

# Phenolic content and antioxidant activity of the Croatian red clover germplasm collection

Polifenoli i njihova antioksidacijska aktivnost u hrvatskoj kolekciji crvene djeteline

**Horvat, D., Tucak, M., Viljevac Vuletić, M., Čupić, T., Krizmanić, G., Kovačević Babić, M.**

**Poljoprivreda/Agriculture**

ISSN: 1848-8080 (Online)

ISSN: 1330-7142 (Print)

<https://doi.org/10.18047/poljo.26.2.1>



**Fakultet agrobiotehničkih znanosti Osijek, Poljoprivredni institut Osijek**

Faculty of Agrobiotechnical Sciences Osijek, Agricultural Institute Osijek

# PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF THE CROATIAN RED CLOVER GERMPLASM COLLECTION

Horvat, D., Tucak, M., Viljevac Vuletić, M., Čupić, T., Krizmanić, G., Kovačević Babić, M.

Original scientific paper  
Izvorni znanstveni članak

## SUMMARY

*The leaf samples of two varieties, twenty breeding populations, and seven local populations of the Croatian red clover were collected in the full flowering stage, with an aim to evaluate their phenolic content and antioxidant activity by using the spectrophotometric methods. A significant variation among the varieties/populations in the content of total phenolics and flavonoids was determined. The results demonstrated that the red clover is a rich source of phenolics and flavonoids, ranging from 38.67 to 59.96 mg GAE/g of DM and 21.19 to 51.48 mg CE/g of DM, respectively. The high levels in both phenolics and flavonoids were found in breeding populations/variety CD-8, Rc-11/7, Rc-11/8, Rc-11/15 and OS Osiris. The leaf extracts manifested a strong antioxidant activity towards diphenyl-1-picrylhydrazyl (DPPH), with a variation from 31.50 to 63.14%. A significant correlation was found between the antioxidant activity of the extracts and their total phenolic and flavonoid content ( $r = 0.737$  and  $0.839$ , respectively). Considering the obtained results, the crude extracts of red clover manifested a significant antioxidant potential and can be used as a natural source of biologically active components in the human and animal nutrition.*

**Keywords:** red clover (*Trifolium pratense*), total phenolics, total flavonoids, antioxidant activity, DPPH

## INTRODUCTION

Red clover (*Trifolium pratense* L.) is a short-lived perennial flowering herbaceous plant from the *Fabaceae* family, widely used fresh, dried, or conserved in the animal diet because of its richness in proteins, vitamins, and minerals (Phelan et al., 2015; Petrović et al., 2016). The red clover's importance in the agricultural production and its positive effects on the environmental conservation are well known (Vasilev et al., 2005), as well as its role in the plants' defense against the biotic and abiotic stresses (Saviranta et al., 2010). The natural populations of the *Trifolium* genus are a rich source of secondary metabolites, which find an application in the traditional

and clinical medicine as well as in pharmacy. Because of a strong biological activity of secondary metabolites, these components significantly improve the quality of both human and animal diet (Bustamante-Rangel et al., 2018; Kumar et al., 2018). The phenolic compounds are a large class of plants' secondary metabolites, demonstrating a diversity of chemical structures.

The plant phenolics include the phenolic acids, flavonoids, tannins, and the less common stilbenes and

Daniela Horvat, Ph. D., Marijana Tucak, Ph. D. (marijana.tucak@poljin.hr), Marija Viljevac Vuletić, Ph. D., Tihomir Čupić, Ph. D., Goran Krizmanić, Ph. D., M. Sc. Marija Kovačević Babić — Agricultural Institute Osijek, Južno predgrađe 17, 31000 Osijek, Croatia

lignans (Dai and Mumper, 2010). Various epidemiological studies have confirmed the anti-inflammatory, estrogenic, antimicrobial, antiallergenic and antitumor properties of the phenolic compounds in the *Trifolium* genus (Khorasani Esmaeili et al., 2015; Petrović et al., 2016). Isoflavones, the major phenolics present in the red clover, are categorized as phytoestrogens. Due to their structural similarity to  $\beta$ -estradiol, isoflavones may have a role in cancer prevention and the moderation of menopausal symptoms (Lipovac et al., 2012; Kowalska et al., 2013; Shakeri et al., 2015). The main isoflavones in the red clover leaves are formononetin and biochanin A, while daidzein and genistein, as well as their glycosidic conjugates and some other aglycones, are also present, but are findable in the minor quantities (Tsao et al., 2006; Tucak et al., 2018). Biological and health effects of isoflavones in animals can be both the positive and the negative ones. The animal red clover feed with a high daidzein and formononetin content promote the sheep and lambs growth (Mustonen et al., 2018), while an animal feed rich in isoflavones, especially in formononetin, can disrupt the reproductive cycle in sheep and cows and cause their infertility (Little et al., 2017; Mustonen et al., 2018). Although the red clover is mostly commercially used as a dietary supplement due to its estrogenic effect, its antioxidant properties have also been proven due to other phenolics, such as the flavonoids (quercetin, hyperoside, kaempferol, luteolin and myricetin), polyphenolic amides (clovamides) and phenolic acids (protocatechuic *p*-hydroxybenzoic, caffeic, *p*-coumaric, ferulic and salicylic) and their derivate (phasesic acid) (Tsao et al., 2006; Petrović et al., 2016; Tava et al., 2019). Since the phenolics are able to scavenge the free radicals and neutralize the reactive oxygen species (ROS) through the hydrogen atoms or electron donation or/and chelate metal cations, they possess a strong antioxidant potential useful for the plants and human body (Antonescu et al., 2019). It is widely accepted that the plant leaves have a higher amount of phenolic compounds than the flowers due to the abundance of pathway precursors in the leaves because of photosynthesis (Saboonchian et al., 2014). The presence of phenolic compounds and their distribution in the red clover vary, based on plant origin (a genetic factor), cultivation influences (soil type, fertilization), weather conditions, plant parts, growing stages, geographical location, time and harvesting season (Papadopoulos et al., 2006; Tsao et al., 2006; Du et al., 2012).

Our current work was aimed to evaluate the content of phenolics in the red clover germplasm collection in order to identify the most favorable materials as a potential source of phenolics, which will be used in our future breeding processes for the improvement of the new varieties' nutritional quality. The study presents the data pertaining to the total phenolic and flavonoid content in the *in vitro* methanol leaf extracts, as well as their antioxidant potential.

## MATERIAL AND METHODS

### Research site characteristics

The field experiment was carried out in Osijek at a lowland location in the eastern part of Croatia (altitude 90 m, lat. 45°32'N, long. 18°44'E). The site soil was a eutric cambisol clay loam texture with a neutral pH reaction. During the red clover growing season (March – October), the long-term (1971–2000) average air temperatures amounted to 15.4°C, while the total amount of monthly precipitation amounted to 467.8 mm. During this research's experimental phase (March – October 2014), the average air temperature and a sum of precipitations amounted to 16.5°C and 650.6 mm, respectively, which indicates that the weather conditions during the 2014 red clover growing season were much more humid and moderately warm. The detailed weather conditions during this research's implementation are described earlier in the paper by Tucak et al. (2019).

### Crop establishment and experimental design

Twenty-nine diploid red clover varieties/populations (two varieties, twenty breeding populations, seven local populations) were created within the framework of the forage crops breeding program at the Agricultural Institute Osijek and evaluated in this paper (Table 1). The experiment was sown on 21 March 2014 using a randomized complete block (RCB) design with four replications. The plots sizes amounted to 1.4 m x 6 m and consisted of the 6-meter drill rows with a 0.2 m row spacing. The seeding rate was 18 kg/ha for the OS Viva, which was used as a base for the adjustment of all other varieties/populations according to their seed size and germination percentage. The herbicides and fertilizers were not applied in the trials.

### Sample preparation

In the second red clover cut (in the second half of July 2014) in a full flowering stage, the average leaf samples (including healthy, young, fully developed leaves) from two replications of all varieties/populations were randomly collected from the plants in the middle of each plot. The collected leaf samples were stored in a refrigerator (-80°C), lyophilized, and grounded by an oscillating mill into a fine powder prior to the extraction procedure.

### Extraction of phenolic compounds

The extraction of phenolics in the red clover was performed using 25 mg of a lyophilized plant tissue by 5 ml of methanol in a 15 ml plastic cuvette. The mixture was homogenized by vortexing it for 2 mins., placed in a Sonorex Digitec ultrasonic bath (Model RK510H, Bandelin, Germany) at room temperature, and sonicated for 60 mins. The mixture was then centrifuged at 9000 rpm for 5 mins. at 4°C (Universal 320RHettich, Germany). Subsequent to the centrifugation, the super-

natant was removed, and the extraction procedure of the residues was repeated with additional 5 ml of methanol during 30 mins. of sonification. After centrifugation, the supernatants were pooled and used for further analyses. All extractions were performed in duplicate, and two aliquots of each extraction were used for the determination of bioactive compounds.

#### Determination of total phenolic content

The total phenolic content in the red clover extracts was determined by the Folin-Ciocalteu reagent, using the method described by Singleton and Rossi (1965) with some modifications. The reaction mixture consisted of 0.1 ml of the sample aliquot, 0.1 ml of Folin-Ciocalteu's phenol reagent (1:1), and 1.5 ml of the distilled water. It was well-vortexed, and after leaving it for 5 mins., the 0.3 ml of 20% Na<sub>2</sub>CO<sub>3</sub> solution was added. Then mixture was thoroughly shaken and left for incubation for 30 mins. at room temperature in the dark place. The total phenolics were quantitated by a conventional spectrophotometric method at 765 nm (Specord 200, Analytic Jena, Germany). The results were expressed as the milligrams of gallic acid equivalents per a gram of dry matter (mg GAE/g of DM), using a calibration curve constructed with the gallic acid (25–500 µg/ml).

#### Determination of total flavonoid content

The red clover's total flavonoid content was assayed using the modified aluminum chloride (AlCl<sub>3</sub>) colorimetric method (Xu and Chang, 2007). A one-ml aliquot of the red clover extract was mixed with the 4 ml of water and 0.3 mL of NaNO<sub>2</sub> solution (5%; w/v diluted with water). After 5 mins., 0.3 mL of AlCl<sub>3</sub> (10%; w/v diluted with water) were added and let to stand for 6 mins. prior to mixing them with 2 ml of 1M NaOH and 2.4 mL of water. The absorbance was determined at 510 nm against a reaction blank by MeOH instead of the sample. A total flavonoid content in the red clover extracts was expressed as the milligrams of catechin-hydrate equivalent (mg CE/g of DM), based on the catechin hydrate calibration curve (0.01–0.4 mg/ml).

#### Antioxidant activity assay

Antioxidant activity was determined using the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The antioxidant activity was measured using a modified version of the method explained by Brand-Williams et al. (1995). This involved the use of a DPPH free radical solution in the methanol. The sample extract aliquot (0.2 ml) was reacted with 1 ml of a 0.5 mmol/l DPPH methanolic solution and 2 ml of MeOH. The reaction mixture was shaken and incubated in the dark together with a control, consisted of 2.2 mL of MeOH and 1 ml of DPPH solution. The absorbance (A) of the reaction mixtures and control mixture were measured against a MeOH blank at 517 nm after 30 mins. A lower absorbance of the reaction mixture indicates a higher free radical scavenging activity. The antioxidant activity was calculated as an inhibition of free radical DPPH in a percentage (%), using the following equation:

$$\% \text{ of antioxidant activity} = (1 - (A \text{ of sample}_{t=30} / A \text{ of control}_{t=0})) \times 100$$

#### Statistical analysis

The results were subjected to an analysis of variance (ANOVA), and significant differences between the mean values of varieties/populations were compared by Fisher's protected LSD test at the 0.05 and 0.01 probability level. The statistical tool STATISTICA v8.0 (StatSoft, Inc., Tulsa, OK, USA) was utilized for the ANOVA test and Pearson's correlation coefficient (*r*) calculation.

## RESULTS AND DISCUSSION

Red clover is a reach source of phenolic compounds beneficial for human health. The analysis of variance demonstrated a statistically significant variation among the varieties/populations in the content of total phenolics and flavonoids. The results were figured as a mean value ± standard deviation. The total phenolic and flavonoid content were in the range from 38.67 in Rc-15/9 to 59.96 mg GAE/g of DM in CD-8 and from 21.19 in Rc-15/17 to 51.48 mg CE/g of DM in Rc-13/19, respectively (Table 1).

**Table 1. Phenolic and flavonoid content in the red clover leaves of a red clover**

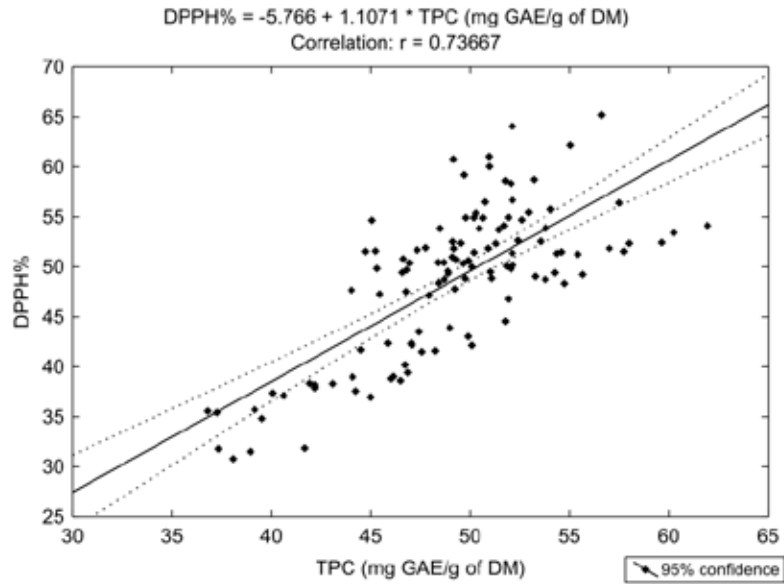
Tablica 1. Sadržaj ukupnih fenola i flavonoida u listovima crvene djeteline

No.	Variety/Population Sorta/Populacija	Status	Total phenolic content Ukupni fenoli (mg GAE/g of DM / mg/g u ST)	Total flavonoid content Ukupni flavonoidi (mg CE/g of DM / mg/g u ST)	DPPH inhibition DPPH inhibicija (%)
1	CD-12	LP	51.83 ± 2.79	43.01 ± 2.57	51.69 ± 2.56
2	CD-13	LP	54.92 ± 2.97	48.67 ± 2.26	53.23 ± 3.54
3	PopMp	LP	48.87 ± 2.53	49.51 ± 2.05	50.61 ± 0.87
4	Rc-11/7	BP	53.00 ± 1.19	51.46 ± 1.82	56.31 ± 3.34
5	CD-1	BP	46.21 ± 1.32	43.94 ± 2.39	42.50 ± 0.76
6	CD-9	BP	49.66 ± 0.42	49.64 ± 2.82	51.00 ± 1.02
7	Rc-11/8	BP	51.66 ± 0.93	49.80 ± 2.73	53.43 ± 1.43
8	OS Viva	V	49.89 ± 0.71	46.80 ± 2.80	49.71 ± 1.77
9	Rc-11/13	BP	52.56 ± 1.8	46.48 ± 1.73	52.00 ± 2.46
10	CD-10	LP	49.50 ± 3.05	48.70 ± 3.35	55.38 ± 0.80
11	Rc-11/15	BP	53.68 ± 2.59	49.75 ± 1.83	63.14 ± 1.87
12	Rc-13/6	BP	50.40 ± 1.19	47.24 ± 1.46	59.67 ± 0.6
13	CD-11	LP	47.13 ± 2.28	44.03 ± 1.96	47.51 ± 2.53
14	Rc-13/19	BP	50.13 ± 1.97	51.48 ± 2.42	52.41 ± 1.21
15	Rc-13/23	BP	46.58 ± 1.49	43.07 ± 1.42	50.56 ± 0.88
16	Rc-13/24	BP	50.73 ± 2.74	48.76 ± 1.48	51.95 ± 1.53
17	Rc-13/25	BP	47.89 ± 3.16	44.56 ± 1.75	48.32 ± 1.50
18	OS Osiris	V	51.20 ± 3.24	50.20 ± 3.17	51.13 ± 1.96
19	Rc-13/40	BP	51.10 ± 3.45	48.64 ± 1.39	52.29 ± 3.48
20	Rc-13/51	LP	47.94 ± 0.84	45.65 ± 1.40	48.38 ± 1.47
21	Rc-15/16	BP	45.56 ± 1.17	29.50 ± 1.35	38.25 ± 1.17
22	Rc-15/17	BP	39.00 ± 1.90	21.19 ± 0.55	31.50 ± 0.50
23	Rc-15/9	LP	38.67 ± 1.94	30.11 ± 1.39	36.38 ± 1.01
24	Rc-15/10	BP	43.61 ± 1.85	26.64 ± 1.31	38.48 ± 0.52
25	Rc-15/12	BP	47.72 ± 1.64	24.20 ± 0.94	40.62 ± 1.56
26	Rc-15/21	BP	54.84 ± 1.96	39.40 ± 0.75	50.57 ± 1.78
27	CD-6	BP	49.24 ± 2.04	31.07 ± 0.70	42.85 ± 1.29
28	CD-7	BP	40.92 ± 1.89	34.47 ± 1.63	36.81 ± 1.80
29	CD-8	BP	59.96 ± 1.63	49.68 ± 2.57	53.09 ± 0.85
Average			49.12 ± 4.94	42.68 ± 9.06	48.61 ± 7.43
LSD 0.05			2.97	2.81	2.54
LSD 0.01			3.93	3.72	3.36

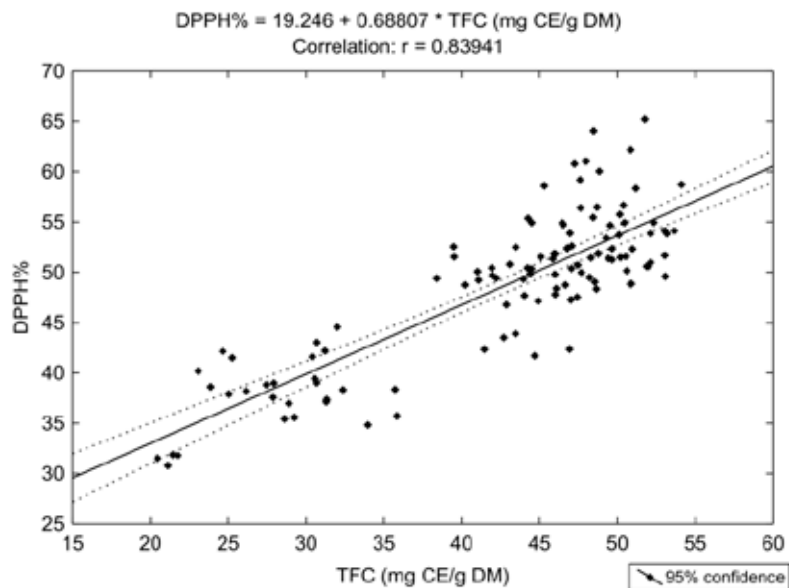
\*Variety-V, Breeding population – BP, Local population – LP / Sorta – V, Oplemenjivačka populacija – BP, Lokalna populacija – LP

The high levels of both the phenolics and flavonoids were found in the breeding populations/variety CD-8, Rc-11/7, Rc-11/8, Rc-11/15 and OS Osiris. A variation between the minimum and the maximum content of total phenolics and flavonoids in the red clover collection was amounted to 35.49% and 58.84%, respectively. Our results showed a great variation in total phenolics and flavonoids among the tested samples, which indicates an important effect of the genetic factor on their accumulation in the plant, which is in accordance with the previous researches (Little et al., 2017; Mustonen et al., 2018; Tucak et al., 2020). Nurguin et al. (2013) found 52.30 mg/g of total phenolic content in methanol extracts of *T. pratense* var. *pratense* and 48.60 mg/g in the extract of *T. pratense* var. *sativum*. Küçükboyacı et al. (2013) found 52.30 mg/g of total phenolic content in the methanol extracts of *T. pratense* var. *pratense* and 48.60 mg/g in the extract of *T. pratense* var. *sativum*. Khorasani Esmaeili et al. (2015) reported total phenolics in the *in vitro* grown red clover in the amount of 31.94 mg GAE/g. Petrović et al. (2016) studied 16 natural populations of three species of the genus *Trifolium* (*T. repens*, *T. alpestre* and *T. pannonicum*), collected from the central Balkan region, and reported total phenolics and flavonoids in the leaf extracts in the range of 29.0–120.1 mg GAE/g of DM and 31.2–351.6 mg RU/g of DM, respectively. Vlaisavljević et al. (2017) investigated the influence of growth phases on phenolics and their biological activity in one red clover variety and found the highest value of total phenolics at a 30-cm plant height phase (41.96 mg GAE/g) and the lowest one at 50 cm of height (30.99 mg GAE/g). Compared to our results, these authors reported the significantly lower total flavonoids (3.87–7.32 mg QE/g). Tava et al. (2019) evaluated the presence and concentration of phenolic compounds and their antioxidant activity in the leaves and flowers of a set of *Trifolium* species originating from the lowland and mountain sites and concluded that a lowland germplasm showed a higher concentration of total phenolics in the leaves than the mountain accessions (50.30 vs. 34.19 mg/g of DM), followed by a similar trend in flowers (114.16 vs. 57.44 mg/g of DM). Analyzing the phytochemical composition of *Trifolium pratense* L., Antonescu et al. (2019) reported total phenolics in the amount of 46.56 mg GAE/100 g of dry extracts and 3.44 mg QE/ml of total flavonoids. As we mentioned, the most abundant phenolic compounds in a red clover with the antioxidant properties are the flavonoids, polyphenolic amides (clovamides) and phenolic acids (protocatechuic acid, *p*-hydroxybenzoic, gentisic, caffeic, *p*-coumaric, ferulic, and salicylic) (Tsao et al., 2006). Currently, liquid chromatography (HPLC), alone or coupled with mass spectrometry (LC–MS), are the widely used techniques for individual phenolics identification and quantification. These methods are more expensive than an ordinary UV-VIS spectrophotometric method, but they are also more selective and precise. Tava et al. (2015) found

15.6 mg/g of clovamides, 13.2 mg/g of total flavonols, and 24.5 mg/g of total isoflavones, producing a total of 53.3 mg/g in the *Trifolium pratense* leaves. Tava et al. (2019) reported that a lowland germplasm of the *Trifolium* species demonstrated a higher concentration of phenolic acids (2.86 mg/g), clovamides (7.62 mg/g), isoflavones (24.19 mg/g) and other flavonoids (14.82 mg/g) in leaves, producing the total phenolics amounting to 50.30 mg/g, more than mountain accessions, having 34.19 mg/g of total phenolics. Antonescu et al. (2019) found ferulic and chlorogenic acid and rutin in the highest concentrations in a *T. pratense* extract, 2.38, 1.59, and 0.86 mg/g, respectively, while other polyphenols, cinnamic, caffeic and syringic acid and catechin were detected in the amounts lower than 0.40 mg/g. As we mentioned above, the phenolic content in red clover may be affected by the genetic factors, growing conditions and plant maturity, and plant parts, as well as by the sampling and extraction methodology (Tsao et al., 2006). The temperature and water regimes are two of the most significant abiotic factors influencing phenolics accumulation in the legumes (Sivesind and Seguin, 2005). Tucak et al. (2019) determined isoflavones in the red clover varieties/populations, and their content in the leaves averagely amounted to 8.43 mg/g of DM. These authors concluded that the weather conditions in 2014 favored the synthesis of isoflavones as the dominant group of red clover phenolics, and consequently it is to be expected that the accumulation of other phenolics was also favored. A DPPH radical scavenging assay is widely used to assay the antiradical activity of plant extracts *in vitro*. A DPPH scavenging activity was expressed as a percentage of DPPH discoloration, and a higher value means signifies a higher antioxidant potential. In this research, significant differences ( $p < 0.05$ ) between the antioxidant activities among the red clover varieties/populations were found (Table 1). The DPPH radical scavenging in the tested samples varied from the lowest 31.50 to the respectable 63.14%. Breeding populations Rc-11/15, CD-10, Rc-11/7, Rc-13/6 and the local population CD-10 manifested the most pronounced ability to scavenge the DPPH free radicals, in contrast to the Rc-15/17, Rc-15/9, CD-7, Rc-15/16 and Rc-15/10, which the lowest values (Table 1). Several other studies reported a significant antioxidant capacity of *Trifolium pratense* by using the different solvents or an antioxidant test, such as DPPH free radical scavenging test, thiobarbituric acid and trolox-equivalent antioxidant capacity assays (Tundis et al., 2015; Antonescu et al., 2019). DPPH radical scavenging activity demonstrated a significant ( $p < 0.05$ ) positive correlation with both the total phenolic and the flavonoid content ( $r = 0.737$  and  $0.839$ , respectively) (Figs. 1 and 2). Similar significant correlations between a radical scavenging activity and the total phenolics were found in the plant extracts from various natural sources (Sahreem et al., 2010; Khorasani Esmaeili et al., 2015; Antonescu et al., 2019).



**Figure 1. Pearson's correlation coefficients ( $r$ ) between total phenolic content (TPC) and DPPH scavenging activity**  
Slika 1. Pearsonov koeficijent korelacije ( $r$ ) između TPC i DPPH aktivnosti



**Figure 2. Pearson's correlation coefficients ( $r$ ) between total flavonoid content (TFC) and DPPH scavenging activity**  
Slika 2. Pearsonov koeficijent korelacije ( $r$ ) između TFC i DPPH aktivnosti

## CONCLUSION

The observed levels of phenolics and the antioxidant properties of leaves extract indicate that the red clover can be used as a natural source of biologically active components in the human and animal nutrition, as well as in the pharmaceutical industry. Several promising populations that could be further used for the improvement of nutritional quality in our breeding program have been identified in the researched collection.

## REFERENCES

1. Antonescu, I. A., Jurca, T., Gligor, F., Craciun, I., Fritea, L., Patay, E. B., Muresan, M., Udeanu, D. I., Ioniță, C. A., Antonescu, A., & Bodog, F. (2019). Comparative phytochemical and antioxidative characterization of *Trifolium pratense* L. and *Ocimum basilicum* L. *Farmacia*, 67(1), 146-153. <https://doi.org/10.31925/farmacia.2019.1.20>
2. Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant

- activity. *LWT - Food science and Technology*, 28(1), 25-30. [https://doi.org/10.1016/S0023-6438\(95\)80008-5](https://doi.org/10.1016/S0023-6438(95)80008-5)
3. Bustamante-Rangel, M., Delgado-Zamarreno, M. M., Pérez-Martin, L., Rodriguez-Gonzalo, E., & Dominguez-Alvarez, J. (2018). Analysis of isoflavones in foods. *Comprehensive Reviews in Food Science and Food Safety*, 17(2), 391-411. <https://doi.org/10.1111/1541-4337.12325>
  4. Dai, J., & Mumper, R. J. (2010). Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules*, 15(10), 7313-7352. <https://doi.org/10.3390/molecules15107313>
  5. Du, W. H., Yue, Y., & Tian, X. H. (2012). Variation of isoflavones production in red clover as related to environment, growth stage and year. *Acta Alimentaria*, 41(2), 211-220. <https://doi.org/10.1556/Alim.41.2012.2.8>
  6. Khorasani Esmaeili, A., Mat Taha, R., Mohajer, S., & Banisalam, B. (2015). Antioxidant activity and total phenolic and flavonoid content of various solvent extracts from *in vivo* and *in vitro* grown *Trifolium pratense* L. (red clover). *BioMed Research International*, 2015, Article Number 643285. <https://doi.org/10.1155/2015/643285>
  7. Kowalska, I., Jedrejek, D., Ciesla, L., Pecio, L., Masullo, M., Piacente, S., Oleszek, W., & Stochmal, A. (2013). Isolation, chemical and free radical scavenging characterization of phenolics from *Trifolium scabrum* L. aerial parts. *Journal of Agricultural and Food Chemistry*, 61(18), 4417-4423. <https://doi.org/10.1021/jf400153c>
  8. Kumar, M. A., Sravanthi Pammi, S. S., Sukanya, M. S., & Giri, A. (2018). Enhanced production of pharmaceutically important isoflavones from hairy root rhizocloned of *Trifolium pratense* L. *In Vitro Cellular & Developmental Biology - Plant*, 54(1), 94-103. <https://doi.org/10.1007/s11627-017-9873-y>
  9. Küçükboyacı, N., Kadioğlu, O., Adigüzel, N., Tamer, U., Güvenç, A., & Bani, B. (2013). Determination of isoflavone content by HPLC-UV method and *in vitro* antioxidant activity of red clover (*Trifolium pratense* L.). *Turkish Journal of Pharmaceutical Sciences*, 10(3), 463-472.
  10. Lipovac, M., Chedraui, P., Gruenhut, C., Gocan, A., Kurz, C., Neuber, B., & Imhof, M. (2012). The effect of red clover isoflavone supplementation over vasomotor and menopausal symptoms in postmenopausal women. *Gynecological Endocrinology*, 28(3), 203-207. <https://doi.org/10.3109/09513590.2011.593671>
  11. Little, V., Reed, K. F. M., & Smith, K. F. (2017). Variation for concentrations of various phytoestrogens and agronomic traits among a broad range of red clover (*Trifolium pratense*) varieties and accessions. *Agronomy-Basel* 7(2), Article Number 34. <https://doi.org/10.3390/agronomy7020034>
  12. Mustonen, E. A., Tuori, M., Kurki, P., Isolahti, M., Taponen, J., & Vanhatalo, A. (2018). Variety, time of harvest and conditions during growing season have impact on red clover isoflavone content. *Agricultural and Food Science*, 27(2), 102-109. <https://doi.org/10.23986/afsci.69781>
  13. Nurgiiin, K. U., Kadioğlu, O., Adigüzel, N., Tamer, U., Güvenç, A., & BANI, B. (2013). Determination of isoflavone content by HPLC-UV method and *in vitro* antioxidant activity of red clover (*Trifolium pratense* L.) *Turk J Pharm Sci*, 10(3), 463-472.
  14. Papadopoulos, Y. A., Tsao, R., McRae, K. B., Mellish, A. E., & Fillmore, S. A. E. (2006). Genetic variability of principal isoflavones in red clover. *Canadian Journal of Plant Science*, 86, Special Issue, 1345-1347. <https://doi.org/10.4141/P06-154>
  15. Petrović, M. P., Stanković, M. S., Anđelković, B. S., Babić, S. Ž., Zornić, V. G., Vasiljević, S. Lj., & Dajić-Stevanović, Z. P. (2016). Quality parameters and antioxidant activity of three clover species in relation to the livestock diet. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 44(1), 201-208. <https://doi.org/10.15835/nbha44110144>
  16. Phelan, P., Moloney, A. P., McGeough, E. J., Humphreys, J., Bertilsson, J., O'Riordan, E. G., & O'Kiely, P. (2015). Forage legumes for grazing and conserving in ruminant production systems. *Critical Reviews in Plant Sciences*, 34(4), 281-326. <https://doi.org/10.1080/07352689.2014.898455>
  17. Saboonchian, F., Jamei, R., & Sarghein, S. H. (2014). Phenolic and flavonoid content of *Elaeagnus angustifolia* L. (leaf and flower). *Avicenna Journal of Phytomedicine*, 4(4), 231-238.
  18. Sahreen, S., Khan, M. R & Khan, R. A. (2010). Evaluation of antioxidant activities of various solvent extracts of *Carissa opaca* fruits, *Food Chemistry*, 122(4), 1205–1211. <https://doi.org/10.1016/j.foodchem.2010.03.120>
  19. Saviranta, N. M. M., Julkunen-Tiitto, R., Oksanen, E., & Karjalainen, R. O. (2010). Leaf phenolic compounds in red clover (*Trifolium pratense* L.) induced by exposure to moderately elevated ozone. *Environmental Pollution*, 158(2), 440-446. <https://doi.org/10.1016/j.envpol.2009.08.029>
  20. Shakeri, F., Taavoni, S., Goushegir, A., & Haghani, H. (2015). Effectiveness of red clover in alleviating menopausal symptoms: a 12-week randomized, controlled trial. *Climacteric*, 18(4), 568-573. <https://doi.org/10.3109/13697137.2014.999660>
  21. Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, 16(3), 144-158.
  22. Sivesind, E., & Seguin, P. (2005). Effects of the environment, cultivar, maturity, and preservation method on red clover isoflavone concentration. *Journal of Agricultural and Food Chemistry*, 53(16), 6397-6402. <https://doi.org/10.1021/jf0507487>
  23. Tava, A., Pecio, Ł., Lo Scalzo, R., Stochmal, A., & Pecetti, L. (2019). Phenolic content and antioxidant activity in *Trifolium* germplasm from different environments. *Molecules*, 24(2), Article Number 298. <https://doi.org/10.3390/molecules24020298>
  24. Tava, A., Pecio, Ł., Stochmal, A., & Pecetti, L. (2015). Clovamide and flavonoids from leaves of *Trifolium pratense* and *T. pratense* subsp. *nivale* grown in Italy. *Natural Product Communications*, 10, 933-936. <https://doi.org/10.1177/1934578X1501000635>
  25. Tsao, R., Papadopoulos, Y., Yang, R., Young, J. C., & McRae, K. (2006). Isoflavone profiles of red clovers and



- their distribution in different parts harvested at different growing stages. *Journal of Agricultural and Food Chemistry*, 54(16), 5797-5805. <https://doi.org/10.1021/jf0614589>
26. Tucak, M., Čupić, T., Horvat, D., Popović, S., Krizmanić, G., & Ravlić, M. (2020). Variation of phytoestrogen content and major agronomic traits in alfalfa (*Medicago sativa* L.) populations. *Agronomy*, 10(1), Article Number 87. <https://doi.org/10.3390/agronomy10010087>
  27. Tucak, M., Horvat, D., Cupic, T., Krizmanic, G., Tomas, V., Ravlic, M., & Popovic, S. (2018). Forage legumes as sources of bioactive phytoestrogens for use in pharmaceuticals: A review. *Current Pharmaceutical Biotechnology*, 19(7), 537-544. <https://doi.org/10.2174/1389201019666180730165917>
  28. Tucak, M., Popović, S., Horvat, D., Čupić, T., Krizmanić, G., Viljevac Vuletić, M., & Ravlić, M. (2019). The characterization of isoflavone content in the Croatian red clover collection. *Poljoprivreda*, 25(1), 1-11. <https://doi.org/10.18047/poljo.25.1.1>
  29. Tundis, R., Marrelli, M., Conforti, F., Tenuta, M. C., Bonesi, M., Menichini, F., & Loizzo, M. (2015). *Trifolium pratense* and *T. repens* (Leguminosae): Edible flower extracts as functional ingredients. *Foods*, 4(3), 338-348. <https://doi.org/10.3390/foods4030338>
  30. Vasilev, E., Vasileva, V., Mihovsky, Tz., & Goranova, G. (2005). Assessment of legume based mixture swards constrained by the environmental conditions in Central North Bulgaria - COST Action 852. In: Wachendorf, M., Helgadottir, A., & Parente, G. (eds.). Sward dynamics, N-flows and forage utilisation in legume-based systems. Proceedings of the 2<sup>nd</sup> COST 852 workshop, 10-12 November 2005, Grado, Italy.
  31. Vlaisavljević, S., Kaurinović, B., Popović, M., & Vasiljević, S. (2017). Profile of phenolic compounds in *Trifolium pratense* L. extracts at different growth stages and their biological activities. *International journal of food properties*, 20(12), 3090-3101. <https://doi.org/10.1080/10942912.2016.1273235>
  32. Xu, B. J., & Chang, S. K. C. (2007). A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. *Journal of Food Science*, 72(2), S159-S166. <https://doi.org/10.1111/j.1750-3841.2006.00260.x>

## POLIFENOLI I NJIHOVA ANTIOKSIDACIJSKA AKTIVNOST U HRVATSKOJ KOLEKCIJI CRVENE DJETELINE

### SAŽETAK

**Listovi crvene djeteline uzorkovani su u fazi pune cvatnje s dvije sorte, dvadeset oplemenjivačkih populacija i sedam lokalnih populacija crvene djeteline u Hrvatskoj s ciljem određivanja ukupnih fenola i flavonoida i njihove antioksidacijske aktivnosti spektrofotometrijskim metodama. Pronađena je statistički značajna varijabilnost među sortama/populacijama u ukupnome sadržaju fenola i flavonoida. Rezultati su pokazali da je crvena djetelina bogat izvor fenola s rasponom koncentracija od 38,67 do 59,96 mg GAE/g u ST te flavonoida od 21,19 do 51,48 mg CE/g u ST. Visoka koncentracija fenola i flavonoida izmjerena je u oplemenjivačkim populacijama/sortama CD-8, Rc-11/7, Rc-11/8, Rc-11/15 i OS Osiris. Biljni ekstrakt dobiven iz lista crvene djeteline imao je snažnu antioksidacijsku aktivnost prema difenil-1-pikrilhidrazilu (DPPH) s varijabilnošću od 31,50 do 63,14%, koja statistički značajno korelira sa sadržajem ukupnih fenola ( $r = 0,737$ ) i flavonoida ( $r = 0,839$ ). Dobiveni rezultati pokazuju da sirovi ekstrakti crvene djeteline imaju značajan antioksidacijski potencijal, pa se stoga mogu koristiti kao prirodan izvor biološki aktivnih tvari u prehrani ljudi i ishrani životinja.**

**Ključne riječi:** crvena djetelina (*Trifolium pratense*), ukupni fenoli, ukupni flavonoidi, antioksidativna aktivnost, DPPH

(Received on June 17, 2020; accepted on September 30, 2020 – Primljeno 17. lipnja 2020.; prihvaćeno 30. rujna 2020.)