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ORIGINAL ARTICLE, PHARMACY

Antioxidant Activity of Dry Birch (*Betula Pendula*) Leaves Extract

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Background: Betula pendula is widespread in Europe and Asia. It has been used in traditional medicine since ancient times. Birch leaf extracts are known to exhibit a number of pharmacological activities. Antioxidant activity has also been reported.

Aim: The aim of this work was to investigate the antioxidant activity of a dry leaf extract from Betula pendula Roth.

Materials and methods: The total flavonoid content was determined. Some of the most commonly used methods were applied to evaluate the antioxidant capacity of the extract in vitro and in vivo. The ability of the extract to scavenge DPPH free radicals was evaluated by the method described by Brand-Williams with suitable modifications. ABTS decolorization assay was also applied. The in vivo assay was performed after acute and chronic administration of the extract into white albino rats, in a dose of 100 and 500 mg/kg bw. The antioxidant potential of the plasma was determined using FRAP reagent.

Results: A total flavonoid content of 42.5 mg/g was found, expressed as quercetin. The antioxidant activity against ABTS was concentration and time dependent. For example the concentration of 200 μg/ml led to 70.95% – 99.46% scavenging activity. DPPH scavenging activity was found to be about 98% at a concentration of 80 μg/ml. The extract possesses antioxidant potential, comparable with that of Trolox, in acute application. In chronic application, poorer results are observed, probably due to biotransformation and elimination processes.

Conclusion: Dried birch leaf extract has a relatively high antioxidant potential and could be used as a natural source of antioxidants.

BACKGROUND

Silver birch (*Betula alba, Betula pendula*, Betulaceae) comes from the temperate cold northern areas of Europe, Asia Minor, Caucasus and Siberia. The healing properties of birch extracts have been known since ancient times in the traditional medicine of different nations. Data has been found on the use of spirit and water extracts from birch bark, leaves and leaf buts. Preparations containing birch extracts were used in ancient Greece, Italy, France, Romania and other European countries. Nowadays, birch leaf extract is used in supportive therapy for bacterial infections of the urinary tract, kidney sand, and for increasing the amount of urine.[1]

The Committee on Herbal Medicinal products (EMA, 573240/2014), defines Birch leaf as the common name of the leaves of *Betula pendula*, Roth and/or *Betula pubescens*, Ehrh. or hybrids of both types. Leaves for medical use are obtained from both cultivated and wild trees. Products of birch leaf are obtained by grinding or shredding dry leaves or using dry and liquid extracts.

Birch leaf extracts are known to exhibit a number of pharmacological activities such as anticancer, antifungal, diuretic, antimicrobial, anti inflammatory etc.[2-4] Antioxidant activity has also been reported, mainly due to the terpenes and flavonoids contained.[3] In our previous studies various model extracts of birch leaf have been developed by varying the type of solvent and the extraction method. An HPLC method was developed for the simultaneous determination of routine, quercetin, hyperoside, betulin and betulic acid in the proposed extracts.[5] Dry extract was obtained by the method of spray-drying and its biological activity was investigated.[6] Many data have been published, showing the good antioxidant activity of the present in the extract pentacyclic triterpenes betulin and betulic acid as well as the

flavonoids rutin, quercetin and hyperoside.[7-9] This gives reason to suppose a high antioxidant activity for the dry birch leaf extract.

AIM

The aim of this work was to investigate the antioxidant activity of a dry leaf extract from *Betula pendula* Roth.

MATERIALS AND METHODS

All materials used in the research such as 2,2-diphenyl-1-picryl hydrazyl (DPPH), Folin-Ciocalteu's reagent, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), methanol, potassium persulfate, fluorescein, Trolox, ascorbic acid, and gallic acid are products of Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany and they are with analytical and pharmaceutical grade.

A number of *in vitro* and *in vivo* methods have been found in the literature to investigate the antioxidant activity of plant extracts and other compounds having an antioxidant effect. They are based on the ability of the antioxidants to deliver electrons, thus eliminating free radicals, reducing them to an inactive or less reactive form.[10] In order to fully characterize the antioxidant potential of the extract, several methods were applied.

Total phenolic compound analysis

Total phenolics were determined according to the method of Singleton & Rossi with Folin-Ciocalteu's reagent.[11] Gallic acid was employed as calibration standard and results were expressed as gallic acid equivalents (GAE) per litre.

DPPH (2,2-Diphenyl-1-picryl hydrazyl) method

The DPPH method is an electron transfer based antioxidant test that causes violet staining in methanol solution. The ability of the plant extract to reduce free radicals is determined against a stable 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical. DPPH reacts with the antioxidant compounds in the composition that act as a donor of hydrogen and lead to DPPH reduction. The change in colour (from deep violet to light yellow) is measured by UV-spectrophotometry.[12,13]

A 0.1M methanol solution of DPPH was prepared. 1 ml of the solution was added to 3 ml of the methanol solution of the test extract in different concentrations (0.5 – 200 μg/ml). Solution was shaken vigorously and stored at room temperature and in the dark for 30 minutes. The change in colour was measured by UV - spectrophotometry at wavelength λ = 517 nm. Ascorbic acid was used as a reference. Percent inhibition is calculated by the formula:

$$\% I = \frac{A0 - A1}{A0} \times 100$$

where:
A0 – absorption of the control (blank)
A1 – absorption in the presence of the test extract or reference (ascorbic acid)

All measurements were repeated three times and the results are presented as mean ± SD.

ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) method

The ABTS radical decolorization method is based on the reduction of ABST+ radicals induced by the antioxidant components in the plant extract. The mechanism of the reaction includes the ability of the antioxidants to donate an electron, which leads to decolorization of the radical.[14]

ABTS was initially dissolved in deionized water to a concentration of 7 mM. A solution of potassium persulfate ($K_2S_2O_8$) at a concentration of 2.45 mM was prepared. The solutions are mixed in a 1:1 ratio (v/v) and stored in the dark for 12 – 24 hours. ABTS solution was diluted to obtain an absorbance λ = 740 nm. 900 μl of the solution was mixed with 100 μl of the tested extract at a different concentration (50 – 800 μg/ml). Absorbance was measured at λ = 734 nm at the beginning of the experiment, at the second, fifteenth, and thirty minutes after mixing. All solutions were used on the day of their preparation. The reduction potency of the extract to the ABTS + radical was compared to that of ascorbic acid. The percent inhibition of the ABTS + radical is expressed by the formula:

$$I\% = (A_{t=0} - A_t)/ A_{t=0} \times 100$$

where:
I = $ABTS^{\bullet+}$ inhibition (%)
$A_{t=0}$ = absorbance of the sample at t = 0
A_t = absorbance of the sample at t = 5 min, t = 10 min, and t = 30 min.

All measurements were repeated three times and the results are presented as mean ± SD.

ORAC (Oxygen Radical Absorbance Capacity) assay

ORAC was measured according to the method of

Ou, Hampsch-Woodill & Prior (15) with some modifications described in details by Denev et al.[16] The method measures the antioxidant scavenging activity against peroxyl radical induced by 2,2'-azo-bis (2-amidinopropane) dihydrochloride (AAPH) at 37°C. Fluorescein (FL) was used as the fluorescent probe. The loss of fluorescence of FL was an indication of the extent of damage from its reaction with the peroxyl radical. The protective effect of the antioxidant was measured by assessing the area under the fluorescence decay curve (AUC) as compared to that of blank in which no antioxidant is present. Solutions of AAPH, fluorescein and Trolox were prepared in a phosphate buffer (75 mmol/l, pH 7.4). Samples were diluted in phosphate buffer as well. Reaction mixture (total volume 200 µl) contained FL – (170 µl, final concentration $5.36x10^{-8}$ mol/l), AAPH – (20 µl, final concentration 51.51 mmol/l), and sample – 10 µl. FL solution and sample were incubated at 37°C for 20 min, and AAPH (dissolved in 37°C buffer) was added. The mixture was incubated for 30 s before the initial fluorescence was measured. After that, the fluorescence readings were taken at the end of every cycle after shaking. For the blank, 10 µl of phosphate buffer was used instead of a sample. Antioxidant activity was expressed in Trolox equivalents. Trolox solutions (6.25; 12.5; 25; 50 and 100 µmol/l) were used for defining the standard curve. One ORAC unit is assigned to the net protection area, provided by a Trolox solution with concentration of 1 µmol/l. The final ORAC values were calculated using a regression equation between the Trolox concentration and the net area under the curve. Results were expressed as micromole Trolox equivalents per litre.

HORAC (Hydroxyl Radical Averting Capacity) assay

HORAC measures the metal-chelating activity of antioxidants under the conditions of Fenton-like reactions employing a Co(II) complex and hence the protecting ability against formation of hydroxyl radical.[17] Hydrogen peroxide solution of 0.55 mol/l was prepared in distilled water. 4.6 mmol/l Co(II) was prepared as follows: 15.7 mg of $CoF_2.4H_2O$ and 20 mg of picolinic acid were dissolved in 20 ml of distilled water. Fluorescein – 170 µl (60 nmol/l, final concentration) and 10 µl of sample were incubated at 37°C for 20 min directly in the FLUOstar plate reader. After incubation, 10 µl H_2O_2 (27.5 mmol/l, final concentration) and 10 µl of Co(II) (230 µmol/l, final concentration) solutions were added. The initial fluorescence was measured, after which the readings were taken every minute after shaking. For the blank sample, a phosphate buffer solution was used. 100, 200, 400, 500 and 600 µmol/l gallic acid solutions (in phosphate buffer 75 mmol/l, pH 7.4) were used for building the standard curve. The final HORAC values were calculated using a regression equation between gallic acid concentration and the net area under the curve. One HORAC unit was assigned to the net protection area provided by 1 µmol/l gallic acid and the activity of the sample is expressed as µmol gallic acid equivalents (GAE) per litre. ORAC and HORAC analyses were carried out using a FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany), excitation wavelength of 485 nm and emission wavelength of 520 nm were used.

FRAP (Ferric reducing ability of plasma)

Thirty-six white male Wistar rats were used (weight 200 – 250 g). Animals were grown under standard laboratory conditions with free access to food and water. They were divided into 4 groups of 9 animals each, as follows:

Group I - control group - saline (0.9% NaCl)

Group II - reference group - Trolox - 160 mg/kg bw

Group III - test group 1 - extract 100 mg/kg bw

Group IV - test group 2 - extract 500 mg/kg bw

The assay was performed in acute and chronic manner. In the chronic experiment, the test solutions were administered ones daily for 14 consecutive days by gavage, in equal volumes. On the 14th day test animals were decapitated and a blood collection was performed. The samples were centrifuged and blood plasma was collected to test the antioxidant activity. In the acute experiment, test animals were probed once and after 60 minutes were decapitated. Blood collection and blood plasma separation were performed.

The FRAP reagent was prepared as a 10:1:1 mixture (acetate buffer pH 3.6:10 mM TPTZ solution: 20 mM $FeCl_3.6H_2O$ solution). The solution of 2,4,6-tripyridyl-triazine (TPTZ 10 mM) was prepared by dissolving 31 mg of TPTZ in 10 ml of diluted 40 mM hydrochloric acid. Freshly prepared FRAP reagent was stored at 37°C for 10 min. 2 ml of FRAP reagent was mixed with 100 µl of the test plasma and 900 µl H_2O and kept for 30 min in the dark. The absorbance was measured at $\lambda = 593$ nm.

Animal testing was approved by the Bulgarian Food Safety Agency, licence No 147, valid until 05.08.2021.

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RESULTS AND DISCUSSION

DPPH method

The biologically active substances with antioxidant potential in the dry extract results in a reduction of the stable 2,2-diphenyl-1-picryl hydrazyl radical. Spectrophotometric analysis showed a decrease in the absorbance of the test samples with the increase of the concentration. Ascorbic acid was used for comparison, as a reference with high antioxidant potential. At low concentrations (from 0.04 to 5 μg/ml), the extract showed a tendency to increase the radical-reducing activity (%I), but with very slight changes. When the concentration increases above 5 μg/ml, a proportional increase in the reducing activity is observed, reaching its maximum of 97% at 80 μg/ml. For comparison, ascorbic acid reduces actively the stable radical at concentrations above 0.5 μg/ml, reaching its maximum reductive activity at concentration about 5 μg/ml. A linear relationship between the concentration of the applied substance and %I was observed, at certain concentration intervals: for the dry extract it is between 2.5 and 80 μg/ml ($y = 18.154x + 0.6359$, $R^2 = 0.9967$); for the ascorbic acid it is between 0,5 and 5 μg/ml ($y = 14.57x + 6.612$, $R^2 = 0.9952$) (**Fig. 1**).

ABTS method

ABTS radical scavenging assay was performed at extract concentrations of 10, 50, 100, 200, 400 and 800 μg/ml. Results show a similar trend, as the observed with the DPPH radical scavenging test. The anti-radical activity of the applied dry extract is concentration and time dependent (**Fig. 2**). The low concentration of 10 μg/ml resulted in antioxidant activity reaching 41.03%, after 30 min of reaction time. Increasing the concentration of the extract leads to an increase of the antioxidant activity. At concentration of 200 μg/ml it is 98.47% after 15 minutes and 99.46% after 30 minutes of reaction time. Concentrations of 400 and 800 μg/ml (not shown in **Figure 2**) show maximum anti-radical activity at the second minute of the reaction time (99%). The results are consistent with the data presented by Shalaby et al. in a study of plant extracts with similar polyphenol content.[18]

Total phenolic content, ORAC and HORAC methods

A number of methods have been developed to determine the antioxidant activity. Most of them are based on the generation of different radicals. In order to fully characterize the antioxidant potential of the test substances, it is advisable to evaluate it by several methods.[16,19] The selected methods cover different aspects of the antioxidant action and give a full picture of the antioxidant potential of the extract. Some of them assess the activity against various radicals ($ABTS^{\bullet+}$, $DPPH^{\bullet+}$, peroxy radical ($RXOO^{\bullet}$), etc.), while the HORAC method measures the metal chelating activity of the antioxidants in the composition in the Fenton-like reaction, therefore the protective ability against the formation of highly reactive hydroxyl radicals.

A number of authors have reported the relationship between polyphenol content and antioxidant activity of the extracts studied. **Table 1** presents the results of the polyphenol content of the tested dry extract and the antioxidant activity of the extract, measuring the ability of the extract to neutralize peroxyl radicals (ORAC) and its complex-forming ability (HORAC). The high polyphenol content found in the dry birch leaf extract is a prerequisite for a high antioxidant activity, with the ORAC and HORAC values expressed in Trolox equivalent and gallic acid equivalent per gram dry extract, being consistent with the data reported for different plant extracts with similar polyphenolic content.[20]

FRAP – method

After single and repeated administration of Trolox in dose 160 mg/kg bw the animals showed significant increase in plasma antioxidant activity, compared to the control group ($p<0,0001$). The rats repeatedly treated with both the extracts 100 mg/kg bw and 500 mg/kg bw increased significantly serum antioxidant activity compared with the control ($p<0.001$ and $p<0.0001$ respectively). After single administration the results were similar: the extracts in dose 100 mg/kg bw and 500 mg/kg bw increased antioxidant activity compared with saline with level of significance $p<0.0001$. The higher dose of the extract showed significantly higher antioxidant activity compared with the lower dose ($p<0.0001$) both after acute and chronic administration. In all experiments the antioxidant activity of Trolox was significantly higher compared with the two experimental groups treated with the extract in dose 100 mg/kg bw and 500 mg/kg bw ($p<0.0001$). (**Tables 2, 3**).

Higher values in the chronic Trolox treated group compared to the acute treatment are probably due to cumulative processes and increase in plasma concentration after repeated administration. However, in the groups treated with the studied extract the trend differs – higher values are observed in the

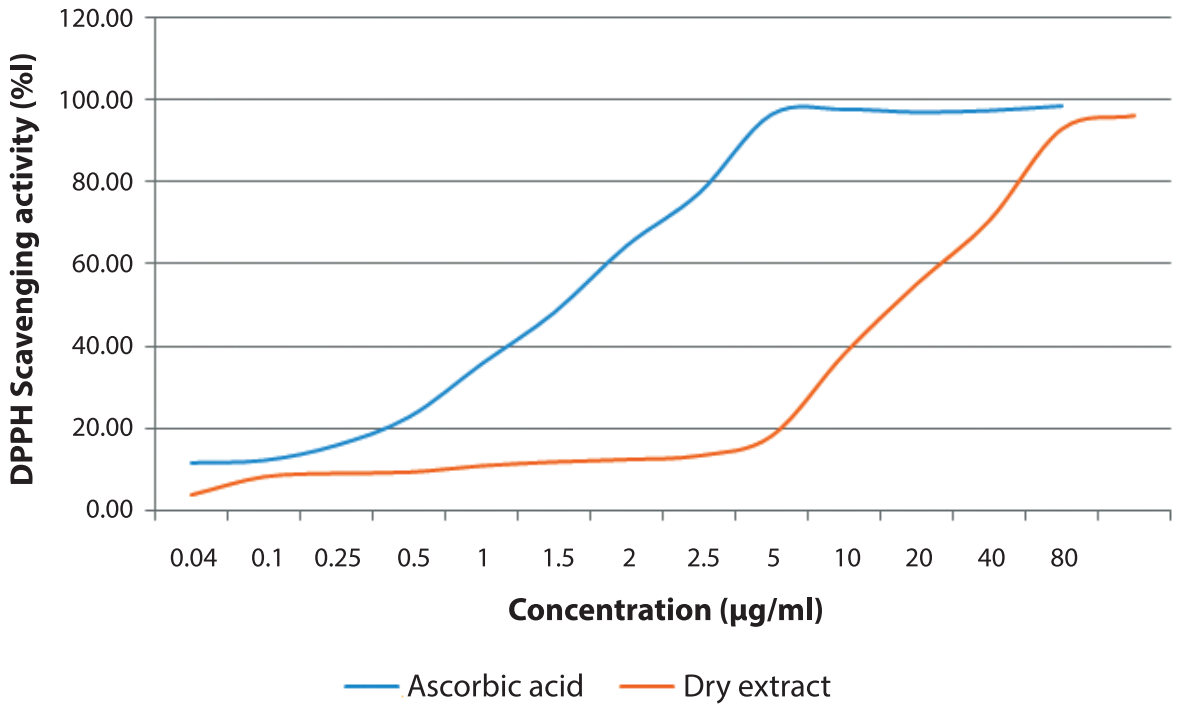

Figure 1. DPPH reducing activity of ascorbic acid and dry extract.

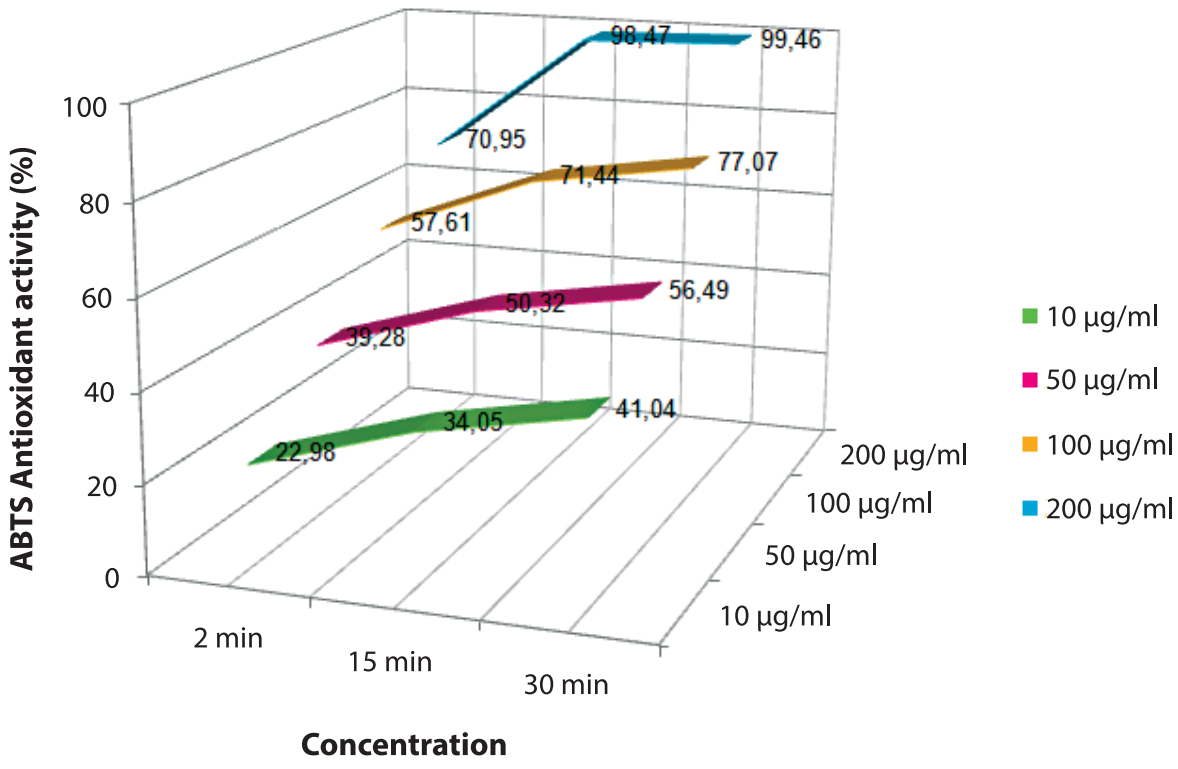

Figure 2. Antiradical activity of dry extract at concentrations of 10, 50, 100 and 200 µg/ml against ABTS.

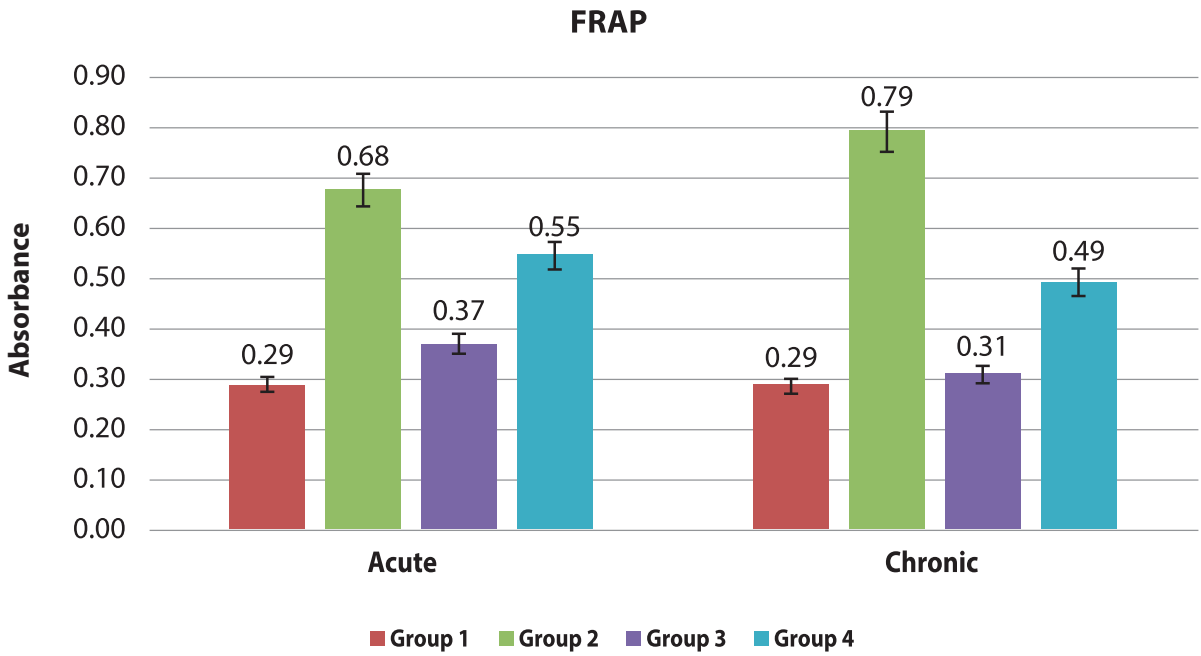

Figure 3. Ferric reducing ability of plasma in acute and chronic administration.

Table 1. Polyphenol content, values for ORAC and HORAC (Trolox equivalent (TE/g) and gallic acid equivalent (GAE/g))

Dry birch leaf extract	Polyphenols GAE mg /lg	ORAC µmol TE/g	HORAC µmol GAE/g
	128.45	1654.2	754.8

Table 2. Serum antioxidant activity after single administration of the extracts

Groups	N	Mean ± SD	t	P
Control	9	0.29 ± 0.009	99.714	<0.0001
Trolox 160 mg/kg bw	9	0.68 ± 0.008		
Control	9	0.29 ± 0.009	17.665	<0.0001
Extract 100 mg/kg bw	9	0.37 ± 0.012		
Control	9	0.29 ± 0.009	62.644	<0.0001
Extract 500 mg/kg bw	9	0.55 ± 0.009		
Trolox 160 mg/kg bw	9	0.68 ± 0.008	64.651	<0.0001
Extract 100 mg/kg bw	9	0.37 ± 0.012		
Trolox 160 mg/kg bw	9	0.68 ± 0.008	31.961	<0.0001
Extract 500 mg/kg bw	9	0.55 ± 0.009		
Extract 100 mg/kg bw	9	0.37 ± 0.012	36.239	<0.0001
Extract 500 mg/kg bw	9	0.55 ± 0.009		

Table 3. Serum antioxidant activity after repeated administration of the extracts

Groups	N	Mean ± SD	t	P
Control	9	0.29 ± 0.012	32.214	<0.0001
Trolox 160 mg kg bw	9	0.797 ± 0.046		
Control	9	0.29 ± 0.012	4.272	<0.0001
Extract 100 mg kg bw	9	0.31 ± 0.008		
Control	9	0.29 ± 0.012	39.915	<0.0001
Extract 500 mg kg bw	9	0.49 ± 0.01		
Trolox 160mgkg bw	9	0.797 ± 0.046	31.478	<0.0001
Extract 100 mg kg bw	9	0.31 ± 0.008		
Trolox 160mgkg bw	9	0.797 ± 0.046	19.401	<0.0001
Extract 500 mg kg bw	9	0.49 ± 0.01		
Extract 100 mg kg bw	9	0.31 ± 0.008	43.594	<0.0001
Extract 500 mg kg bw	9	0.49 ± 0.01		

acute treated groups compared to the chronically treated. This might be explained by the biotransformation and elimination processes taking place in the body, leading to a relatively rapid decrease in the concentration of biologically active substances, responsible for the antioxidant effect of the applied extract. These results confirm the suggestion that the development of a modified release formulation would be rational, in order to maintain a high plasma concentration over a longer period of time (6-8 hours).

CONCLUSIONS

Results from the study demonstrate the antioxidant capacity of the dry birch leaves extract. It exhibits a relatively strong antioxidant activity, therefore it can be used as a natural source of antioxidants, presenting the potential opportunity to prepare products with high value, helpful in preventing various oxidative stress related conditions.

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Антиоксидантная активность экстракта сухих берёзовых листьев (*Betula Pendula*)

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Ключевые слова: листья betula pendula, сухой экстракт, антиоксидантная активность

Введение: Betula pendula широко распространена в Европе и Азии. Применяется в народной медицине с древних времён. Известно, что экстракт берёзового листа обладает рядом фармакологических свойств. Сообщалось также об антиоксидантной активности.

Цель: Целью данного исследования было исследование антиоксидантной активности экстракта сухого берёзового листа (Betula pendula Roth).

Материалы и методы: Определено общее содержание флавоноидов. Некоторые из наиболее часто используемых методов были использованы для оценки антиоксидантной способности экстракта in vitro и in vivo. Способность экстракта захватывать свободные радикалы DPPH оценивали в соответствии со способом, описанным Brand-Williams, с необходимыми модификациями. Был также применен тест окрашивания АБТС (ABTS). Анализ in vivo проводили после острого и хронического применения экстракта на белых крысах-альбиносах в дозе 100 и 500 мг / кг массы тела. Антиоксидантный потенциал плазмы определяли реагентом FRAP.

Результаты: Общий флавоноидный состав составлял 42,5 мг / г, выраженный как кверцетин. Антиоксидантная активность АБТК была зависимой от концентрации и времени. Например, концентрация 200 мкг / мл приводила к 70,95% - 99,46% радионуклидной способности. Радионуклидная способность DPPH составляла около 98% при концентрации 80 мкг / мл. Экстракт обладает анти-

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оксидантным потенциалом, сравнимым с потенциалом Тролокса при остром применении. При хроническом применении наблюдались худшие результаты, вероятно, из-за процессов биологической трансформации и элиминации.

Вывод: Сухой экстракт берёзового листа обладает относительно высоким антиоксидантным потенциалом и может быть использован в качестве природного источника антиоксидантов.