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Chemical Composition, Antioxidant Activity and Phytotoxic Properties of Silver Birch Leaves

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Abstract

In the present study, silver birch leaves were analyzed for chemical composition, antioxidant activity and phytotoxic properties. Polyhenols, triterpenic compounds and vitamin C were determined by means of spectrophotometric and HPLC-MS/HPLC-DAD methods. The scavenger activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and the reducing power assay were used for antioxidant activity evaluation. Triticum assay was employed for phytotoxicity estimation. It was found that birch leaves are a considerable source of flavonoids (1.6278 g%), phenolcarboxylic acids (2.8548 g%) and triterpenic compounds (11.7817 g%). Using HPLC-MS, several phenols were determined: hyperoside (386 mg%), quercitrin (157.57 mg%), quercetin (154.67 mg%), p-coumaric acid (51.64 mg%) and sinapic acid (2.6015 mg%). Antioxidant activity of birch leaves (EC50 = 0.4651mg/mL - DPPH method and EC50 = 0.5172 mg/mL - reducing power assay) was weak compared to that of standards (chlorogenic acid, sinapic acid, hyperoside and catechin). Leaves showed strong inhibition of root elongation in 5%-1.33% concentration range. The herbal product represents a rich source of active substances with moderate antioxidant activity. Gentisic and sinapic acids are new compounds, unmentioned by scientific literature. Phytotoxic effects were observed only for high concentrations.

Keywords: *polyphenols, sinapic acid, gentisic acid, Triticum assay*

1. Introduction

The *Betulaceae* family includes numerous genera (six according to China's and North America's Flora, two according to Pakistan's Flora) and over 100 species natives of northern parts of Europe, China, North and South America (A.R.BRANCH & al. [1]). The family is divided into two subfamilies: *Betuloideae* (*Betula* and *Alnus* genera*)* and *Coryloideae* (*Corylus*, *Carpinus*, *Ostrya* genera) (BLAMEY [2]). *Betula pendula* Roth. (silver birch) has a wide arial, being spread in Europe, Africa, Asia and North America. Buds (*Betulae gemmae*),

leaves (*Betulae folium*) and bark (*Betulae cortex*) are used for therapeutic purposes (BLAMEY [2]). Silver birch leaves are a rich source of flavonoids (hyperoside – 0.8 g%, quercitrin – 0.14 g%, avicularin – 0.57 g%, quercetin-3-O-glucuronide -0.25 g%, myricetin-3-O-galactoside – 0.37 g%, isoquercitrin – 0.13 g%, kaempferol-3-O-glucoside – 0.29 g%) (V. OSSIPOV & al. [3], K. DALLENBACH-TÖLKE & al. [4], M.P. GERMANÒ & al. [5]), proanthocyanidins – 0.44 g% (M. TĂMAŞ & al. [6]), phenolcarboxylic acids (gallic acid – 16 mg%, caffeic acid – 22 mg%, chlorogenic acid – 38 mg%, protocatehuic acid – 26 mg% and ferulic acid – 59 mg%) (A.C MIŠAN. & al. [7]). *Betulae folium* are also a source of gallo- and ellagitannins (casuarictin, potentilin and pedunculagin) (J.P. SALMINEN & al. [8]), lupeolic saponins (betulinic acid, betulin) (ISTUDOR [9]) and dammaranic esters (WICHTL [10]). Fatty alcohols, fatty acids (miristic, palmitic, linoleic and linolenic) (E.E. SHULT'S & al. [11]), essential oil (rich in caryophyllene oxide, betulenol, mirtenol, geraniol) (C.K.H. BAŞER & al. [12]), minerals (selenium, zinc, manganese, potassium) (ISTUDOR [9]), vitamins (C, E) (O. ZYRANOVA & al. [13]) and carotenoids (S. SILLANPÄ & al. [14]) are also found in birch leaves. Silver birch leaves have anti-inflammatory (C. GRÜNDEMANN & al. [15]), antimicrobial (D. WOJNICZ & al. [16]), diuretic (J. HAVLIK & al. [17]), antioxidant (M. KRATCHANOVA & al. [18]) and detoxification properties (T.I. BOKAVA & al. [19]).

The aim of our research consists of: phytochemical screening (by means of qualitative and quantitative methods), antioxidant activity (scavenger activity against 2,2-diphenyl-1-picrylhydrazyl free radical – DPPH and reducing power assay) and phytotoxicity evaluation (*Triticum assay*) of silver birch leaves.

2. Material and methods

Plant material

Silver birch leaves were collected in the month of May 2012, from Morăreşti village (coordinates 45°00'28"/N 24°33'48" E), Argeş district, Romania. Leaves were air-dried in the shade and stored in laboratory conditions. For ascorbic acid determination fresh leaves have been used. Herbarium voucher samples (no. 225) of both fresh and dried leaves were deposited in the Department of Pharmacognosy, Phytochemistry, Phytotherapy, School of Pharmacy, "*Carol Davila*" University of Medicine and Pharmacy, Bucharest, Romania.

Reagents and solvents

All reagents and solvents were purchased from Karl Roth (Germany) unless otherwise stated. Betulin and diphenylboryloxyethylendiaamine (DFBOA) were from Sigma-Aldrich (Germany) and vitamin C, trichloroacetic acid were acquired from Merck (Germany).

Thin layer chromatography (TLC)

Thin layer chromatography was used for identification of phenolic and triterpenic compounds. It was performed on aluminium-coated TLC plates (20 x20 cm, kept for 1h at 100°C). Several eluent systems were used: ethyl acetate : acetic acid : formic acid : water = 100:11:11:26 (v/v/v/v) (eluent system A)(WAGNER [20]); toluene : dioxane : acetic acid = 90:25:4 (v/v/v) (eluent system B) (A. TOIU & al [21]) and chloroform : acetone = 80:20 (v/v) (eluent system C) (M.L. POPESCU & al. [22]). Eluent systems A and B were used for phenolic compounds identification, while eluent system C for triterpenic compounds. Plates were spotted with methanolic, hydrolysed methanolic solutions of birch leaves (prepared as

described below) and 0.1 mg/mL methanolic solutions of rutin, quercitrin, caffeic acid, chlorogenic acid, quercetin, kaempferol, ursolic acid, oleanolic acid, betulinic acid and betulin.The plates were developed over a path of 15 cm, air-dried and sprayed with a 10 g/L solution of DFBOA (in ethylacetate) (for eluent systems A, B) or with acetic anhydride and a mixture of ethanol : sulphuric acid = 1:1 (v/v) followed by heating at 100°C for 5 min. (for eluent system C). The plates were examined in UV light (λ = 365 nm) at a CamagReprostar Lamp with Epson Photo PC850, before and after spraying with the detection reagents.

Preparation of test solutions for TLC analysis: 1 g of dried birch leaves were heated under a reflux condenser with 10 mL of methanol for 5 min (solution M). 5 mL of solution M were treated with 5 mL of 2N HCl and heated on a reflux condenser for 1 h (solution MH). Half of MH solution was brought in a separation funnel and treated with 3 quantities (each of 15 mL) ethyl ether. The organic layers were evaporated on a water bath and the residue was dissolved in 2 mL methanol (solution MHE).

Spectrophotometric determinations

Total phenols (expressed as gallic acid equivalent) were determined with Folin-Ciocalteu reagent according to V.L. SINGLETON & al. [23] method, that is very much used for herbal products analysis (A.B. ALIYU & al. [24]). The assay for phenolcarboxylic acids (PCA, expressed as caffeic acid equivalent) was performed according to European Pharmacopoeia 7th edition (EUROPEAN PHARMACOPOEIA 7th edition [25]), based on the formation of oxymes with Arnow reagent. The flavonoid content (expressed as quercetin equivalent) was estimated based on the chelating reaction with aluminium chloride according to B. CHRIST & al. [26]. Tannins (expressed as pyrogallol equivalent) were determined based on their precipitation with skin powder (FARMACOPEEA ROMÂNĂ ed-a-X-a [27]). Triterpenic compounds (free, glycosidic and esterified forms – expressed as betulinic acid equivalent) were determined based on their dehydration in the presence of perchloric acid and vanillin acetate with formation of compounds with a maximum absorption at λ = 540-560 nm (J. LU & al. [28]). For all determinations a Jasco V-530 spectrophotometer (Jasco, Japan) was used. Calibration curves of: gallic acid (1.22-2.44 μg/mL, R^2 = 0.9982, n=6), caffeic acid (5.55-35.52 μg/mL, R^2 = 0.9964, n = 6), quercetin (2.06-14.42 μg/mL, R^2 = 0.9983, n = 8) and betulinic acid (6.25-31.25 μg/mL, R^2 = 0.9990, n=9) were used to calculate the percentage of active substances.

Preparation of samples for polyphenols determination: for PCA and total phenolic content, 5 g of dried birch leaves were heated twice on a reflux condenser with 50 mL 50% ethanol (v/v) for 30 min. ($M_{et50\%}$). For flavonoids and tannins determination, samples were prepared according to methods previously mentioned (B. CHRIST & al. [26], FARMACOPEEA ROMÂNĂ ed. a-X-a [27]).

Preparation of samples for triterpenic compounds determination: **free forms**: 0.5 g dried herbal product were heated twice on a reflux condenser with 25 mL chloroform. The solutions were brought in a 50 mL volumetric flask and evaporated on a water bath (Raypa, Spain). The residue was dissolved in ethanol and brought to 10 mL with the same solvent in a volumetric flask; **glycosidic forms**: 0.5 g dried herbal product was heated twice on a reflux condenser with 25 mL 50% ethanol (v/v) for 30 min. The ethanolic solutions were filtered in a 50 mL volumetric flask. 10 mL of this solution were treated with 20 mL 2 N HCl on a reflux condenser for 4 h. The hydrolysed solution was treated with 3 quantities (each of 15 mL) chloroform; the organic layers were filtered over anhydrous sodium sulphate and evaporated on a water bath. The residue was dissolved in 10 mL ethanol (volumetric flask);

esterified forms: 0.5 g dried herbal product was heated on a reflux condenser with 20 mL ethanol and 5 mL 33% potassium hydroxide for 1 h. The solution was brought into a separating funnel and 20 mL water were added. The mixture was extracted with 3 quantities (each of 15 mL) chloroform. The organic layers were combined, filtered over anhydrous sodium sulphate and evaporated on a water bath. The residue was dissolved in 10 mL ethanol (volumetric flask).

HPLC-MS/HPLC-DAD analysis

- **Determination of phenolic compounds.** HPLC analysis was performed on Agilent 1100 HPLC Series System (Agilent, SUA) using the chromatographic conditions previously described. Calibration curves in 0.5-50 mg/mL range with good linearity ($R^2 > 0.99$, n = 5) have been used for determination of polyphenols (L. VLASE & al. [29]). Chlorogenic acid and caffeic acid were not quantified in the present chromatographic conditions, due to overlapping. For polyphenols determination $M_{et50\%}$ solution (prepared as described above) was used. 10 mL of $M_{et50\%}$ solution was treated with 10 mL 2N HCl and heated on a reflux condenser at 80°C for 1h ($MH_{et50\%}$).
- **Determination of ascorbic acid.** HPLC analysis was carried out using a Varian HPLC (SUA). The separation was achieved on a C18, 150x4.6 mm i.d., 5 μm particle columm. The mobile phase consisted of methanol (A) and water (B). The gradient used was 75% A and 25% B 0-10 min. The flow rate was 1 mL/min and the injection volume 10 μL. The ascorbic acid content was determined based on a calibration curve (0.05-0.3 mg/mL, R^2 = 0.986, n= 5). For ascorbic acid determination 30 g of fresh leaves were mixed with 30 mL 50% ethanol (v/v) for 1 h, using a magnetic stirrer (Kartell, Italy).

Antioxidant activity

a. DPPH radical scavenger capacity

DPPH free radical scavenger capacity was determined according to M. OHNISHI & al. [30] method. Briefly 0.5 mL of $M_{et50\%}$ (0.10 mg/mL-1.46 mg/mL) was mixed with 3 mL ethanolic solution 0.1 mM of DPPH. The mixture was kept in the dark, at room temperature and the absorbance of the DPPH solution was measured at λ = 517 nm, before (A_{start}) and 30 min. after adding the extractive solutions (A_{end}). Ethanol was used as blank. Chlorogenic acid, sinapic acid, hyperoside and catechin (dissolved in 50% ethanol) were used as positive controls. The ability to scavenge the DPPH free radical was calculated using the following formula:

$$\textbf{DPPH radical scavenging activity (\%)} = (A_{start} - A_{end}) / A_{start} \times 100.$$

The concentration of birch leaves/positive controls that inhibited 50% of the DPPH free radical (EC_{50}, mg/mL) was determined graphically from the linear regression curve plotted between percent (%) of inhibition and extract/positive controls concentrations. All measurements were done in triplicate.

b. Reducing power assay

The reducing power assay was determined according to M. OYAIZU [31] method. Briefly, 2.5 mL of different dilutions of $M_{et50\%}$ (0.10 mg/mL-1.46 mg/mL) were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. Samples were kept at 50°C in a water bath (Raypa, Spain) for 20 min. After, 2.5 mL of 10%

trichloroacetic acid was added and the mixture was centrifuged at 2500 rpm for 5 min. (Universal 16 centrifuge). The upper layer (2.5 mL) was mixed with 2.5 mL water and 0.5 mL of a 0.1% ferric chloride solution. The absorbance was measured at λ = 700 nm, after 10 min., against a blank that contained all reagents except for herbal sample/positive controls. A higher absorbance indicates stronger reducing power. Chlorogenic acid, sinapic acid, catechin and hyperoside (dissolved in 50% ethanol) were used as positive controls. The herbal product/ positive controls concentration providing 0.5 of absorbance (EC50 mg/mL) was determined graphically from the linear regression curve plotted between absorbance and extract/positive controls concentration (mg/mL). All determinations were performed in triplicate.

Phytotoxicity evalution

The phytotoxic activity was investigated using Gr. Constantinescu method (Triticum assay), which is used to determine the maximum dilution of extractive solutions, that depending on exposure time, influence root elongation and kariokynetic film (D. Gr. CONSTANTINESCU & al. [32]). The research was conducted on roots of wheat germ (*Triticum aestivum* Mill., *Boia* type, SC Adaflor, Tulcea district, Romania).

Preparation of samples: 2.5 g dried leaves were heated with 50 mL of water on a reflux condenser for 30 min. After cooling, the extract was filtered through a plug of absorbent cotton in a 50 mL volumetric flask (5% stock solution - STM_1). Volumes of 10; 7.5; 5;1;0.1 STM_1 solution were diluted to 15 mL with distilled water. The following concentrations were obtained: 3.33%; 2.5%; 1.66%; 0.33%; 0.033% (STM_2-STM_6). For the determination, 12 germinated wheat caryopses with embryonic root lenght of 1 cm (kept for 24 h in a Sanyo MLR-351H germination room) where brought to Petri dishes (d = 50 mm) and treated with 15 mL of each solution (STM_1-STM_6). During the whole experiment, the length of embryonic roots was determined by linear method. Results were compared to a negative control, which contained distilled water, instead of extractive solutions. Changes of kariokynetic film for each concentration were investigated after 24h. Microscopic sections (vegetative cone of an embryonic root of each sample) were stained with a solution of diluted acetic orcein and analyzed at a Labophot 2 Nikon microscope.

Statistical analysis. Results for spectrophotometric and antioxidant determinations represent the average ± SD of three independent determinations and were calculated in Microsoft Office Excel 2007. Results obtained after 72 h of contact between extractive solutions and wheat caryopses have been used for statistical analysis, since they have the advantage of enough contact time and avoid measurement errors, that occur after 96 and 120 h. Statistical analysis was performed using Minitab 15.1.1.0 (Minitab Inc., 2007) software. Normality distribution was assessed using Ryan-Joiner, Darlington-Anderson and Kolmogorov-Smirnov tests. The statistical analysis used Kruskal-Wallis test (at a 95% confidence interval) to compare root elongation among multiple groups and Mann-Whitney test (at a 99% confidence interval) for pairwise *post hoc* comparisons, as the assumption of normality was not satisfied and the ANOVA could not be applied. The inhibitory effect was determined using the following formula:

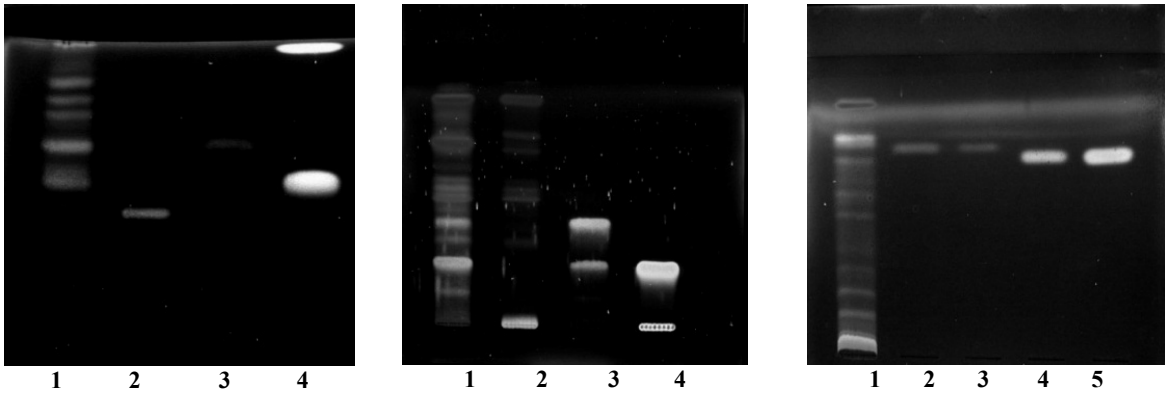
$$I_{inh} = 100 - \frac{T-1}{M-1} \times 100$$

where: T = test solution median, M = control median, 1 = initial value of embryonic roots (cm).

3. Results and discussion

Thin layer chromatography (TLC)

TLC analysis of polyphenols showed the presence of: chlorogenic acid (R_f = 0.45), hyperoside (R_f = 0.56) (fig. 1A, eluent system A) and quercetin (R_f = 0.20), kaempferol (R_f = 0.34) (fig. 1B, eluent system B). Although scientific literature (WICHTL [10], A.C. MIŠAN & al. [7]) foresees the presence of rutin in silver birch leaves, this compound was not identified by us. Analysing the chromatograms (fig. 1A, 1B) one can note the presence of other spots corresponding to compounds with flavonoidic behaviour (yellow fluorescence after spraying with the detection reagent) or phenolcarboxylic derivatives (blue fluorescence after spraying with the detection reagent). Spots with red fluorescence correspond to chlorophyll. TLC analysis of triterpenic compounds (fig.1C, eluent system C) showed the presence of one spot with a similar fluorescence and R_f value (0.82) corresponding to betulinic acid. Spots corresponding to ursolic/oleanolic acid (R_f = 0.73) were also identified. Analysing the chromatogram (fig. 1C), one can note the presence of other spots, corresponding to triterpenic compounds or sterols, that were not identified due to lack of standards.



A
Legend: 1 – M solution, 2 – rutin, 3 – hyperoside, 4 – chlorogenic acid + caffeic acid (from downwords to top;

B
Legend: 1 – MHE solution, 2 – MH solution, 3 – quercetin + kaempferol (from downwords to top), 4 – chlorogenic acid

C
Legend: 1 – M solution, 2 – betulin, 3 – betulinic acid, 4 – oleanolic acid, 5 – ursolic acid

Fig.1. TLC analysis of phenols and triterpenic compounds

Spectrophotometric determinations

The spectrophotometric assays (table 1) revealed a considerable amount of flavonoids (g% quercetin) and tannins (g% pyrogallol), similar to ones stipulated by scientific literature (M. TĂMAŞ & al. [6], FARMACOPEEA EUROPEANĂ ed. a-7-a [25], O. ZYRANOVA & al. [13]). The phenolic content is low compared to A.C MIŠAN. & al. [7] results that found 13.9 g% for silver birch leaves collected from Serbia. Regarding triterpenic compounds, one can note the presence of free forms mainly (11.7817 g%).

Table 1. Results for spectrophotometric determinations

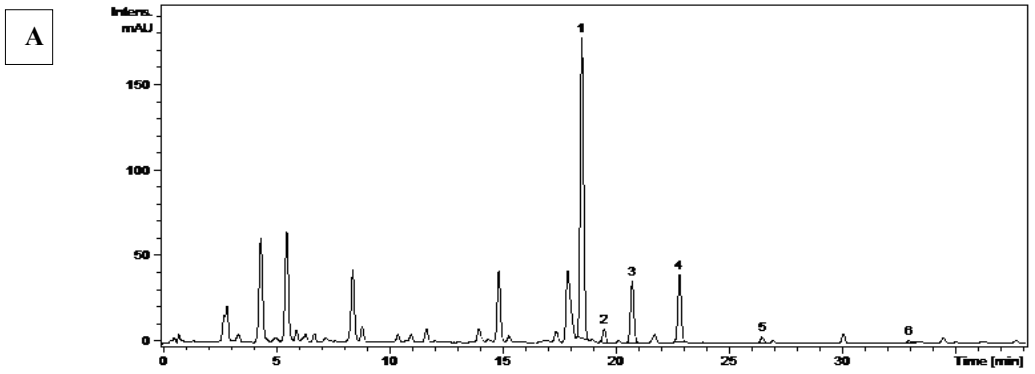
Betulae folium		
Total phenolic content (g% gallic acid)		7.5147 ± 0.5535
Flavonoids (g% quercetin)		1.6279 ± 0.0854
PCA (g% caffeic acid)		2.8548 ± 0.1108
Tannins (g% pyrogallol)		3.2100 ± 0.1058
Triterpenic compounds (g% betulinic acid)	free forms	11.7817 ± 1.0202
	esterified forms	10.9276 ± 0.2571
	glycosidic forms	4.3568 ± 0.5138

HPLC-MS/HPLC-DAD analysis

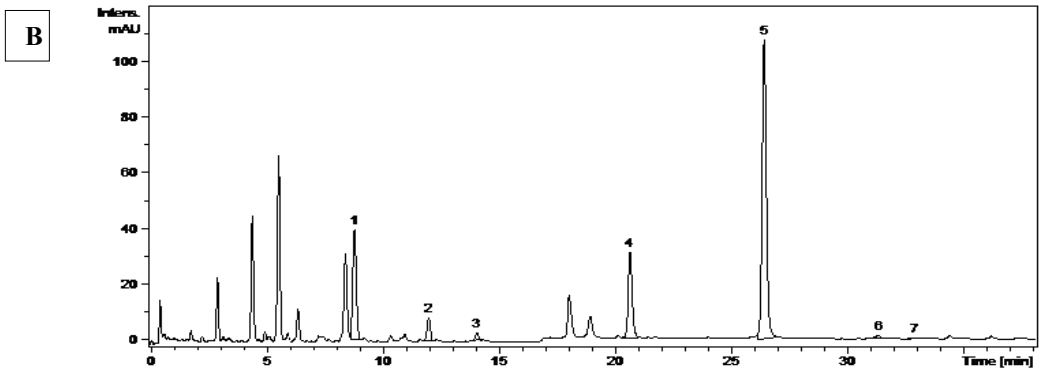
HPLC results for polyphenols (table 2, fig. 2A, B) pointed out that flavonoids are the major compounds from silver birch leaves. Hyperoside content (0.36 g%) is low compared to scientific data (0.8 g%), while the isoquercitrin content (0.15 g%) is similar (0.14 g%) (A. CARNAT & al. [33]). The isoquercitrin content found by us is low compared to M.P. GERMANÒ & al. [5] results (0.12 g%). Quercetin, kaempferol and apigenin contents are low compared to A.C. MIŠAN & al. [7] results. Quercetin content increased significantly after hydrolysis, since it results from hyperoside, quercitrin, isoquercitrin and probably other heterosides (avicularin, quercetin-3-O-glucuronide) (M.P. GERMANÒ & al. [5]), that were not identified due to lack of standards. Myricetin and kaempferol probably result from myricetin-3-O-rhamnoside, myricetin-3-O-galactoside, kaempferol-3-O-glucoside or kaempferol-3-O-glucuronide (M.P. GERMANÒ & al. [5]). Our results are different from A.C. MIŠAN & al. [7] that founds among free aglycones, only apigenin in unhydrolysed solutions. The apigenin content decreased significantly after hydrolysis, probably due to heat or pH degradation.

Table 2. HPLC results for polyphenols

Compound	**UV identification**	**MS identification**	**Concentration (mg/100 g dried herbal product)**	
			$M_{et\ 50\%}$	**$MH_{et\ 50\%}$**
chlorogenic acid	NO	YES	-	-
gentisic acid	NO	YES	-	-
caffeic acid	NO	YES	-	-
ferulic acid	YES	YES	-	9.029
p-coumaric acid	YES	YES	-	51.64
sinapic acid	YES	YES	-	2.6015
hyperoside	YES	YES	386	-
quercitrin	YES	YES	157.57	-
isoquercitrin	YES	YES	25.68	-
quercetin	YES	YES	5.2757	154.67
kaempferol	YES	YES	-	3.2057
myricetin	YES	YES	60	58.711
apigenin	YES	YES	5.2736	1.8125



Legend: 1 - hyperoside, 2 - isoquercitrin, 3 - myricetin, 4 - quercitrin, 5 - quercetin, 6 – apigenin



Legend: 1 - *p*-coumaric acid, 2 – ferulic acid, 3 – sinapic acid, 4 - myricetin, 5 - quercetin 6 - kaempferol, 7 – apigenin

Fig. 2. HPLC-MS chromatograms for polyphenols (A - $M_{et\ 50\%}$, B - $MH_{et\ 50\%}$)

Among PCA, gentisic acid and chlorogenic acid were identifed in both hydrolysed and unhydrolysed solutions, however caffeic, sinapic, ferulic and *p*-coumaric acids were present only in $MH_{et50\%}$, so we concluded that they exist only in glycosidic or esterified forms. *P*-coumaric acid content is high compared to other results (33 mg%) found by M.P. GERMANÒ & al. [5]. To our knowledge, sinapic acid and gentisic acid are new compounds, unmentioned by scientific literature. Recent research have shown that sinapic acid has antiinflammatory, antioxidant, anxiolytic and neuroprotective effects (C. CHEN [34]), while gentisic acid has a dose dependent neuroprotective activity in Parkinson disease (M.P. KABRA & al. [35]).

Ascorbic acid was identified in fresh birch leaves (fig. 3) and the content (3.63 g/100 g fresh herb) was high compared to other results (0.5 g%) (ESCOP MONOGRAPHS [36]). However the comparison with scientific data was difficult, since the collection time is not mentioned.

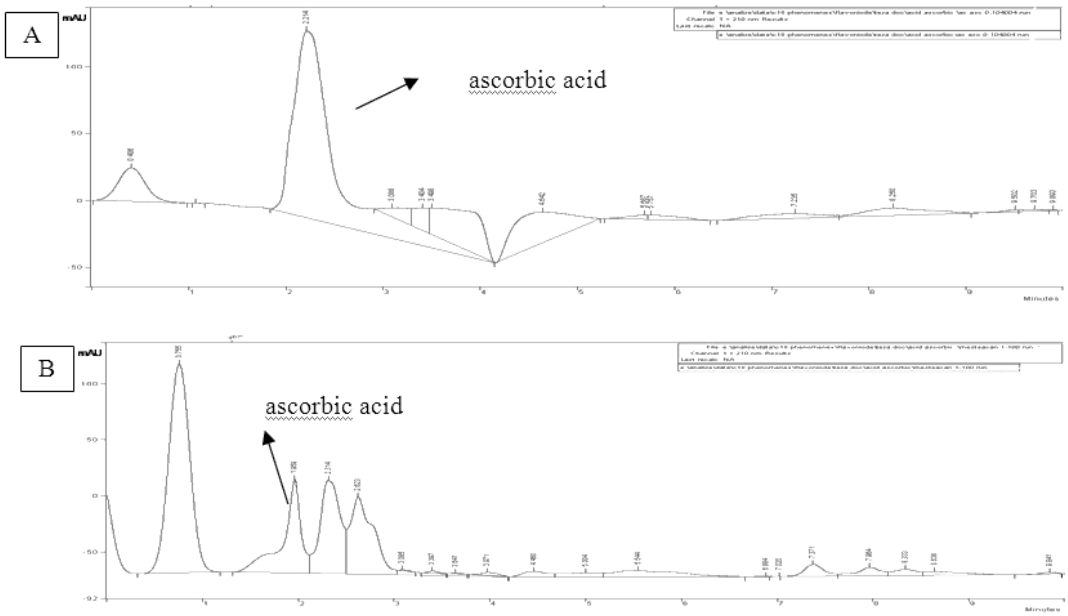


Fig.3. HPLC chromatograms for ascorbic acid
(A – reference standard, B – birch leaves)

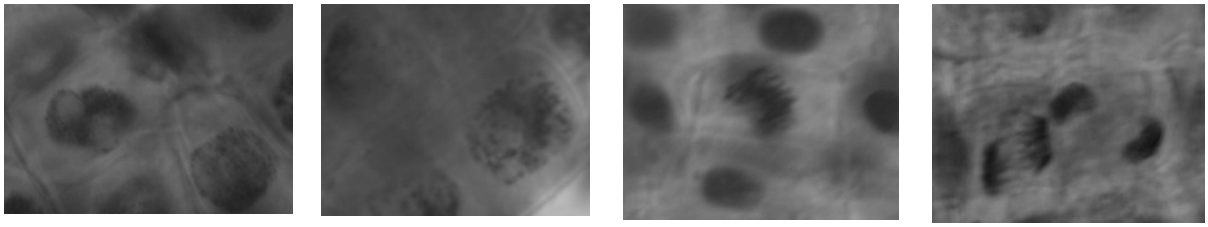
Antioxidant activity

The antioxidant activity of silver birch leaves (table 3) is low compared to positive controls (catechin, hyperoside, sinapic acid, chlorogenic acid), since EC_{50} (mg/mL) values are higher. At 0.1 mg/mL, the highest concentration common for both positive controls and birch leaves, the DPPH free radical inhibition was 24.89% for birch leaves and 92.78% (for chlorogenic acid), 89.40 % (for sinapic acid), 85.57 % (for hyperoside) and 94.93 % (for catechin) respectively. For reducing power assay, at 0.1 mg/ml, the absorbance value at λ = 700 nm was 0.20 for birch leaves; 1.04 for chlorogenic acid; 1.39 for sinapic acid; 0.92 for hyperoside and 1.22 for catechin. Our birch leaves EC50 value for DPPH scavenger activity is lower compared to M.P. GERMANÒ & al. [5] results that found 0.137 mg/mL, while the EC_{50} for reducing power activity is similar.

We assume that the polyphenols are responsible for silver birch leaves antioxidant activity, since these compounds have been shown to exert several biological functions such as free radicals quenching activity and metal chelation (R. RODRIGO & al. [37]).

Table 3. Antioxidant activity of silver birch leaves and positive controls

Sample	Method	
	DPPH EC_{50} (mg/mL)	**Reducing power EC_{50} (mg/mL)**
Betulae folium	0.4651 ± 0.0019	0.5172 ± 0.0223
chlorogenic acid	0.0227 ± 0.0019	0.0267 ± 0.0067
sinapic acid	0.0295 ± 0.0005	0.0220 ± 0.000
hyperoside	0.0325 ± 0.0007	0.0316 ± 0.0021
catechin	0.0242 ± 0.0043	0.0194 ± 0.0001



A **B** **C** **D**

Fig. 4. Kariokynetic film changes. A – hypertrophied nuclei, B – precipitated nuclear material, C – metaphases in tropokinesis, D – telophases in tropokinesis/bridge telophases

Phytotoxicity evaluation

The statistical analysis after 72 h of contact between extractive solutions and wheat caryopses, pointed out that there are significant differences towards the negative control, especially at high concentrations. Our results (table 4) emphasized that silver birch leaves extractive solutions have a strong (I_{inh} = 100%) and significant ($p < 0.01$) inhibitory effect upon root elongation at 5%; 3.33%; 2.5% and 1.66 % concentration. Regarding 0.33% and 0.0033% concentrations, the solutions seemed to stimulate root growth, but the results were not statistically significant compared to negative control. The inhibitory effect of birch leaves upon root elongation is associated with changes in the kariokynetic film. At high concentrations (5% and 3.33%) one can note the presence of hypertrophied nuclei (fig. 4A) and precipitated nuclear material (fig. 4B). For 2.5% and 1.66% concentrations, there are several areas with inhibited kinesis together with metaphases in tropokinesis (fig. 4C). At lower concentrations (0.33%; 0.033%) normal and abnormal divisions (bridge telophases and telophases in tropokinesis) were also seen (fig. 4D).We assume that the chemical composition of silver birch leaves is responsible for these changes, since high concentrations of triterpenic saponins are known for antiproliferative and anticancer effects. It is well known that betulinic acid modulates antiapoptotic BcL_2, BcL_{XL} proteins expression and is responsible for caspase 3,8,9 activation (L. PETCULESCU – CIOCHINĂ & al. [38], S. FULDA [39]).We also consider that high concentration of polyphenols can also inhibit root elongation, through pro-oxidant mechanisms (R.RODRIGO& al. [37]).

Table 4. Statistical comparison (Mann-Whitney test) between root elongation upon *Betulae folium* extractive solutions and negative control

Concentration (%)	Root elongation upon *Betulae folium* extractive solutions (median)	Root elongation upon negative control (median)	Difference of medians	99% confidence interval for difference of medians	p value (Mann-Whitney)	Inhibitory effect (%)
5	1	6.4	- 5.395	(-6.499 – - 2.399)	0.0001	100%
3.33	1	6.4	-5.350	(-6.501 – - 2.401)	0.0001	100%
2.5	1	6.4	- 5.350	(-6.501 – - 2.401)	0.0001	100%
1.66	1.01	6.4	-5.200	(-6.300 – - 2.400)	0.0001	100%
0.33	7.4	6.4	1.150	(-0.701 – 3.600)	0.0681	-18.51%
0.033	7.2	6.4	1.050	(-1.500 - 3.699)	0.3595	-14.81%

3. Conclusions

Among polyphenols, gentisic acid and sinapic acid are new compounds, mentioned here for the first time. To our knowledge no spectrophotometric determinations regarding free and esterified forms of triterpenic compounds in silver birch leaves have been made. *Betulae folium* has a moderate antioxidant activity. Phytotoxic effects were seen at high concentrations, so birch leaves might be used as a herbicide.

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