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REGULAR ARTICLE

COMPARISON OF THE ANTIOXIDANT ACTIVITY OF THE DIFFERENT *BETULA PENDULA* ROTH. EXTRACTS FROM NORTHERN KAZAKHSTAN

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SUMMARY

The *in vitro* and *in vivo* antioxidant activities of ethanolic extracts of the vegetative organs of *Betula pendula* Roth. were studied by evaluating the ability to scavenge the DPPH radical, ABTS cation radical or hydrogen peroxide or by the metal chelation activity or reducing power. The phospholipid oxidation processes were studied using the acute hypoxia model. The changing proportion between acid and neutral phospholipids was used for the estimation of the influence of the birch extract on brain tissue. As an index of the pathological state the information entropy parameters were calculated. It has been found that the rats which had experienced ischemia have a decreased amount of acid PL in the homogenates. Preventive injection of birch extracts brings about normalization of PL level. This fact points out a possibility of applying extracts of *Betula Pendula Roth* in order to regulate the PL level of experimental rats as well as an estimation of the antioxidant effect of exogenous substances.

Key words: *Betula* vegetative organs, Antioxidant activity, Lipid peroxidation, Phosholipids

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1. Introduction

The genonymum *Betula*, family Betulaceae derives from the latin Beatus (blissful). More than 140 *Betula* species are known in the world [1-2]. The most common species in Kazakhstan is *Betula pendula* Roth. (BPR), also well known as the European white birch or silver birch and is mainly growing in the northern part of the country. Birch species have been used since ancient times in folk and traditional medicine in various forms [3]. The extensive data concerning the application of BPR in medicine, as well as the wide availability in good quantity have attracted the attention of scientists worldwide.

The medical applications of birch products are very extensive, because of a wide range of pharmacological and physiological actions [4]. Special attention was focused on medical properties of the birch bark with vascular, antiviral and antitumor activity [5-7]. The birch buds (*Gemmae Betulae*) are widely used as diuretic and cholagogue and as an antiseptic, wound healing agent [8]. The leaf tincture (*Folium betulae*) is commonly used for treatment of the anemia, cardiovascular and kidneys diseases [9]. The betula sap (*Succus Betulae*) assists in treating kidney, and urinary tract diseases, skin diseases, rheumatism and gout and is instrumental in the purification of the body. Birch juice is extracted in early spring from the trunk of birch and thanks to its natural properties it is outstanding in removing toxic substances from the body and it also has high antimicrobial, phagocytosis-influencing, antiphlogixtic and antipyretic activities [10]. *Oleum Betulae empyreumaticum rectificatum* is the oil obtained by the dry distillation of the bark

and wood of Betula and rectified by steam distillation. It is used mainly as an external remedy in cutaneous diseases and has on found many applications in cosmetics and related personal care products [11-12]. The unique properties are caused by the chemical composition of each species. Diverse phytochemical investigations of Betula species have shown that they contain mainly phenolics, flavonoids, tannins, saponins, glycosides, sterols and terpene derivatives [13-17]. The therapeutic benefit of medicinal plants is often attributed to their antioxidant properties [18].

The aim of this article was the study and comparison of antiradical and antioxidant activity of the different Betula organs (such as bines, bark, leaves and aglets) using next assays: 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{+}$) radical scavenging activities, reducing capacity, hydrogen peroxide scavenging activity, ferric ion reduction capacity by the *o*-phenanthroline colour method and determination of total flavonoid, total phenolic content using Folin-Ciocalteu's phenolic reagent. The *in vivo* study of the composition of the phospholipids (PL) level in brain tissue was used for the estimation the effects of some birch extracts (buds and aglets) on the lipid peroxidation process. PL, as universal components of cell membranes can be used as objective indices of the state of a normal or pathological organism [19]. The injection of antioxidant exogenous substances promotes breakage of the lipid oxidation chain with formation of more stable and less harmful radicals. The exogenous phenolic antioxidants play a very important role in the regulation of the organism antioxidant system processes. First of all, it is connected with its ability to control integrity and functional activity of important cellular structures, namely the membranes. It is necessary to note that phenolic compounds are the main reductants of the hydroxyl radical which is the most reactive product of the lipid oxidation. Hence, plant medical products and biologically active additives containing polyphenols in their structure are very effective against reactive oxygen spicies. The plant antioxidants possess a complex action mechanism and simultaneously a more soft influence on the organism: many synthetic antioxidant components have shown toxic and/or mutagenic effects, which have shifted the attention towards naturally occurring antioxidants.

2. Experimental

Plant material and chemicals: Birch tree parts were collected in the Northern Kazakhstan. The plant material was air dried in a shady and aerated room until the weight was stable. The ethanolic plant extracts were prepared according standard methods – about 50 g. of different vegetative organs BPR powders were partitioned by successive extraction with petroleum-ether, EtOAc, EtOH using a Soxhlet apparatus.
1,1-Diphenyl-2-picryl hydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), Folin-Ciocalteu's reagent and Trolox were obtained from Sigma Aldrich, Belgium. The other chemicals used were *o*-phenanthroline, ferric chloride, ascorbic acid, gallic acid, sodium carbonate, sodium hydroxide, hydrogen peroxide, potassium iodide, sodium fluoride, hydrochloric acid, iron alum, ammonium molybdate, sodium thiosulfate, sulfuric acid. All other chemicals and solvents used in the experiment were of analytical grade. The instruments used were UV spectrophotometer (Perkin Elmer Lamda 20).

Animals: Female mice of 1.5-month age (170-200 g.) were used. The animals were housed in plastic cages and maintained in ambient temperature and a normal light/dark cycle. They were fed standard laboratory food and water *ad libitum*. The experimental protocols were approved by the animal care. Animals were divided in the following groups: intact group; control group (the acute hypoxia sustained animals); ethanolic extracts of BPR. (aglets, buds), standard (Vitamin E): received *per os* 5 mg/kg body weight before acute hypoxia procedure for 20 days.

Acute hypoxia model: The acute hypoxia model was reproduced by asphyxia for 5 minutes; the air tube was intubated for

apparent death reanimation according to Korpachev assay [20].
The blood and tissues sampling was carried out one hour after the rehabilitation time. The tissue was perfused in situ immediately with 0.9% NaCl and thereafter removed, and was rinsed in chilled 0.15 M. phosphate buffer (pH 7.4). This was then blotted dry, weighed and homogenized in ice cold 0.15 M. phosphate buffer (pH 7.4) to yield a 10% w/v homogenate.

Determination of the PL in tissues: PL extraction was made according to the Folch assay [21]. The quantitative amount to the phosphor content in tissues was determined with malachite green reagent and calculated according to the literature [22]

Determination of total phenols: The total phenolic content of the extract was determined by the modified Folin-Ciocalteu method [23]. The extract (1 mg mL^{-1}) was mixed with 5 mL Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 4 mL (75 g L^{-1}) of sodium carbonate. The mixture was vortexed for 15 sec and allowed to stand for 30 min at 40°C for colour development. The absorbance was measured at 765 nm using a UV-VIS spectrophotometer. Samples of extract were evaluated at a final concentration of 1 mg mL^{-1}. Total phenolic content was expressed as mg mg^{-1} gallic acid equivalent (GAEC) using the equation obtained from the calibration curve: y = 10.25x+0.228; R^2 = 0.987.

Determination of total flavonoids: Total flavonoid was estimated using the literature method [24]. A 2% $AlCl_3$ ethanol solution (0.5 mL) was added to 0.5 mL of extract. After 60 min at room temperature, the absorbance was measured at 420 nm. The extract was evaluated at a final concentration of 1 mg·mL^{-1}. Total flavonoid content was calculated as quercetin equivalent (mg g^{-1}) (QEC) using the equation obtained from the calibration curve: y = 1.723x + 0.048 R^2 = 0.996

The DPPH assay: The radical scavenging capacities of BPR ethanolic extracts in concentrations (0,01-1,0 mg/ml) were estimated according to the method [25]. Aliquots of the sample (0.1 mL) extracts were added to 3 mL of a $6 \cdot 10^{-5}$mol/L DPPH· methanolic solution. The decrease in absorbance at room temperature was determined at 520 nm. The IC_{50} parameter was determined as the amount of ethanolic spice extract necessary to decrease the initial DPPH· concentration by 50%.

ABTS radical cation decolorization assay: To generate $ABTS^{+}$, the protocol according to Re et al. was used [26]. The 5 mL 14 mM $ABTS^{+}$ (0.0385 g. $ABTS^{+}$in 5 mL deionized water) and 5 mL potassium persulfate (0.0066 g. in 5 mL deionized water) were mixed together and stand in the dark for 12-16 h before use. To determine scavenging activity of different birch extracts, 10 µL of extract was added to 990 µL of $ABTS^{+}$ solution (adjusting the absorbance at 734 nm to 0.700±0.020 before use) and the decrease of A_{734} was recorded every 1 min until stable. The percent of scavenging activity at 1 min of reaction was calculated by the equation:

$$Inhibition\ of\ A_{734}(\%) = \frac{A_0 - A_t}{A_0} \cdot 100$$

where A_0 is the absorbance at t= 0 min and A_t is the absorbance measured after 1 min.

Reduction of ferric ions by the o-phenanthroline colour method: The reaction mixture of plant extract and 1.0 mL of *o*-phenanthroline stock solution (0.198 g of o-phenanthroline was dissolved with some heating in 30-40 mL of distilled water. To 0.2892 g of iron alum in other vessel was added 2.0 mL of 1.0 M hydrochloric acid and these mixture was dissolved in 30-40 mL of distilled water. Both solutions were combined and the total volume of complex solution was diluted to 100 mL. After 1 h 2.0 mL of 0.5 M of sodium fluoride were added. The absorption at 490 nm was measured after 30 min incubation at room temperature. The antioxidant activity values were obtained in ascorbic acid equivalents (mg/ml) from the calibration curve y =1.896x - $5 \cdot 10^{-2}$ (R^2 = 0.999) [27].

Reducing power assay: The different concentration of the extracts (0,01-2,0 mg/mL) in 1 mL of deionized water were mixed with phosphate buffer (2.5 mL, 0.2M, pH 6.6) and 1% potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL). The mixture was incubated at 50°C for 20 minutes. The reaction was stopped by adding trichloroacetic acid (2.5 mL, 10%) to the mixture, which was then centrifuged for at 1000 x g for 10 minutes. The centrifugate layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm [27]. Gallic acid and quercetin were taken as a reference.

Statistical analysis: The experimental results were expressed as mean standard deviation (SD) of three replicates. The *in vivo* experiment data were expressed using Student statistics for 15 replicates.

3. Results and Discussion

Many literature reports show a simple relationship between the content of phenolic compounds and the antioxidant capacity of plant extracts [28-29] allowing them to act as a reducing agent and a hydrogen donor in the two assays (DPPH, ABTS). Thus, phenolic compounds are major antioxidants in plants. The relatively low level of total phenols for some vegetative organs of BPR might account for the weak activity observed in the DPPH radical scavenging assay. The total flavonoid content was high compared to phenolic content (Table 1). A positive linear correlation was observed between the total phenol and flavonoid contents (R^2= 0.790) thus indicating a significant relationship between these parameters.

Table 1: Polyphenolic, flavonoid content and the radical scavenging activity of BPR vegetative organs. IC_{50} (mg/mL)

Compds	Total flavonoid[a]	Total phenol[b]	DPPH	ABTS
Buds	35.4±1.94	0.124±0.00	0.511±0.01	0.29±0.03
Aglets	51.2±0.15	0.150±0.01	0.034±0.01	0.18±0.02
Bines	76.1±2.05	0.214±0.04	0.026±0.01	0.20±0.04
Leaves	22.8±1.14	0.047±0.00	ND*	3.86±0.72
Bark	70.7±2.48	0.317±0.01	0.041±0.00	0.05±0.00

Data given are the mean of three replicates± standard derivations. [a] Expressed as mg quercetin g^{-1} of dry plant material, [b] Expressed as mg gallic acid mg^{-1} of dry plant material.), *ND: Not Determined

The free radical-scavenging activities of BPR extracts along with reference standards, such as Trolox, were determined by the DPPH radical method, and the results are shown in Table 2. The decrease in absorbance of the DPPH radical caused by the antioxidant was due to the scavenging of the radical by hydrogen donation. It is visually noticeable as a color change from purple to yellow. A lower value of IC_{50} indicates a higher antioxidant activity. Extracts obtained from birch bines registered the highest DPPH radical scavenging activity and the significant differences between them and the other samples are directly proportional to the total flavonoid concentration of the respective samples. The time depending scavenging activity of the silver birch species and the standard antioxidant Trolox was investigated. It was found that a 1 min reaction of bines and bark extracts has the similar RSA of the DPPH radical as Trolox. The lowest activity was observed for the leaves extract.

The scavenging effect of the $ABTS^{+\cdot}$ radical by several extracts of silver birch was determined. The highest value of the inhibition was observed at a concentration 0.5 mg/ml for all extracts, excepted aglets. The absorbance at 734 nm strongly decreased in the first minute of the reaction and then slightly decreased until stable. The bark extract showed a higher scavenging rate of $ABTS^{+\cdot}$. Among these bark extracts the ones derived of the completely removed free radical respectively 1 and 2

min after the start of the reaction. The percentage scavenging activity and IC_{50} of the investigated extracts at 1 min of the reaction time were calculated. The highest percentage activity at 91.73% (0.2 mg/ml) was found for bines extract. The lowest activity was again demonstrated for the birch leaves extract. The results of the IC_{50} parameter for ABTS radical scavenging activity are presented in Table 1.

The antioxidant action of a majority of bioactive substances is connected to their ability to easily oxidize. Coordination compounds of transition metals, such as iron or copper, molybdenum, rhenium could be used as indicator of the redox activity. For example: Fe (III) – pyridine-2,6 dicarboxylic acid, Cu (II)- neocuproine [30-32]. Addition of a *o*-phenanthroline solution to the investigated system lead to an increase of the redox potential of the $\mathbf{Fe(Phen)_3]^{3+}+\bar{e}\leftrightarrow Fe(Phen)_3]^{2+}}$ reaction. Several concentrations ranging from 0.25-5.0 mg/ml for the extracts of BPR were tested for their antioxidant activity. The change of antioxidant activity of the investigated extracts in ascorbic acid equivalent have been given in Fig.1. According to the assay used, *o*-phenanthroline forms a pink colored complex with Fe^{2+}, which gets disrupted in the presence of other chelating agents. The ethanolic extracts of the investigated plants interfered with the formation of the ferrous-*o*-phenanthroline complex, thereby suggesting that the extracts have metal chelating activity.

Fig. 1: The relative AOA in ascorbic acid equivalents

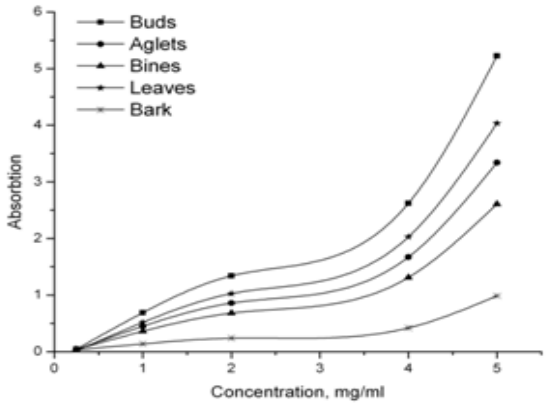
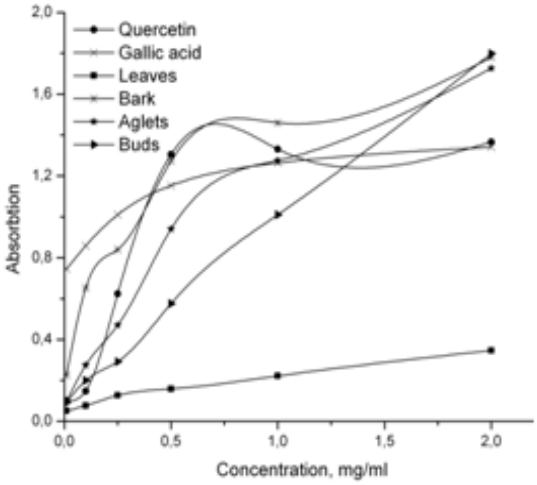


Fig. 2: Reducing power of BPR ethanolic extracts



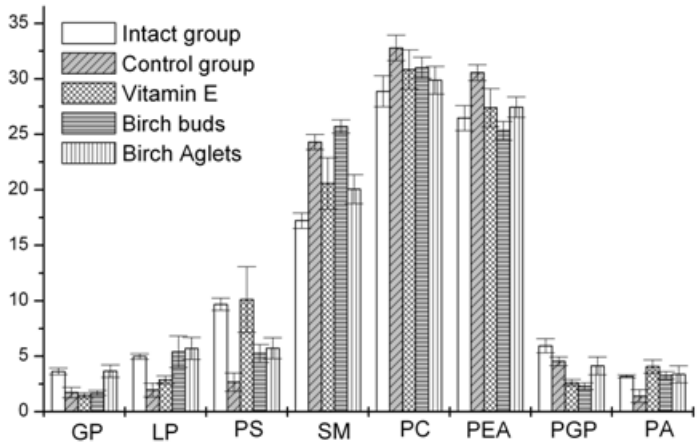
The FRAP (ferric reducing antioxidant power) assay is based on the formation of the colored complex of antioxidant and potassium ferricyanide, trichloroacetic acid and ferric chloride. The presence of polyphenolic compounds in the crude extracts is assumed because of their active participation as reducing agents for the ferric ions in our model system. The widely known antioxidants gallic acid and quercetin were used as standard compounds.

For the measurements of the reducing ability, the Fe^{3+}– Fe^{2+} transformation was investigated in the presence of the BPR extracts. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxide and prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [33]. Figure 2 depicts the reductive effect of BPR extracts. Similar to the antioxidant activity, the reducing power of the vegetation organs of the birch increased with increasing dosage. All the doses showed significantly higher activities than the control, exhibiting greater reducing power, indicating that the different vegetation organs of the silver birch contain of hydrophilic polyphenolic compounds that cause a greater reducing power.

Lipid oxidation is an old but still very important topic in food stability and in human health. Oxidative damage to cellular components such as lipids and cell membranes by free radicals and other reactive oxygen species is believed to be associated with the development of a range of degenerative diseases [34]. The changing nature of the quantitative composition of PL is an indication of the intensity of oxidative stress as well as the efficiency of exogenous plant antioxidants [35].

The antioxidant action of the buds extract of *Betula Pendula* Roth. was practically identical to standard antioxidant compound Vitamin E. The changing of the PL amount in the brain tissue demonstrates the influence dynamics of the plant extracts on the peroxidation processes. The analysis of the PL content in brain tissues is indicating that in the PL series the biggest fractions are SM, PC and PEA. The information about the allocation of the PL fractions is illustrated in diagram 1.

Diagram1: The changing of the PL fractions in brain tissue
GP−α- glycerophosphate; LP–lysophosphatidylcholine; PS−phosphatidyl serine; SM–sphingomyelin; PC–phosphatidylcholine; PEA−phosphatidyl ethanolamine; PGP–polyglycerophosphatids; PA−phosphatidic acids



In a group of experimental animals with acute hypoxia different dynamics of some PL fractions were observed. The SM fraction showed the clearest change of PL composition. Thus, the amount of SM in the intact group is 17.22±0.70 and in the control group 24.3±0.63 ($P<0.01$). In the experimental groups (birch aglets and birch buds) the same rate is significantly reduced. As result of lipid oxidation processes intensification, the PL spectra were depleted for the easily oxidizable fractions like PS, PGP and was concentrated for the SM and PS fractions, stable to oxidation. The composition of the PGP fraction has cardiolipin, which takes part in the activation of electron transfer enzymes at some stage of the breathing cycle. The decrease of the PGP in brain tissue confirms that acute hypoxia is accompanied by tissue respiration abnormality.

For a determination of the pathologic process tendency, the informational entropy was calculated (Table 2). The decrease of the informational entropy relative to the control group value is an indication of the stronger inhibition properties of plant samples.

Table 2: The informational entropy (IE) data for the POL processes in brain tissues

Groups	IE
Intact group	2,19
Control group	2,58
Vitamin E (standard)	2,40
Birch Buds	2,37
Birch Aglets	2,50

The analysis of the presented data confirms the increase of the IE as an indicator of pathologic process enhancement. The IE stature in the control group (without antioxidant addition) is a sign of the pathology of the animals with apparent death (acute hypoxia). In all experimental groups (with addition of birch extracts) the same indexes are lower than in the control group. The birch buds extract has the most favorable effect (comparable with the effect of the classic antioxidant – vitamin E) on the lipid peroxidation processes in brain tissue. It has been found that the rats which had experienced ischemia have a decreased amount of acid PL in the homogenates. Preventive injection of birch extracts brings about normalization of PL level. This fact points out a possibility of applying extracts of *Betula Pendula* Roth in order to regulate the PL level of experimental rats as well as an estimation of the antioxidant effect of exogenous substances.

4. Conclusions

The results of present study show hat from all vegetative organs of *Betula pendula* Roth growing in Northern Kazakhstan the bines extract has the highest amount of flavonoids, which exhibit the greatest antioxidant activity through the scavenging of DPPH radical. Bark extract has the greatest IC50 parameter for the scavenging activity of the $ABTS^{\cdot+}$ radical, that directly connected with content of total phenols in composition of the extract. The action of the birch buds on inhibition of lipid oxidation in tissues is comparable with effect of the standard antioxidant Vitamin E. It could be concluded that the different extracts of the silver birch vegetative organs through in vitro and in vivo experiments possesses antioxidant activity which might be helpful in preventing or slowing the progress of various oxidative stress related diseases.

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