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Composition of Proteins and Phenolics in the Leaves of Different Mulberry Species (*Morus alba* L., *M. alba* × *rubra, M. australis* Poir., *M. nigra* L.)

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ABSTRACT

The leaves of the mulberry (*Morus* sp.) have a variety of medicinal, culinary, industrial and agricultural applications. In our study, we compared the protein and phenolic contents of different mulberry species (*Morus alba* L., *M. alba* × *rubra, M. australis* Poir., *M. nigra* L.) from the mulberry germplasm collection to determine species-specific differences. The possibility of using mulberries as animal feed and for pharmacological purposes was reviewed. Total phenols of all genotypes were analysed using the Folin-Ciocalteu method, while total protein content was determined using the Lowry's method. The individual phenols were analysed by high-performance liquid chromatography with UV/VIS detection. The total protein content ranged from 162.03 mg BSA/g DW (*M. australis*) to 239.42 mg BSA/g DW (*M. alba*). Significantly higher contents of total proteins were determined in the leaves of *M. alba*. The highest mean concentrations of total phenols (21.51 mg GAE /g DW), chlorogenic acid (18.05 mg/g DW), 4-caffeoylquinic acid (4.36 mg/g DW), 5-*p*-coumaroylquinic acid (2.01 mg/g DW), quercetin glycoside (0.74 mg/g DW) and kaempferol acetyl hexoside (4.42 mg/g DW) were determined in *M. alba* × *rubra* and *M. nigra*. In contrast, white mulberry (*M. alba*) genotypes contained on average the most rutin (2.63 mg/g DW) and quercetin-malonyl-hexoside (1.59 mg/g DW). It can be concluded that the leaves of the white mulberry are best suited as animal feed due to their high protein content, while the black mulberry and the hybrid *M. alba* × *rubra* have pharmacological potential due to their high phenolic content.

Key words: *Morus* sp., leaves, proteins, phenolic acids, flavonoids

INTRODUCTION

Mulberry bioactivity has been widely described, but mostly refers to the fruits, while studies on the leaves are scarce (Polumackanycz et al., 2021). In this study, the leaves of three mulberry species (*Morus alba* L., *M. australis* Poir., *M. nigra* L.) and a hybrid *M. alba* × *rubra*, were compared in terms of their protein and phenolic composition. Mulberry leaves are considered to be an excellent food source with high content of proteins, carbohydrates, vitamins, trace elements, and dietary fibre. Some reports indicate that mulberry leaves are high in bioactive compounds, including phenolic acids, flavonoids γ-aminobutyric acid (GABA) and alkaloids (Butt et al., 2008; Devi et al., 2013). These compounds have been confirmed to be involved in antioxidation, lowering blood glucose levels, lowering blood pressure, preventing atherosclerosis, and inhibiting inflammation (Park et al., 2013; Jeszka-Skowron et al., 2014; Yu et al., 2018).

Mulberry is a woody perennial plant in the family Moraceae and genus *Morus*, native to China. Mulberry is a deciduous plant that grows quickly, has a short propagation period and grows in a wide range of environmental conditions, i.e. in the tropics, subtropics and temperate zones. The plants are preferred for their foliage yield, as the leaves are the only food for the silkworm *Bombyx mori* L. to produce silk, and are used for various activities due to their strong adaptability to the environment. About 12-16 species of the genus *Morus* are found in subtropical, warm and temperate regions of Asia, Africa and North America (Ramesh et al., 2014). The nutritional composition of mulberry leaves varies depending on soil and other environmental factors of the site as well as genotypic characteristics (Kumar et al., 2010).

Mulberry leaves have high protein content (18-25 g/100 g DW (dry weight)). In addition to proteins and their unique composition of amino acids dominated by aspartic acid, glutamic acid, phenylalanine, lysine, and arginine, the

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major notable constituents of the leaves are carbohydrates, calcium, iron, ascorbic acid, carotenoids, thiamine, folic acid, and phenols (Al-Kirshi et al., 2009; Thaipitakwong et al., 2018; Urbanek et al., 2019). Yu et al. (2018) determined a crude protein content of 27.63-37.36 g/100 g DW in white mulberry leaves, which was generally higher than that of common leafy vegetables listed in Chinese food composition tables, such as Chinese cabbage (27.78 g/100 g DW), bok choy (27.27 g/100 g DW), cabbage (22.06 g/100 g DW), spinach (29.54 g/100 g DW), shepherd's purse (30.85 g/100 g DW), purple amaranth (25.00 g/100 g DW) and water spinach (30.96 g/100 g DW). Sánchez-Salcedo et al. (2017) compared protein content between white and black mulberry leaves and concluded that white (19.4% DW) mulberries generally had higher protein content than black (13.4% DW) mulberries. This result is in agreement with the results previously observed by Gueven (2012) and Srivastava et al. (2003). The proteins of mulberry leaves are of high quality and are used in India, Pakistan and Bangladesh along with wheat flour to make parathas. The addition of mulberry powder also improves the storage stability of wheat for up to two months (Srivastava et al., 2003).

Phenolic compounds are aromatic alcohols with at least one hydroxyl group on the benzene ring and belong to the secondary metabolites, i.e., metabolites of plants, fungi, or lichens formed from primary metabolites, with different functions, e.g., as defence or signalling compounds, inhibitors, or toxins, and are usually not directly involved in growth and development processes (Botanical Terminology Dictionary, 2011). Iqbal et al. (2012) determined the chemical composition of white (*M. alba*), red (*M. rubra*), and black (*M. nigra*) mulberry leaves grown in the hilly areas of Azad Kashmir and Chitral, Pakistan. The content of total phenols ranged from 16.21 ± 1.34 mg GAE/g DW for *M. alba* to 24.37 ± 2.14 mg GAE/g DW for *M. nigra*. Significant differences ($p = 0.004$) were found in total phenolic content among leaves of the three mulberry cultivars. These results are much higher than those reported for oil palm leaves (Han & May, 2010) and Iranian medicinal plants (Bouayed et al., 2007).

Sánchez-Salcedo et al. (2015) concluded that among the clones studied, *M. alba* had the lowest phenolic content (12.81–15.50 mg GAE /g DW), while *M. nigra* clones had slightly higher values (from 13.48 to 16.13 mg GAE /g DW). These results are in agreement with a previous report on mulberry leaves from Pakistan (Iqbal et al., 2012), which also found higher values for total phenolic content of *M. nigra* than for *M. alba*. Radojković et al. (2012) reported higher values of total phenolics in mulberry leaves, from 66.76 (*M. alba*) to 115.23 (*M. nigra*) mg GAE /g DW. However, Thabti et al. (2012) recorded lower values of total phenols in mulberry leaves (from 3.45 to 6.31 mg GAE /g DW). One explanation for the large variability of phenolic content in mulberry leaves could be found in the different extraction procedures and analytical methods used. In addition, it has been suggested that phenolic substances in leaves vary according to conditions such as drought, temperature fluctuations, pollution, UV light and pathogen attack (Pinhero et al., 1997). A certain diversity of phenolic compounds has been found in mulberry leaves, which are said to have multiple biological effects, including antioxidant activity. The major phenolic acids were identified as chlorogenic acid, followed by other caffeoylquinic acid derivatives and *p*-coumaric acid derivatives. The flavonol fraction contained rutin, quercetin as well as kaempferol glycosides (Flaczyk et al., 2013; Choi et al., 2015; Urbanek et al., 2022).

The concentrations of total protein, total phenols and individual phenolic acids (chlorogenic acid, *p*-coumaric acid hexoside, 4-caffeoylquinic acid, 5-caffeoylquinic acid, 5-coumaroylquinic acid, *p*-coumaroylquinic acid) and individual flavonoids (rutin, quercetin malonyl hexoside, kaempferol acetyl hexoside, quercetin-dirhamnosyl-glycoside, quercetin-rhamnosyl-hexoside, quercetin-glucoside, quercetin-acetyl-rhamnosyl-hexoside, quercetin-acetyl-hexoside, kaempferol-dirhamnosyl-hexoside, kaempferol-rhamnosyl-hexoside, kaempferol-acetyl-rhamnosyl-hexoside) in mulberry leaves of three species (*M. alba*, *M. nigra*, *M. australis*) and the hybrid *M. alba* × *rubra* grown in the mulberry collection plantation at Vila Pohorski dvor (φ: 46° 50' 88.33''; γ: 15° 62' 20.73'') were studied. We hypothesised that the white mulberry contains higher protein concentrations and lower phenolic concentrations than the black mulberry. We also hypothesised that there are statistically significant differences in the concentrations of total proteins, total phenols and individual phenols between the species.

MATERIALS AND METHODS

Plant material

In our study, we included leaves of three different mulberry species (*M. alba*, *M. australis*, *M. nigra*) and one hybrid *M. alba* × *rubra*. Altogether we analyzed 28 different genotypes, 24 white mulberry genotypes, which were 5-7 years old trees propagated from old local mulberry varieties from different eco-geographic regions of Slovenia, Hungary and Italy and three of each other species (Table 1). For biochemical analyses, five to seven fully developed sun-exposed leaves (5th to 7th leaf below the apex) from a one-year branch were collected randomly per each sampled tree (3–5 trees per selected genotype) and used as one sample. The trees were sampled 23rd of June 2021 and grown in the mulberry collection plantation under Vila Pohorski dvor (φ: 46° 50' 88.33''; γ: 15° 62' 20.73''). Samples were immediately stored on dry ice, later transferred to a freezer at −80 °C, and subsequently freeze-dried and grounded. The prepared

samples were then stored in airtight vials at −20 °C before biochemical analyses. The concentrations of the analyzed nutrients are calculated on a dry weight (DW) basis.

Determination of total protein content

Total proteins were quantified using the Lowry's method (Lowry et al., 1951). Previously, the proteins were precipitated

Table 1: List of mulberry genotypes analysed in our study

Group name/ Identification No.	Species	Region	Detailed regionalization	Detailed location	Latitude	Longitude
Slovenian mulberry genotypes						
SE 5	*M. alba*	South-Eastern region	Bela krajina	Castle Dragatus	45.521	15.159
SE 8	*M. alba*	South-Eastern region	Bela krajina	Marindol	45.506	15.329
SM 101.1	*M. alba*	Submediterranean region	Kras	Castle Školj	45.661	14.007
SM 137	*M. alba*	Submediterranean region	Koprska brda	Monastery Krog Sečovlje	45.466	13.647
SM 214	*M. alba*	Submediterranean region	Goriška brda	Medana - cemetery	45.982	13.520
SP 12	*M. alba*	Subpannonean region	Slovenske gorice	Drvanska 6, Benedikt	46.614	15.887
SP 256	*M. alba*	Subpannonean region	Murska ravan	Dobrovnik	46.655	16.354
SP 303	*M. alba*	Subpannonean region	Dravska ravan	Prepolje 42	46.446	15.765
SP 304	*M. alba*	Subpannonean region	Dravska ravan	Župečja vas 56	46.376	15.767
Hungarian mulberry genotypes						
BE 1264.2	*M. alba*	Southern Great Plain	Bekes	Bekescsaba	46.684	21.088
GMS 2286	*M. alba*	Western Transdanubia	Gyor-Moson-Sopron	Bosarkany	47.714	17.223
GMS 2329	*M. alba*	Western Transdanubia	Gyor-Moson-Sopron	Lebeny	47.738	17.387
GMS 2532	*M. alba*	Western Transdanubia	Gyor-Moson-Sopron	Nagycenk	47.610	16.672
GMS 2533	*M. alba*	Western Transdanubia	Gyor-Moson-Sopron	Nagycenk	47.610	16.672
SO 1013	*M. alba*	Southern Transdanubia	Somogy	Fö utca, Nagybajom	46.396	17.500
SO 1035	*M. alba*	Southern Transdanubia	Somogy	Mernye, Fö utca	46.517	17.819
TO 1131	*M. alba*	Southern Transdanubia	Tolna	Sárszentlőrinc	46.665	18.599
VE 2706	*M. alba*	Central Transdanubia	Veszprem	Tihany	46.922	17.825
ZA 1060	*M. alba*	Western Transdanubia	Vas	Bajánsenye	46.807	16.390
ZA 1070	*M. alba*	Western Transdanubia	Vas	Csákánydoroszló	46.968	16.470
ZA 2084	*M. alba*	Western Transdanubia	Zala	road Kiliman - Gelse	46.628	16.995
Reference sericultural varieties		origin	obtained			
'Florio'	*M. alba*	Italy	mulberry gene bank CREA Padua, Italy			
'Giazzola'	*M. alba*	Italy	mulberry gene bank CREA Padua, Italy			
'Kokusou'	*M. alba*	Japan	mulberry gene bank CREA Padua, Italy			
'Morettiana'	*M. alba*	Italy	mulberry gene bank CREA Padua, Italy			
Fruit varieties						
M. alba × rubra	*M. alba × rubra*	hybrid	Hubmann, Austria			
M. australis	*M. australis*	unknown	Hubmann, Austria			
M. nigra	*M. nigra*	East Styria, Austria	Pucher, Austria		46.971	15.703

with trichloroacetic acid (TCA) according to Kumar et al. (2018). 25 mg of the dry leaf powder was homogenised in 5 mL of 80% ethanol. The supernatant was discarded and the pellet was suspended in 5 mL of 10% TCA for 30 min. After centrifugation, the supernatant was discarded. The pellet was then washed with 5% TCA to remove interfering amino acids and phenols. The protein precipitate was then dissolved in 1 M sodium hydroxide in a hot water bath for 30 minutes. The proteins extracted in 1 M NaOH were diluted 10-fold with distilled water. Five hundred µL of the diluted protein sample was placed in a test tube and 700 µL of the Lowry reagent was added. After a 20-minute incubation at room temperature, 100 µL of the Folin-Phenol reagent was added to the samples. After incubation at room temperature for 30 minutes, the absorbance was measured spectrophotometrically at 750 nm. The total protein content in each sample was calculated using a standard curve generated with bovine serum albumin (BSA, 0.05-0.25 mg/mL) and expressed as milligrames of BSA equivalent per gram of dried mulberry leaves (mg BSA/g DW).

Extract preparation for phenolic analysis

For the analysis of total phenols and individual phenols, 20 mg ± 0.3 of the ground lyophilised mulberry powder was homogenised with 1.5 mL of 3% formic acid in 70% methanol according to the procedure of Ainsworth and Gillespie (2007). Throughout the analytical process, the samples were kept in an ice bath due to the sensitivity of the bioactive compounds. The samples were then incubated for 30 minutes in an ultrasonic bath with ice insert. Samples were centrifuged at 4 °C for 15 min at 12.000 rpm. We then transferred 100 µL of the supernatant to new dark microcentrifuges. The microcentrifuges containing the remaining samples were centrifuged for HPLC (high performance liquid chromatography) analysis for a further 45 min at 4 °C and 12.000 rpm. After centrifugation, 700 µL of the supernatant was pipetted into vials and the samples were stored in a freezer at -20 °C until liquid chromatography for single phenol analysis. To new microcentrifuges containing 100 µL supernatant for total phenol analysis, 200 µL of freshly prepared Folin's reagent [1 mL Folin's reagent and 9 mL ddH_2O] was added to each with an electronic pipette and vortexed in each case. Another 800 µL Na_2CO_3 solution [18.5 g Na_2CO_3 with 200 mL ddH_2O] was added with an electronic pipette. The samples were then shaken in the dark at 30 rpm for two hours at room temperature.

Determination of the total phenol content

Total phenols were determined using the Folin-Ciocalteu method according to the procedure of Ainsworth and Gillespie (2007). We measured the absorbance using a spectrophotometer (Varian Cary 50 Bio) at a wavelength of 765 nm. First, the spectrophotometer was calibrated with the negative control (zero), then the absorbance of all standards and finally all samples was measured in two replicates. Using the absorbance and the known concentrations of the standards, we determined the concentration of total phenols in our sample. The content of total phenols in the samples is expressed as mg gallic acid equivalents per gram of dried mulberry leaves (mg GAE /g DW). All samples were measured in duplicate. If the deviations between the two replicates were too large, the sample analysis was repeated.

Determination of individual phenolics

Individual phenols in the samples were analyzed by high-performance liquid chromatography (HPLC) as described in Urbanek Krajnc et al. (2019), with a Waters Alliance 2695 HPLC system. Phenolic compounds were detected with a 2996 PDA detector at 280 nm (phenolic acids) and 350 nm (flavonoids). All considered phenolic compounds were previously identified by a mass spectrometer (Thermo Finigan, San Jose, CA) with an electrospray interface (ESI) operating in negative ion mode. The procedures were previously described by Mikulič-Petkovšek et al. (2013). The concentrations of individual phenols in the extracts of different mulberry genotypes were identified by comparing the retention time with standard solutions, the areas of the chromatographic peaks were measured and the results were expressed in mg/g of dry matter. First, we diluted and determined the retention times of all standards (described in Table 2).

Table 2: Known concentrations [mg/g] and retention times of the phenolic standards

	Chlorogenic acid	5-*p*-coumaroyilquinnic acid	Rutin	Quercetin-3-glucoside	Kaempferol-3-glucoside	4-caffeoylquinic acid
Retention time	12.82 min	12.3 min	19.8 min	21.1 min	22.72 min	13.97 min
S1	0.031	0.003	0.013	0.001	0.001	0.010
S2	0.062	0.007	0.026	0.005	0.004	0.075
S3	0.094	0.013	0.035	0.010	0.021	0.150
S4	0.125	0.020			0.015	0.015
S5	0.156	0.066			0.020	0.020

Mobile phase A consisted of 97% ddH_2O, 3% acetonitrile and 0.1% formic acid. Mobile phase B consists of 97% acetonitrile, 3% ddH_2O water and 0.1% formic acid. The gradient method was set as follows: 0–10 min = 95% A, 5% B; 10-15 min = 80% A, 20% B; 15-30 min = 70% A, 30% B; 30-35 min = 10% A, 90% B, 35-45 min = 0% A, 100% B, 45 min: 95% A, 5% B.

For liquid chromatography we used Gemini C18 110A, 150 x 4.6 mm, 3 µm column and the temperature was set at 25 °C. The flow rate of the mobile phase was 0.6 mL/min, and the injection volume of extracts and standards was 20 µL. All samples were measured in two repetitions, if there was a large deviation between the two repetitions, the analysis was repeated.

Statistical analysis

Statistical analysis was performed using the program IBM SPSS Statistics 25 (New York; USA; 2017), StatSoft, Inc. Statistica 8.0 (Victoria; Australia; 2007). For each mulberry species, we calculated the mean value and standard deviation. We checked the assumptions for the Analysis of Variance (ANOVA), performed the Levene's test for homogeneity of variances, the Kolmogorov-Smirnov test for the normal distribution of the variable and checked for the presence of outliers. The nonparametric equivalent of an ANOVA, called the Kruskal-Wallis test, was conducted because the assumption of homogeneity of variance and normality was violated. The results of this test indicated that there was a difference between the medians of at least one pair of groups, so we proceeded with the Dunn-Bonferroni post-hoc test. We rejected the null hypothesis at a risk level of $\alpha = 0.05$.

RESULTS AND DISCUSSION

Total protein content in leaves of different mulberry species

White mulberry leaves are characterised by a high content of proteins (13-31%) and amino acids, especially glutamic acid and aspartic acid, followed by leucine (Al-Kirschi et. al, 2012; Urbanek et. al, 2019; Andadari et al, 2021). The total protein content of mulberry leaves examined in our study ranged from 162.03 mg BSA/g DW (M. australis) to 239.42 mg BSA/g DW (*M. alba*). Compared to M. australis, *M. nigra* and *M. alba* × *rubra*, significantly higher total protein contents were determined in the leaves of *M. alba* × *rubra* (Figure 1). We can explain the large standard deviation of protein content in *M. alba* by the fact that a variety of white mulberry genotypes originating from different geographical regions were included in the study.

The Kruskal-Wallis test showed statistical significance of mulberry species on total protein content ($p < 0.05$). We also performed a multiple comparison test, which revealed a statistically significant difference in total protein content between the medians of *M. alba* and *M. nigra*, *M. alba* × *rubra* and M. australis, while no statistically significant differences were found between *M. nigra* and *M. alba* × *rubra* (Figure 1). It has already been confirmed that higher protein content in certain mulberry cultivars is significantly correlated with larval growth and has a positive effect on silk gland development and cocoon characteristics of silkworms (El-Banna et al., 2013; Adeduntan, 2015; Kumar et al., 2018).

All the white mulberry genotypes studied contained an average of 208.28 ± 17.05 mg BSA/g DW of total proteins. The protein levels are in accordance with those of Kumar et al. (2010) who investigated the total protein content of leaves of different white mulberry cultivars using the Lowry method and found that protein levels in the leaves ranged from 176 to 209 mg BSA/g DW.

Urbanek et al. (2019) investigated the effects of pruning on total protein content in white mulberry leaves and found significantly higher total protein levels in pruned trees compared to unpruned trees. Protein levels ranged from 95 mg BSA/g DW in unpruned trees to 135 mg BSA/g DW in pruned trees, which is lower than in our study. Due to the high protein content, whose quality is comparable to or even superior to that of soybean meal, mulberry leaves have been recognised as a rich source of high-protein feed for sheep, Vietnamese cattle and young pigs (Gryn-Rynko & Olszewska-Slonina, 2016; Tesfay et al., 2018; Hassan et al., 2020).

Total phenol content in leaves of different mulberry species

The content of total phenols differed significantly between the species *M. alba*, *M. nigra*, *M. alba* × *rubra* and M. australis. Based on our data, white mulberry (*M. alba*) contained statistically significantly less total phenols than black mulberry (*M. nigra*), which is consistent with the studies of other authors (Iqbal et al., 2012; Radojković et al., 2012; Sánchez-Salcedo et al., 2015). We can assume that the higher content of phenols in black mulberry leaves is one of the reasons why silkworms (*Bombyx mori*) prefer white mulberry leaves; because some flavonoids serve as antifeedants and can reduce the nutritional value of mulberry leaves by binding amino acids and proteins. They may even interfere with silkworm digestion by binding to digestive enzymes in the gut (Thabti et al., 2012; Asanaga et al., 2015; Samuel & Humtap, 2019). In advanced studies with silkworm diets, animals fed with black mulberry leaves were unable to form cocoons (Urbanek et al., 2022). Figure 2 shows the mean, minimum and maximum values of the total phenolic contents of different mulberry species.

In our study, the mean value of total phenolic content in the leaves of 24 different genotypes of white mulberry ranged from 15.23 to 21.23 mg GAE/g DW. The results are in agreement with those of Urbanek et al. (2019) who reported total phenolic content in the range of 7 to 20 mg GAE/g DW.

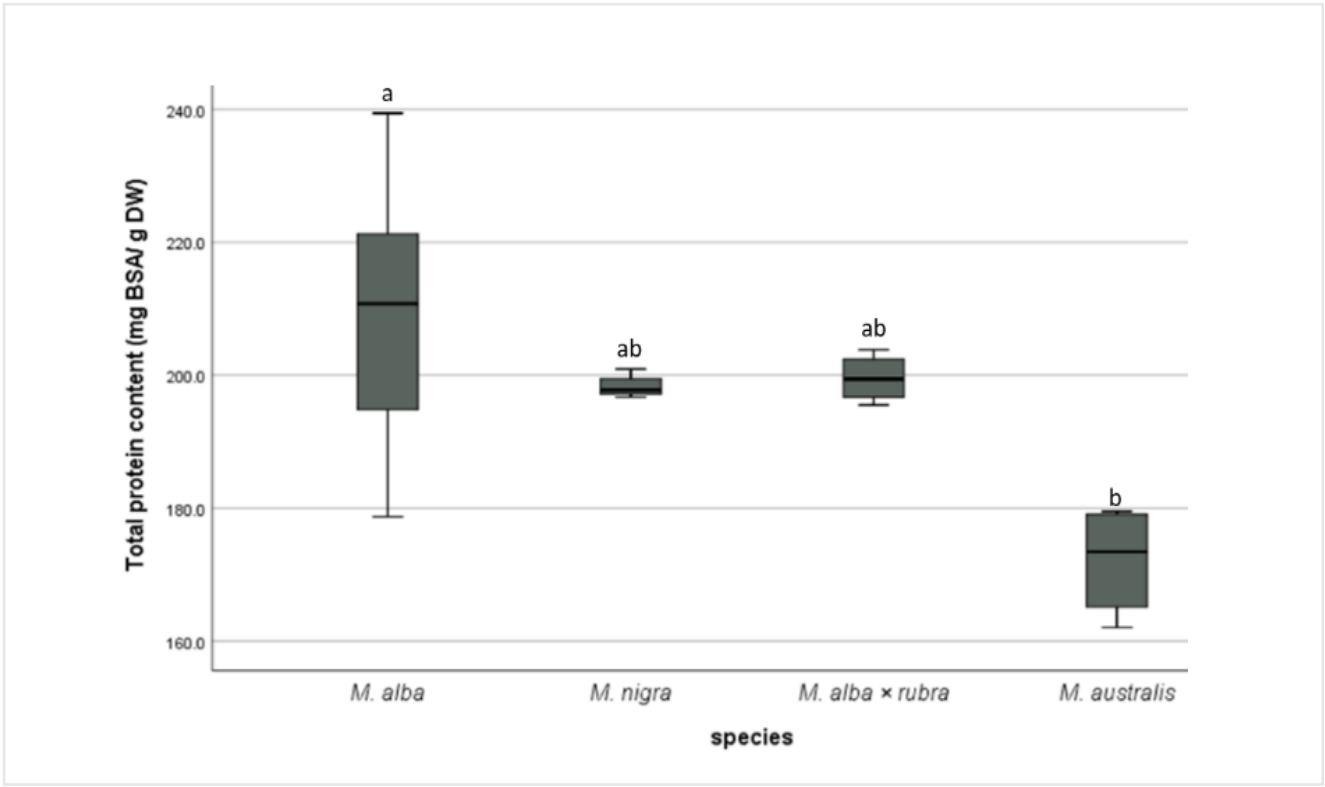

Figure 1: Mean, minimal and maximal values of total protein contents in *M. alba*, *M. nigra*, *M. alba* x *rubra* and *M. australis* leaves. Different letters (a,b) indicate significant differences ($p < 0.05$) between the mulberry species, which were determined using Dunn-Bonferroni post hoc test.

Iqbal et al. (2012) compared the content of total phenolics between white mulberry (16.21 ± 1.34 mg GAE/g DW) and black mulberry (24.37 ± 2.14 GAE/g DW) leaves and found that black mulberry had the best potential for preventive treatment of diseases as it contained the most total phenolics. Among the clones examined in the study by Sánchez-Salcedo et al. (2015), the *M. alba* clones had the lowest phenolic content (12.81–15.50 mg GAE/g DW), while the *M. nigra* genotypes had slightly higher values (13.48–16.13 mg GAE/g DW). Similarly, Radojković et al. (2012) measured lower levels of total phenols in white mulberry leaves than in black mulberry leaves.

Individual phenol content in leaves of different mulberry species

We determined statistically significant differences between mulberry species for all individual phenolics except for rutin and quercetin-malonyl-hexoside. In most cases, *M. nigra* and the hybrid *M. alba* × *rubra* contained the highest levels of most individual phenols, except for rutin and quercetin-malonyl-hexoside, where the highest values were obtained from the white mulberry genotypes. The mean values of the individual phenolic acid contents of the different mulberry species are shown in Figure 3A.

Chlorogenic acid is the predominant phenolic acid of the mulberry leaves with an average value of 10.29 mg/g DW (considering all species). The sample SM 214 (*M. alba*) had a minimum value of 5.55 mg/g DW, and the maximum value was found in the sample *M. nigra* with 18.25 mg/g DW. Memon et al. (2010) confirmed that regardless of the mulberry species (*M. alba*, *M. nigra* and *M. laevigata*), mulberry leaves contain the most chlorogenic acid, in their case between 60.5% and 67.2%, with *M. laevigata* having the highest content and *M. alba* the lowest.

In our study, *M. nigra* and *M. alba* × *rubra* contained statistically significantly more chlorogenic acid than *M. alba* and *M. australis*. Sánchez-Salcedo et al. (2015) analysed the phenolic profile of the leaves of four different genotypes of white mulberry and four genotypes of black mulberry and confirmed the highest content of chlorogenic acid among all individual phenols analyzed; from 5.29 ± 0.24 mg/g DW (*M. alba*) to 8.39 ± 1.94 mg/g DW (*M. nigra*). The results of other authors (Memon et al., 2010; Sánchez-Salcedo et al., 2015; Pothinuch and Tongchitpakdee, 2019) are in agreement with the results of our study, as chlorogenic acid is dominant among the individual phenols and black mulberry contains more chlorogenic acid than white mulberry.

Of all the dominant individual phenols, there was the lowest amount of 4-caffeoylquinic acid, on average 1.15 mg/g DW, of all the extracts analysed, SP 12 (*M. alba*) had the lowest content and the highest *M. alba* × *rubra*, namely 4.54 mg/g DW. Sánchez-Salcedo et al. (2015) measured 0.17 to 0.42 mg/g

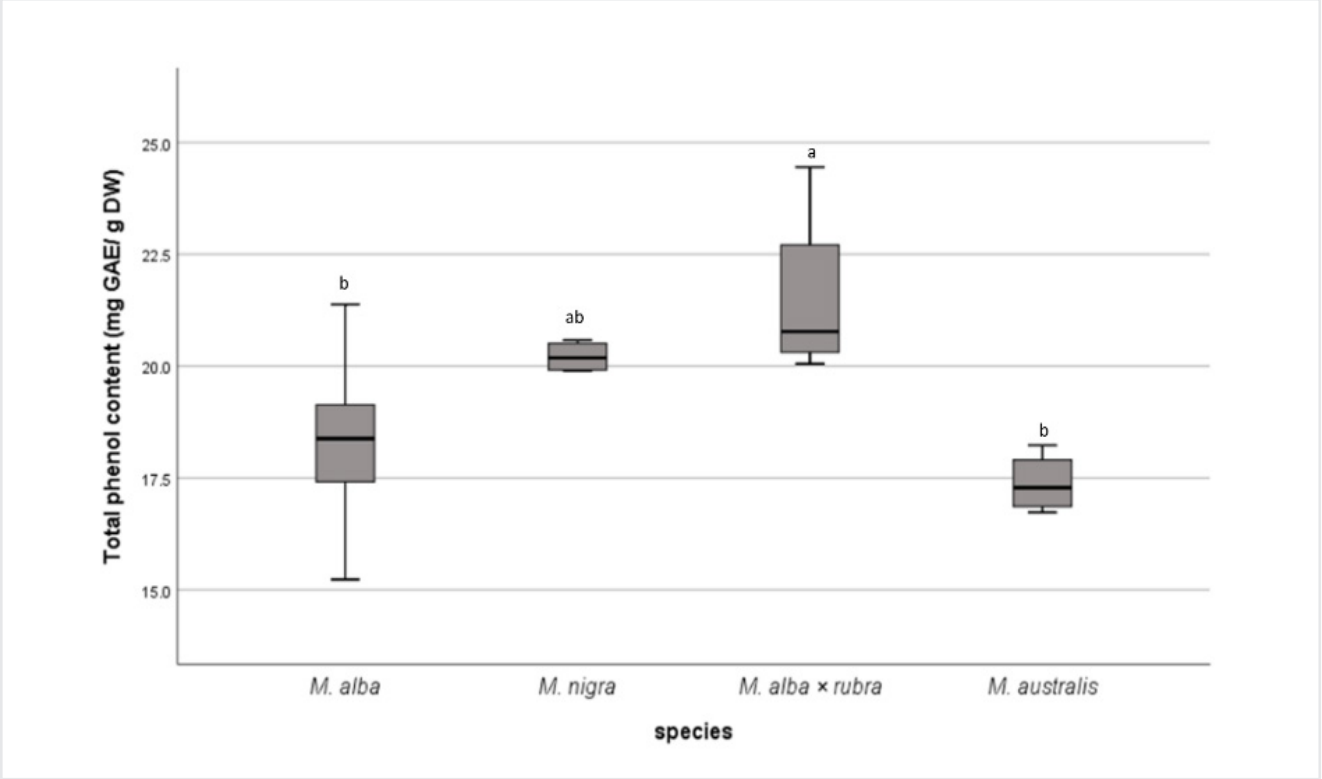

Figure 2: Mean, minimal and maximal values of total phenol contents in *M. alba*, *M. nigra*, *M. alba* x *rubra* and *M. australis* leaves. Different letters (a,b) indicate significant differences ($p < 0.05$) between the mulberry species, which were determined using Dunn-Bonferroni post hoc test.

DW of 4-caffeoylquinic acid in white mulberry leaves and 0.36 to 0.58 mg/g DW in black mulberry leaves. In comparison, we determined higher mean levels of 4-caffeoylquinic acid, 0.95 ± 0.25 mg/g DW in white mulberry and 1.26 ± 0.03 mg/g DW in black mulberry leaves. Pothinuch and Tongchitpakdee (2019) measured higher levels of 4-caffeoylquinic acid (1.3-2.4 mg/g DW) in three white mulberry cultivars and identified it as the dominant phenol, along with chlorogenic acid and rutin.

The average value of *p*-coumaroylquinic acid (*cis*) in all genotypes studied was 1.33 mg/g DW, of all samples SM 6 (*M. alba*) had the lowest value (0.45 mg/g DW) and the highest *M. nigra*, which contained 3.28 mg/g DW. Memon et al. (2010) could not detect *p*-coumaroylquinic acid in white mulberry leaves, while black mulberry leaves contained 4.42 mg/g DW, which is four times higher than our values.

The mean values of the individual flavonols of the different mulberry species are shown in Figure 3B. In the mulberry samples analysed, the second most represented phenol was rutin with an average value of 2.54 mg/g DW in all genotypes. Of all the extracts analysed, SM 214 (*M. alba*) had the lowest content (1.2 mg/g DW) and the highest TO 1131 (*M. alba*) namely 4.54 mg/g DW. Sánchez-Salcedo et al. (2015) measured lower values for rutin, ranging from 0.59 to 0.95 mg/g DW. Pothinuch and Tongchitpakdee (2019) measured 1.0 to 4.4 mg/g DW in mulberry leaves, which is consistent with our analytical results.

Quercetin-malonyl-hexoside was measured with an average value of 1.57 mg/g DW, genotype GMS 2533 (*M. alba*) contained the lowest amount (0.84 mg/g DW), while the highest amount (2.25 mg/g DW) was measured in 'Kokosou' (*M. alba*). Pothinuch and Tongchitpakdee (2019) measured 2 mg/g DW in the young leaves of the *M. alba* cultivar 'Buriram 60' and 1.46 mg/g DW in the old leaves of quercetin- malonyl-hexoside, but failed to detect quercetin-malonyl-hexoside in the other two sericulture cultivars 'Sakonnakhon' and 'Khunphai'.

The average value of kaempferol-acetyl-hexoside was 2.24 mg/g DW, the lowest value (1.24 mg/g DW) was detected in *M. nigra* and the highest in *M. alba* × *rubra*, which contained an average of 4.42 mg/g DW. Pothinuch and Tongchitpakdee (2019) measured lower levels of kaempferol hexoside in all three sericultural cultivars, ranging from 0.14 to 0.73 mg kaempferol-3-rutinoside equivalent/g DW.

CONCLUSION

The results of our research showed us a great diversity in the content of proteins and phenols between the mulberry species. We also found that the different mulberry species bear specific biochemical traits. We can conclude that the leaves of the white mulberry have the highest total

protein content and a low total phenolic content and are therefore best suited for animal feed. We also found the highest levels of total and individual phenols in the leaves of black mulberry and the hybrid *M. alba* × *rubra*, which are therefore best suited for pharmacological purposes. The leaves of *M. australis* contain the lowest amounts of both proteins and phenols. The results obtained would help us to select and propagate superior mulberry genotypes with high total protein or phenolic contents as a valuable source for silkworms and other animal feeds as well as for pharmacological purposes.

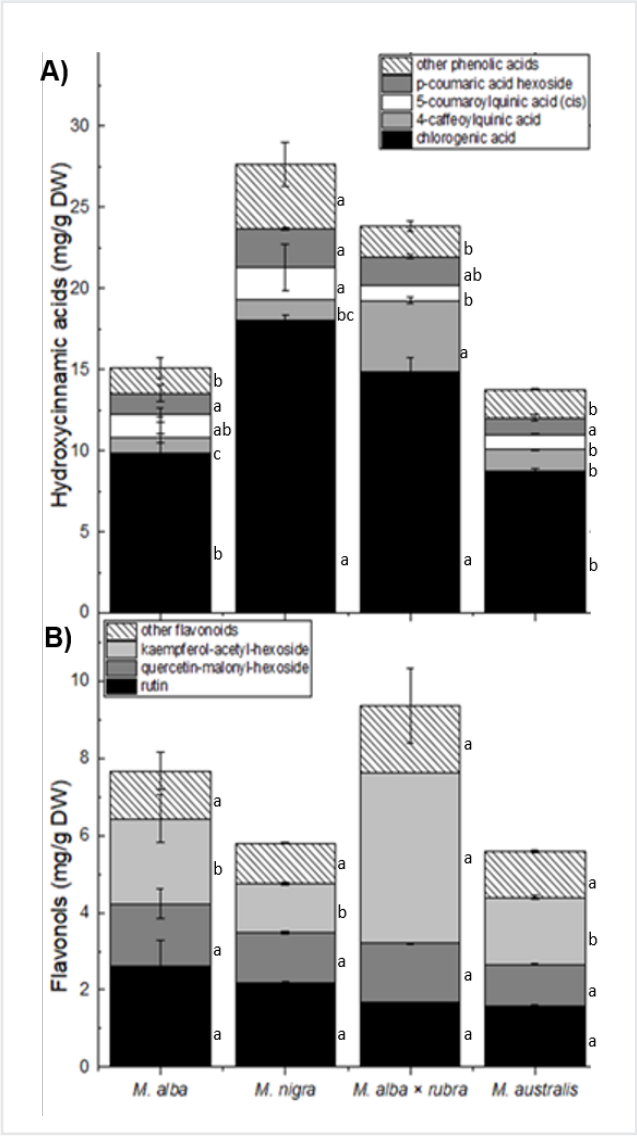

Figure 3: Content levels of predominant (A) hydroxycinnamic acids and (B) flavonols of different mulberry species. Each value represents mean ± standard deviation. Different letters (a–c) indicate significant differences ($p < 0.05$), which were determined using Dunn-Bonferroni post hoc test. Other phenolic acids are: 5-caffeoylquinic acid, p-coumaric acid, 5-coumaroyquinic *trans*, *p*-coumaroylic acid. Other flavonoids are: quercetin-dirhamnosylglycoside; quercetin-rhamnosylhexoside; quercetin-glucoside; quercetin-acetyl-rhamnosyl-hexoside; quercetin-acetyl-hex.; kaempferol-dirhamnosyl-hexoside; kaempferol-rhamnosyl-hexoside; kaempferol-acetyl-rhamnosyl-hexoside).

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REFERENCES

1. Adeduntan, S. A. (2015). Influence of different varieties of mulberry leaves (*Morus alba*) on growth and cocoon performance of biovoltine strain of silkworm (*Bombyx mori*). *International Journal of Biological and Chemical Sciences, 9*(2), 751-757. Retrieved from: https://doi.org/10.4314/ijbcs.v9i2.15
2. Ainsworth, E. A., & Gillespie, K. M. (2007). Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. *Nature Protocols, 2*, 875-877. Retrieved from: https://doi.org/10.1038/nprot.2007.102
3. Al-Kirshi, R. A., Alimon, A. R., Zulkifli, I., Zahari, M. W., & Sazili, A. Q. (2009). The chemical composition and nutritive value of mulberry leaf as a protein source in poultry diets. *First International Seminar and the 7th Conference of Indonesian Association of Nutrition and Feed Science (AINI).* Purwokerto, Indonesia.
4. Andadari, L., Minarningsih, M., & Suwandi. T. (2021). The effect of feeding various species of mulberry (*Morus* spp.) on the growth of silkworm and quality of cocoon hybrid BS 09. *IOP Conference Series: Earth and Environmental Science, 914*(1), 012017). Retrieved from: 10.1088/1755-1315/914/1/012017
5. Botanical Terminology Dictionary. (2011). Retrived from https://isjfr.zrc-sazu.si/sl/terminologisce/slovarji/botanicni
6. Bouayed, J., Piri, K., Rammal, H., Dicko, A., Desor, F., Younos, C., & Soulimani, R. (2007). Comparative evaluation of the antioxidant potential of some Iranian medicinal plants. *Food Chemistry, 104*(1), 364-368.
7. Butt, M. S., Nazir, A., Sultan, M. T., & Schroën, K. (2008). *Morus alba* L. nature's functional tonic. *Trends in Food Science & Technology, 19*(10), 505-512. Retrieved from: https://doi.org/10.1016/j.tifs.2008.06.002
8. Choi, S. W., Lee, Y. J., Ha, S. B., Jeon, Y. H., & Lee, D. H. (2015). Evaluation of biological activity and analysis of functional constituents from different parts of mulberry (*Morus alba* L.) tree. *Journal of the Korean Society of Food Science and Nutrition, 44*(6), 823-831. Retrieved from: https://doi.org/10.3746/jkfn.2015.44.6.823

9. Devi, B., Sharma, N., Kumar, D., & Jeet, K. (2013). *Morus alba* Linn: A phytopharmacological review. *International Journal of Pharmacy and Pharmaceutical Science, 5*(2), 14-18.
10. El-Banna, A. A., Moustafa, M. N., Mahmoud, S. M., El-Shafei, A. M., & Moustafa, A. A. (2013). Effect of feeding different mulberry varieties on some biological characteristics of the silkworm, *Bombyx mori* L. *Egyptian Journal of Pure and Applied Science, 51*, 055-060. Retrieved from: https://doi.org/10.21608/ejaps.2013.18597
11. Flaczyk, E., Kobus-Cisowska, J., Przeor, M., Korczak, J., Remiszewski, M., Korbas, E., & Buchowski, M. (2013). Chemical characterization and antioxidative properties of Polish variety of *Morus alba* L. leaf aqueous extracts from the laboratory and pilot-scale processes. *Agricultural Sciences, 4*(05), 141. Retrieved from: https://doi.org/10.4236/as.2013.45b026
12. Gryn-Rynko, A., Bazylak, G., & Olszewska-Slonina, D. (2016). New potential phytotherapeutics obtained from white mulberry (*Morus alba* L.) leaves. *Biomedicine & Pharmacotherapy, 84*, 628-636. Retrieved from: https://doi.org/10.1016/j.biopha.2016.09.081
13. Güven, İ. (2012). Effect of species on the nutritive value of mulberry leaves. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi, 18*(5), 865-869.
14. Han, N., & May, C. (2010). Determination of antioxidants in oil palm leaves (*Elaeis guineensis*). *American Journal of Applied Sciences, 7*(9), 1243-1247. Retrieved from: https://doi.org/10.1016/j.foodchem.2006.11.069
15. Hassan, F. U., Arshad, M. A., Li, M., Rehman, M. S. U., Loor, J. J., & Huang, J. (2020). Potential of mulberry leaf biomass and its flavonoids to improve production and health in ruminants: Mechanistic insights and prospects. *Animals, 10*(11), 2076. Retrieved from: https://doi.org/10.3390/ani10112076
16. Iqbal, S., Younas, U., Chan, K. W., Sarfraz, R. A., & Uddin, K. (2012). Proximate composition and antioxidant potential of leaves from three varieties of Mulberry (*Morus* sp.): a comparative study. *International Journal of Molecular Sciences, 13*(6), 6651-6664. Retrieved from: https://doi.org/10.3390/ijms13066651
17. Jeszka-Skowron, M., Flaczyk, E., Jeszka, J., Krejpcio, Z., Król, E., & Buchowski, M. S. (2014). Mulberry leaf extract intake reduces hyperglycaemia in streptozotocin (STZ)-induced diabetic rats fed high-fat diet. *Journal of Functional Foods, 8*, 9-17. Retrieved from: https://doi.org/10.1016/j.jff.2014.02.018
18. Kumar, K., Mohan, M., Tiwari, N., & Kumar, S. (2018). Production potential and leaf quality evaluation of selected mulberry (*Morus alba*) clones. *Journal of Pharmacognosy and Phytochemistry, 7*(2), 482-486. Retrieved from: https://www.phytojournal.com/archives/2018/vol7issue2/PartG/7-1-319-836.pdf
19. Kumar, K., Mohan, M., Tiwari, N., & Kumar, S. (2018). Production potential and leaf quality evaluation of selected mulberry (*Morus alba*) clones. *Journal of Pharmacognosy and Phytochemistry, 7*(2), 482-486.
20. Kumar, L., & Arunachalam, G. (2009). Genetic variability in *Morus alba* L by biochemical and bioassay methods for increased silk productivity. *Journal of Biomedical Science and Research, 1*(1), 11-18.
21. Kumar, R. V., Chauhan, S., Kumar, D., & More, N. (2010). Nutritional composition in leaves of some mulberry varieties:-A comparative study. *International Conference on Bioinformatics and Biomedical Technology* (pp. 438-442), Chengdu, China. Retrieved from: 10.1109/ICBBT.2010.5478923
22. Lowry OH, Rosenbrough NJ, Farr AL., & Randall RJ. (1951). Protein measurement with folin phenol reagent. *Journal of Biological Chemistry, 193*, 265-275.
23. Memon, A. A., Memon, N., Luthria, D. L., Bhanger, M. I., & Pitafi, A. A. (2010). Phenolic acids profiling and antioxidant potential of mulberry (*Morus laevigata* W., *Morus nigra* L., *Morus alba* L.) leaves and fruits grown in Pakistan. *Polish Journal of Food and Nutrition Sciences, 60*(1), 25-32.
24. Mikulic-Petkovsek, M., Slatnar, A., Schmitzer, V., Stampar, F., Veberic, R., & Koron, D. (2013). Chemical profile of black currant fruit modified by different degree of infection with black currant leaf spot. *Scientia Horticulturae, 150*, 399-409. Retrieved from: https://doi.org/10.1016/j.scienta.2012.11.038
25. Park, E., Lee, S. M., Eun Lee, J., & Kim, J.-H. (2013) Anti-inflammatory activity of through Inhibition of NF-kB. *Journal of Functional Foods, 5*(1), 178-186. Retrieved from: https://doi.org/10.1016/j.jff.2012.10.002
26. Pinhero, R. G., Rao, M. V., Paliyath, G., Murr, D. P., & Fletcher, R. A. (1997). Changes in activities of antioxidant enzymes and their relationship to genetic and paclobutrazol-induced chilling tolerance of maize seedlings. *Plant Physiology, 114*(2), 695-704. Retrieved from: https://doi.org/10.1104/pp.114.2.695
27. Polumackanycz, M., Wesolowski, M., & Viapiana, A. (2021). *Morus alba* L. and *Morus nigra* L. Leaves as a promising food source of phenolic compounds with antioxidant activity. *Plant Foods for Human Nutrition, 76*(4), 458-465. Retrieved from: https://doi.org/10.1007/s11130-021-00922-7
28. Pothinuch, P., & Tongchitpakdee, S. (2019). Phenolic analysis for classification of mulberry (*Morus* spp.) leaves according to cultivar and leaf age. *Journal of Food Quality, 2019*, 2807690. Retrieved from: https://doi.org/10.1155/2019/2807690
29. Radjabi, R. (2010). Effect of mulberry leaves enrichment with amino acid supplementary nutrients on silkworm, *Bombyx mori* L. at north of Iran. *Academic Journal of Entomology, 3*(1), 45-51.
30. Radojković, M. M., Zeković, Z. P., Vidović, S. S., Kočar, D. D., & Mašković, P. Z. (2012). Free radical scavenging activity and total phenolic and flavonoid contents of mulberry (*Morus* spp. L., Moraceae) extracts. *Hemijska industrija, 66*(4), 547-552. https://doi.org/10.2298/HEMIND111111002R
31. Ramesh, H. L., Sivaram, V., & Yogananda Murthy, V. N. (2014). Antioxidant and medicinal properties of mulberry (*Morus* sp.): A review. *World Journal of Pharmaceutical Research, 3*(6), 320-343.
32. Samuel, Y. G., & Humtap, O. T. (2019). Evaluation of the antinutrients, nutrients, phytochemicals and metals content of

five leafy vegetables in Dengi metropolis. *International Journal of Biochemistry Research & Review, 24*(3), 1-8. Retrieved from: https://doi.org/10.9734/IJBCRR/2018/44652

33. Sánchez-Salcedo, E. M., Amorós, A., Hernández, F., & Martínez, J. J. (2017). Physicochemical properties of white (*Morus alba*) and black (*Morus nigra*) mulberry leaves, a new food supplement. *Journal of Food and Nutritional Research, 5*, 253-261.
34. Sánchez-Salcedo, E. M., Mena, P., García-Viguera, C., Hernández, F., & Martínez, J. J. (2015). (Poly) phenolic compounds and antioxidant activity of white (*Morus alba*) and black (*Morus nigra*) mulberry leaves: Their potential for new products rich in phytochemicals. *Journal of Functional Foods, 18*, 1039-1046. Retrieved from: https://doi.org/10.1016/j.jff.2015.03.053
35. Srivastava, S., Kapoor, R., Thathola, A., & Srivastava, R. P. (2003). Mulberry (*Morus alba*) leaves as human food: a new dimension of sericulture. *International Journal of Food Sciences and Nutrition, 54*(6), 411-416. Retrieved from: https://doi.org/10.1080/09637480310001622288
36. Tesfay, G., Tamir, B., & Berhane, G. (2018). Substitution of mulberry leaf meal on feed intake, body weight and carcass characteristics of Tigray highland lambs. *Jurnal Ilmu Ternak dan Veteriner, 23*(1), 28-37.
37. Thabti, I., Elfalleh, W., Hannachi, H., Ferchichi, A., & Campos, M. D. G. (2012). Identification and quantification of phenolic acids and flavonol glycosides in Tunisian *Morus* species by HPLC-DAD and HPLC–MS. Journa*l of Functional Foods, 4*(1), 367-374. Retrieved from: https://doi.org/10.1016/j.jff.2012.01.006
38. Thaipitakwong, T., Numhom, S., & Aramwit, P. (2018). Mulberry leaves and their potential effects against cardiometabolic risks: a review of chemical compositions, biological properties and clinical efficacy. *Pharmaceutical Biology, 6*(1), 109-118. Retrieved from: https://doi.org/10.1080/13880209.2018.1424210
39. Urbanek Krajnc, A., Bakonyi, T., Ando, I., Kurucz, E., Solymosi, N., Pongrac, P., & Berčič, R. L. (2022). The effect of feeding with central european local mulberry genotypes on the development and health status of silkworms and quality parameters of raw silk. *Insects, 13*(9), 836. Retrieved from: https://doi.org/10.3390/insects13090836
40. Urbanek Krajnc, A., Ugulin, T., Pausic, A., Rabensteiner, J., Bukovac, V., Mikulic Petkovsek, M., ... & Felicijan, M. (2019). Morphometric and biochemical screening of old mulberry trees (*Morus alba* L.) in the former sericulture region of Slovenia. *Acta Societatis Botanicorum Poloniae, 88*(1), 3641. Retrieved from: https://doi.org/10.5586/asbp.3614
41. Yang, Y., Wang, G., & Pan, X. (2009). *China Food Composition*, 2nd ed. Peking University Medical Press: Beijing, China.
42. Yu, Y., Li, H., Zhang, B., Wang, J., Shi, X., Huang, J., ... & Deng, Z. (2018). Nutritional and functional components of mulberry leaves from different varieties: Evaluation of their potential as food materials. *International Journal of Food Properties, 21*(1), 1495-1507. Retrieved from: https://doi.org/10.1080/10942912.2018.1489833

Sestava beljakovin in fenolov v listih različnih vrst murv (*Morus alba* L., *M. alba* × *rubra*, *M. australis* Poir., *M. nigra* L.)

POVZETEK

Listi murve (*Morus sp.*) so vse splošno uporabni v medicini, kulinariki, industriji in kmetijstvu. V naši raziskavi smo primerjali vsebnost beljakovin in fenolov različnih vrst murv (*Morus alba* L., *M. alba* × *rubra*, *M. australis* Poir., *M. nigra* L.), iz kolekcijskega nasada z namenom določitve specifičnih vrstnih razlik. Skupne fenole vseh genotipov smo analizirali z metodo Folin-Ciocalteu, vsebnost skupnih beljakovin pa z Lowryjevo metodo. Posamezne fenole smo analizirali s tekočinsko kromatografijo visoke ločljivosti z UV/VIS detekcijo. Celotna vsebnost beljakovin je znašala od 162,03 mg BSA/g DW (*M. australis*) do 239,42 mg BSA/g DW (*M. alba*). Bistveno višje vsebnosti skupnih beljakovin so bile ugotovljene v listih *M. alba*. Najvišje povprečne koncentracije skupnih fenolov (21,51 mg GAE/g SS), klorogenske kisline (18,05 mg/g SS), 4-kafeoilkininske kisline (4,36 mg/g SS), 5-*p*-kumaroilkininske kisline (2,01 mg/g SS), kvercetin glikozid (0,74 mg/g SS) in kaempferol acetil heksozid (4,42 mg/g SS) smo določili v *M. alba* × *rubra* in *M. nigra*. Nasprotno pa so genotipi bele murve (*M. alba*) vsebovali v povprečju največ rutina (2,63 mg/g SS) in kvercetin-malonil-heksozida (1,59 mg/g SS). Sklepamo lahko, da so listi bele murve najprimernejši za živalsko krmo, saj vsebujejo največ beljakovin medtem, ko imata črna murva in hibrid *M. alba* × *rubra*, zaradi visoke vsebnosti fenolov farmakološki potencial.

Ključne besede: *Morus* sp., listi, beljakovine, fenolne kisline, flavonoidi