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RESEARCH ARTICLE

Total Phenolics and Total Flavonoids, Nitrate Contents and Microbiological Tests in Dry Extract of Bulgarian White Birch Leaves (*Betula pendula*)

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Abstract

Recently, the interest in nutritional supplements plant based has increased. Plant extracts of the leaves of white birch (*Betula Pendula*) have shown the health-promoting properties. Leaves of birch (*Betula Pendula*) were collected during the period 2005-2008, with a view to their use as food additives and in pharmacy. The total phenolic and total flavonoid contents of dry extract of *Betula pendula* leaves were evaluated using the Folin-Ciocalteu methods and aluminum chloride colorimetric assay. The determination of nitrates in leaves of birch *Betula Pendula* has performed using HPLC method for nitrates in fruits and vegetables since a method of non-edible plants is not available. Microbiological contaminants were investigated using the International Pharmacopoeia methods. The following microbiological indicators were covered: total aerobic mesophyllic microorganisms (vegetative and spore forms), *Coloforms, Esherichia coli, Salmonella species, Staphylococcus aureus,* yeasts and moulds. Current ISO standard methods were used. The dried material from the foliage of *Betula Pendula* has shown a significant amount of phenols and flavonoids, and compliance with regulatory requirements for nitrates and microbiology.

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INTRODUCTION

In recent years, interest in plant-derived food additives has grown. Furthermore plant extracts of Bulgarian white birch (*Betula pendula*) leaves have been shown to possess health-promoting properties. The white birch leaves extract were strong diuretic and have effect at nephrolithiasis and urinary bladder lythiasos, sedative effect on spasms of smooth muscle. It might be used in following conditions: kidney diseases, ischia nerve inflammation and podagra and atherosclerosis and also it has an antimicrobial effect (Harbone J.B., 1993). The extracts from the leaves of the white birch significantly increase diuresis, and with this and the emission of sodium and chloride ions, ie act as salidiuretik. Until recently it was assumed that the diuretic action is due to the presence of resinous substances. Therefore birch buds were preferred because they are rich in resins. However, it is clear that flavonoids have a greater role in the diuretic effect. They are contained mainly in the leaves. In addition, the leaves contain potassium nitrate, which enhances the diuretic effect of the flavonoids. This effect was related to total flavonoids (Neoretal Monography, 2006). Nitrate pollution is probably caused by agricultural practices. An excess of nitrogen was applied in order to increase crop yield. Animal waste is released into the environment. Industrial processes and air pollution, especially from automobile exhaust, also contribute significantly to the nitrate pollution (Hallberg GR, 1989, Puckett LJ, 1995; Smil V, 1997; Vitousek PM. et al., 1997; Campbell WH, 1999). To balance the need for increased crop production with the control of pollution caused by underutilization of applied nutrients is an active area of research (Shaffer MJ et al., 1991; Matson PA et al., 1998; Pang XP et al., 1998; Campbell WH, 1999).

Nevertheless, nitrate/nitrogen and other nutrient pollution has become a major ecological problem worldwide (Smil V., 1997; Vitousek PM et al., 1997; Campbell WH, 1999). Nitrate, the major source for higher plants, is reduced to ammonium in a two-step process catalyzed by NR (nitrate reductase) and NiR (nitrite reductase). Whereas NR is a cytosolic enzyme, NiR is a nuclear-encoded chloroplast protein that is synthesized in the cytoplasm and imported info the plastid (Friemann A. et al., 1992). The appearance of nitrate assimilatory enzymes is regulated by the plant. In plants, the regulation of NiR expression shares some common features with that of NR, which is thought to be the key enzyme with respect to regulation of the nitrogen flux from nitrate to amino acids: both enzymes are highly regulated by nitrate and light (Friemann A. et al., 1992). Nitrate reductase (NR; EC 1.6.6.1-3) catalyzes NAD(P)H reduction of nitrate to nitrite. NADH-specific NR forms (EC 1.6.6.1) exist in higher plants and algae; NAD(P)H-bispecific forms (EC 1.6.6.2) are found in higher plants, algae, and fungi; and NADPH-specific forms (EC 1.6.6.3) are found in fungi. NR serves in plants, algae, and fungi as a central point for integration of metabolism by governing flux of reduced nitrogen by several regulatory mechanisms. NR catalyzes the first step of nitrate assimilation in all these organisms, which appears to be a rate-limiting process in acquisition of nitrogen in most cases. Since nitrate is the most significant source of nitrogen in crop plants, understanding the role of NR in higher plants has potential economic importance, especially in light of recent studies qualifying the enzyme as one focal point for integration of control of carbon and nitrogen metabolism (Campbell WH, 1999).

The accumulation of nitrate in some surface and groundwaters of the United States, Canada, and Europe had become a serious enough threat to human health and in most countries was adopted a Maximum Contaminant Limit (MCL) (Smil V, 1997; Campbell WH, 1999). The high concentrations of nitrate in drinking water and food caused methemoglobinemia or bluebaby syndrome, associated with the strong binding of nitrite to hemoglobin and oxidation of the iron center, which is more serious in infants, since fetal hemoglobin binds nitrite more strongly and can result in death (Campbell WH, 1999).

The coordinate appearance of the bispecific NAD(P)H-nitrate reductase (NR; EC 1.6.6.2) and nitrite reductase (NiR; EC 1.7.7.1) was investigated in leaves and roots from European white birch seedlings (Betula bendula Roth) (Friemann A. et al., 1992). Water extracts from the leaves of birch in vitro have shown virostatic and weak cytotoxic action (Petkov V., 1982). Phenolic compounds regularly increase in slowly growing stressed plants. Therefore, it is natural that phenolics also are sensitive to different forms of pollution (Loponen J et al., 1998). Total phenolics are secondary metabolites synthesized by plants, both during normal development (Harborne JB, 1982) and in response to stress conditions such as infection, wounding and UV radiation, among others (Beckman CH, (2000). On the basis of the number of phenol subunits, the modern classification forms two basic groups of phenolics – simple phenols and polyphenols. The group of simple phenols contains also the so called ¨phenolic acids¨ or phenols with carboxyl group underlying the specificity of their function (Harborne JB and Turner BL, 1984). Polyphenols contain at least two phenol rings.

Flavonoids are a subject of comprehensive studies in recent years. More than 4000 flavonoids have been identified in the different higher and lower plant species (Harborne JB and Turner BL, 1984). Total flavonoids are the largest class of polyphenols. They have a common structure with diphenyl propanes and consist two aromatic rings linked through three carbons (Saraf S et al., 2007). Flavonoids are nearly ubiquitous in plants and are recognized as the pigments, which are responsible for the colors of leaves, especially in autumn (Shahidi F and Naczk M, 2004). The distribution of the phenols in the tissues and cells of the leaves is not uniform (Naczk M and Shahidi F, 2006). Phenols have a wide spectrum of biochemical activities such as antioxidant activity, anti-mutagenic, anti-carcinogenic, and the ability to alter gene expression (Tapiero H et al., 2002); Nakamura Y et al., 2003). Numerous epidemiological studies have confirmed their significant correlation with decrease in cardiovascular and carcinogenic risk (Cook NC and Samman S, 1996). Formulation of preventive action and implementation of health effects requires information on total phenolic and total flavonoid composition in plants.

The aim of this study is focused on the determination of the total phenolic and flavonoid contents and total nitrates (2005-2008) and microbiological suitability (2008) in dry extract of Bulgarian white birch (*Betula Pendula*) leaves.

MATERIALS AND METHODS

Plant material

The study covers the period 2005-2008 in dry extract of Bulgaria birch (*Betula Pendula*) leaves. The sampling lasted one year according to the seasonality of harvesting for individual species. All samples data are stated in the sampling protocol. The birch leaves were picked up on May-September. Primarily have been used the youngest leaves because they are the most aromatic and tender. Samples were immediately dried in an oven-dried at 400 ° C or in air. The dried leaves were then ground to a powder and extracted in a Soxhlet extractor with ethanol for one hour. Then the extract was filtered, dried and stored in a dark and dry place until further analysis.

Chemical reagents

Gallic acid, (+)-catechin and Folin-Ciocalteu's phenol reagent were purchased from Sigma Chem. Co. All other chemicals were of analytical grade.

Sample preparation

For analysis of total phenolic and total flavonoid compounds the powered dry extract sample of 0.5 g was weighted and extracted with 50 ml 80% aqueous methanol on an ultrasonic bath for 20 min. An aliquot (2 mL) of the extracts was ultracentrifugated for 5 min at 14 000 rpm.

Determination of total phenolic

The total phenolic contents of the dry extract of white birch (*Betula pendula*) leaves were determined using the Folin-Ciocalteau assay. An aliquot (1 mL) of extracts or standard solution of gallic acid (20, 40, 60, 80 and 100 mg/L) was added to 25 mL volumetric flask, containing 9 mL of distilled deionised water (dd H_2O). A reagent blank using dd H_2O was prepared. One milliliter of Folin-Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 min, 10 ml of 7% Na_2CO_3 solution was added to the mixture. The solution was diluted to volume (25 mL) with dd H_2O and mixed. After incubation for 90 min at room temperature, the absorbance against prepared reagent blank was determined at 750 nm with an UV-VIS Spectrophotometer Lambda 5. Data of total phenolic contents of white birch leaves are expressed as milligrams of gallic acid equivalents (GAE) per 100 g dry weight (mg GAE/100 g dw). All samples were analyzed in triplicate (Marinova D et al., 2005; Christova-Bagdassarian VL et al, 2013).

Determination of total flavonoid

Total flavonoid content was measured by aluminum chloride colorimetric assay. An aliquot (1 mL) of extracts or standard solution of (+)-catechin (20, 40, 60, 80 and 100 mg/L) was added to 10 mL volumetric flask, containing 4 mL of distilled deionised water (dd H_2O). To the flask was added 0.3 mL 5% $NaNO_2$. After 5 min, 0.3 mL 10% $AlCl_3$ was added. At sixth minutes, 2 mL 1 M NaOH was added and the total volume was made up to 10 mL with dd H_2O. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm with an UV-VIS Spectrophotometer Lambda 5. Data of total flavonoid contents of white birch leaves are expressed as milligrams of (+)-catechin equivalents (CE) per 100 g dry weight (mg CE/100 g dw). All samples were analyzed in triplicate (Marinova D et al., 2005; Christova-Bagdassarian VL et al, 2013).

HPLC method for determination of nitrates (EN 12014-2:2001).

The method was based on European Standard specifies a high performance liquid chromatography (HPLC)/ion-exchange high performance liquid chromatography (IC) method for the determination of nitrate contents of vegetables and vegetable products. This method is applicable to nitrate contents in the range of 50 mg/kg to 3000 mg/kg.

Sample preparation

Powered dry extract sample of 10 g was weighted, nitrate compounds was extracted by water for 30 min on an water bath at 90°C and on an ultrasonic bath for 3 min, was ultracentrifuged for 5 min at 14 000 rpm. An aliquot of the extracts was filtrated through 0.45 µm filter.

Chemical reagents

$NaH_2PO_4.H_2O$; $NaHSO_4.H_2O$; $NaNO_3$ or KNO_3; H_3PO_4; NaOH were purchased from Sigma Chem. Co. All other chemicals were of analytical grade.

High-Performance Liquid Chromatography

The analysis were performed on a Hewlett-Packard chromatograph system (series 1050), equipped with a quaternary pump, and UV-Vis detector (Hewlett-Packard, series 1050) and column thermostat (Hewlett-Packard, series 1100). Data analyses were carried out using HP ChemStation software (Agilent Technologies). Separation was conducted on a RP Prevail C_{18}, 5 µm (150 x 4.6 mm i.d., Alltech) guard column. The solvent system was composed according to buffer (pH=3.3) of $NaH_2PO_4.H_2O/NaHSO_4.H_2O$ (0.06:0.0033, v/v) (eluent). The column was then returned to the initial conditions and equilibrated for 15 min. The flow rate was 0.5mL/min. The column temperature was set at 35°C. Injection volume was 20 µL. The detection was done at the wavelength of 210 nm. The LOQ was 0.5 mg/kg and the LOD was 2.5 mg/kg. The recovery was 94-100%.

Microbiology (microbiological contaminants)

Sample preparation

ISO 6887 – 4 Microbiology of food and animal feeding stuffs – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 4: specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products.

Microbiological examination

ISO 4833 Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of microorganisms – Colony-count technique at 30ºC.

ISO 4831 Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *coliforms* – Most probable number technique.

ISO 7251 Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of presumptive *Esherichia coli* - Most probable number technique.
ISO 6579 Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella spp*.
ISO 6888-3 Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) – Part 3: detection and MPN technique for low number.
ISO 7954 Microbiology of food and animal feeding stuffs – General guidance for enumeration of yeasts and moulds - Colony-count technique at 25ºC.

RESULTS AND DISCUSSION

The determined analytical parameters of the total phenolics method was as follows: limit of detection - 0.4 mg GAE/100 g dry weight; limit of determination – 1.2 mg GAE/100 g dry weight; recovery – 97% and reproducibility (RSD) – 2.7%. The determined analytical parameters of the total flavonoids method was as follows: limit of detection - 0.6 mg CE/100 g dry weight; limit of determination – 1.8 mg CE/100 g dry weight; recovery – 96% and reproducibility (RSD) – 3.7%. These results proved the viability of the both used methods to determine total phenol and total flavonoid compounds in dry extract of white birch (*Betula pendula*) leaves.
The determined analytical parameters of the nitrates method was as follows: limit of detection - 25 ng; limit of quantification – 5 ng, recovery – 94-100% and reproducibility (RSD) – 2.8%.
The results for total phenolics and total flavonoids, and nitrates in the studies white birch (*Betula pendula*) leaves are presented in Table 1.
The Table 1 presents the results of analyzes done to common phenols, flavonoids and total nitrates in different seasons over four years. Thus, the studies cover the period of the early spring and summer and mid-autumn to study in 2005. The content of total phenols was the highest in March and the lowest (5720 mg CE/100 g) in June. In flavonoids content remained low in June, but showed the highest value (2401 mg CE/100 g) in October. These interesting results have given us good reason to link changes in the levels of biologically active substances examined the power of sunshine. Since our samples are few in our study there will be some inaccuracies. To avoid this next year 2006 analyzed over three consecutive months when the sun is in your power. Those are: July, August and September, and two determinations were made for the month of November, which is late autumn. The variation of total phenols in the summer months is greater than the total flavonoids. This is probably due to the extremely large range of phenolic compounds. In late autumn were recorded lower values for total phenolics compared to the summer months. Strong impression extremely low values in autumn, to wit for November, which are: 821 mg CE/100 g for flavonoids.
The study of the samples during the winter months (from December to February) of 2007 and 2008 showed similar values for the total phenols and the content of the total flavonoids. Here the levels of the flavonoids is the highest compared to all other samples tested. Does low temperature factors at this stage we are too hard to explain. In the literature there is ample evidence for the protective role of the flavonoids against the various external influences (Cook NC and Samman S, 1996; Beckman CH, 2000; Tapiero H et al., 2002; Nakamura Y et al., 2003; Shahidi F and Naczk M, 2004; Naczk M and Shahidi F, 2006; Saraf S et al., 2007).
Analysis of samples at the same time (May 2008) shows significant differences in the content of total phenols, as its levels of total flavonoids. These data allow us to emphasize the crucial importance of the variability of biologically active substances, which is reflected in our results.
In the United States, the Maximum Contaminant Limits (MCLs) for nitrate as 10 ppm nitrate-N and nitrite as 1 ppm nitrite-N were set by the Clean Water and Safe Drinking Water Acts of 1974. The linkage between other human health risks such as cancer and long-term exposure to high nitrate concentrations in drinking water and foods also are not well enough documented to justify further restriction of the nitrate Maximum Contaminant Limit (MCL) (Campbell WH, 1999). In the legislation are not established standards for nitrates in dried leaves of white birch (*Betula pendula*), but the data can be attributed to the limitations of nitrates in leafy vegetables - lettuce or spinach in Ordinance 31/2004 of the Ministry of Health for setting maximum levels for contaminants in foodstuff. This regulation set maximum levels for nitrates in leafy vegetables - fresh spinach 2500-3000 mg/kg depending on the season and fresh lettuce - 2500-4500 mg/kg depending on the season and method of manufacture - in the field or in the greenhouse. Data obtained for dried birch leaves showed variations for different seasons and different years in a wide range - 151-2590 mg/kg. The highest values were found in August 2006, while the lowest was in May 2008. As apart from the peaks, in winter there is usually a tendency to lower levels, which correlates with the requirements of Ordinance 31/2004 (Ordinance № 31/29.07.2004 of Ministry of Health of Bulgaria) to lower levels of nitrates in leafy vegetables in this season. Low values also show that regardless of processing (drying), which can be reason for concentrations of nitrate due to loss of moisture, the content in Bulgarian white birch leaves is in accordance with

Ordinance № 31/2004 (Ordinance № 31/29.07.2004 of Ministry of Health of Bulgaria). Nitrate content in white birch (*Betula pendula*) leaves are safe for consumers, but is likely to have some impact on the diuretic effect of flavonoids.

Table 1. Total phenolics, total flavonoids and nitrates in white birch (*Betula pendula*) leaves

Year of samples	Total phenolics, mg CE/100 g	Total flavonoids, mg CE/100 g	Nitrates, mg/1000 g
March 2005	9259 ± 12	1509 ± 7	1342 ± 7
June 2005	5720 ± 13	702 ± 4	2062 ± 11
October 2005	5998 ± 9	2401 ± 9	1247 ± 10
July 2006	5257 ± 11	2245 ± 6	1020 ± 11
August 2006	9739 ± 12	1989 ± 7	2590 ± 11
September 2006	8513 ± 12	2663 ± 11	810 ± 7
November 2006	5333 ± 13– (**A**)	821 ± 7– (**A**)	582 ± 7 – (**A**)
October 2006	6186 ± 13– (**B**)	821 ± 7–(**B**)	786 ± 6– (**B**)
December 2007	10340± 12	8710 ± 12	1256 ± 7
February 2008	10740 ± 12	8110 ± 12	444 ± 6
May. 2008	5540 ± 14– (**I**)	3430 ± 13– (**I**)	306 ± 7 – (**I**)
May 2008	10340 ± 12 – (**II**)	8340 ± 12 – (**II**)	151 ± 7 – (**II**)

In the Table 2 are shown the microbial limits or the absence of specified microorganisms in extracts of white birch leaves (Betula pendula). The total aerobic microbial count and the total yeast and mould count (presented as colony-forming units per gram CFU/g of dry material), the absence of Staphyloccous aureus, Esherichia coli and Gram-negative bacterial species have been used as indicators of microbiological quality. Microbial count is just of white birch leaves quality indicators. The extracts of Betula pendula must be clear of bacterial pathogens such as Salmonella species.

Provided samples for microbiological analysis of dry birch leaves extract, free of pathogenic and indicator microorganisms and microbiological indicators researched the requirements of Ph. Eur. III, section 5.1.4. for medicinal products for oral use (for human).

Table 2. Microbiological tests of white birch (*Betula pendula*) leaves

Year of samples	Aerobic bacteria: Total plate count, CfU/g	Coliforms *Esherichia coli* MPN /1 g	*Pseudomonas aeruginosa*	*Salmonella species* in 25.0 g	*Staphyloccous aureus*	Yeasts CfU/g	Moulds CfU/g
2008	< 10	< 0.30	Absent in 1.0 g	Absent in 25.0 g	Absent in 1.0 g	< 10	< 10

CONCLUSIONS

Summarized data about the content of total phenols, flavonoids, nitrates and microbiological contamination of dried leaves of white birch are presented. Extracts of the studied material are required for use for the production of medicines and herbal supplements by Bulgarian pharmaceutical industry. The data show that the extracts of the leaves of white birch have some content as a total phenols and flavonoids. The levels of these biologically active substances demonstrated dependence on the season, weather conditions typical for the region and last but not least - the biological variability. Forthcoming in-depth study of individual flavonoid representatives would provide a more accurate characterization of the biological activity of the product, given their use as food additives and in pharmacy. Nitrate levels are within acceptable limits. The studies reveal the absence of pathogenic microorganisms, mold and fungi in the preparation of materials for medicinal use. The lack of a tendency to microbiological contamination allows to prepare medicines by these extracts for oral human use.

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