HPLC ANALYSIS OF PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY OF *RIBES* FRUIT LEAVES

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Abstract: Berry fruit leaves are recognized as potential medicaments which are rich in different phenolic compounds, and have been used in folk medicine for centuries. In order to evaluate phenol composition of fruit leaves from red currant (*Ribes rubrum*) and black currant (*Ribes nigrum*) species were subjected to the spectrophotometric and HPLC analysis. The antioxidant activity was estimated using DPPH free radical scavenging assay. Flavonols, flavan-3-ols and phenolic acids were the main phenol classes found in the investigated leaf extracts. Investigated extracts showed significant antioxidant activity and a correlation with total phenol content. *Ribes* leaf extracts, rich in phenolic content, with significant antioxidant activity, can be used as medical supplements.

Key words: berry fruit leaves, phenolic compounds, antioxidant activity

Introduction

Phenolic compounds are produced by plants, both edible and inedible, as a response to the environmental stress and pathogens. They are present in all plant parts in different quantities, depending on the stage of plant development and the environment influence.

Berry fruits are recognized as plants which are rich in different phenolic compounds and have been used in folk medicine for centuries.

Phenolic compounds are mainly represented by anthocyanins, phenolic acids, flavan-3-ols and flavonols. These compounds are recognized as potential antioxidant agents with possible applications as food and medical ingredients.

Berry fruits, such as grape, blueberry, chokeberry, bilberry, cranberry, blackberry, raspberry, blackcurrant, strawberry, etc. are a particularly rich source of phenolic antioxidants (Buricova et al., 2011; Deighton et al., 2000; Nowaka and Gawlik-Dzikib, 2007; Ordogh et al., 2010).

There are also studies on the beneficial effects of phenolic compounds on the heart and other chronic diseases, as well as their anticancer, anti-inflationary and antimicrobial activities (Andjelkovic et al., 2013, Deighton et al., 2000; Radovanovic et al., 2013).

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However, there is less research of polyphenolic antioxidants of berry fruit leaf extracts (Buricova et al., 2011; Gudej and Tomczyk, 2004; Vagiri et al., 2012).

Berry fruit leaves are traditionally used for easing childbirth-related muscle spasms, morning sickness, for colds, sore throats, diarrhea, threat wounds, colic pain, uterine relaxant (Puupponen-Pimia et al., 2005; Ryan et al., 2001; Venskutonis et al., 2007).

The objectives of this study were first to identify phenolic compounds from red currant (*Ribes rubrum*) and black currant (*Ribes nigrum*) species fruit leaves and then determine their antioxidant activity.

Material and methods

Chemicals

Methanol, acetonitrile and formic acid of HPLC-grade were obtained from Merck (Darmstadt, Germany). The standard phenolic compounds and 2,2`-diphenyl-1-picrylhydrazyl (DPPH) free radical and all other chemicals were supplied from Sigma Chemical Co. (St. Louis, MO). The reagents used were of analytical quality.

Samples

The berry fruit leaves were collected from the southern Serbia region after harvest. Samples of berry leaves were washed and dried at 60 °C. Dried leaves were crushed in a grinder for 2 min and then used for extractions.

The samples of dry leaves (0.5 g DW, dry weight) were extracted with 40 mL solvent system of methanol/acetone/water/acetic acid (30/42/27.5/0.5) by stirring continuously at room temperature in the dark for 30 min, and then centrifuged for 10 min at 2500 x g. The extracts were evaporated under vacuum rotary evaporator and diluted in 10 mL methanol. Extracts were filtered through a 0.45 μm syringe filter before analysis.

Spectrophotometric assay

Total phenols, hydroxycinnamoyl tartaric acids and flavonols in tested extracts were determined according to the spectrophotometric method previously described (Radovanović et al., 2013). Results were expressed as milligrams (mg) of gallic acid equivalents (GAE) for total phenols, mg of caffeic acid equivalents (CAE) for total hydroxycinnamoyl tartaric acids, and mg of quercetin equivalents (QE) for total flavonols per gram (g^{-1}) of extract dry matter (DM).

HPLC assay

HPLC apparatus used for separation and determination of individual polyphenols from leaf extracts was an Agilent Technologies 1200 chromatographic system, equipped with a photodiode array detector (DAD) and fluorescence detectors (FD). The column was thermostated at 30 °C. The separation was performed on an Agilent-Eclipse XDB C-18 4.6 × 150 mm column. The HPLC grade solvents used were formic acid / water (5 : 95 v/v) as solvent A and acetonitrile / formic acid / water (80 : 5 : 15 v/v) as solvent B. The injection volume was 5 μL and the flow rate was 0.8 mL min^{-1}. The detection wavelengths were 280, 320 and 360 nm for UV, and 275/322 nm ($\lambda_{Ex}/\lambda_{Em}$) for fluorescence-detection. The different phenolic compounds were identified by comparing their retention times and spectral characteristics with data of original reference standard

compounds and with data given in the literature (Gudej and Tomczyk, 2004). Results were expressed as mg g^{-1}extract DM.

DPPH test

Antioxidant activity of all investigated extracts was estimated, determining the radical scavenging activity of extracts by DPPH test previously described (Andjelković et al., 2013). The antiradical activities of investigated extracts were expressed as median efficient concentrations (EC_{50}).

Statistical analysis

Three analytical replictes were carried out on each sample. Measurements were averaged, and results are given as mean ± standard deviation (SD).

Results and discussion

The results of the spectrophotometric assay of investigated berry leaf extracts are shown in Table 1.

Tabela 1. Sadržaj ukupnih fenola, estara hidroksicimetnih i vinskih kiselina i flavonola (mg g^{-1}DM ± SD) i antioksidativne aktivnosti ekstrakata voćnih listova, EC_{50} (mg mL^{-1} ± SD)

Table 1. Total phenol, hydroxycinnamoyl tartaric acid and flavonol contents (mg g^{-1}DM ± SD) and antioxidant activity of fruit leaf extracts, EC_{50} (mg mL^{-1} ± SD))

Fenolni sastav *Phenolic composition*	Ekstraki listova crvene ribizle *Extract of red currant leaves*	Ekstrakti listova crne ribizle *Extract of black currant leaves*
Ukupni fenoli (mg_{GAE} g^{-1}DM) *Total phenols (mg_{GAE} g^{-1}DM)*	109.14 ± 0.93	9.75 ± 0.09
Hidroksicimetne i vinske kiseline ((mg_{CAE} g^{-1}DM) *Hydroxycinnamoyl tartaric acids (mg_{CAE} g^{-1}DM)*	105.78 ± 0.89	8.19 ± 0.13
Flavonoli (mg_{QE} g^{-1}DM) *Flavonols (mg_{QE} g^{-1}DM)*	36.71 ± 0.25	35.48 ± 0.29
Antioksidativna aktivnost, EC50 (mg mL^{-1}) *Antioxidant activity, EC_{50} (mg mL^{-1})*	0.50 ± 0.08	0.69 ± 0.07

GAE - Gallic acid equivalent; CAE - Caffeic acid equivalent; QE - Quercetin equivalent

The results showed high content of total phenols in investigated red and black currant leaf extracts, from 105.78 to 109.14 mg GAE g^{-1} DM extract. Also, significant amounts of flavonols were found in tested leaf extracts. Their content was ranged from 35.48 to 36.71 mg QE g^{-1} DM in red currant extract.

In order to determine more precisely phenolic composition in investigated extracts, the HPLC assay was used. Results (Table 2) are in good agreement with those obtained by spectrophotometric determination of investigated extracts (Table 1).

The results showed quite different phenolic composition, which belongs mainly to the three classes of phenols: phenolic acids, flavonols and flavan-3-ols. Other authors also found the present of these phenolic classes in some berry leaf extracts (Buricova et al., 2011; Nowaka and Gawlik-Dzikib, 2007; Vagiri et al., 2012).

Phenolic acids (hydroxybenzoic and hydroxycinnamoyl acids), as well as gallic, caffeic, ellagic and chlorogenic acid were present in all tested leaf extracts.

The epicatechin was predominantly flavan-3-ol in tested extracts, followed by catechin, epicatechin gallate and procyanidin B2. The presence of these compounds was reported by Vagiri et al. (2012) in black currant leaf extracts.

The quercetin-3-glucoside, rutin, kaempherol-3-glucoside and quercetin were predominant flavonols while luteolin-3-glucoside and myricetin were less abundant.

Tabela 2. HPLC analiza fenolnih jedinjenja u ispitivanim ekstraktima vocnih listiva
Table 2. HPLC analysis of phenolic compounds in investigated extracts of fruit leaves

Fenolni jedinjenja (mg g^{-1} DM) *Phenolic compounds (mg g^{-1} DM)*	Ekstrakt listova crvene ribizle *Extract of red currant leaves*	Ekstrakt listova crne ribizle *Extract of black currant leaves*
Gallic acid	0.22 ± 0.02	0.18 ± 0.01
Ellagic acid	4.30 ± 0.04	4.15 ± 0.01
Caffeic acid	0.39 ± 0.01	nd
Chlorogenic acid	1.26 ± 0.04	0.21 ± 0.01
Quercetin-3-glucoside	9.07 ± 0.15	7.14 ±0 .21
Rutin	6.14 ± 0.10	5.84 ± 0.17
Luteolin-3-glucoside	0.62 ± 0.05	nd
Myricetin	2.74 ± 0.05	nd
Kaempferol-3-glucoside	3.11 ± 0.09	4.10 ± 0.06
Quercetin	2.28 ± 0.08	3.52 ± 0.11
(+)-catechin	2.08 ± 0.08	0.92 ± 0.02
(-)-epicatechin gallate	1.14 ± 0.04	0.46 ± 0.02
(-)-epicatechin	3.76 ± 0.11	1.27 ± 0.05
Procyanidin B2	nd	2.78 ± 0.09
∑ Phenolic acids	6.17	4.54
∑ Flavan-3-ols	6.98	5.43
∑ Flavonols	23.96	20.60
∑ Total phenols	37.11	30.57

The results of antioxidant activity of extracts are shown in Table 1, expressed as EC_{50} values (mg mL^{-1}). Higher radical scavenging activity showed red currant than black currant leaf extracts. Strong radical scavenging activity of leaf extracts, corresponding to their high phenol content. It is possible that these constituents may

interact to produce synergistic or antagonistic antioxidant effects with each other compounds.

Conclusion

Both methods, spectrophotometric and HPLC analysis confirmed high phenol content in investigated *Ribes* fruit leaf extracts. *Ribes* leaf extracts, rich in phenolic content, with significant antioxidant activity, can be used as medical supplements.

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