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**RESEARCHES UPON INDIGENOUS HERBAL
PRODUCTS FOR THERAPEUTIC
VALORIFICATION IN METABOLIC DISEASES.
NOTE II. POLYPHENOLS CONTENT,
ANTIOXIDANT ACTIVITY AND
CYTOPROTECTIVE EFFECT OF *BETULAE
FOLIUM DRY EXTRACT***

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Abstract

Among natural compounds, polyphenols are well-known antioxidants with beneficial effects towards endothelial dysfunction, the main cause of vascular complications in type 2 diabetes mellitus (DM 2). In the present study, a *Betulae folium* dry extract was evaluated for its polyphenols content, antioxidant activity and protective effect against OH• free radicals (generated by H₂O₂ and FeSO₄ reaction) induced oxidative stress in EA.hy926 endothelial human cell line. Spectrophotometric and LC-MS analysis were used for polyphenols determination, 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) for antioxidant activity and cellular viability (MTT assay) for evaluating the cytoprotective effect. *Betulae folium* dry extract is a rich source of polyphenols, with antioxidant activity (IC₅₀ = 0.1841mg/mL) and has a modest cytoprotective effect in a narrow concentration range (0.03-0.05 mg/mL).

Rezumat

Dintre compușii naturali, polifenolii sunt agenți antioxidanți, cu rol benefic în disfuncția endotelială, principala cauză a complicațiilor vasculare din diabetul zaharat tip II (DZ II). Obiectivele cercetărilor noastre au constat în determinarea conținutului în polifenoli a extractului de *Betulae folium*, a activității antioxidante și a efectului protector asupra celulelor endoteliale EA.hy926, supuse stresului oxidativ în prezență de sulfat feros și apă oxigenată (cu formare de radicali liberi OH•). Conținutul în polifenoli a fost determinat spectrofotometric și prin LC-MS. Acțiunea antioxidantă a fost determinată utilizând radicalul liber 2,2-difenil-1-picrilhidrazil (DPPH), iar efectul citoprotector pe baza viabilității celulare (testul MTT). Extractul reprezintă o sursă de polifenoli cu efect antioxidant (IC₅₀ = 0,1841mg/mL). Acesta prezintă acțiune citoprotectoare doar la concentrații cuprinse între 0,03-0,05 mg/mL.

Keywords: polyphenols, endothelial cells, oxidative stress, *Betulae folium dry extract*

Introduction

Type 2 Diabetes mellitus (DM 2) is a common metabolic disease responsible for 6.8% of global mortality [24]. It is characterized by insulin resistance, which is associated with dyslipidaemia, hypertension, impaired fibrinolysis and atherosclerosis [7]. Hyperglycaemia, the hallmark of diabetes, is the main cause of endothelial dysfunction (responsible for future vascular complications) through: overproduction of advanced glycation products (AGE), increased production of adhesion molecules (SVCAM1) and excessive generation of reactive oxygen species (ROS) [21].

Polyphenols (flavonoids, phenolcarboxylic acids, proanthocyanidins, tannins) are well-known for their antioxidant activity, *insulin like* effects, HMG-CoA and aldoreductase inhibitory properties [13, 20].

Betula pendula Roth. (birch leaves) are used for anti-inflammatory [26], antibacterial [22], diuretic [13] and antioxidant [11] properties. Moreover, Molokovskiĭ D.S. *et. al* (1989) showed that birch leaf infusion has hypoglycaemic activity [15].

Regarding their chemical composition, silver birch leaves contain high amounts of: flavonoids (2.5-3% represented by hyperoside, quercitrin, avicularin, kaempferol-3-O-glucoside or glucuronide and myricetin derivatives), phenolcarboxylic acids (gallic, chlorogenic, caffeic, ferulic, *p*-coumaric), gallo and ellagotannins (1-9%), proanthocyanidins (3.9%), oligoelements (selenium, manganese, vanadium) [1, 2, 14, 25].

The aim of our study was the evaluation of polyphenols content, antioxidant activity and protective effects of a *Betulae folium dry extract* against OH• free radicals (generated by H₂O₂ and FeSO₄ reaction) induced oxidative stress, in EA.hy926 endothelial human cell line.

Materials and Methods

Plant material

Birch leaves (*Betula pendula* Roth., *Betulaceae*) were harvested in May 2011, from Morărești, Argeș District; dried in shadow and stored in laboratory conditions.

Reagents and solvents

All chemicals were purchased from Sigma-Aldrich (Germany), unless otherwise stated. Ascorbic and gallic acids were from Roth (Germany), fetal bovine serum (FBS) was purchased from EuroClone (Italy) and hydrogen peroxide was from Chemical Company (Romania). EA.hy926

cells (ATCC, CRL-2922) were acquired from LGC Standards (U.K.). They are hybridomas obtained by fusing primary human umbilical vein cells with a thioguanine resistant clone of A549 (lung cancer cell line), being a useful tool in angiogenesis, inflammation and atherosclerosis research [3].

Preparation of birch dry extract (BE): 300 g of powdered birch leaves were heated twice with ethanol 40% (v/v) under a reflux condenser for 30 min., using 1:20 herbal product/ solvent ratio for the first extraction and 1:10 for the second one. After cooling, the combined filtrates were concentrated using a rotary evaporator (Buchi R 210-215) to remove the solvent, and then freeze-dried (using a Christ Alpha 1-2/B Braun, Biotech-International lyophilizer) to yield 51 g extract.

Spectrophotometric determination of total polyphenols = PT (g% tannic acid), tannins = TA (g% tannic acid), flavonoids = FL (g% hyperoside), phenolcarboxylic acids = AFC (g% chlorogenic acid) and proanthocyanidins = PR (g% cyanidin chloride) were carried out using a Jasco V-530 spectrophotometer (Jasco, Japan) and methods previously described [6].

HPLC-MS analysis was performed on an Agilent 1100 HPLC Series system (Agilent, SUA) using the chromatographic conditions previously described [4, 15, 17]. Quantitative determinations were performed using an external standard method. Calibration curves, in 0.5-50 mg/mL range, with good linearity ($R^2 > 0.999$, $n = 5$) were used for quantitative determination of polyphenols [4, 16, 18].

Preparation of samples: 0.25 g BE were dissolved in 25 mL ethanol 40% (volumetric flask) (SB). For extraction of free aglycones 1 mL HCl 2N was added to 1 mL SB and heated on a reflux condenser at 80°C for 60 min. (SBH).

Antioxidant activity was determined with the well-known DPPH free radical [4, 8, 23], using the method described by Ohnishi *et al.* (1995) [17]. BE was dissolved in ethanol 40% in different concentrations ranging from 0.02-0.4 mg/mL. Each dilution (0.5 mL) was mixed with 5 mL of a 0.1 mM DPPH ethanolic solution and left in the dark for 30 (original method) and 60 min. Absorbances were measured at $\lambda = 517$ nm, using ethanol as a blank. Gallic acid and vitamin C (concentration ranging from 0.008-0.05 mg/mL and 0.01-0.05 mg/mL), dissolved in the same solvent, were used as standard references. The absorbance of the DPPH solution was measured before (A_{control}) and 30, 60 min. after adding the extracts or standard references (A_{sample}). DPPH radical scavenging activity (%) was calculated using the formula: $(A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}) \times 100$. The IC₅₀ (mg/mL) value, which is the concentration of BE or standard references that reduces the radical activity with 50%, was calculated through linear interpolation using inhibition (%) vs. concentration (mg/mL) standard curves.

In vitro tests on EA.hy926 cell line

Cell culture. EA.hy926 cells were grown in culture medium (Dulbecco's modified Eagle's medium supplemented with: 8.9 mg/L L-alanine, 15 mg/L asparagine, 13.3 mg/L aspartic acid, 14.7 mg/L glutamic acid, 11.5 mg/L proline, 10 mg/L ascorbic acid, 0.4 mg/L biotin, 1 mL sodium selenite, 2.2 mg/L sodium bicarbonate, 110 mg/L pyruvic acid, 4.76 mg/L HEPES = 4-(2-hidroxyethyl)-1-piperazineethanesulfonic acid, 10% fetal bovine serum - FBS, 100 U/mL penicillin and 100 µg/mL streptomycin). Confluent cells were trypsinised, centrifuged and subcultured in culture medium, in a humidified 5% CO₂ / 95% air atmosphere, at 37°C. For experiments, cells were seeded at a density of 8 x 10³ cells in 96 well-culture plates using culture medium and allowed to adhere for 72 h.

Evaluation of cytotoxicity. When endothelial cells reached confluence (after 72 h) the culture medium was replaced and cells were incubated with serum-free medium. After 24 h, concentrations ranging from 0.005-0.3 mg/mL BE were prepared from 1 mg/mL aqueous sterile, filtered solution (0.22 µm Millex, Millipore), by serial dilutions in serum-free medium to give a volume of 200 µL in each well of a microtiter plate (96-well), following incubation for 24h. Viability (%) was determined using MTT (dimethylthiazol-diphenyltetrazolium bromide), a water-soluble tetrazolium salt, which is converted to an insoluble purple formazan by mitochondria dehydrogenases from the live cells. Untreated cells were used as control.

MTT assay. Briefly, the culture medium, was replaced with 200 µL 0.5 mg/mL MTT and cells were incubated at 37°C. After 2h, the water-insoluble formazan was solubilized in 100 µL 0.1 M HCl in isopropanol. The absorbance was measured at $\lambda = 570$ nm with a background subtraction at $\lambda = 690$ nm, on a microplate reader (Tecan, Austria) [9]. Results were reported as percentage from the untreated cell control, considered as 100% viable cells.

Determination of H₂O₂ concentration that reduced cell viability by 50%. After incubation in serum-free medium for 24h (incubation in serum-free medium prior to exposure to oxidative agents, was needed because FBS contains antioxidants that may interfere the results [9]), cells were incubated in Hanks' balanced saline solution (HBSS) containing various concentrations of H₂O₂ (100 µM-1mM), obtained from 30% stock solution, and a fixed concentration of 100 µM FeSO₄ (H₂O₂/FeSO₄). After 30 min. at 37°C, cells were washed with HBSS and further incubated in culture medium containing 0.5% FBS for 24h. Control consisted of incubation of the cells in the same conditions, except H₂O₂ was omitted [5]. Viability (%) was determined using MTT assay, as previously described.

Evaluation of cytoprotective effect. Cells were pre-treated with 0.005-0.05 mg/mL BE in serum-free medium for 24 h, then exposed to H₂O₂/FeSO₄ for 30 min., washed with HBSS and further incubated in culture medium with 0.5% FBS. Untreated cells (not exposed to H₂O₂/FeSO₄ or BE = M₁) and cells only treated with H₂O₂/FeSO₄ without extract pre-treatment = M₂, were used as controls. After 24 h, viability was determined using MTT assay and results were reported as percentage (%) from the untreated cell control M₁ (considered as 100% viable cells).

Light microscopy. Cells were photographed using a Carl Zeiss Axio Microscope equipped with a digital camera Axio Cam ERC5S (Carl Zeiss, Germany).

Statistical analysis. The data analysis was performed using Microsoft Excel 2003 software and GraphPad version 5.00 for Windows. Data are reported as mean $\pm$ standard deviation (SD). The type of distribution (normal/abnormal) was established with D'Agostino & Pearson test. Comparison of control with and each sample (for cell culture studies) and BE antioxidant activity for 30 and 60 min. was carried out using Student t-test. Significant statistical differences were considered for p value < 0.05.

Results and Discussion

Betulae folium dry extract is a rich source of polyphenols (Table I).

Table I
Spectrophotometric determination of *Birch dry extract* polyphenols

<i>Birch dry extract</i>	PT (g% tannic acid)	TA (g% tannic acid)	FL (g% hyperoside)	AFC (g% chlorogenic acid)	PR (g% cyanidin chloride)
	22.794 $\pm$ 2.5170	9.92 $\pm$ 0.1691	13.088 $\pm$ 0.6918	8.592 $\pm$ 0.3103	2.0144 $\pm$ 0.1434

PT = total polyphenols, TA = tannins, FL = flavonoids, AFC = phenolcarboxylic acids, PR = proanthocyanidins
Results represent mean $\pm$ SD (n = 3)

Using LC-MS, we have identified phenolic compounds (Table II, Figure 1), also mentioned by scientific literature, except for sinapic and gentisic acids, which are mentioned here for the first time. Hyperoside, isoquercitrin and apigenin content is low compared to Germano *et al.* (2012) results on an ethanolic 96% birch dry extract (2468 mg / 100 g hyperoside, 126 mg / 100 g isoquercitrin, 0.60 mg / 100 g apigenin), but quercitrin, quercetin and myricetin quantities are higher [11].

Due to the lack of standards we couldn't establish if kaempferol comes from kaempferol-3-O-glucoside or kaempferol-3-O-glucuronide. Caffeic, ferulic, *p*-coumaric and sinapic acids were identified only in SBH solution, so we concluded that they exist only in glycosidic or esterified

forms. Chlorogenic, caffeic and gentisic acids weren't quantified, in the present chromatographic conditions, due to overlapping.

Table II
LC-MS results

Compound	$t_R \pm SD$ (min.)	SB (mg/100g dry extract)	SBH (mg/100g dry extract)
gentisic acid	2.15 ± 0.07	+	+
chlorogenic acid	5.62 ± 0.05	+	+
caffeic acid	5.60 ± 0.04	-	+
<i>p</i> -coumaric acid	8.7 ± 0.08	-	155.18
ferulic acid	12.2 ± 0.10	-	28.20
sinapic acid	14.3 ± 0.10	-	6.69
hyperoside	18.60 ± 0.12	1227	-
isoquercitrin	19.60 ± 0.10	78.54	-
quercitrin	23.00 ± 0.13	55.90	-
myricetin	20.70 ± 0.06	188.19	192
quercetin	26.80 ± 0.15	20.73	807.82
apigenin	33.10 ± 0.15	9.843	11.83
kaempferol	31.60 ± 0.17	-	19

"+" - MS identified, "-" - MS not identified

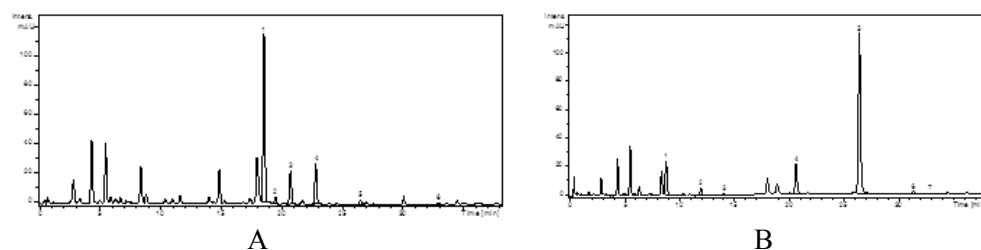


Figure 1
LC-MS chromatograms (A – SB, B – SBH)

Antioxidant activity of birch dry extract at 30 and 60 min. is low, compared to standard references. Our IC₅₀ values (0.1841 / 0.1654 mg/mL) (Table III) are similar to the one reported by Germano *et al.* (2012) (0.137 mg/mL) for an ethanolic 96% birch dry extract [11].

Table III
IC₅₀ values for BE and standard references

<i>Birch dry extract (BE)</i>	IC ₅₀ (mg/mL)	
	30 min.	60 min.
	0.1841 ± 0.000	0.1654 ± 0.0030
vitamin C	0.0253 ± 0.006	0.0244 ± 0.0002
gallic acid	0.0073 ± 0.006	0.0035 ± 0.0014

Results represent mean $\pm$ SD (n = 3)

At 0.05 mg/mL (the highest concentration, common for both BE and standards) the DPPH inhibition was 18.75% (BE), 95% (gallic acid) and 94.69% (vitamin C) for 30 min. incubation time (Figure 2). No significant differences ($p > 0.05$) occurred between 30 and 60 min., although Ozgen *et al.* (2006) foresaw that a longer reaction time could increase the antioxidant activity, due to polymerisation of phenolic compounds [19].

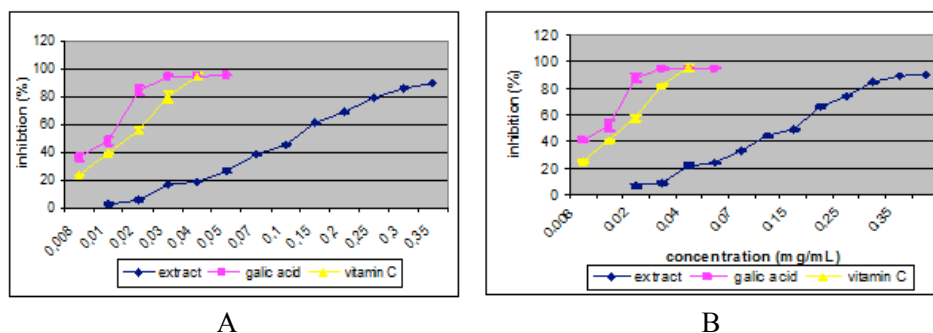


Figure 2
DPPH inhibition (%) (A – 30 min, B – 60 min)

At low concentrations (0.005-0.05 mg/mL) BE didn't influence cell viability (Figure 3).

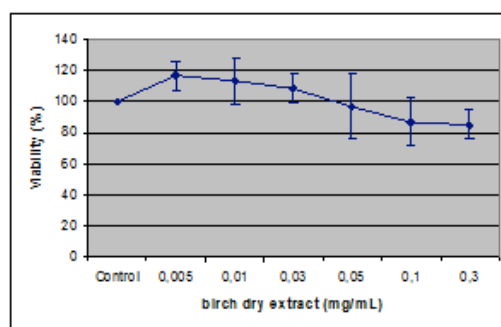


Figure 3
BE cytotoxicity treatment

Error bars = mean $\pm$ SD (n = 8)

However, starting with 0.1 mg/mL, the viability decreased under 90% (Figure 3, Figure 5B), probably due to the reaction between culture medium and phenolic compounds, that leads to quinones with cytotoxic activity [12]. Lupeolic saponins (identified by us in BE using chemical reactions) are also known for their antiproliferative activity on both normal and cancer cell lines [10]. Regarding this, BE cytoprotective effect was

evaluated only for 0.005-0.05 mg/mL. The H_2O_2 concentration, which reduced cell viability by 50%, was 500 μM (Figure 4, Figure 5C).

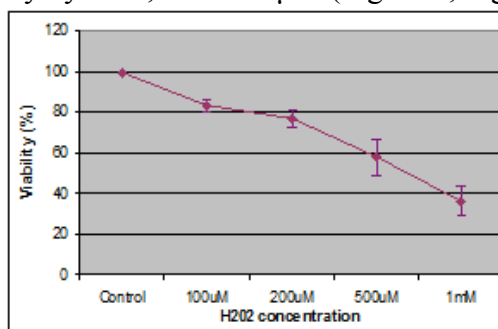


Figure 4

H_2O_2 concentration that reduced cell viability by 50%

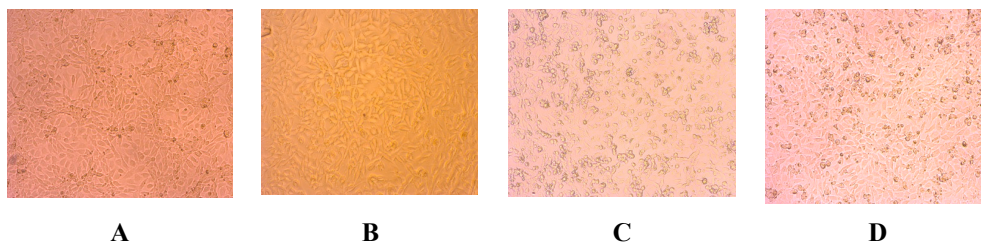
The BE cytoprotective effect was significant at 0.03 and 0.05 mg/mL ($p < 0.05$) compared to cells treated with $\text{H}_2\text{O}_2/\text{FeSO}_4$ (Table IV, Figure 5D). Among BE polyphenols, quercetin and kaempferol aglycones or glycosidic forms together with AFC can pass through the membrane, by passive or active (specific transporters) mechanisms, being able to increase cell glutathione level and decrease ROS production [20].

Table IV

Viability of cells after pre-treatment with BE extract

Sample Viability (%)	M_1	M_2	Pre-treatment of cells (exposed to oxidative stress) with BE			
			0.005 mg/mL	0.01 mg/mL	0.03 mg/mL	0.05 mg/mL
Mean $\pm$ SD (n = 8)	100 $\pm$ 0.000	56.61 $\pm$ 3.8385	64.89 $\pm$ 2.3443	68.21 $\pm$ 2.5597	76.3 $\pm$ 0.7910	81.97 $\pm$ 6.3112
Normal distribution (D'Agostino and Pearson test)	yes	yes	yes	yes	yes	yes
t-test, IC = 95%, degrees of freedom (n-1 = 7)						
Treatment vs. M_1		$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
Treatment vs. M_2			ns	ns	$p < 0.05$	$p < 0.05$

M_1 = untreated cells, M_2 = cells treated with 500 μM H_2O_2 + 100 μM FeSO_4 , ns – not significant

**Figure 5**

EA.hy926 cells: A - control M₁ (untreated cells), B - cells treated with 0.1 - 0.3 mg/mL BE, C - cells treated with 500 µM H₂O₂ + 100 µM FeSO₄ (M₂), D - protective effect of pre-treatment with 0.05 mg/mL BE

Conclusions

Betulae folium dry extract is a source of polyphenols with antioxidant activity. To our knowledge, no previously research regarding protective effect of silver birch leaves, against OH• free radicals-induced oxidative stress on EA.hy926 cell line, has been performed.

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