Antioxidant Activity of Dry Birch (Betula Pendula) Leaves Extract

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Received: 29 Mar 2018
Accepted: 02 May 2018
Published Online: 31 July 2018
Published: 29 Nov 2018

Key words: Betula Pendula leaves, dry extract, antioxidant activity

Citation: Penkov D, Andonova V, Delev D, Kostadinov I, Kassarova M. Antioxidant activity of dry birch (Betula pendula) leaves extract. Folia Med (Plovdiv) 2018;60(4):571-9. doi: 10.2478/folmed-2018-0035

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.
flavonoids rutin, quercetin and hyperoside.\textsuperscript{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-sulfonic 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 \( \lambda = 517 \) nm. Ascorbic acid was used as a reference. Percent inhibition is calculated by the formula:

\[
\% I = \frac{A_0 - A_1}{A_0} \times 100
\]

where:

- \( A_0 \) – absorption of the control (blank)
- \( A_1 \) – 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-ETHYL BENZOTHIAZOLINE-6-SULPHONIC ACID) METHOD**

The ABTS radical decolorization method is based on the reduction of ABTS+ 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.\textsuperscript{14}

ABTS was initially dissolved in deionized water to a concentration of 7 mM. A solution of potassium persulfate (K\(_2\)S\(_2\)O\(_8\)) 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 \( \lambda = 740 \) 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 \( \lambda = 734 \) 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:

\[
I\% = \frac{A_{t=0} - A_t}{A_{t=0}} \times 100
\]

where:

- \( I \) = ABTS •+ 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...
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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 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 FeCl3.6H2O 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 H2O and kept for 30 min in the dark. The absorbance was measured at λ = 593 nm.

Animal testing was approved by the Bulgarian Food Safety Agency, licence No 147, valid until 05.08.2021.
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, R² = 0.9967); for the ascorbic acid it is between 0.5 and 5 μg/ml (y = 14.57x + 6.612, R² = 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 polyphenolic 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•⁺, DPPH•⁺, peroxy radical (RXOO•), 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 peroxy 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  | 99.714  | <0.0001 |
| Control                 | 9  | 0.29 ± 0.009  | 99.714  | <0.0001 |
| Extract 100 mg/kg bw    | 9  | 0.37 ± 0.012  | 17.665  | <0.0001 |
| Control                 | 9  | 0.29 ± 0.009  | 99.714  | <0.0001 |
| Extract 500 mg/kg bw    | 9  | 0.55 ± 0.009  | 62.644  | <0.0001 |
| Trolox 160 mg/kg bw     | 9  | 0.68 ± 0.008  | 62.644  | <0.0001 |
| Extract 100 mg/kg bw    | 9  | 0.37 ± 0.012  | 64.651  | <0.0001 |
| Trolox 160 mg/kg bw     | 9  | 0.68 ± 0.008  | 64.651  | <0.0001 |
| Extract 500 mg/kg bw    | 9  | 0.55 ± 0.009  | 31.961  | <0.0001 |
| Extract 100 mg/kg bw    | 9  | 0.37 ± 0.012  | 36.239  | <0.0001 |
| Extract 500 mg/kg bw    | 9  | 0.55 ± 0.009  | 36.239  | <0.0001 |
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 | 31.478 | <0.0001 |
| Control                 | 9  | 0.29 ± 0.012 | 39.915 | <0.0001 |
| Extract 100 mg kg bw    | 9  | 0.31 ± 0.008 | 4.272  | <0.0001 |
| Control                 | 9  | 0.29 ± 0.012 | 31.478 | <0.0001 |
| Extract 500 mg kg bw    | 9  | 0.49 ± 0.01  | 19.401 | <0.0001 |
| Trolox 160 mg kg bw     | 9  | 0.797 ± 0.046 | 31.478 | <0.0001 |
| Extract 100 mg kg bw    | 9  | 0.31 ± 0.008 | 43.594 | <0.0001 |
| Extract 500 mg kg bw    | 9  | 0.49 ± 0.01  | 19.401 | <0.0001 |

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.

ACKNOWLEDGMENTS

This work was supported by University Project SDP – 02/2016, Medical University of Plovdiv, Bulgaria.

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Антиоксидантная активность экстракта сухих берёзов листьев (Betula Pendula)

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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: Определенно общее содержание флавоноидов. Не которые 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). Анализы in vivo проводили после острого и хронического применения экстракта на белых крысах-альбино сах в дозе 100 и 500 мг / кг массы тела. Антиоксидантный потенциал плазмы определяли реагентом FRAP.

Результаты: Общий флавоноидный состав составил 42,5 мг / г, выраженный как кверцетин. Антиоксидантная активность АБТК была зависимой от концентрации и времени. Например, концентрация 200 мкг / мл привела к 70,95% - 99,46% радионуклидной способности. Радионуклидная способность DPPH составляла около 98% при концентрации 80 мкг / мл. Экстракт обладает анти-
Образец цитирования: Penkov D, Andonova V, Delev D, Kostadinov I, Kassarova M. Antioxidant activity of dry birch (Betula Pendula) leaves extract. Folia Med (Plovdiv) 2018;60(4):571-9. doi: 10.2478/folmed-2018-0035

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

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