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Original paper

Vaccinium corymbosum leaves, a potential source of polyphenolic compounds

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Abstract

Studies carried out so far on Blueberry leaves have shown a complex composition in these leaves, but there are great differences among different cultivars. The aim of this study was to optimize the extraction method in order to avoid compounds' degradation during extraction and to find a simple HPLC method for the analysis of *V. corymbosum* leaves collected from a culture in Mureș county, Romania. First extraction was carried out with methanol in an ultrasound water-bath, and the concentrated extract was applied on a Sephadex LH-20 column. The second extraction was performed using a mixture of quartz sand and ground leaves. The fractions were then analyzed by TLC and HPLC-UV-Vis. Spectrophotometric determination of total polyphenols, tannins and proanthocyanidins was carried out, too. TLC revealed that the acetone fraction contained mostly tannin polymers. The HPLC analysis revealed several compounds, among them cyanidin and quercetin glycosides. Small qualitative differences between the two types of extracts were observed. The blueberry leaves extract showed high polyphenolic content. This study confirmed that blueberry leaves collected from our area contain a high concentration of important polyphenolic compounds, which could be further used in the development of new natural medicines.

Keywords Blueberry, HPLC, Leaves, Polyphenols, Sephadex LH-20, TLC.

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Introduction

Phenolic compounds have received an increasing attention in the last few years because they have shown promising effects on human health, including antioxidant, antiproliferative, and antibacterial effect, and they seem to have beneficial effects on metabolic disorder symptoms and preventing obesity (SOUSA, 2016 [1], NORBERTO, 2013 [2], PERVIN, 2013 [3]). Analysis of phenolic compounds usually implies a few problems, because of their sensitivity to environmental conditions, such as light, temperature, and solvents (WELCH, 2008 [4]). *Vaccinium corymbosum* L. also called high bush blueberry is a cultivated species and is a rich source of phenolic compounds. Recent studies have shown that changes in the synthesis of phenolic compounds in plant organs appear along geographical gradients and there are differences in composition between different cultivars (MARTZ, 2010 [5], HABANOVA, 2013 [6], WANG, 2015 [7]). Until now there have been identified three important classes of compounds in the leaves: anthocyanins, flavonoids and phenolcarboxilic acids, in different proportions and in different concentrations (FERLEMI, 2016 [8], MATSUO, 2010 [9]). The leaves from the culture are not being exploited at this moment, although they are a very valuable source for future development of nutraceuticals. A rapid and efficient method for extraction and analysis is still needed, knowing that the optimization studies are very important for the extraction of unadulterated compounds, which could be further isolated from plant material. The aim of the study was to make a preliminary analysis of the leaves from *Vaccinium corymbosum* L. collected from a cultivar in Mureș county, Romania, in order to develop an optimized method for extraction and analysis. The evaluation of the chemical profile in this plant material adds significance to this study, offering a background for the future use of these leaves as a source of phenolic compounds.

Materials and Methods

Plant material: Blueberry leaves (*Vaccinium corymbosum*) were collected from a culture in Mureș county, România in October 2014 when the leaves were red. The leaves were air-dried under darkness at room temperature, and preserved in normal conditions. A sample of this specimen was stored at the Department of Pharmacognosy and Phytotherapy from the University of Medicine and Pharmacy Tîrgu Mureș.

Chemicals: Reference substances were Cyanin chlorid – CNCL, Isoquercitrin – ISOQ, Rutin – RUTN (Carl Roth GmbH), Kuromanin – KRMN, Kaempferol – KMPH, Caffeic acid – CAFA and Chlorogenic acid – CLRA (Cayman Chemical Company). Other chemicals used, methanol, acetonitrile, acetone, chloroform, trifluoroacetic acid, were of analytical or HPLC grade. A solution of 0.5% vanillin solution in methanol containing 4% (v/v) HCl was used for TLC detection.

Extraction using quartz sand: About one gram of ground leaves was mixed with two grams quartz sand for five minutes in a glass mortar using a glass pistil. The powder was then introduced in a glass column and extracted by gravity with small portions of methanol, collecting 5 ml-fractions (Q) and then with acetone 70% at room temperature (Andersen & Markham [10]). The extraction has been interrupted when TLC analysis indicated that the colorless fractions do not contain any compound. These fractions were excluded from further analysis.

Ultrasound-assisted extraction: Dried blueberry leaves were ground in a commercial coffee mill. Phenolic compounds were extracted from ground leaves in an ultrasonic water-bath at a temperature not exceeding 55°C by using a solid to solvent ratio of 1:10 (w/v). The cooled slurry was filtered through filter paper and the residue was re-extracted once more in the same conditions. The combined extracts were evaporated to dryness under vacuum below 50°C using an Ika rotary evaporator RV 05-ST with a vacuum controller.

Separation of phenolic compounds using Sephadex LH-20 column chromatography: Column chromatography was performed using Sephadex LH-20 (GE Healthcare Bio-Science AB, Uppsala). The crude extract (C) was suspended in 5 ml methanol, and then applied to a chromatographic column packed with Sephadex LH-20 that had been equilibrated with methanol. Phenolic fractions were eluted from the column using small portions of methanol followed by acetone 70%, by collecting 5 ml-fractions (ANDERSEN & MARKHAM, 2006 [10]).

Thin-layer chromatogaphy (TLC): The fractions obtained after quartz sand extraction (Q fractions) and Sephadex LH-20 separation (S fractions) were analysed on pre-coated TLC sheets ALUGRAM® Xtra SIL G (Macherey-Nagel GmbH & Co.KG, Duren) 20 x 10 cm using the following solvent system: water-methanol-chloroform (10:35:65 v/v/v). Plates were developed after a path of 8 cm. Spots were detected under ultraviolet light and by spraying with vanillin solution, followed by heating (AMAROWICZ, 2005 [11]).

Preparative TLC: The fourth methanolic fraction (Q4) from quartz sand extraction was separated on pre-coated TLC glass plates Sil G25 with the solvent system consisted of water-methanol-chloroform (10:40:60) into five sub-fractions (P1, P2, P3, P4, P5). The fractions were chemical revealed on the plate by spraying the margins of the plate with Natural Product Reagent (according to NEU) for guidance. The sprayed sections were then cut from the plate and excluded from further analysis. The subfractions were eluted from the gel with methanol and were further analysed by HPLC.

HPLC analysis: The HPLC analysis for phenolic compounds was performed using an Agilent 1100 series system with UV detector on Inertsil ODS, 150 x 4.6 mm, 3 μm column (GL Sciences Inc.). The mobile phase consisted of 0.1% trifluoroacetic acid (A), acetonitrile (B) and methanol (C) and the runs were made at a flow rate of 0.8 mL/min. Temperature of the column was set to 35°C.

Methanolic solutions of chlorogenic acid, caffeic acid, cyanin chloride, isoquercitrin, rutin, kuromanin and kaempherol, alone and in mixtures, were used for

qualitative analysis. Mixtures composition: mixture I – chlorogenic acid 19.8 μg/ml, caffeic acid 20.6 μg/ml, cyanin chloride 20.8 μg/ml, isoquercitrin 20 μg/ml and rutin 20 μg/ml; mixture II – chlorogenic acid 14.14 μg/ml, caffeic acid 14.71 μg/ml, cyanin chloride 14.85 μg/ml, isoquercitrin 14.28 μg/ml, rutin 14.3 μg/ml kuromanin, 7.34 μg/ml and kaempherol 14.28 μg/ml. The volume of injection was 20 μl. Two different gradient elution compositions were used. First gradient elution program was: 87% A, 11% B, 2%C at 0 min, 74% A, 22% B, 4% C at 15 min, 72% A, 23% B, 5% C at 20 min, 87% A, 11% B, 2% C at 25 min, 87% A, 11% B, 2% C at 28 min. The monitoring wavelength was set at 280, 350 and 525 nm and the tested solutions were Q4, S4, P1, P2, P3, P4, P5. For the second method, the following gradient elution program was applied: 87% A, 8% B, 5% C AT 0 min, 73% A, 22% B, 5% C at 15 min, 72% A, 23% B, 5% C at 20 min, 15% A, 80% B, 5% C at 25 min, 15% A, 80% B, 5% C at 30 min, 87% A, 8% B, 5% C at 31 min, 87% A, 8% B, 5% C at 35 min. The monitoring wavelength was set at 280 nm and the tested solutions were Q4, S4.

Determination of tannins, proanthocyanidins and total polyphenols: Spectrophotometricdetermination of total polyphenols and tannins was carried out according to the method described in European Pharmacopoeia 7th edition [12]. Tannin content was expressed as g pyrogallol/100 g dry weight. Total polyphenolic content was expressed in g gallic acid equivalents/100g dry weight (GAE), using the equation of the calibration curve of gallic acid ($y=4.1445x+0.0456$, $R^2 > 0.999$). The proanthocyanidins determination was performed by the spectrophotometric technique of Lebreton [13], with small modifications and was expressed as g cyanidin chloride equivalents/100 g dry weight (C3GE). For this purpose, 0.2 g leaf powder was treated with 25 ml HCl 2N in a round flask with ascending condenser and was kept 40 minutes on a boiling water bath. After cooling, the solution was filtered and extracted three times with 20 ml n-butanol in a separation funnel. The butanol solutions were reunited and diluted to 100 ml, and then the absorbance was determined at 550 nm. All determinations were performed on a SPECORD 210 spectrophotometer (Analytik Jena AG). All samples were analyzed in three replicates. In each case, the mean value ± standard deviation was calculated.

Results and Discussion

The *Vaccinium corymbosum* leaf extract was found to be rich in polyphenolic compounds. Our results (Table 1) are in accordance with the data from the literature, the concentration of tannins in blueberry leaves is very similar with the concentration of tannins in bilberry leaves collected from Poland (7,48 g/100 g DW) (ROSLON, 2011 [14]). The total polyphenolic content determined in our study is in the range of the concentration determined in a study conducted in China on *Vaccinium formosum* (6,715-34,917 g GAE / 100 g DW) and slighty higher than the concentration of total polyphenols determined in Canada from *Vaccinium corymbosum* leaves collected in october (15,583 g GAE / 100 g DW) (DENG, 2014 [15], ROUTRAY, 2014 [16]).

Table 1. Cuantification of components from *Vaccinium corymbosum* leaves extract

Metabolites	(% ± S.D.)*	Standard used
Total polyphenols	18.65 ± 0.63	Gallic acid
Tannins	6.7 ±1.31	Pyrogallol
Proantocyanidins	15.47 ±0.99	Cyanidin-3-glucoside

*Values are expressed as g per 100 g of the dry extract (%, w/w), and all data are means ± S.D. (n=3).

For the qualitative analysis we performed a simple method of extraction using quartz sand, because phenolic compounds are easily degraded by heat, pH changes and light. The sand extraction is a suitable sample preparation method for glycosides protection and to prevent the hydrolysis. The sand method or sea sand disruption method (SSDM) is a matrix solid phase dispersion (MSPD) which combines multiple steps (disruption of plant material, extraction and purification) into a single one (WIANOWSKA, 2015 [17]). Five methanolic fractions and four acetonic fractions after quartz sand extraction were collected. Column chromatography separation on Sephadex LH-20 provided 4 methanolic fractions and 5 acetonic fractions. The TLC plates (Fig. 1.) were analyzed in visible light, after derivatization with 0.5% vanillin solution in methanol containing 4% HCl. Three pink spots have been separated from the methanolic fractions.

The TLC analysis revealed the presence of flavan-3-ols (pink spots) after derivatization with vanilin/HCl reagent, seen on both TLC plates, and condensed tannins, which were eluted with acetone on the Sephadex column chromatography as previously described (PEGG, 2008 [18]).

At least three different classes of compounds are extracted and the best wavelength of detection was selected after monitoring the fractions at three different wavelengths. As it could be seen from the chromatograms for the forth fraction (Q4) presented in Fig. 2.A, the wavelength 280 nm is suitable for the identification of all reference compounds. The peaks seen at 280 nm are visible at 350 nm, too, with slight intensity differences. The wavelength 350 nm amplifies three peaks between 16 and 19 minutes and the wavelength 525 nm is very sensitive and specific only for the two peaks from 9 and 11 minutes.

The subfractions obtained from preparative TLC (Fig. 2. B, C, D, E, F) were analyzed at 280 nm, too. The results indicated no further improvement of the extract's chemical profile. Being a time-consuming method, this procedure was not further investigated.

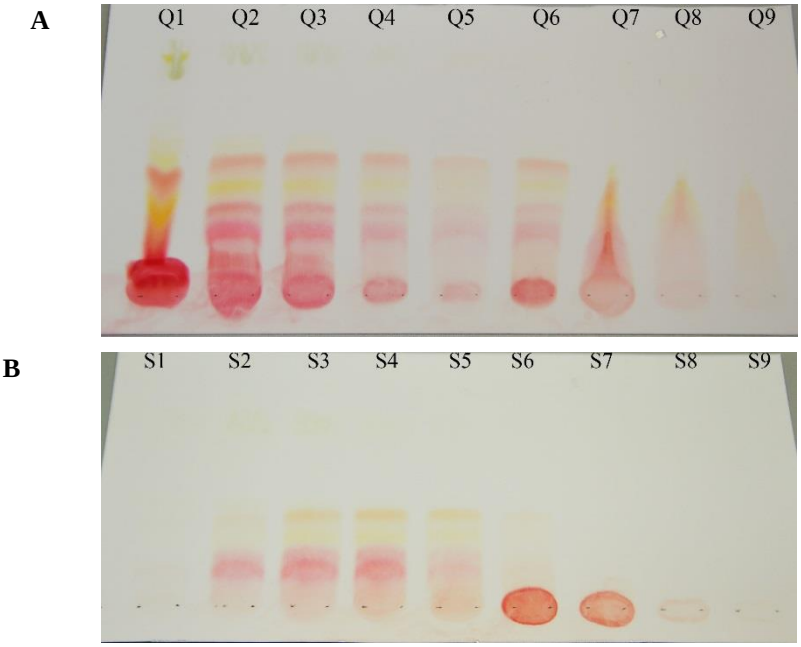

Figure 1. TLC chromatoplates of fractions obtained after quartz sand extraction (**A**) and Sephadex LH-20 separation (**B**)

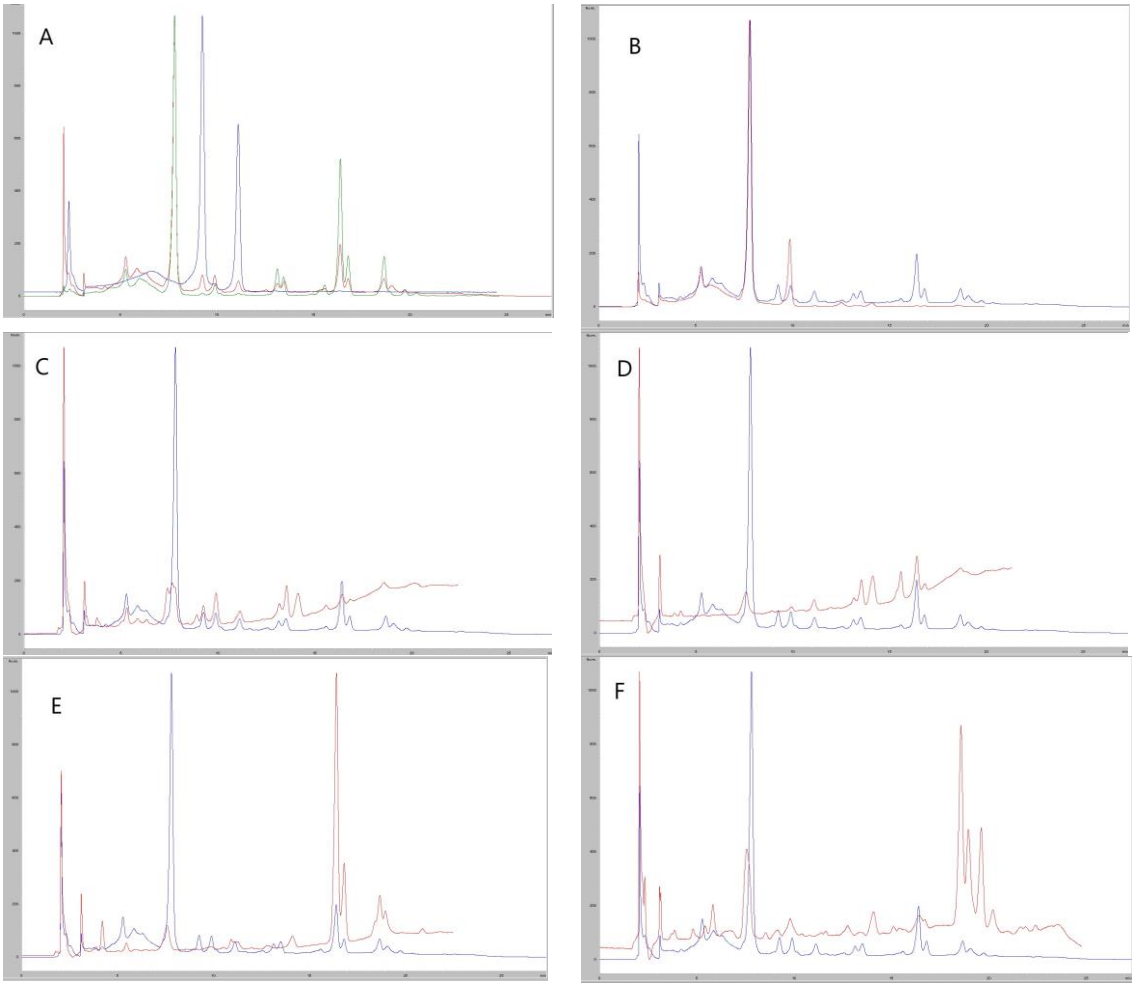

Figure 2. Normalized chromatograms of (**A**) Q4 fraction at different wavelenghts – 280 nm, 350 nm and 525 nm, and overlaid chromatograms at 280 nm of (**B**) Q4 fraction and P1 subfraction; (**C**) Q4 fraction and P2 subfraction, (**D**) Q4 fraction and P3 subfraction, (**E**) Q4 fraction and P4 subfraction, (**F**) Q4 fraction and P5 subfraction

Four compounds were identified by HPLC using the second gradient elution: two phenolic acids - chlorogenic acid and caffeic acid and two quercetin glycosides - rutin and isoquercitrin (Fig. 3). The chromatographic profile of both fractions is almost identical with two differences: S4 fraction contains an extra peak at t_R=4 min and Q4 fraction has an extra peak at t_R=33,4 min. The extra peak on the Sephadex fraction chromatogram could be explained by the type of extraction method which needs high temperature for a proper extraction of all compounds or the extra compound could be an aglycon obtained after heating the extract. Also the extra peak of the quartz fraction could be related only with the extraction method, the risk of heat-related degradation being avoided in this case by working at room temperature. The S4 fraction contains a higher concentration of the identified compounds than Q4, although we used the same quantity of solvents, but, as it was clearly seen on the TLC analysis, the first three sand extraction fractions were more concentrated due to the different extraction capacity of sand in comparison with Sephadex, and the compounds were eluted according to their weight. The high concentration of compounds proved by TLC analysis confirms the fact that the sand is an abrasive material, which exposes the cells to the solvent and allows a higher amount of compounds to be extracted (TEIXEIRA, 2005 [19], MANHITA, 2006 [20]).

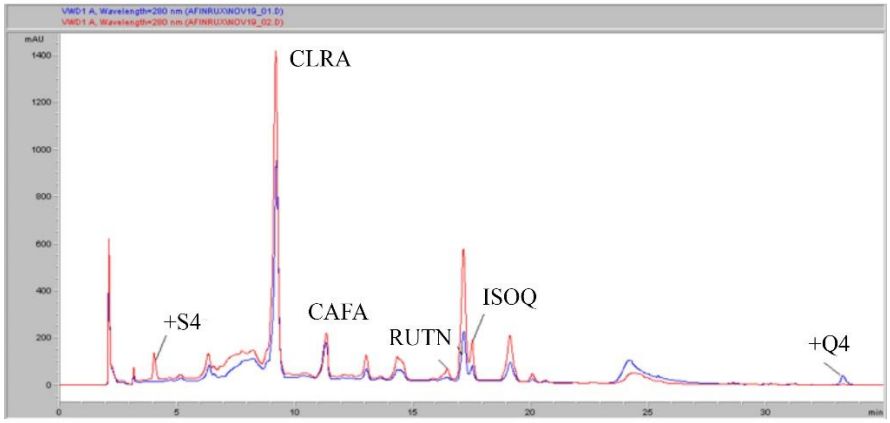

Figure 3. Chromatograms of Q4 and S4 fraction at 280 nm

Conclusions

In conclusion, a methodology as a rapid preliminary analysis of blueberry leaves composition is proposed. The preparative TLC is a time consuming process with no beneficial contributions in this step. The sand extraction method is preferable for the qualitative and quantitative analysis and Sephadex separation should only be used when the fraction containing condensed tannins is needed. Further studies of all fractions are necessary for a better evaluation of the two extraction methods. *Vacciunium corymbosum* leaves contain a large amount of polyphenols and proanthocyanidins, this is why we consider it to be a good resource for obtaining valuable compounds with biological activity, a process that could have a beneficial effect for the blueberry cultivars.

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