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ANTIOXIDANT ACTIYITY OF LEAF AND FRUIT EXTRACTS FROM *RUBUS FRUTICOSUS, RUBUS IDAEUS* AND *RUBUS LOGANOBACCUS* GROWING IN THE CONDITIONS OF THE REPUBLIC OF MOLDOVA

Alexandru MÎRZA

National Botanical Garden (Institute) "Alexandru Ciubotaru", 18, Pădurii Street., MD 2002, Chişinău, Republic of Moldova. Phone/Fax: + 37322550443, Email: alexandrumirza1@gmail.com

Corresponding author: alexandrumirza1@gmail.com

Abstract

The identification of alternative crops that require less water and produce high yields of organic matter is an important step towards a sustainable agriculture. This research was focused on determining the level of antioxidant activity of leaf and fruit extracts of some Rubus species, growing under the climatic conditions of the Republic of Moldova: Rubus fruticosus L., Rubus idaeus L. and Rubus loganobaccus L.H. Bailey. We evaluated the total phenol content (Folin-Ciocalteau assay): in vitro antioxidant capacity employing DPPH; ABTS methods and ferrozine test for iron chelating capacity. In leaf extracts, the total phenolic content ranged from 28.70 to 90.84 GAE/g DW and in fruit extracts – 13.97 to 45.08 GAE/g DW. In all assays, the leaf extracts of studied Rubus species showed the highest values of antioxidant activity (DPPH — IC_{50}) = 45.39 — 68.11 µg/ml; ABTS — 42.57 µM TE/g dry matter, iron chelating capacity — 53.06 %). A high correlation was found between the values for the total phenolic content and the antioxidant activity. Our results confirmed that leaf extracts of Rubus species can prevent activity of free radicals by scavenging or by inhibiting them.

***Key words*:** *antioxidant activity, fruit and leaf extracts, Rubus fruticosus, Rubus idaeus, Rubus loganobaccus, total phenols*

INTRODUCTION

In recent years, a lot of attention has been paid to improving the live quality and reducing the use of synthetic ingredients in food and strengthening health, which is why there has been an increase in the interest in fruits, which are rich in natural compounds, and are indispensable for a healthy diet, promoted by the World Health Organization (WHO). Healthy eating involves the daily consumption of fruits rich in antioxidants, of a phenolic nature and last but not least, those rich in various vitamins.

There has always been a permanent concern for horticulturists to mobilize and expand the fruit assortment to meet the demands of consumers for fresh fruits throughout the year, but also – of the food and pharmaceutical industry, regarding the creation of a diversified assortment of food and raw materials for obtaining various biologically active compounds for maintaining and strengthening human health [1, 2, 4, 7, 9].

The notions of free radicals or reactive oxygen species (ROS) are used to describe chemical compounds that contain one or more unpaired electrons, because of which, they are highly unstable and are capable of causing damage to other molecules by taking electrons from them in order to stabilize themselves. Free radicals are created during normal metabolic processes in the cells and play a dual role in the body, with both harmful and beneficial effects. Excess production of reactive oxygen species (ROS) and/or a decrease in antioxidant levels may lead to the tissue damage and different diseases. Oxidative stress has been linked to many human diseases, as either a cause or an effect. Antioxidants are substances that can prevent the damage caused by free radicals and it is considered that a sufficient intake of antioxidants can protect against many diseases. The human body produces several antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, which are able to neutralize many

types of free radicals. Antioxidants can act as free radical scavengers, by inhibiting their formation (e.g. by blocking activation of phagocytes) and preventing formation of OH and/or decomposition of lipid hydroperoxides, by repairing the caused damage or by any combination of the above. Recent studies suggest that the antioxidants derived from plant materials, such as vegetables, fruits etc., with free-radical scavenging properties, may have great therapeutic effects in diseases associated with oxidative stress, such as cancer, diabetes, neurodegenerative disease, cardiovascular diseases, arthritis and gastrointestinal diseases and may slow down the aging process. Many synthetic antioxidants have shown toxic and/or mutagenic effects, while plant-based remedies have usually fewer side effects than the synthetic drugs [4, 7, 11, 12, 22].

The genus *Rubus*, family *Rosaceae*, includes about 1500 species, 10 species of *Rubus* are found in the spontaneous flora of the Republic of Moldova: (*R. caesius* L., *R. canescens* DC., *R. constrictus* Lef. et P.J. Mull., *R. hirtus* Waldst. et Kit., *R. idaeus* L., *R. montanus* Libert ex Lej., *R. nessensis* Hall., *R. serpens* Weihe ex Lej. et. Court. *R. tereticaulis* P.J.Mull., *R. ulmifolius* Schott.)[21].

Species, cultivars, mutant forms and new hybrid populations of the genus *Rubus* can provide a source of healthy food and a valuable source of raw materials for the phytotherapeutic industry [1, 2, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 19, 20, 23, 24, 25].

Raspberry, *Rubus idaeus* L., began to be grown in England in the mid-1500s. It is a perennial, thorny shrub, 1-2 m tall, with straight stem, arched towards the top, with fine thorns, which are dense at the bottom and rare or even absent at the top. The leaves are compound, of 3 leaflets on the fertile branches or 7 leaflets on sterile branches, ovate lanceolate, with irregular incisions on the edge, on the underside of the leaf – whitish, because of the hairs. The inflorescences are located at the top of the branches of the previous year or at the axils of the leaves, with white flowers, pentamerous, the petals being equal in size to the sepals, of 1-1.4 cm, with numerous pistils and stamens. The fruits are red, juicy polydrupes with persistent calyx. It is found in clearings, on rocky sides, especially in deforestation zones or fellings on hills and mountains, which are invaded in 3-5 years. It is grown in individual households but also in agricultural plantations for fruit. Along with other berries, raspberries occupy 3100 ha in the Republic of Moldova.

Blackberry, *Rubus fruticosus* L., grows spontaneously in the forest areas, on the edges. The shoots at the top are canaliculate, long, slender. The leaves are quite varied, generally narrow, with elliptical terminal leaflets, ovate or obovate, often small, usually with an elongated tip, upper leaves ovate lanceolate. Lower leaflets - short petiolate. Inflorescence - simple racem, rarely panicle. The flowers are white or pale pink, rarely of a bright pink. It blooms in June-July, bears fruit in August.

Hybrid berry, *Rubus loganobaccus* L.H. Bailey. The original plant was selected from seedlings resulting from the hybridisation of the octoploid blackberry Aurora and a tetraploid raspberry, made in 1969 at the Scottish Horticultural Research Institute, Dundee, UK. The cultivar 'Aurora', which was bred at Corvallis, Oregon, served as the maternal parent. The pollen parent was also bred at the Scottish Horticultural Research Institute, a tetraploid raspberry, 626/67. The plants are characterized by vigorous and sturdy shoots, which, in young plants, are spreading, but in more mature plants – tend towards a more semi-erect habit. The spines are elliptical in shape, dense, and highly pigmented at their base and tip. The leaves are mostly deep green, but there is also some red pigmentation, especially in young leaves, around the margins of older leaves and in the petioles. The compound leaves usually have five leaflets, which are very slightly convex and have a distinct relief between the veins. Suckering in the true botanical sense does not occur in this species, but mature plants commonly produce from approximately 5 to 9 replacement canes from root-stock buds. The fruits are produced on fruiting lateral shoots of about 30 cm in length. They are usually deep red or purplish red and become deep purple when over-ripe, large and of long

conical shape. Tayberry fruits are darker than those of Loganberry and are purpler than those of Loganberry, which fruits are redder. The fruits appearance is glossy, with a slight downiness. The plug remains attached when separated from the plant. Ripening starts early and lasts over a long period.

This research was aimed at determining the level of antioxidant activity of leaf and fruit extracts from *Rubus fruticosus* L., *Rubus idaeus* L. and *Rubus loganobaccus* L.H. Bailey, grown under the climatic conditions of the Republic of Moldova.

MATERIALS AND METHODS

The leaves and fruits samples of *Rubus idaeus, Rubus fruticosus* and *Rubus loganobaccus* ′Tayberry Medana′ were collected from the experimental plot of the "Alexandru Ciubotaru" National Botanical Garden (Institute) Chişinău, Republic of Moldova, N 46°97′32.0″ latitude and E 28°88′77.4″ longitude. The collected plant products (leaves) were fixed by dehydration, then ground. They were dried under natural conditions, in the shade. As for the fruits, they were fresh (Figs. 1, 2, 3, 4, 5, 6, 7).

Fig. 1. *Rubus idaeus* with fruits
Source: Own photograph. Original.

Fig. 2. *Rubus idaeus* fruits
Source: Own photograph. Original.

Fig. 3. *Rubus fruticosus* leaves
Source: Own photograph. Original.

Fig. 4. *Rubus fruticosus* fruits
Source: Own photograph. Original.

Fig. 5. *Rubus loganobaccus* fruits
Source: Own photograph. Original.

Fig. 6. Tayberry with flowers and fruits
Source: Own photograph. Original.

Fig. 7. Tayberry with fruits
Source: Own photograph. Original.

The leaves and fruit extracts from *Rubus* sp. were extracted with 60% aqueous ethanol, at room temperature, after 30 min of permanent shaking, the extract was filtered through Whatman no. 2 filter paper by vacuum suction, using Buchner funnel. The procedure was repeated 6 times. The combined extracts were evaporated under reduced pressure to dryness at 40°C and stored at -4°C until analysis.

The total phenolic content of extracts was measured by employing the Folin-Ciocâlteu assay [18]. An aliquot of 250 µl of Folin-Ciocâlteu phenol reagent (10 x diluted), 50 µl of the extract, and 500 µl water, were mixed and for 1 min left to react.

Then *20%* Na_2CO_3 solution, (800 µl) was added and left to react for 2 h (the time is reduced to 30 min at 40^{o}C), and then the absorbance of the mixture was measured at 760 nm (the control was the solution without extract). The total phenolic content was indicate as mg gallic acid per gram of dry plant material.

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), which is stable one, was used for the determination of free radical-scavenging activity of the extracts. This radical at an average temperature of 23^{O}C colours the methanol in violet and in the presence of an antioxidant is reduced, producing an uncoloured solution. The use of DPPH provides an easy and rapid way to evaluate antioxidants. Sample stock solutions (1mg/ ml) were diluted to final concentration of 200, 100, 50, 25, 10, 5 and 1µg/ml in methanol.

At an equal volume (0.75ml) of methanolic solution of DPPH (1.5 ml, 20 mg/1), different concentrations of each extract were added. The solutions were kept for 15 min at about 23^{o}C, the absorbance was measured at 517 nm. As control solution served methanol. As negative control, methanol (0.75 ml), and DPPH solution (1.5 ml, 20mg/1) were used. The IC_{50} value was calculated graphically and it denoted the sample concentration, which was required to collect 50% of DPPH free radicals [3].

The antioxidant activity measurement by ABTS assay is a method based on the sample capacity to inhibit the $ABTS^+$ (2.2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) and as control serves Trolox (the standard antioxidant)[14]. The $ABTS^+$ is obtained as a result of potassium persulfate ($K_2S_2O_8$) reaction. To obtain this, 0.1 ml of $K_2S_2O_8$ (70mM) is put in to reaction with10 ml of ABTS 2mM and left in the darkness for about 14 h at 23^{o}C. By taking 1 ml of the previous solution and thinning it - out in 24 ml of ethanol, the working solution is obtained, the absorbance at λ = 734 nm must be 0.70±0.02.

The reaction is produced in the measuring cuvette by adding 10 µl of standard to 0.99 ml of $ABTS^+$ radical, as a result, the antioxidants present in the standard inhibit the radical, and reduce the absorbance, and the reduction process is in a quantitative relationship with the concentration of antioxidants present in the sample.

Meanwhile, was prepared a Trolox calibration curve for a concentration range of 2.5 - 30µM and the calculation of the concentration in Trolox equivalents (µM TEAC) was done by the insertion of the inhibition percentage acquired for the sample.

The method of Dinis et al. was used to evaluate the chelation of ferrous ions by the extracts [5].

Briefly, 50 µl of 2 mM FeCl was added to 60 µl of samples (10 mg/ml). The reaction was initiated by the addition of 200 µl of 5 mM ferrozine solution.

The reaction between the samples - 60 µl (10 mg/ml) and 50 µl of 2 mM FeCl was initiated by 200 µl of 5 mM ferrozine solution.

After what the mixture was shaken, kept at 23°C for 10 min, the absorbance of the solution was taken at 562 nm.

The formula [(A_0- A_S)/A_S] x 100, was used to calculate the percentage inhibition of ferrozne-Fe^{2+} complex formation, the absorbance of the control is marked by A_0, the extract standard as well. As a positive control EDTA was used.

Data were expressed as mean of three replicates and standard error (SE). Statistical significance ($P<0.05$) was evaluated by the Student's test. AII analyses were performed using GraphPad Prism; version 6.01, 2012.

RESULTS AND DISCUSSIONS

Phenolics are a class of secondary metabolites, which consist of one or more aromatic rings with variable degrees of hydroxylation, methoxylation and glycosylation, influencing the fruit colour, astringency and bitterness. Flavonoids, phenolic acids, tannins and stilbenes are the main categories of phenolic compounds found in the researched berries. The established total phenolic compound concentration in the examined *Rubus* extracts was in the range from 13.97 to 90.84 GAE/g DW. As a result of the research carried out, it was established that total phenolic compounds in the leaf extracts from the studied *Rubus* species varied from 28.70 to 90.84 GAE/g DW, but in fruit extracts – from 13.97 to 45.08 GAE/g DW (Figs. 8 and 9). A high index for the total phenolic compounds was found in the leaf and fruit extracts from *Rubus loganobaccus* 'Tayberry Medana'.

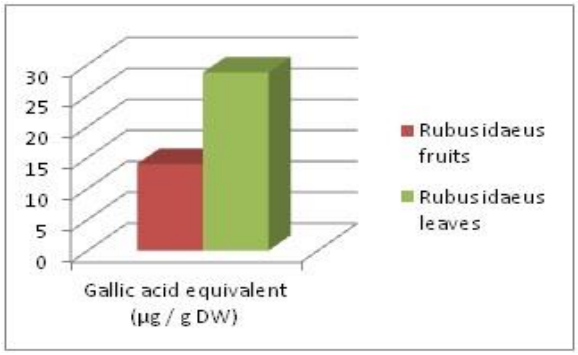

Fig. 8. The total content of phenolic compounds in *Rubus idaeus.*
Source: Own determination.

Many researchers have found a link between the structure of phenolic compounds and their antioxidant properties. The total phenol content of plants has been associated with their antioxidant activity due to their redox properties, acting as hydrogen donors and oxygen unpaired electron acceptors. Flavonoids have the ability to transfer electrons to free radicals, chelation of metal catalysts, activation of antioxidant enzymes and mitigation of oxidative stress caused by nitric oxide.

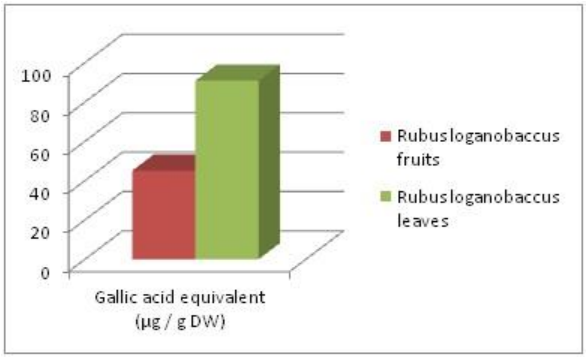

Fig. 9. The total content of phenolic compounds in *Rubus loganobaccus* 'Tayberry Medana'.
Source: Own determination.

Different assays are used to measure antioxidant capacity in foods and biological samples. Currently, the most commonly used methods for measuring antioxidant capacity are: 1,1-diphenyl-2- picrylhydrazyl (DPPH) assay, 2,20 -azino-bis-3- ethylbenzthiazoline-6-sulphonic acid (ABTS) assay, Iron chelating capacity.

Scientists consider that the effect of antioxidants on DPPH radical scavenging is due to their hydrogen-donating ability. In the specialized literature, it has been mentioned that DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. A commonly used parameter to measure the antioxidant activity is the IC_{50}, which stands for concentration of antioxidant needed to decrease the initial DPPH concentration by 50%. The higher antioxidant power has the extract, the lower value IC_{50} have.

All results on DPPH radical scavenging of *Rubus* species leaves and fruits extracts are shown in Figure 10, 11 and Table 1. In general, in all assays, the leaf extracts of

Rubus species showed higher values of antioxidant activity than fruit extracts. A high antioxidant activity was found in the leaf extracts of *Rubus fruticosus* and the fruit extracts of *Rubus loganobaccus* 'Tayberry Medana'. It was determined that, the extracts of the *Rubus loganobaccus* 'Tayberry Medana' showed a higher level of free-radical sequestering than the respective extracts of *Rubus idaeus.*

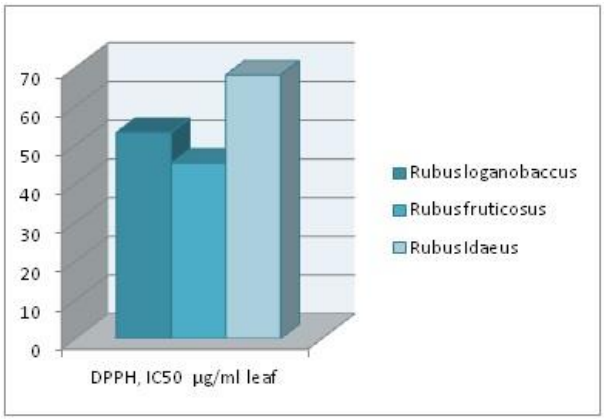

Fig. 10. The antioxidant activity of *Rubus* sp. leaf extracts, DPPH, IC_{50} µg/ml.
Source: Own determination.

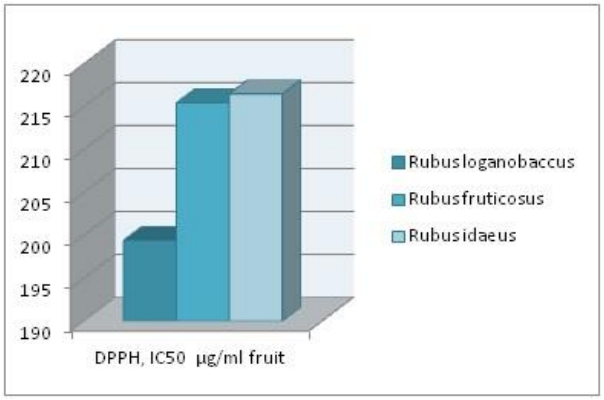

Fig. 11. The antioxidant activity of *Rubus* sp. fruit extracts, DPPH, IC_{50} µg/ml.
Source: Own determination.

The results for scavenging of ABTS radical represented in Figure 12, 13 and Table 1 were in the range from 1.50 ± 0.16 to 42.57 ± 0.45 µM TE/g DW. The ABTS method showed that the extract of hybrid berry (*Rubus loganobaccus*) leaves has the highest antioxidant properties (42.57 µM TE/g DW) and fruits (15.47 µM TE/g DW).

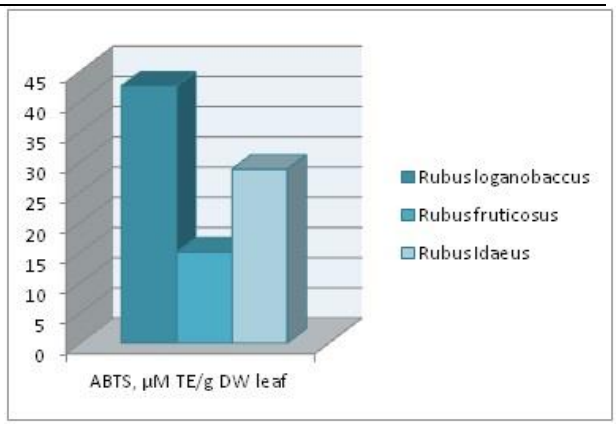

Fig. 12. The antioxidant activity of *Rubus* sp. leaf extracts, ABTS, µM TE/g DW.
Source: Own determination.

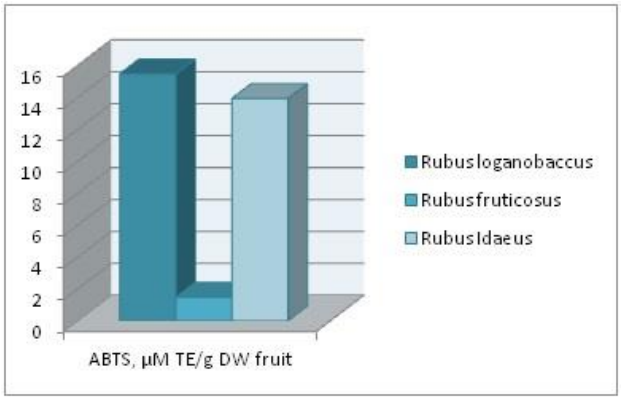

Fig. 13. The antioxidant activity of *Rubus* sp. fruit extracts, ABTS, µM TE/g DW.
Source: Own determination.

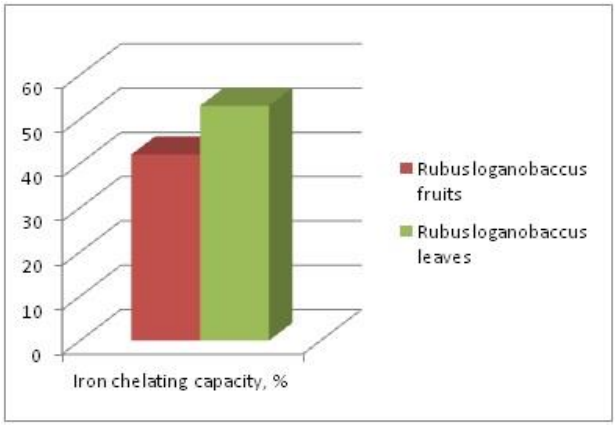

Fig. 14. The antioxidant activity of *Rubus loganobaccus* extracts, Iron chelating capacity, %.
Source: Own determination.

It was found that the iron chelating capacity of *Rubus loganobaccus* 'Tayberry Medana' extracts ranged from 53.06±2.15%, in leaf extract, to 42.07±2.56 (fig.14, tab.1) and was slightly lower in comparison with the Standard EDTA (99.98 ±0.19).

Table 1. The antioxidant activity of *Rubus* sp. extracts

Dried extract	DPPH, IC_{50} µg/ml	ABTS, µM TE/g DW	Iron chelating capacity, %
Rubus loganobaccus leaf	53.27±3.20[a]	42.57±0.45 [a]	53.06±2.15 [b]
Rubus loganobaccus fruit	199.39±2.16[a]	15.47±1.44[a]	42.07±2.56[a]
Rubus fruticosus leaf	45.39±0.94 [a]	15.10±0.96	
Rubus frutcosus fruit	215.44=1.54[b]	1.50±0.16	
Rubus idaeus leaf	68.10±0.74	28.79±0.43[b]	
Rubus idaeus fruit	216.50±0.39	13.93±0.20[c]	
Standard Gallic acid	1.50±0.3	-	-
Standard Trolox	5.28±0.9	-	-
Standard EDTA	-	-	99.98±0.19

[1]Mean of three replications ± standard error
[2]Means followed by the different small letters within a column denote significant differences (P<0.05)
Source: Own results.

Some authors mentioned various findings about phytochemical potentials of *Rubus* species. Ekbatan Hamadani et al. [6]. remarked that the total phenolic contents in *Rubus loganobaccus* leaves grown in the field were higher (66.63 ± 1.31 GAE/g) compared with those grown in the greenhouse (65.30 ± 2.56 mg GAE/g), the plants grown in the field contained higher amounts of flavonoids than those grown in the greenhouse (29.35 ± 8.53 and 22.44 ± 3.32 mg QE/g, respectively).

The amount of ascorbic acid of the field grown *Rubus loganobaccus* leaves were higher (EC_{50} - 2.82 ± 0.70 µg/mL) compared to those grown in the greenhouse (EC_{50} - 2.41 ± 0.75 µg/mL).

Veljkovic et al. [23] reported that the total phenolic compounds of wild raspberry, *Rubus idaeus*, leaf methanolic extracts ranged from 59.68 to 96.83 mg GA/g, the flavonoid concentration was 7.02-7.53 mg Ru/g, total tannins in the methanol extracts 0.73-1.27 mg/mL, anthocyanins 4.43 to 9.00 µg/L, antioxidant activity 110.17-199.18 µg/mL, inhibitory activity between 2.5-20.00 mg/mL.

Sharma & Kumar [16] reported that the reducing power of *Rubus ellipticus* fruits extracts was significant and fluctuate from 81% to 93% antioxidant activity. The measurement of antioxidant activity in the *Rubus ellipticus* ethanolic fruit extracts showed a DPPH radical scavenging ranging from 28.68% to 68.66%.

Zeidan & Oran [25] found that the *Rubus sanguineus* leaves ethanolic and methanolic extracts showed the highest DPPH activity, about 99% (at the concentration of 15 mg/ml). The ethanolic fruit extract of the same species showed a similar percentage, but the DPPH scavenging activity of methanolic extract were 95%.

The aqueous leaf extract was the lowest (83% DPPH activity) at 15 mg/ml in comparison to the organic extracts. The fruit extracts showed 90% DPPH activity, still less than that of the organic extracts.

According to the results obtained by Orhan et al. [11] "the total phenolic content in *Rubus sanctus* flower extract was 31.01 GAE/g extract, in leaf extract 26.27.01 GAE/g extract and lowest level was observed in shoot extract 25.54 mg GAE/g extract, the radical scavenging activity of studied extracts (DPPH inhibition) were 81.4%, 85.6%, 87.2% respectively".

Moon et al. [8] found that "*Rubus crataegifolius* fruit methanol extract showed strong antioxidant activity (75.04%, 50%), as compared with vitamin C (79.9%, 54.1%), by the DPPH and H_2O_2 method, respectively.

Zayova et al. [24] mentioned that, free radical scavenging activity was 38% higher in *Rubus loganobaccus* fruit extracts of *in vitro* propagated plants, compared with extracts of traditionally cultivated plants.

'Tayberry' cultivar of the same species showed similar trend for *in vitro* propagated plants regarding water-soluble antioxidant capacity of fruit extracts. Traditionally cultivated plants had WS-AOC 46% lower in their extracts.

In vitro cultivated plants of 'Tayberry' cultivar showed a higher content of total phenols by 23% in their fruit extracts compared to the content of fruits derived from traditionally cultivated plants. For the flavonoids the percentage was by 34% higher.

Studies conveyed by Ştefănuţ et al. [20] showed a content for the *Rubus fruticosus* fruit extracts as follows: anthocyanins -1,343 mg/L, phenolics - 3,284 mg GAE/L and antioxidant activity - 17.3 (µM TE/gFM)

Najda & Labuda [10] reported that *Rubus fruticosus* fruits had a content of total phenolic-101,947, anthocyanin contents - 38,021 and flavonoid contents - 4,291 per 100 grams of fruits. The values for antioxidant activity of fresh fruits for DPPH,

FRAP and ABTS were 1,293 μMTE/g, 971 μMTE/g and 517 μMTE/g respectively.

CONCLUSIONS

The results of our research indicate that all *Rubus* plant extracts exhibit a significant free radical scavenging activity.

The leaf extracts of *Rubus* sp. showed the highest values of antioxidant activity (DPPH — IC_{50}) = 45.39 — 68.11 μg/ml; ABTS — 42.57 μM TE/g dry matter, iron chelating capacity — 53.06 %).

This study suggests that *Rubus* sp. leaf extracts exhibit great potential for antioxidant activity and may be useful for medicinal purposes.

We concluded that *Rubus loganobaccus* ′Tayberry Medana′ is an important source of natural antioxidants, which might be helpful in preventing some negative effects caused by oxidative stress.

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