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ANTIOXIDANT AND CYTOTOXIC ACTIVITY AND POLYPHENOLS ANALYSIS USING HPLC-DAD - MS/MS OF *BETULA RADDEANA* GROWING IN GEORGIA

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ABSTRACT

According to modern pharmacological study the genus Betula has choleretic, diuretic, antibacterial, antifungal, antioxidant and anti-inflammatory effects. Species of the genus Betula that grow in Georgia are not sufficiently studied from the point of view of Pharmacognosy. The qualitative and quantitative composition and activity of the polyphenols sum, their factions and individual substances is not determined. It should be noted that there is great interest among scientists in the search for natural compounds with antioxidant and cytotoxic effect. Studies have shown that the main pharmacological a role of polyphenols is in the treatment of cardiovascular diseases and malignant tumors. Based on the presented above, the authors have carried out the fractionation of polyphenols of *Betula raddeana* using solvents of different polarity by liquid-liquid extraction. The cytotoxic and antioxidant effects of the methanol extract and the organic fractions were studied *in vitro* with the DPPH and WST-1 reagent respectively. Methanol extract (LC50 8.5 ± 0.1 μg / ml) and ethyl acetate fractions (LC50 9.8 ± 0.1 μg / ml) have a high cytotoxic activity. Their antioxidant activity is (IC50 0.29 ± 0.02 mg / ml) and (IC50 0.34 ± 0.02 mg / ml), respectively. This is due to a high content of polyphenolic compounds.

KEYWORDS: Betula, cytotoxic and antioxidant action, polyphenol, LC-DAD-MS/MS.

INTRODUCTION

The genus *Betula* of the family *Betulaceae*, has a wide distribution in the northern hemisphere from Canada to Japan. *Betula* can be deciduous trees or shrubs, usually colouring well in autumn and often with striking white, pink, or peeling brown bark; separate male and female catkins open before or with the leaves in spring.

Among the woody threatened species of the Red list of Georgia genus *Betula* is represented by three species *Betula medwedewii Regel, Betula megrelica Sosn., Betula raddeana Trautv.*[2] All three species are stated as vulnerable and were included in the Red List of Georgia, issued in 2006 on the basis of assessments made earlier and conferring to the requirements of IUCN Red List Categories and Criteria, Version 3.1, 2001, and IUCN Guidelines for National and Regional Red Lists, 2003.[13,14]

The Birch tree has a long history of medicinal use in different countries and cultures to cure skin diseases especially eczema, infections, inflammations, rheumatism and urinary disorders.

The medical applications of birch products are very extensive, because of a wide range of pharmacological and physiological actions.[5,11] Special attention was focused on medical properties of the birch bark with vascular, antiviral and antitumor activity.[6] The birch buds (*Gemmae Betulae*) are widely used as diuretic and cholagogue and as an antiseptic, wound healing agent.[9]

Diverse phytochemical investigations of Betula species have shown that they contain mainly phenolics, flavonoids, tannins, saponins, glycosides, sterols and terpene derivatives.[3,4,10,15] The therapeutic benefit of medicinal plants is often attributed to their antioxidant properties.[1,8,17]

This present work covers the flavonoids chemistry as well as biological activities of the main components isolated from *Betula raddeana* investigated by our group.

Plant Description

B. raddeana grows from the lower part of the subalpine belt (in pine, beech and mixed forests) to the mountainous forest belt (in birch forests together with *B. litwinowii* at altitudes of 1500-2500 meters above sea level. It also forms pure stands. *B. raddeana is* rare plant that has a significant range, but a small population, occurs sporadically, endemic to the Caucasus.

A small tree height of 7-8 meters. The bark of the trunk is white or pinkish, the branches are thin, dark brownish, with lentils. Young one-year-old branches are yellowish-brown, with whitish velvety pubescence.

Leaves 3–4.5 cm long, ovate or oblong-ovate, with a pointed tip and a wedge-shaped or rounded base. The edge of the sheet is unequally sharp-toothed. Above the leaf blade is bare, green, lighter below, with pubescence along the veins and in their corners. Petioles about a centimeter long, densely pubescent.

Anther earrings thin, cylindrical, 4-6 cm long, assembled in brushes of 2-3 pieces. The pistillate catkins are solitary, ovate-elliptical, 2-2.5 cm long.

The fruit is an obovate nut, about 3 mm long, pubescent at the apex, with wide wings that are almost equal in width to the nut.[7]

EXPERIMENTAL

Materials. The leaves of *Betula raddeana* Trautv. were collected in Georgia in april 2010 and identified by Dr. Tsiala Gviniashvili, a botanist from the Institute of Botany. Voucher specimens N 9527 were deposited in the Herbarium at the Department of Pharmacognosy and Botany, Faculty of Pharmacy, Tbilisi State Medical University. 1,1-Diphenyl-2-picryl hydrazyl (DPPH), Folin-Ciocalteu's reagent and Trolox were obtained from Sigma Aldrich.

Sample Preparation for LC/MS/MS and HPLC Analysis. The leaves of *B. raddeana* were dried and ground into powder. Two grams of powdered leaves were mixed with 100 ml methanol, and then placed on a rotating shaker at 200 rpm for 6 h. Finally, the filtrate was

collected, and filtered through a 0.45 µm nylon filter. A 10 µl volume extract was injected into the HPLC column for analysis by LC-DAD-MS/MS.

Extraction, purification and identification of active compounds. A 500 g leaves of the dried *B. raddeana* powder was mixed in methanol (2.5 L) and kept in the shaking incubator at 25 °C for 7 days and filtered in vacuum using Whatman filter paper and the filtrate thus obtained was concentrated by evaporation at room temperature. A portion (10.0 g) of the concentrated methanol extract was fractionated to afford n-hexane (HF) (869 mg), dichloromethane (DMF) (1.84 g), ethyl acetate (ETF) (2.1 g), and aqueous (AQF) (5.19 g) soluble materials. After solvent fractionation, organic fractions and methanolic crude extract (MEEX) were evaluated for in vitro pharmacologic activities.

Total polyphenol contents. Total phenolic content of *B. raddeana* was measured by employing the method described by Singleton, V.L.[12] involving Folin-Ciocalteu reagent as an oxidizing agent and gallic acid as standard. To 0.5 ml of extract mixed with 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of sodium carbonate (7.5% w/v) solution were added. After 20 minutes of incubation at room temperature the absorbance was measured at 760 nm using a UV-visible spectrophotometer. Total phenolic content was quantified by calibration curve obtained from measuring the known concentrations of gallic acid (0-100µg/ml). The phenol content of the sample was expressed as mg of GAE (gallic acid equivalent)/100 g of the dried extract.

LC-DAD/ESI-MS/MS analysis. Quantification of phenolics was performed by using an Agilent technologies 1290 Infinity LC system consisting a DAD and coupled to a Agilent technologies 6460 Triple quadrupole LC/MS.The column was a 200 mm X 4 mm 3 µm particle size Zorbax Eclipse C18, maintained at 35^0C and protected with a UHPLC GUARD Zorbax Eclipse column of the same material using 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B). A stepwise gradient from 10% to 90% solvent B was applied at a flow rate of 0.5 ml/min for 46 min, followed by washing and reconditioning the column. The HPLC profiles were monitored at 210, 254, 325 and 340 nm. Electrospray mass spectra data were recorded on a negative ionisation mode for a mass range *m/z* 100 to *m/z* 1000.

The identification of phenolic compounds were obtained out by using authentic standards and by comparing the retention times and UV and MS, MS/MS spectra, while quantification was performed by external calibration with standards.

Free DPPH radical-scavenging activity on. DPPH scavenging potential of different *B. raddeana* fractions was measured based on scavenging ability of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by *B. raddeana* antioxidants. The method was employed to investigate the free radical scavenging activity (1). Freshly prepared 2mL DPPH (3×10^{-5} M in DMSO) solution was thoroughly mixed with 2mL of different *B. raddeana* fractions. The reaction mixture was incubated for 1h at room temperature. Absorbance of the resultant mixture was recorded at 517nm using UV-VIS spectrophotometer. Results were expressed as percentage decrease with respect to control values. MEEX, HFR, DMF and ETF samples were evaluated at final concentration of 90 µg/ml, and All tests were performed in triplicate.

Calculation of 50% Inhibition Concentration (IC50). The concentration of the extract (mg/mL) that was required to scavenge 50% of radicals was calculated by using the percent scavenging activities of five different extract concentrations. Percent scavenging activity was calculated as $[1- (A_1 - A_2)/Ac] \times 100$. Where: A_1 is the absorbance measured with *B. raddeana* fractions in the particular assay with a DPPH; A_2 is the absorbance measured with different *B. megrelica Sosn* fractions in the particular assay but without a DPPH; A_c is the absorbance of control with particular solvent (without *B. raddeana* fractions).

Cell lines and culture medium. Hela (ephitheloid cervix carcinoma, human) cell lines were obtained from the American Type Culture Collection (Rockville, MD). Hela cells were maintained in continuous culture in DMEM (Dulbecco's Modified Eagle Medium) medium (Bio Whitthaker) grown at 37°C in humidified 5% CO_2 and 95% air atmosphere. Medium was supplemented with 10% heat-inactivated foetal bovine serum (Bio Whitthaker), 1% L-glutamine (200 mM)(Bio Whitthaker) and antibiotics: penicillin (100 UI/ml)-streptomicin (100 µg/ml) (Pen-strep^R, Bio Whitthaker).

Cytotoxicity assay. 96-well tissue culture microplates (Micro Test –96 Falcon, Becton-Dickinson) were seeded with 100 µl medium containing x cells in suspension (x= 7000 cells/well for Hela). Twenty four hours incubation later, cells were treated with a dilution of different *B. raddeana* fractions in culture medium. After 48 hours incubation at 37°C in presence of compounds, mitochondrial dehydrogenase activity in viable cells was measured

by adding WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) reagent and reading absorbance at 450 nm with a scanning multiwell spectrophotometer after one hour delay. The absorbance was directly correlated to the viable cell number. Experiments were performed in triplicate and the results were expressed as cell proliferation in comparison to control. Colchicine were used as the reference samples.

Statistical analysis: Three replicates of each sample were used for statistical analysis and the values are reported as mean ± SD.

RESULTS AND DISCUSSION

Purification of the methanol extract from *B. raddeana* by repeated column chromatography yielded fractions rich in phenolic compounds, which were studied by LC/MS/MS. The optimum conditions were applied to the identification of the compounds. The purpose of this research was to identify phenolic compounds, quantify main flavonoids and phenolic acids and to isolate a flavonoid glycoside that was found to be characteristic for *B. raddeana.* A sensitive, accurate and specific method coupling high performance liquid chromatography (HPLC) with diode array detector (DAD) and electrospray ionization mass spectrometry (MS) was developed for the separation and identification of phenolic acids, flavonoid glycosides and aglycones in the methanolic extract of *B. raddeana.* The molecular masses of phenolic acids and flavonoids were assigned by electrospray ionization mass spectrometry. The subsequent structure characterization was carried out by a tandem mass spectrometric method. Fragmentation behavior of flavonoid glycosides and phenolic acids was investigated using mass spectrometry in negative mode. Fragmentation of aglycones provided characteristic ions for each family of flavonoids. In the spectra of the flavanoid glycosides present both ions – the deprotonated molecule $[M-H]^-$ of the glycosides and the ion corresponding to the deprotonated aglycone $[A-H]^-$. The MS, MS/MS and UV data together with HPLC retention time (*TR*) of phenolic acids and flavonoids allowed structural characterization of these compounds.

After LC-DAD and MRM analysis Apigenin, rutine, luteolin, kaempferol, myricetin, naringenin, hyperozide, quercetin, p-coumaric-, ellagic-, ferulic, gallic-, vanillic acides were determined in *B. raddeana* (Table 1). The HPLC-DAD chromatograms and total ion chromatograms of the extracts of *B. raddeana* are shown in Figure 1.

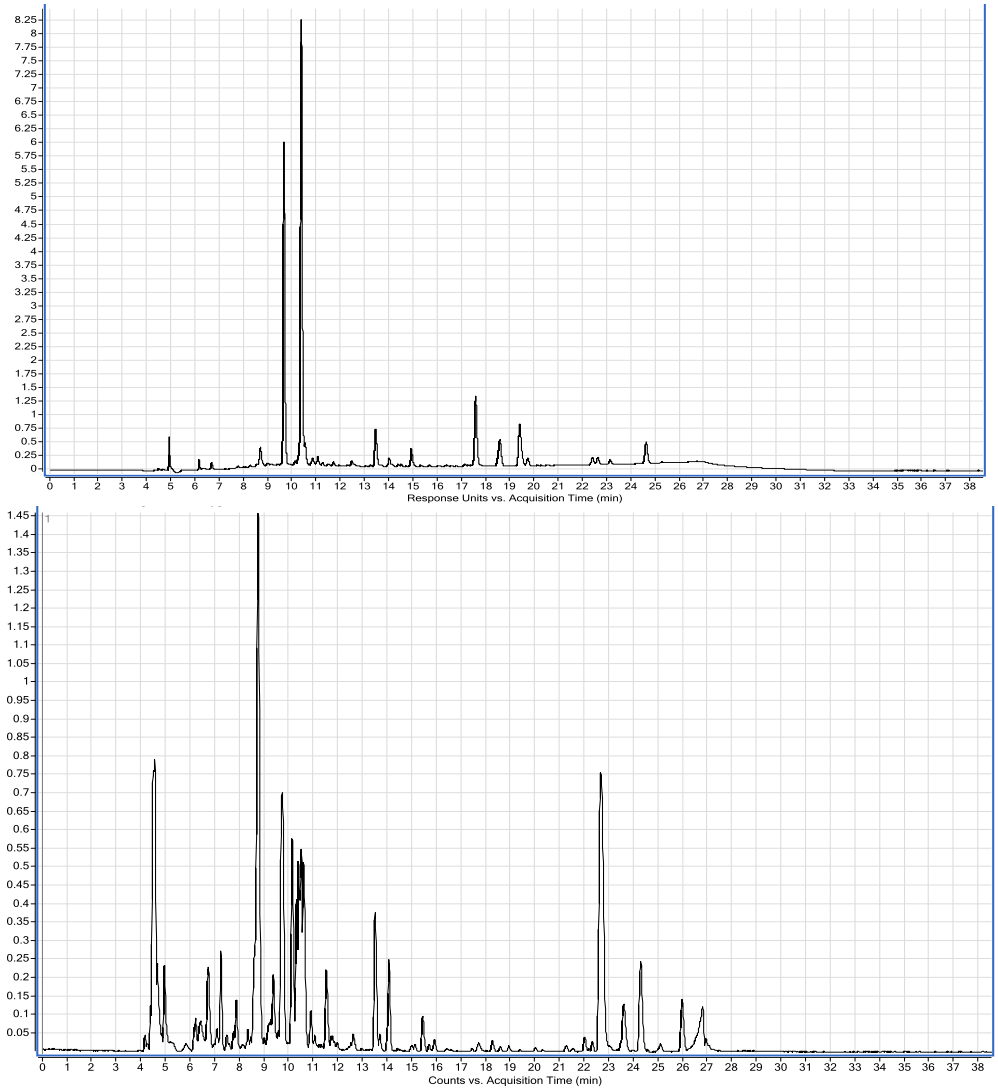

Figure 1: HPLC-(-) ESI-MS -TIC and HPLC-DAD chromatogram of methanolic extract of *B. raddeana.*

Table I: LC-MS-MS characteristics of flavonoids and phenolic acids.

Flavonoids	***MS m/z [M-H]-***	***MS/MS ions, m/z (rel. Int. %)***	***CV/CE* Cone Voltage / Collision Energy**	**Content (mg/g, dw)**
Apigenin	**269**	**151 (100), 117 (80)**	**-35/-15**	**1.8**
Rutine	**609**	**609 (100), 301 (40)**	**-40/-30**	**1.1**
Luteolin	**285**	**285 (100), 151 (85), 133 (50)**	**-40/-30**	**0.7**
Kaempferol	**285**	**93 (100), 285 (40)**	**-60/-55**	**1.8**
Myricetin	**317**	**317 (50), 151 (100), 137 (70)**	**-40/-25**	**7.1**
Naringenin	**271**	**119 (80), 151 (100), 271 (20)**	**-40/-30**	**0.8**
Hyperozide	**463**	**463 (5), 301(100)**	**-40/-38**	**2.4**
Quercetin	**301**	**151 (100), 121 (40), 301(20)**	**-35/-25**	**10.7**
Phenolics	**-------**	**-------**	**-------**	**-------**
p-Coumaric	**163**	**119 (100), 163 (40)**	**-30/-15**	**9.2**
Ellagic	**301**	**145 (100), 129 (70), 301(35)**	**-40/-30**	**12.8**

Ferulic	**193**	**134 (100), 149 (30), 178 (55)**	**-30/-20**	**8.6**
Gallic	**169**	**169 (10), 125 (100)**	**-25/-15**	**15.7**
Vanillic	**167**	**91 (100), 123 (30)**	**-40/-20**	**3.6**

The present study was undertaken to evaluate the polyphenols content and cytotoxicity activities of different organic soluble materials of the methanolic extract of *B. raddeana*. The results are shown in tables 2. The amount of total phenol content was found to differ for different fractions and ranged from 34.9 mg to 304.8 mg of GAE/g of extract of *B. raddeana*. Among all fractions, the highest phenolic content was found in ETF (304.8 mg of GAE/100g of extract) followed by MEEX (264.7 mg of GAE/100g of extract). Significant amount of phenolics were also seen in DMF (108.5 mg of GAE/100g of extract), HF (41.3 mg of GAE /100g of extract) and AQF (34.9 mg of GAE /100g of extract).

Table 2 show the results of the in-vitro cycotoxic testing after 48 hours of exposure to the samples and the positive control, Colchicine (CC). The LC50 values of MEEX, HF, ETF, DMF, AQF were found to be 8.5 ± 0.1; 19.2 ± 0.2; 9.8 ± 0.1; 17.3 ± 0.2 and 28.4 ± 0.3 (µg/ml), respectively (Table-1) as compared to 0.25 ± 0.01 µg/ml exhibited by CC. The MEEX and DMF showed strong cytotoxic activity while ETF, HF, AQF demonstrated significant cytotoxic activities.

The cytotoxic activities were increased with an increased content of total phenolics in organic fractions. Further, all fractions showed higher cytotoxic activities and positively correlated with total phenolic content.

Table 2: The total phenolic content and cytotoxic activities of different fractions of *B. raddeana*.

Sample	Total Phenolic Content (mg of GAE/100 g of dried extract)	Cytotoxic activity (LC50 in µg/ml)	DPPH free radical scavenging activity (IC50 (µg/ml)
MEEX	264.7±3.9	8.5 ± 0.1	0.29± 0.02
DMF	108.5 ± 1.5	17.3 ± 0.2	0.46± 0.03
ETF	304.8 ±5.2	9.8 ± 0.1	0.34 ± 0.02
HF	41.3 ±1.8	19.2 ± 0.2	0.59± 0.03
AQF	34.9 ±1.1	28.4±0.3	0.79± 0.03
CC	-	0.25 ± 0.01	-
Trolox	-	-	0.30±0.02

Total phenolic content of different *B. raddeana* fractions were solvent dependent. The content of total phenolics in aqueous fractions decreased in the order of ethyl acetate

(304.8±5.2 100mg/g) > methanol (264.7±3.9 mg/100g) > dichlormethane (108.5±1.5 mg/100g) > n-hexane (41.3±1.8 mg/100g) > aqueous (34.9±1.1 mg100/g) fractions. As different *B. raddeana* fractions exhibited free radical- scavenging activities, there may be different kinds of total phenolic compounds (hydrophilic and hydrophobic) in different *B. raddeana* fractions.

From the above results, it is evident that the methanolic crude extract and ETF soluble fraction revealed strong cytotoxicity which also suggest the presence of secondary metabolites in these extractives.

The methanol extract showed significant activities in antioxidant assays (IC50 0.29 ± 0.02 mg/mL) and contained a high level of total phenolic content. The highest DPPH radical scavenging effect was detected in organic ethyl acetate fraction (IC50 0.34 ± 0.02 mg/mL) followed by dichlormethane, n-hexane and aqueous fractions (IC50 0.46± 0.03 mg/mL, 0.59± 0.03 mg/mL, 0.79± 0.03 mg/mL respectively (Table 2). Also, DPPH radical scavenging activities were increased with an increased content of total phenolics in organic fractions.

B. raddeana could be subjected for extensive chromatographic separation and purification processes to isolate bioactive compounds for the discovery of novel therapeutic agents.

CONCLUSION

An HPLC–DAD-ESI-MS/MS based method was proven suitable and sensitive to determine different flavonoids in *B. raddeana* extract and fractions. We have proposed an HPLC–UV-MS/MS based method for the determination of several flavonoids, which can offer quite good linearity, accuracy, precision and low limit of detection. All results demonstrate this method is suitable for the quality control of *B. raddeana* leaves.

The results of present study show that from vegetative organs of *Betula raddeana.* growing in Georgia the leaves extract has the highest amount of total phenolics, which exhibit the greatest antioxidant and cytotocic activity. It could be concluded that the different extracts of the *B. raddeana* might be helpful in preventing or slowing the progress of various oxidative stress related diseases.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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