperature are key factors determining isoflavone recovery.

The extraction method is a critical determinant of the yield and composition of bioactive compounds obtained from herbal raw materials. Numerous studies have confirmed that extraction efficiency strongly depends on the choice of solvent, extraction time, temperature, and solid-to-liquid ratio. The study by Drużyńska et al. [23] demonstrated that extraction parameters, such as ethanol concentration, temperature, and time, significantly influence the polyphenol yield and antioxidant activity of *T. pratense* extracts. Ethanolic extractions ($\approx 60\%$ ethanol, $80\text{ }^{\circ}\text{C}$, 60 min) provided the highest total polyphenol content and radical-scavenging capacity. The work by Verhulst et al. [24] optimized polyphenol extraction from *Trifolium pratense* using response surface methodology and showed that 80% ethanol, 45 min, $40\text{ }^{\circ}\text{C}$ provided the highest yield (14.85 mg GAE/g DW). The authors identified biochanin A and formononetin as major constituents and highlighted that mild, energy-efficient extraction conditions can maximize recovery while supporting sustainable processing of red clover for nutraceutical and pharmaceutical use. Luo et al. [25] also optimized the ultrasound-assisted extraction of *Trifolium pratense* isoflavones (using 86% ethanol and a 1:29 solid–liquid ratio) and demonstrated that the obtained extract strongly inhibited LPS-induced inflammation in macrophages by suppressing the NF- κ B and p38 MAPK pathways. Consistent with these reports, the present study further demonstrates that rational optimization of extraction parameters using a Box–Behnken design enables efficient enrichment of formononetin and biochanin A.

Cultivar selection is also an important factor influencing the phytochemical profile of *Trifolium pratense*. Numerous studies have shown that the content of isoflavones, particularly formononetin and biochanin A, varies considerably among cultivars due to genetic differences, growth conditions, and harvest stage [18,26,27]. The isoflavone content obtained in this study was consistent with or higher than values reported for red clover cultivars described in the literature. Mikulić et al. [6] observed significant genotype-dependent variation in isoflavone profiles among 30 *Trifolium pratense* genotypes, with biochanin A as the dominant compound. They reported that the total isoflavone content among different *Trifolium pratense* genotypes ranged from 0.87 to 13.05 mg/g DW, with biochanin A and formononetin as dominant compounds, highlighting strong genotype-dependent variability in phytochemical composition. Similarly, Lemežienė et al. [28] reported that at the flowering stage of *Trifolium pratense* the concentrations of formononetin ranged from 2.61 to 4.40 mg/g DW and biochanin A from 1.79 to 3.32 mg/g DW, underscoring the considerable variability in isoflavone content among cultivars. The results obtained after extraction optimization in this study fall within or slightly above these ranges, confirming that both the selected plant material and the applied extraction conditions provided an isoflavone-rich extract comparable to high-yielding *T. pratense* varieties described previously.

PCA demonstrated that the variability among *T. pratense* cultivars is primarily driven by total isoflavone abundance, with formononetin and biochanin A contributing jointly to

the separation of high- and low-isoflavone chemotypes. The observed cultivar-dependent variation in formononetin and biochanin A content among *T. pratense* genotypes aligns with previous reports showing significant differences in isoflavone profiles between red clover cultivars and ploidy levels, with tetraploid genotypes often exhibiting higher isoflavone concentrations than diploids [6].

The antioxidant activity of the optimized red clover extract is consistent with the well-documented redox-modulating properties of isoflavones and accompanying phenolic constituents present in *Trifolium pratense* L. Antioxidant mechanisms play a key role in both systemic and local biological contexts by limiting oxidative stress, which is known to impair tissue regeneration and accelerate extracellular matrix degradation and what is important for longevity. Previous studies have demonstrated that red clover extract exhibit moderate but biologically relevant antioxidant activity, contributing to the neutralization of reactive oxygen species and protection of cellular components involved in tissue repair [18,23]. In addition to their antioxidant effects, enzyme inhibition represents a crucial functional aspect of plant extracts in wound-related applications. In Esmaeili et al. [29], the in vitro methanol extract of *Trifolium pratense* exhibited DPPH radical-scavenging activity with an EC₅₀ of approximately 205 µg/mL, indicating weaker antioxidant capacity compared with in vivo extracts. Notably, biochanin A has been shown to mitigate oxidative and inflammatory damage in skin by activating the Nrf2/HO-1 signaling pathway and enhancing endogenous antioxidant defenses, thereby supporting the role of isoflavone-rich extracts in protecting skin from oxidative stress and promoting tissue resilience [30].

Elastase is a key protease involved in the degradation of elastin and other extracellular matrix proteins, and its excessive activity has been associated with delayed wound healing and chronic inflammation [14]. The observed elastase inhibition by the red clover extract aligns with literature reports indicating that polyphenols and isoflavones can modulate elastase activity, thereby supporting the preservation of extracellular matrix integrity [31,32]. In contrast, the weaker inhibition of hyaluronidase is in agreement with previous findings, which show that this enzyme is less susceptible to inhibition by isoflavone-rich extracts, suggesting a more selective enzyme modulation profile [33]. The combination of antioxidant activity and enzyme inhibition indicates that the red clover extract may contribute to the regulation of oxidative and proteolytic processes relevant to tissue repair, tissue regeneration and skin aging. While earlier reports have described antioxidant or skin-related activities of red clover or isolated isoflavones, the present work directly links cultivar-specific isoflavone enrichment with functional biological outcomes evaluated in relevant skin cell models.

The scratch assay showed that fibroblast migration was higher in cultures treated with the red clover extract than in the untreated control, indicating that the extract directly supports a process that is essential for skin regeneration. Similar effects have been reported for red clover isoflavones and other phytoestrogens, which were shown to stimulate fibroblast motility, collagen production and wound closure through estrogen-dependent and oxidative stress-related signaling pathways. For example, formononetin has been reported to enhance fibroblast proliferation and migration in damaged skin models, while biochanin A has been linked to improved cellular resistance to oxidative stress, both of which are relevant to tissue repair [9]. Moskot et al. [34] have shown that isoflavone compounds can enhance fibroblast migration in scratch wound models—genistein accelerated fibroblast movement in a dose-dependent manner in an in vitro scratch assay. A hydrogel containing *Ocimum basilicum* and *Trifolium pratense* extracts was used, which at a concentration of 50 µg/mL led to complete reconstruction of the fibroblast monolayer, indicating the ability to improve dermal cell migration [35]. In the context of skin aging, reduced fibroblast activity and impaired matrix remodeling are major limiting factors for regeneration, therefore

the increased migration observed here suggests that the isoflavone-rich red clover extract may also have anti-aging relevance.

The present results highlight the strong interconnection between cultivar-dependent isoflavone composition, extraction optimization, and biological activity. The integration of chemometric optimization with phytochemical profiling enabled the selection of extracts enriched in formononetin and biochanin A, which translated into pronounced antioxidant capacity, inhibition of extracellular matrix-degrading enzymes, and enhanced fibroblast migration. This multi-level approach underscores the relevance of combining extraction design, chemical characterization, and biologically relevant in vitro models when evaluating plant-derived materials for skin-related applications, providing a coherent framework that bridges phytochemistry with functional outcomes. Nevertheless, comprehensive safety evaluation, including topical tolerance and long-term exposure studies, will be required before the development of advanced formulations can be considered.

5. Conclusions

Red clover (*Trifolium pratense* L.) is a rich source of isoflavones that have attracted increasing interest for their antioxidant, anti-aging, and skin-regenerative properties. This study shows that red clover cultivars differ strongly in their content of formononetin and biochanin A. The Box–Behnken design enabled the selection of extraction conditions that maximized the recovery of formononetin and biochanin A, providing a reproducible basis for the biological evaluation of the red clover extract. Among the tested cultivars, Hammon contained the highest amount of both compounds. Skin aging is closely associated with oxidative stress, degradation of extracellular matrix components, and impaired fibroblast migration. The extract obtained from this high-isoflavone cultivar showed antioxidant activity, inhibited elastase and hyaluronidase and increased fibroblast migration in the scratch test. These effects are important for maintaining skin structure and supporting tissue repair, both of which are reduced during skin aging. Therefore, higher isoflavone content may contribute to preserving skin functionality and resilience, which can be conceptually described as supporting skin longevity. Cultivars rich in isoflavones, such as Hammon, appear to be particularly suitable for use in skin-related applications, providing a solid basis for further formulation development and in-depth safety and efficacy studies.

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Abbreviations

The following abbreviations are used in this manuscript:

ANOVA	Analysis of variance
BBD	Box–Behnken design
CO ₂	carbon dioxide
CTAB	cetyltrimethylammonium bromide
DMEM	Dulbecco’s Modified Eagle Medium
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power
HPLC-DAD	High-performance liquid chromatography with diode-array detection
IC _{0.5}	Concentration required to reach 0.5 absorbance
IC ₅₀	Concentration required to inhibit 50% of activity
PBS	Phosphate-buffered saline
PCA	Principal component analysis
pH	Potential of hydrogen
PTFE	Polytetrafluoroethylene
RC	Red clover
TPTZ	2,4,6-tripyridyl-s-triazine

Appendix A

- Milena—a diploid variety developed by Małopolska Hodowla Roślin Sp. z o.o. (Kraków, Poland), entered into the National Register in 2008. It exhibits good winter hardiness and a low tendency to lodging. Regrowth rate in early spring and after mowing is assessed as average. The variety shows good resistance to clover canker and powdery mildew. It is intended for hay production under open-field cultivation. Performs well in pure stands as well as in grass mixtures. Pure sowing provides high yields of fresh and dry matter with very good quality parameters. Plants are characterized by a high total protein content in dry matter. Seed production performance is satisfactory, with seed yields ranging from 300 to 600 kg per hectare.
- Pasieka—a diploid, semi-erect, mid-early variety with good winter hardiness and low lodging susceptibility. It is distinguished by elongated flower heads with shortened corolla tubes, facilitating pollination by honeybees and ensuring good seed set. As a result, nectar is more accessible to honeybees compared to other red clover varieties. Pasieka can be cultivated both as forage and as a melliferous plant and is valued for its high honey yield (up to 100–200 kg of honey per hectare). Due to improved flower accessibility for bees, it is recommended for seed production in regions where natural clover pollinators (bumblebees) are scarce. It provides high yields of green and dry matter in regions with sufficient precipitation during the growing season and allows for two to three harvests per year. The variety shows rapid regrowth in spring and after successive cuts.
- Pyza—a diploid variety with a medium flowering time. Plants exhibit good winter hardiness and rapid regrowth during the early vegetation period, while regrowth after mowing is moderate. Pyza shows a low tendency to lodging and low susceptibility to clover canker. Under conditions favorable for powdery mildew development, disease severity is reduced. The variety is intended for mowing use and produces high yields of green and dry matter. Dry matter is characterized by a high crude protein content, resulting in a high protein yield.

- **Milvus**—a diploid red clover variety developed by DSV, characterized by very high yield potential, particularly in the second year of use. The average dry matter yield reaches approximately 17.0 t/ha. The variety shows high tolerance to unfavorable environmental conditions and forms a dense sward with strong competitiveness, making it well suited for mixtures with fast-growing grasses. Milvus does not perform well on peat soils, where its deep root system (up to 2.5 m) may be damaged during winter. It grows best on water-retentive but not waterlogged soils.
- **Nemaro**—a German diploid red clover variety developed by Saatzucht Steinach, with medium-early maturity, entered into variety lists in 1986. It is characterized by high dry matter yield, very good persistence, and moderate frost tolerance. Nemaro is well suited for green manure production and forage use. The variety shows good resistance to fungal diseases and rapid regrowth after cutting. Best yields are obtained in regions with a temperate climate and deep, humus-rich soils with moderate calcium content.
- **Maro**—A Polish, mid-early diploid red clover variety characterized by high yield potential, good winter hardiness, and rapid regrowth after winter and following the first cut. It is valued for its high protein content and is commonly used for green forage and silage. The variety is resistant to clover canker and powdery mildew and shows low lodging susceptibility during the growing season. Maro was bred by *Małopolska Hodowla Roślin* and received a gold medal at the Poznań International Fair in 2003.
- **Larus**—a tetraploid variety developed by DSV, characterized by high yield potential, particularly for dry matter, good plant health, and low to moderate susceptibility to winter damage. It is persistent and produces high yields in the second year of use. Larus shows moderate resistance to lodging and is characterized by early flowering.
- **Hammon**—a tetraploid, highly stable-yielding variety developed by DLF, with excellent agronomic traits. It demonstrates very high tolerance to thermal and soil moisture stress, high winter hardiness, and long persistence in the sward. The variety shows no pronounced weaknesses in evaluated agronomic traits and exhibits high competitiveness, making it suitable for grass mixtures and ensuring uniform regrowth after each cut.
- **Vesna**—a Czech, early-maturing tetraploid variety, reaching maturity 5–7 days earlier than traditional red clover varieties. Early maturity enables earlier forage harvesting. Vesna is characterized by exceptional tolerance to thermal and soil-related stress and rapid regrowth after cutting. High stress resistance and excellent disease tolerance ensure stable yields throughout the growing season, even under challenging climatic conditions. Compared to diploid varieties, Vesna has larger leaf blades and produces higher biomass yields. It performs well in early silage mixtures with grasses and alfalfa and is particularly suitable for mixtures with alfalfa due to its early maturity.
- **Fregata**—a very early tetraploid red clover variety characterized by high yield potential, rapid initial growth, strong frost resistance, long persistence in the sward, and resistance to anthracnose. It is widely used for the production of high-quality forage under intensive management. Fregata is the first tetraploid red clover variety selected for low formononetin content, an estrogenic compound. The concentration of this compound in plant biomass is approximately 50% lower than in comparable varieties.
- **Carbo**—a tetraploid variety combining the favorable agronomic traits of Larus and Corvus, developed through crossing these genotypes. It has been included in recommended forage crop lists since 2009 and outperforms standard diploid varieties in yield. Carbo exhibits high resistance to clover leaf spot, powdery mildew, and anthracnose, as well as high tolerance to low winter temperatures and drought, contributing to excellent persistence. Strong competitiveness ensures reduced weed infestation throughout the utilization period.

- Forelia—a Swiss tetraploid variety characterized by high disease resistance (particularly to anthracnose) and high yield potential. It is a forage variety recommended in national variety lists since 2016. Forelia was developed by crossing the low-formononetin variety Fregata with the diploid variety Formica, resulting in reduced formononetin content. It is considered a variety with an excellent resistance and productivity profile, particularly suitable for intensive forage production on arable land.
- Osmia—tetraploid variety distinguished by high persistence and yield stability. It shows rapid early growth after sowing and a very uniform distribution of yield throughout the growing season. High durability and resistance to fungal diseases ensure stable yields in the second year of use. Strong early growth improves soil coverage and competitiveness against weeds, reducing the need for chemical plant protection. Low susceptibility to powdery mildew, clover canker, and anthracnose results in above-average plant health.
- Elanus—a very early tetraploid variety with moderate early growth after sowing. It shows high resistance to clover canker, anthracnose, and powdery mildew. Elanus is characterized by moderate protein content, high yields, and high dry matter production in the second year of use. Winter hardiness is assessed as moderate, while tolerance to difficult site conditions and drought is very high.

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