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EVALUATION OF TOTAL PHENOLS IN ALFALFA (*Medicago sativa* L.) COLLECTED FROM DIFFERENT LOCALITIES IN REPUBLIC OF MACEDONIA

Valentina Butleska Gjoroska[1]*, Marija Krstik[2], Ivana Jovanovska Klincarska[2], Ana Cvetanovska[3], Lenka Cvetanovska[2], Liljana Koleva Gudeva[1]

[1]Faculty of Agriculture, Goce Delcev University - Stip, Krste Misirkov Str., No 10-A, 2000 Stip, Republic of Macedonia
[2]Faculty of Natural Science and Mathematics, Arhimedova Str., No 3, 1000 Skopje, Ss. Cyril and Methodius University in Skopje, Republic of Macedonia
[3]Faculty of Veterinary Medicine, Lazar Pop Trajkov Str., No 5, 1000 Skopje, Ss. Cyril and Methodius University in Skopje, Republic of Macedonia
**Corresponding author: tina_valentina2@yahoo.com*

Abstract

Phenols are secondary biomolecules, which in their structure contain an aromatic ring attached to one or more substituents, such as flavonoids, which are one of the largest class plant phenols.

Phenolic compounds participate in biochemical processes that are important for the protection of plants against infections caused by fungi and viruses, mechanical damage, regulation of metabolism, and more. Phenolic compounds are oxidized by phenol oxidases to quinones, by releasing hydrogen and thus directly participating in the exchange of matter. The intensity of the biosynthesis of phenolic compounds increases after plant infection with pathogenic microorganisms. Therefore, the amount of total phenolic in plants can be used as biochemical parameter and indicator in the selection of plants for resistance to fungi, bacteria and viruses. Phenolic compounds are intensively synthesized in mechanical damage to plants, whereby they build protective film with active condensation in damaged areas. In this way they prevent cell wall degradation in infections caused by the necrotropic pathogens that destroy the cells in order to build their colonies on the dead tissues.

This paper presents the results of the research on the determination of the total phenols determined in three regions of the Republic of Macedonia in a total of 20 different locations. The content of the total phenols in the dry plant material of alfalfa in three mowings was determined by routine method in Folin-Ciocalteu (1927). The quantitative determination of total phenols in the plant ethanol extract was performed spectrophotometrically at a wavelength of 765nm.

Key words: *Lucern mowings, spectrophotometry, phenolic compounds, forage crop*

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a perennial fodder culture and is one of the many species of the *Medicago* genus. Of all species, the widespread production is common or blue alfalfa belonging to the family Fabaceae (Василиченко, 1949). It is one of the oldest cultivated species. It belongs to the oldest and most widely used forage crops in many parts of the world, but also in our country, but it is one of the most important forage crops.

Alfalfa, also called lucerne is characterized by high nutritional value (Julier et al., 2000), both in green mass, also in hay, silage, sage or alfalfa flour. Alfalfa is a fodder culture that ensures high yield and quality of protein foods (Dinic et al., 2005), which makes it one of the most important forage crops. The high yield of this crop comes from the ability of alfalfa to regenerate continuously through the year.

In addition to the nutritional components that make alfalfa useful as an animal feed or food supplement (Hatfield, 1992), the plants produce a variety of secondary metabolites showing biological activity. Many of these compounds help protect the plant against herbivores (Cambier et al., 2000; Awmack and Leather, 2002) and can influence the choise of food sources by insect herbivores (Shonle and Bergelson, 2000; Lankau, 2007; Mosleh et al., 2008).

In agricultural production, it has a huge influence and has a dominant role in the intensification of fodder production, due to the presence of vitally important vitamins, carbohydrates, mineral elements and other active components essential for the growth and development of plants that enable the ability for high productivity, quality and ability for strong regeneration (Hao et al., 2008).

Alfalfa has a powerful and developed root system, which improves the soil structure and deeper depths, but is also responsible for its resistance to drought on one side and very low temperatures on the other side (Markovic et al., 2007a). Through symbiotic nitrogenfixation with the bacterium *Rhisobium meliloti* var. *medicaginis* fixes large amounts of nitrogen from the air (Јакушкин, 1947); it reaches 300-400kg/ha per year of nitrogen, which corresponds to more than 60 tonnes of manure. In the soil significant amounts of phosphorus and potassium live. All this makes the alfalfa an excellent pre-culture for a range of important crops: cereal, industrial and horticultural.

In addition, alfalfa also contains phenolic compounds that have more significant functions, such as stimulating the growth and development of plants, protecting plants from pathogens, giving the color of flowers, and thus stimulate pollination, give the taste of green fruits and more.

Phenolic compounds are a group of chemical compounds that are widely distributed in nature. They are simple compounds present in most fresh fruits and vegetables, or complex compounds present in bark, roots and leaves of plants. A group of polyphenols, responsible for the color of many fruits, vegetables, and flowers, are known as anthocyanins. There are several important classes, of phenolic compounds. According to the basic skeleton, the structure of natural polyphenols varies from simple molecules, such as simple phenols (volatile phenols), to highly polymerized compounds, such as condensed tannins (Waterman and Mole, 1994).

Phenolic compounds as prevalent active secondary biomolecules that participate in various biochemical processes important for photosynthesis, regulation of metabolism and other, direct research mainly to confirming the quality of this culture as an excellent component in feeding stuffs.

MATERIALS AND METHODS

Plant material

The object of the examination was alfalfa (*Medicago sativa* L.), collected from three different regions on the territory of the Republic of Macedonia, Skopje region with coordinates (42.01^{o} N, 21.24^{o} E), Ovchepolski (41.45^{o} N, 22.11^{o} E) and Tetovo region (42.00^{o} N, 20.58^{o}E) from a total of 20 different locations, in three mowings. Table 1 describes the locations from the examined sites with their altitude (m), latitude (^{o}N) and longitude (^{o}E). The material is collected during the vegetative cycle of alfalfa. The experiments were carried out on dry plant material using modern quantitative methods.

Table 1. Description of the locations from the examined sites altitude (m) and latitude (˚N) and longitude (˚E).

Order number	Locations	Region	Altitude (m)	Latitude (°N)	Longitude (°E)
1.	Bogovinje	Tetovo	531.50	41.9236809	20.9168772
2.	Vrutok	Tetovo	682.41	41.7665300	20.8381550
3.	Dzepciste	Tetovo	474.48	42.0331690	21.0001650
4.	Galate	Tetovo	600.73	41.8381370	20.8813700
5.	Zelino	Tetovo	1605.94	41.9006530	21.1175770
6.	Pechkovo	Tetovo	991.87	41.7843700	20.8311530
7.	Jegunovce	Tetovo	658.34	42.1245655	21.0875064
8.	Avtokomanda	Skopje	246.68	42.0006868	21.4536642
9.	Sopiste	Skopje	1017.16	41.8638490	21.3083500
10.	Drachevo	Skopje	264.41	41.9352675	21.5098515
11.	Saraj	Skopje	424.88	42.0017493	21.2815977
12.	Radishani	Skopje	392.32	42.0732769	21.4479917
13.	Vlae	Skopje	256.07	42.0072938	21.3801924
14.	Glumovo	Skopje	274.74	41.9817742	21.3103747
15.	Dobroshane	Ovce Pole	302.06	42.1066200	21.7540130
16.	Cheshinovo	Ovce Pole	294.00	41.8735350	22.2905610
17.	Karbinci	Ovce Pole	342.98	41.7882100	22.2622460
18.	Obleshevo	Ovce Pole	297.63	41.8639320	22.2622460
19.	Lozovo	Ovce Pole	277.86	41.7806752	21.8995629
20.	Mustafino	Ovce Pole	289.18	41.8407190	22.0789350

The content of the total phenols in dry plant material from alfalfa was determined by the routine method in Folin-Ciocalteu (Singleton et al., 1965). The Folin-Ciocalteu reagent is most commonly used for the quantitative determination of the content of phenols and antioxidant compounds in plant extracts.

The quantitative determination of total phenols in the plant methanol extract was performed spectrophotometricaly at a wavelength of 765nm (Singleton et al., 1999), and the results are presented as mg GAE/g DW (mg Gallic acid equivalent/g dry weight).

Extraction method

The extraction of phenolic compounds from the plant material was carried out with 80% methanol (CH_3OH). Dry plant material (50mg) was macerated and then incubated. Then, the extracted plant extracts were centrifuged and the supernatant obtained was collected and used for quantitative analysis of the content of total phenolic compounds.

Method for determination of total phenols

For the quantitative determination of the content of the total phenolic compounds in the methanolic extracts, samples were taken, a series of standard solutions (in concentration rag from 0-0.4mg**.**ml^{-1}) against a blank test. The reaction mixture of the sample consists of 20μL of methanol extract, 80μL of 80% CH_3OH, 500μL diluted aqueous solution of Folin-Ciocalteu reagent (1:9) and 400μL of 0.7M Na_2CO_3. A series of standard solutions were prepared by diluting a gallic acid solution with a concentration of 0.4 mg**.** ml^{-1}. The blank was used for calibration of the spectrophotometer and contained 100μL of 80% CH_3OH, 500μL dilute aqueous solution of Folin-Ciocalteu reagent (1: 9) and 400μL of 0.7M Na_2CO_3. The prepared samples, the standard solutions and the blank were incubated on a temperature of 50^0C water bath for 5 minutes. Then, the samples were cooled to room temperature (15 minutes) and their absorbance was measured at a wavelength of 765nm. The results were expressed in mg GAE/g DW of plant material. The samples were prepared in triplicate for each analysis and the mean value of absorbance was calculated.

Statistical analysis

The data were statistically processed (XLSTAT 2014) with a one-way variance analysis (ANOVA), in order to determine the significant differences ($p < 0.05$) between the mean values of the samples. Subsequently, the results were Post hoc analyzed using Duncan's multiple ranking test. This test is used to determine statistically significant differences in the content of phenolic compounds within the three mowings.

RESULTS AND DISCUSSION

Content of total phenolic compounds at the first mowing

In this experiment, results of the studies for determining the total phenolic compounds have been presented. Phenolic compounds constitute a large class of plant secondary metabolites characterized by a high degree of structural heterogenity. Phenolic compounds such as 4-hydroxybenzoic, salicylic, r-coumarinic, gallic acid as well as some other phenolic compounds have a certain effect on the growth of plants. It has been found that the plant phenol-linullaric acid acts as a phytohormone and can replace the abscissic acid (Cvetanovska, 2016).

Form the results presented in Figure 1 and Table 2 can be seen that the significantly increased content of phenolic compounds was determined in the following regions and their localities: Tetovo (Dzepciste), Skopje (Glumovo), Ovchepole region (Lozovo) and the smallest content was measured in the Skopje region (Drachevo).

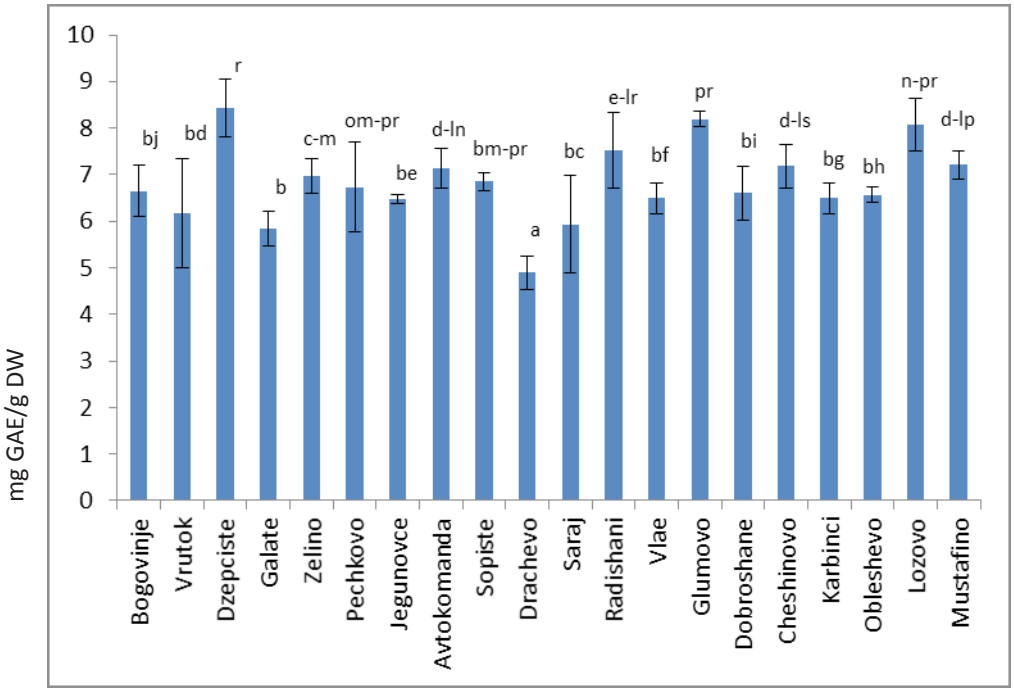

Figure 1. Content of total phenolic compounds in alfalfa (*Medicago sativa* L.), at certain localities on the territory of the Republic of Macedonia in the first mowing, expressed in mg GAE/g DW.

* If the small letters that are above of each column are the same there in no statistical difference, and if they are different there is a statistically significant differences.

Table 2. Content of total phenolic compounds in alfalfa (*Medicago sativa* L.), at certain localities on the territory of the Republic of Macedonia in the first mowing, expressed in mg GAE/g DW.

No.	Locations	First mowing mg GAE/ g DW	Standard deviation
1.	Bogovinje	6.65 bj	± 0.54
2.	Vrutok	6.17 bd	± 1.18
3.	Dzepciste	8.42 r	± 0.62
4.	Galate	5.84 b	± 0.36
5.	Zelino	6.96 c-m	± 0.37
6.	Pechkovo	6.73 om-pr	± 0.97
7.	Jegunovce	6.47 be	± 0.10
8.	Avtokomanda	7.14 d-ln	± 0.43
9.	Sopiste	6.85 bmpr	± 0.18
10.	Drachevo	4.89 a	± 0.37
11.	Saraj	5.93 bc	± 1.05
12.	Radishani	7.52 e-lr	± 0.81
13.	Vlae	6.49 b-p	± 0.33
14.	Glumovo	8.19 pr	± 0.17
15.	Dobroshane	6.6 bi	± 0.59
16.	Cheshinovo	7.18 d-ls	± 0.47
17.	Karbinci	6.49 bg	± 0.33
18.	Obleshevo	6.56 bh	± 0.17
19.	Lozovo	8.07 n-pr	± 0.57
20.	Mustafino	7.21 dlp	± 0.31

The intensity of the biosynthesis of phenolic compounds increases after infection of plants with pathogenic microorganisms. Therefore, the amount of certain phenolic compounds in the plant can be used as a biochemical parameter in the selection of plants resistant to fungi, bacteria and viruses.

Phenolic compounds are intensively synthesized and in mechanical damage to plants and damaged areas they build a protective film (with active condensation).

Content of total phenolic compounds at the second mowing

The content of the total phenolic compounds in alfalfa (*Medicago sativa* L.), collected from different localities of the territory of the Republic of Macedonia in the second mowing is presented in Figure 2 and Table 3.

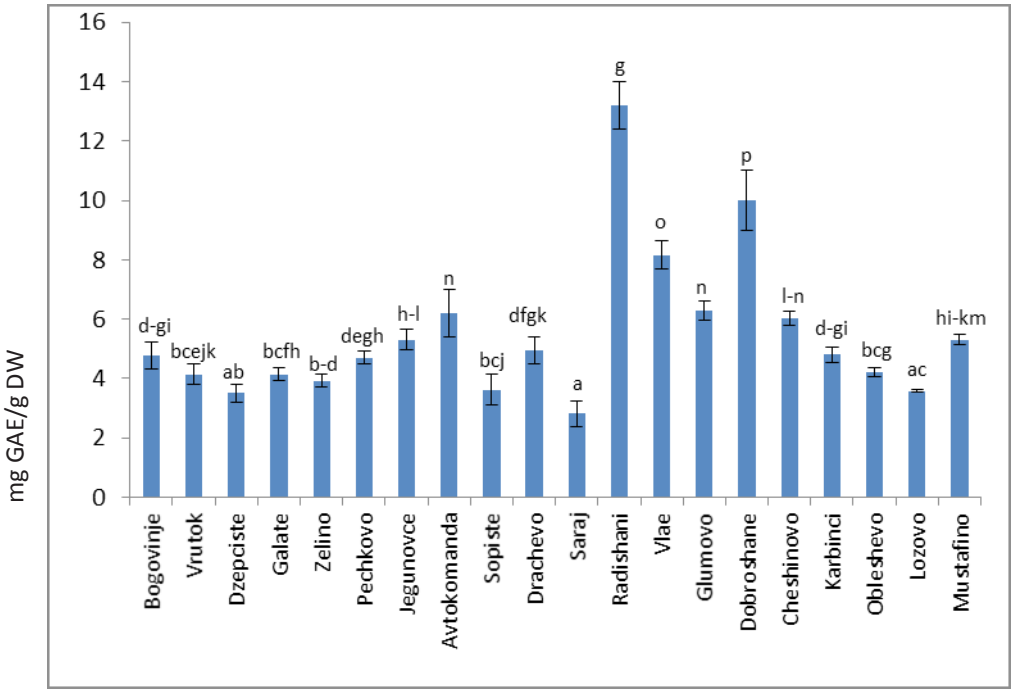

Figure 2. Content of total phenolic compounds in alfalfa (*Medicago sativa* L.), at certain localities on the territory of the Republic of Macedonia in the second mowing, expressed in mg GAE/g DW.
* If the small letters that are above of each column are the same there in no statistical difference, and if they are different there is a statistically significant differences.

Table 3. Content of total phenolic compounds in alfalfa (*Medicago sativa* L.), at certain localities on the territory of the Republic of Macedonia in the second mowing, expressed in mg GAE/g DW.

No.	Locations	Second mowing mg GAE/ g DW	Standard deviation
1.	Bogovinje	4.77 d-gi	± 0.46
2.	Vrutok	4.14 bcejk	± 0.34
3.	Dzepciste	3.51 ab	± 0.30
4.	Galate	4.14 bcfh	± 0.22
5.	Zelino	3.93 b-d	± 0.22
6.	Pechkovo	4.71 degh	± 0.21
7.	Jegunovce	5.31 h-l	± 0.34
8.	Avtokomanda	6.2 n	± 0.79
9.	Sopiste	3.63 bcj	± 0.53
10.	Drachevo	4.95 dfgk	± 0.46
11.	Saraj	2.82 a	± 0.43
12.	Radishani	13.21 q	± 0.80
13.	Vlae	8.16 o	± 0.46
14.	Glumovo	6.29 n	± 0.32
15.	Dobroshane	10.01 p	± 1.02
16.	Cheshinovo	6.04 l-n	± 0.23
17.	Karbinci	4.8 d-gi	± 0.25
18.	Obleshevo	4.21 bcg	± 0.16
19.	Lozovo	3.57 ac	± 0.04
20.	Mustafino	5.31 hi-km	± 0.16

The results show that the highest content of total phenolic compounds was found in the Skopje (Radishani) and the Ovche Pole region (Dobroshane), while the smallest quantity of measured phenolic compounds content was measured in the Skopje region (Saraj). The statistical analysis shows a significant difference between the amount of total phenols between the locations Radishani and Saraj ($p < 0.05$).

This data points to the fact that certain factors contributed to increased production of phenolic compounds in alfalfa grown in the Skopje region. As possible factors for increasing the total content of total phenols can be the following: altitude, external stress factors, climatic factors, etc.

The results of the authors, who analyzed the quantitative and qualitative phytochemical characteristics of plants, show different values. (Bystricka et al., 2010), reported that concentration and dynamics of the polyphenol synthesis in plant depends on the plant species, type of organs and growth stage. The results of some authors, who have comparatively analyzed the concentration of phenolic compounds in plant parts, support the fact that the highest concentration of phenolic compounds was found in leaves.

Content of total phenolic compounds at the third mowing

The obtained results, shown in Figure 3 and Table 4 are showing that there is a significant difference ($p<0.05$) between the Skopje region (Sopiste) and the Tetovo region (Vrutok) materials, taken in the third mowing.

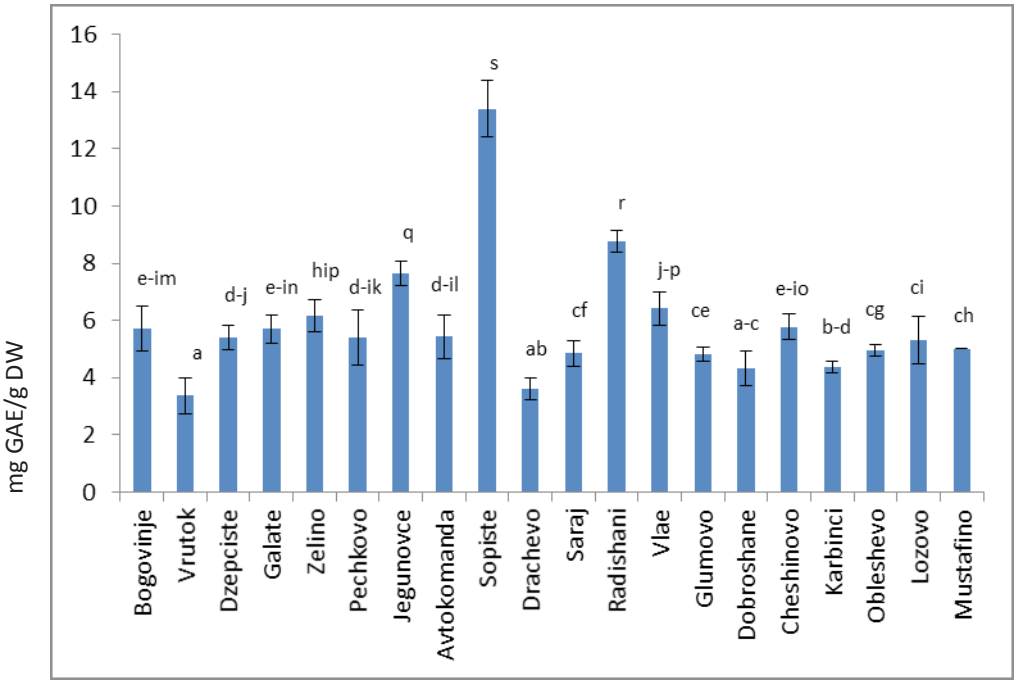

Figure 3. Content of total phenolic compounds in alfalfa (*Medicago sativa* L.), at certain localities on the territory of the Republic of Macedonia in the third mowing, expressed in mg GAE/g DW.

* IF the small letters that are above of each column are the same there in no statistical difference, and if there are different there is a statistically significant differences.

Table 4. Content of total phenolic compounds in alfalfa (*Medicago sativa* L.), at certain localities on the territory of the Republic of Macedonia in the second mowing, expressed in mg GAE/g DW.

No.	Locations	Third mowing mg GAE/ g DW	Standard deviation
1.	Bogovinje	5.7 e-im	± 0.78
2.	Vrutok	3.37 a	± 0.62
3.	Dzepciste	5.4 d-j	± 0.44
4.	Galate	5.71 e-in	± 0.49
5.	Zelino	6.15 hip	± 0.56
6.	Pechkovo	5.41 d-ik	± 0.97
7.	Jegunovce	7.64 q	± 0.43
8.	Avtokomanda	5.43 d-il	± 0.76
9.	Sopiste	13.4 s	± 0.99
10.	Drachevo	3.61 ab	± 0.38
11.	Saraj	4.85 cf	± 0.46
12.	Radishani	8.76 r	± 0.39
13.	Vlae	6.42 j-p	± 0.57
14.	Glumovo	4.8 ce	± 0.25
15.	Dobroshane	4.31 a-c	± 0.60
16.	Cheshinovo	5.78 e-io	± 0.45
17.	Karbinci	4.38 b-d	± 0.21
18.	Obleshevo	4.96 cg	± 0.20
19.	Lozovo	5.30 ci	± 0.83
20.	Mustafino	5.00 ch	± 0.01

The content of the phenolic compounds varies depending on the external conditions. Frequently there is a change in the content of the phenols, whose content is variable, depending on the eco-physiological conditions of the environment. The content may be increased even when it is necessary to absorb harmful ultraviolet radiation or when reducing the growth of surrounding competing plants (Koleva-Gudeva, 2010).

CONCLUDING REMERKS

Phenolic compounds are a large group of the secondary metabolites widespread in the plant kingdom. They are categorized into classes depending on their structure and subcategorized within each class according to the number and position of hydroxyl group and the presence of other substituents. The antioxidant properties of phenolics are mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers. Commonly, the in vitro methods often do not correlate with the ability of phenolic compounds to inhibit oxidative deterioration of foods. Phenolic compounds are counted in such compounds that represent specificity in plant cells (because they contain enzymes responsible for their biosynthesis). Depending on their structure and degree of polymerization, they participate in various biochemical processes that are important for photosynthesis, plant protection against fungi and viruses, mechanical damage, regulation of metabolism, etc. Phenolic compounds are used in plant production in the selection of plants resistant to the action of oxygen radicals and pathogens. In the first mowing, significantly increased content of phenolic compounds in the three regions in certain sites, Skopje, Tetovo and Ovche Pole is confirmed.

In the second mowing, the phenomenon of significance was observed between location Radishani and Saraj ($p < 0.05$). Significant difference regarding the content of total phenolic compounds was observed between the Skopje region at the Sopishte location in

the third mowing and the Tetovo region at the location Vrutok ($p < 0.05$). Within the three mowings, a significant increase in the content of the phenolic compounds has been determined in the first mowing in certain location in all three regions, in the second and the third mowings we have a significant difference between certain locations in the given regions.

The alfalfa in agricultural production has a huge impact and today it is the most important multiannual fodder culture in the intensification of fodder production, which is due primarily to the high productivity, quality and ability for strong regeneration. The listed properties of alfalfa not only give economic but also very high ecological significance, and it has been increasingly applied in sustainable organic production, bio nutritionism and dietetics.

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ОДРЕДУВАЊЕ НА ВКУПНИ ФЕНОЛИ ВО ЛУЦЕРКА (*Medicagosativa* L.) КОЛЕКЦИОНИРАНА ОД РАЗЛИЧНИ ЛОКАЛИТЕТИ ВО РЕПУБЛИКА МАКЕДОНИЈА

Валентина Бутлеска-Ѓороска[1]*, Марија Крстиќ[2], Ивана Јовановска-Клинчарска[2], Ана Цветановска[2], Ленка Цветановска[2], Лилјана Колева-Гудева[1]

[1]Земјоделски факултет, Универзитет „Гоце Делчев" - Штип, ул. „Крсте Мисирков" бр. 10-А, 2000 Штип, Република Македонија

[2]Природно-математички факултет - Скопје, Универзитет „Св. Кирил и Методиј", Република Македонија

[3]Ветеринарен факултет – Скопје, Универзитет „Св. Кирил и Методиј", Република Македонија

**Контакт автор: tina_valentina2@yahoo.com*

Резиме

Фенолните се секундарни биомолекули кои во својата структура содржат ароматичен прстен со еден или повеќе супституенти, а растенијата во својот секундарен метаболизам ги синтетизираат овие соединенија кои содржат една или повеќе фенолнни групи.

Фенолните соединенија учествуваат во биохемиски процеси кои се значајни за заштитата на растенијата од инфекции предизвикани од габи и вируси, механички оштетувања, регулација на метаболизмот и друго. Фенолните соединенија се оксидираат со фенол-оксидази до кинони, при што ослободуваат водород и така директно учествуваат во размената на материјата. Интензитетот на биосинтезата на фенолните соединенија се зголемува по инфекција на растенијата со патогени микроорганизми. Заради тоа и количината на вкупните фенолни во растенијата може да се користи како биохемиски параметар и индикатор во селекцијата на растенијата за отпорност на габи, бактерии и вируси. Фенолните соединенија интензивно се синтетизираат и при механичките повреди кај растенијата, при што на оштетените места градат заштитен филм со активна кондензација. На тој начин ја спречуваат деградацијата на клеточните ѕидови при инфекции предизвикани од некротрофните патогени, кои ги уништуваат клетките за да на изумрените ткива изградат свои колонии.

Во овој труд се презентирани резултати од истражувањата за одредувањето на вкупните феноли одредувани на три региони на Република Македонија на вкупно 20 различни локации. Содржината на вкупните феноли во сув растителен материјал од луцерка во три откоси е одредувана со рутинска метода по Folin-Chioclateau (1927). Квантитативното одредување на вкупните феноли во растителниот етанолен екстракт е вршено спектрофотометриски на бранова должина од 765 nm.

Клучни зборови: *секундарни биомолеули, спектрофотометрија, фенолни соедининија*