SUPPLEMENTARY MATERIAL

A bioanalytical approach of chemical composition, bioactivity and cytotoxicity of *Berteroa incana* L. herb

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Abstract: *Berteroa incana* is a wild herb widespread in temperate zones which was practically not studied for its biological effects. Methanolic and aqueous extracts of *B. incana* were assessed for the content in polyphenols and related antioxidant and antimicrobial activities and the polysaccharide extract for the content in saccharides and the associated cytostatic effect. The results obtained highlighted that methanolic extracts of *B. incana* contain moderate amounts of polyphenols, the most representative been isoquercitrin 4.41±0.02 mg$100g^{-1}$dry weight plant material (DW), quercetin 4.21± 0.05, sinapic acid 5.23±0.12 and ferulic acid 5.05±0.12 mg $100g^{-1}$DW, with correlated moderate antioxidant activities (IC_{50} 13.40±0.01 μg mL^{-1}) and absent antibacterial activity. The polysaccharide fraction showed high content in saccharides, especially in arabinose (312.22±7.54 mg g^{-1} polysaccharide extract) and glucose (279.22±5.59), and promising cytostatic effect.

Keywords: *Berteroa incana; polyphenols; saccharides; cytotoxicity; antimicrobial activity; antioxidant activity*

Experimental Section

Reagents

Folin–Ciocalteu reagent, (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid di-ammonium salt (ABTS) and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) were purchased from Sigma–Aldrich (Steinheim, Germany) and $AlCl_3$ from ICN Biomedicals (Ohio, USA). Caffeic acid (Sigma, C0625), quercetin (Sigma, Q4951), kaempferol (BioChemika, 60010), rutin (Sigma, R5143), luteolin (Fluka, 72511), ferulic acid (Aldrich, 128708), chlorogenic acid (Aldrich, C3878), sinapic acid (Sigma, D7927), p-coumaric acid (Fluka, 28200), syringic acid (Fluka, 86230), naringenin (BioChemika,71155), myricetin (Sigma, M6760) and cinnamic acid (Fluka, 96340) stock solutions, 1 mg mL^{-1}, were prepared by dissolving in methanol the appropriate amount of substance. Sodium tetra borate and sodium phosphate were purchased from Sigma (Germany) and sodium dodecyl sulphate from Fluka (Switzerland).

The standard monosaccharides were purchased from Fluka (Switzerland): L(-) fucose, D(+)xylose, L(+) rhamnose, L(+) mannose, and from Merck (Germany): L(+) arabinose, D(+) galactose, D(-) ribose and D(+) glucose.

Ultra-pure water, 0.1 M and 1 M sodium hydroxide solutions were purchased from Agilent Technologies (Germany). Solvents (Merck, Germany) and solutions were filtered on 0.2 μm membranes (Millipore, Bedford, MA, USA) and degassed prior to use. The stock solutions for each standard were stored at +4°C. The working solutions were prepared daily by diluting the stock solutions in background electrolyte (BGE).

Plant material

The aerial part of the *B. incana* plants were collected in the wild from Bucsani, Dambovita district of Muntenia - Romania (latitude 44.87 and longitude 25.65), during July - August 2013, when the flowers were in blossom period. The vegetal material was taxonomically identified by PhD biologist Eugenia Nagoda and the voucher specimen was recorded at the Herbarium of the `Dimitrie Brandza` botanical garden in Bucharest (Reference No. 400627). The collected plant material was dried up to 10% from the initial weight in a Memmert oven with aeration, powdered by a grinder (Grindo Mix GM 200) and finally stored refrigerated in dark all-plastic containers.

Preparation of extracts

The ultrasound-assisted extraction does not require complex instruments and is considered more effective for polyphenols extraction than the conventional extraction methods (Dai & Mumper 2010; Roidaki et al. 2015). Hence, the extraction of polyphenols was performed using an ultrasound extraction method at RT (the flask was immersed in ice to avoid overheating), over 1 hour, with water (aqueous extract-AE) and a mixture of methanol: water (methanolic extract-ME) (30 vol. %, 50 vol. % and 70 vol. %) in 1:10 ratio (g mL^{-1}). Next, the extracts were centrifuged for 15 minutes at 5000 min^{-1} and the supernatants were collected, adjusted to 10 mL, filtered on 0.2 μm Millipore PTFE and stored at -20 °C.

For polysaccharide separation, 10 grams of crushed plant material were extracted in hexane and chloroform to eliminate fat materials and pigments. After the supernatants were removed, the dry plant material was immersed in 100 mL deionized water for 10 h, and then was heated at 100 ^{o}C for 1.5 h. The resulting extract was filtered through paper and the plant residue was extracted in the same way again. Next, the resulting extracts were pooled, concentrated and precipitated by adding ethanol to a final concentration of 80% v/v. The mixture was left 10 h at 4 ^{o}C and the precipitate was collected by centrifugation 10 min at 6000 rpm, then was reconstituted with 50 mL deionized water and completely deproteinated by several rinses with Sevag solution (chloroform/butyl alcohol v/v 4:1) (Staub 1965; Chen et al. 2015). By adding ethanol to a final concentration of 80% v/v at 4^{o}C for 10 h again, the supernatant was concentrated, precipitated, centrifuged and subsequently dried at 37 ^{o}C.

Phytochemical screening of extracts

The hydroalcoholic and aqueous extracts of *B. incana* were investigated using various chemical tests (Table1S) in order to evaluate the existence or the absence of different phytochemical compounds, respectively phenolic compounds, saponins, sterols, alkaloids, tannins, glycosides, proteins and amino acids (Wani et al., 2011).

The total content of alkaloids

The determination of the total content of alkaloids (TA) was based on the reaction of alkaloids with brome cresol green (BCG) forming a yellow-coloured product extracted in chloroform and measured at 470 nm. The method was reported by Shamsa et al. (2008) and offers the advantages of sensitivity, stability and short time analysis, being frequently used for

the TA estimation. We used the obtained calibration curve of standard atropine: $y=0.00080x+0.02675$, $R^2=0.9952$ ($p < 0.0001$).

Determination of total polyphenols and flavonoids

The total phenolic content (TP) was estimated according to the Folin-Ciocalteu photometric method (ISO 14502-1:2005). The absorbance was measured at 765 nm. TP was assessed using calibration curve of standard Gallic acid: $y=0.0241x+0.0956$, $R^2=0.9991$ ($p < 0.0001$). The results were expressed as mg Gallic acid equivalents per 100g of dry weight of plant material (mg GAE $100g^{-1}$ DW).

The technique most employed for quantification of total flavonoids (TF) is based on the spectrophotometric determination of complex flavonoid-$AlCl_3$ which provides a hyper chromic effect (Lin and Tang 2007). The absorbance of the final mixture was measured at 425 nm. The TF was estimated using a calibration curve of quercetin: $y=0.0141x-0.009$; $R^2=0.9995$ ($p < 0.0001$). TF content of the plant extracts was expressed as mg quercetin equivalents per 100 g of dry weigh of plant material (mg QE $100g^{-1}$ DW).

DPPH and ABTS radical scavenging methods

The antioxidant activity was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH (2,2′-diphenyl-1-picrylhydrazyl). Experiments were carried out according to the method of Blois (1958) with a slight modification (Brand-Williams et al. 1995). A volume of 2.0 mL of the diluted sample extract was put into test tube and 1.0 mL of methanolic solution of DPPH· radicals (0.274 mM) was added. After 30 minutes, the absorbance of mixture was measured at 517 nm. The amount of sample necessary to decrease the absorbance of DPPH by 50% (IC50) was calculated.

The ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation de-colorization method is based on the reduction of ABTS•+ solution by antioxidants found in the tested plant extracts (Erel 20014). Stock solution of ABTS•+ (7 mM) was obtained by mixing the ABTS with potassium persulfate solution 2.5 mM and storing 12 hours in the dark. A volume of 0.5 mL of plant extract was added to 2.5 mL of ABTS•+ solution, and the absorbance was measured at 731 nm 15 min after.

A spectrophotometer Thermo Scientific Evolution 260 Bio was used for all spectrometric analysis. All the measurements were performed in triplicates.

Antimicrobial activity

The study of the different plant extracts effect on microorganisms was performed on referential strains of Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538 and ATCC 25923) and Gram-negative bacteria (*Escherichia coli* ATCC 8739). The antibacterial activity was investigated in triplicates by the qualitative screening of the susceptibility of different microbial strains to the plant extracts using the disk-diffusion method (CLSI 2006a; CLSI 2006b).

HPLC-DAD of polyphenols

The assessment of individual phenolic compounds was performed using a liquid chromatographic system (Agilent, USA) with a diode array detector (DAD). Separation was achieved on a 150 mm x 4.6 mm, 5 µm particle, Fortis C18 (Fortis Technologies Ltd., Cheshire, United Kingdom) column at 30^{o}C. The mobile phase consisted from a gradient based on water and acetic acid at pH 3 (solvent A) and acetonitrile (solvent B). The samples were eluted as follows: Step *1*: 25% to 29% B in 0.00-20.00 min; Step *2*: 29% to 45% B in 20.00-27.00 min; Step *3*: 45% to 54% B in 27.00-40.00; Step *4*: 54% to 62% B in 40.00-45.00 min; Flow rates were initially 0.5 mL min^{-1}, changed to 0.7 mL min^{-1} from 10.00 min to 20.00 min, and then 0.8mL min^{-1}. The injection volume was 10 µL, and UV spectra were recorded between 280-400 nm at a rate of 0.8 spectrum s^{-1} and 4.0 nm resolution. Data were acquired and handled by ChemStation software (Agilent, USA).

The chromatographic method was validated in term of linearity, limit of detection (LoD), limit of quantification (LoQ), precision and accuracy. Linear ranges for the all compounds were between 2.5-50 µg mL^{-1} and all calibration curves expressed a good linearity (R^2 > 0.997) within the test range. The LoDs were between 0.01 and 0.08 µg/mL and LoQs between 0.04 and 0.9 µg/mL Intra-day and inter-day variations expressed as RSD % were lower than 5%, which revealed a good precision of the analytical method. Accuracy was determined by recovery test, values obtained for the analysed compounds were between 94.4% and 113.3%.

Carbohydrates analysis by CZE

20 mg of polysaccharide extract was dissolved in 2mL 1.5 M sulfuric acid solution. The hydrolysis of the polysaccharides was completed at 100 ^{o}C, and then the samples were cooled and neutralized with1 mL 6.0 M NaOH solution.

For 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization, to each test tube, 100 µL 0.5 M PMP methanol solution and 100 µL 0.3 M NaOH were added to 200 µL of mixed working standards solution or the hydrolysed polysaccharide sample. The mixture was left to react for 30 min at 70^{o}C, then was cooled to the room temperature and neutralized with 100 µL 0.3 M HCl solution. The final volume of mixture was supplemented to 1 mL with distilled water and the excess of derivatization reagent was removed with 10 mL chloroform. The upper phase was collected and filtered through a 0.45 µm membrane and degassed by sonication (Honda et al. 2003).

The separation of the PMP derivatized monosaccharides was obtained using an Agilent CE system (software ChemStation) with diode array detector (DAD) and CE standard bare fused-silica capillary (Agilent Technologies, Germany) with internal diameter of 50 µm and effective length of 72 cm. Prior to use, the capillary was washed successively with basic solutions: 10 min with 1M NaOH, 10 min with 0.1M NaOH followed by ultra-pure water for 10 min and background electrolyte (BGE) for 20 min. The capillary was flushed between runs with 0.1M NaOH for 1 min, H_2O for 1 min and BGE for 2 min. The electrolyte was refreshed after 3 consecutive runs.

The selected method was based on Chen et al. (2015) with some changes. The running buffer consisted from 90 mM boric acid adjusted to pH 9.95 with 1 M NaOH solution. The applied voltage was 30 kV and the detection was fixed to 245 nm. The performance characteristics of the method (validated in house) were: linearity between 0.75 and 53 µg mL^{-1}, detection and quantification limits ranged from 0.06 to 0.45 and 0.21 to 1.49 µg mL^{-1}, respectively, relative standard deviation values for repeatability were 5.19 % for intra-day assays and 5.06% for inter-day assays, and recovery values ranged between 85.99 and 112.64%.

Cytotoxicity studies

The cytotoxicity of polysaccharide extracts from *B. incana* was evaluated with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) Cell Proliferation Assay (Mossman 1983). The MTT assessments were done after 24 h and after 72 h on two cell lines: a stabilized cell line, derived from mouse fibroblast cells (NCTC clone 929) and a tumour stabilized cell line of human colorectal adenocarcinoma cells (Caco-2). The normal and tumour cells were cultured in Minimum Essential Medium (MEM) containing10% foetal bovine serum (FBS) and 1% antibiotics (penicillin, streptomycin and neomycin), at 37 °C, in 5% CO_2 humidified atmosphere.

The cell suspensions were obtained by sub confluent culture trypsinization and were seeded into 24-well plates; each well was seeded with $5x10^4$ cells/mL for 24 h and after standing overnight the culture medium was replaced with medium with different concentrations of the analysed samples (polysaccharide extract). The cell viability on normal cell line (NCTC) was assessed with samples concentrations between 100 and 1500 μg mL^{-1}, and for tumour cell line were used concentrations between 100 and 750 μg mL^{-1}.

The culture medium was removed after 24 h and respectively 72 h of exposure of cells to polysaccharide extracts and the cells were washed with 0.2 M PBS, pH 7.4 and then the MTT solution was added to each well (500 μg, 0.25 mg mL^{-1}). After 3 h of incubation in a humidified 5% CO_2/95% air atmosphere, at 37 °C, the MTT solution was removed and the formazan crystals formed in living cells were solubilized with an equal volume of isopropanol. After 15 min of incubation at 37 °C and gentle stirring the absorbance was measured at 570 nm with a Tecan Sunrise (Austria) spectrometer. The cell viability was expressed as a percentage of control treated cells with different concentrations of analysed samples, all of them being analysed in triplicates.

Cell cycle analysis

The cell cycle analysis was performed using a Becton Dickinson LSR II flow cytometer, and the cellular DNA content was quantified using ModFit LT 3.0 software. The observed changes in the dynamics of the cell cycle, between cell fractions in the phases G1, S and G2, are based on quantifying of DNA content in the tumour cell line Caco-2 after 72 hours of incubation with polysaccharide extract. The cell line was seeded in 6 well plates at a cell density of $8x10^4$ cells/mL and was maintained in culture medium MEM (supplemented with 10% foetal bovine serum and 1% antibiotics) at 37 °C in humidified atmosphere with 5% CO_2. At 24 hours after seeding, the cells were incubated in the presence of polysaccharide extract at concentrations of 250, 350, 500 and 750 μg mL^{-1}. After 72 hours of cultivation in the presence of the extracts, the cells were tripsinizated, washed twice with PBS and fixed in 70% vol. ethanol overnight at 4 °C; then, the cells were washed with PBS and incubated with 0.5 mg mL^{-1} RNaseA for 30 minutes at 37 °C. Next, the cells were labelled by incubation with100 μg mL^{-1} propidium iodide for 30 minutes at 4 °C.

Statistical analysis

All the analyses were carried out in triplicate in three different samples at each experimental level and the results are expressed as means values ± standard deviation (SD). The other statistical data were obtained with MaxStat version 3.60. The values representing the means ± SD of three determinations were analyzed using one-way ANOVA Tukey HSD test. Significant differences were assessed at the $p < 0.05$ probability level.

Supplementary Data

Preliminary phytochemical screening

The results of the phytochemical evaluation and the qualitative estimation of the different extracts of *B. incana* are presented in Table S1, some important phytochemicals being evidenced. The presence of bioactive compounds indicates a possible medicinal value for the analysed plant. The phytochemical study revealed the presence of tested phytocompounds in all methanolic extracts of *B. incana*.

Cell cycle analysis

The analysis of the cell cycle for the tumour cell line Caco-2 treated with different concentrations of polysaccharide extract after 72 h of incubation is based on the amount of DNA in each phase of interphase, respectively G1, S and G2. In the control sample of the untreated tumour cells was observed a dissimilar cells distribution, namely 64% for G1, 26% for S and 10% for G2. After the incubation of the cells during 72 hours with the polysaccharide extract in concentrations between 250 μg mL^{-1} and 750 μg mL^{-1}, the number of cells from G1 phase decreased from 54.62% at 250 μg mL^{-1} to 35.67% for 750 μg mL^{-1}. Therefore, the treatment with polysaccharide extract induced a decrease of the cell number in G1 phase and an increase of the cell number in S phase (from 25.23% to 35.09%); these results indicate that polysaccharide extract arrests the cell cycle in the S phase in a dose dependent manner and can suppress the tumor cells proliferation.

Table S1 Phytochemical content of *B. incana* extracts

Chemical components	Performed test	AE	30% vol. ME	50% vol. ME	70% vol. ME
Saponins	Foam test	+	+	+	+
Phenolic compounds	Ferric chloride test	+	+	+	+
Tannins	Ferric chloride test	+	+	+	+
Proteins & amino acids	Biuret and xantoprotein reaction	+	+	+	-
Alkaloids	Dragendorff's test	-	+	+	+
Terpenoids	Salkowski's test	-	+	+	+
Sterols	Liebermann-Burchard's test	-	+	+	+
Glycosides	Borntrager's test	+	+	+	+

Note: +: present, -: absent.

Table S2 TP, TF and oxidant activity of extracts of *B. incana*

Sample	TP content[a,b] mg GAE $100g^{-1}$ DW plant material	TF content[a,***] mg QE $100g^{-1}$ DW plant material	Inhibition[a,***] %	IC_{50}[a,***] ($\mu g\ mL^{-1}$)
AE	34.30 ± 0.68	7.74 ± 0.04	50.97 ± 0.56	31.91±0.07
30% vol. ME	35.32 ± 0.34	18.68 ± 0.16	69.83 ± 0.27	21.34±0.06
50% vol. ME	36.61 ± 1.40	24.57 ± 0.84	66.93 ± 0.08	20.71±0.03
70% vol. ME	39.23 ± 0.22	31.60 ± 0.22	56.36 ± 0.55	13.40±0.01

a, data are expressed as means values ± standard deviation (n=3).

b, within the column, insignificant differences ($P > 0.05$) there are between AE and 30% vol. ME values; significant differences ($P < 0.05$) there are between the rest of the means.

***, very significant differences ($P < 0.001$) there are between all the values obtained in the same column.

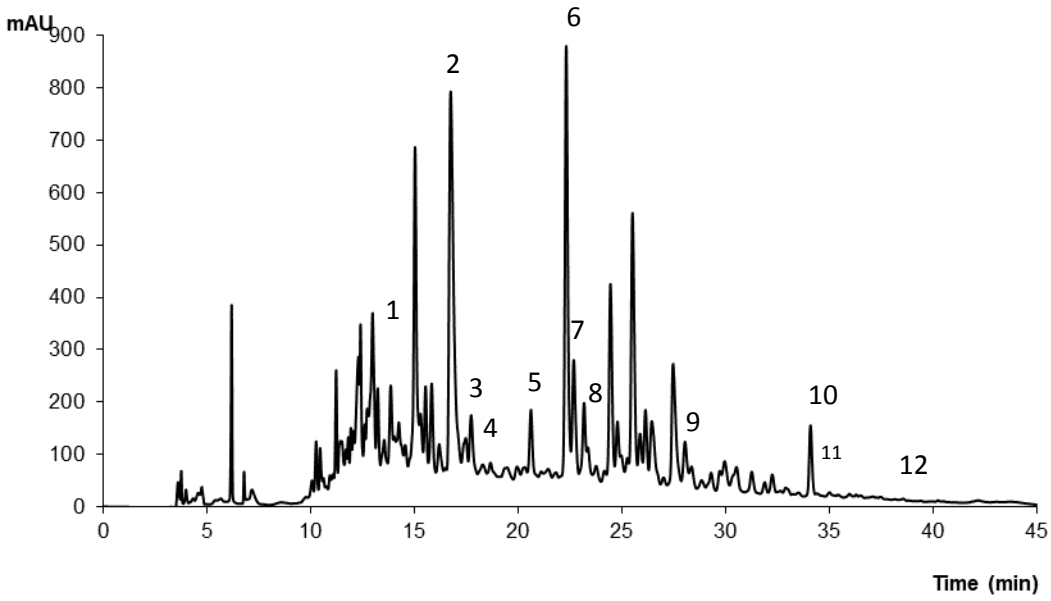
Supplementary figures captions:

Figure S1 *Berteroa incana* (L.) DC and inflorescence in umbelliferous raceme

Figure S2 Chromatogram of *B. incana* ME (70% vol.); the number of compounds corresponds with Table 1

Figure S1

Figure S2



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