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Polyphenols in Bulgarian medicinal fruits

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The aim of the study is to determine the total phenols, total flavonoids and individual representatives of flavonoids – catechin, epicatechin and rutin in decoctions of widely used fruits of medicinal plants in Bulgaria. This would allow to gather new analytical data for their polyphenol content and correspondingly, for their antioxidant potential. In the present study various fruits of medicinal plants were studied: elderberry (*Sambucus nigra*), cranberry (*Vaccinum vitis-ideae*), hawthorn (*Crategus monogyna*), rosehip (*Rosa canina*), dogwood (*Cornus mas*), blackthorn (*Prunus spinosa*), and black chokeberry (*Aronia melanocarpa*). For the analytical determination of the content of total phenols the Folin-Ciocalteu method was used; for total flavonoids – the method with aluminium trichloride and individual flavonoids - catechin, epicatechin and rutin, were determined by HPLC with PDA detection. The results show that decoctions of rosehip have higher contents of total phenols and total flavonoids but no catechin, epicatechin or rutin. The highest amount of rutin was detected in elderberry (10700 µg/100 ml) along with very high contents of total phenols and total flavonoids, suggesting the promotion of *Sambucus nigra* to be used in antioxidant phytochemical formulations.

Keywords: Polyphenols, Medicinal plants, Total phenols, Total flavonoids, Fruit decoctions

INTRODUCTION

Around the world, there is traditional knowledge about the beneficial health effects of a traditionally used variety of medicinal plants. The flora in Bulgaria offers a great variety of plants - over 4000 higher plants are identified, and about 800 of them are known to have healing properties [1]. The presence of such a large variety of plants and a large number of compounds of plant origin predetermines that the phytotherapeutic potential of natural plants is vast and still insufficiently studied. A significant part of the existing studies is related to the antioxidant effect of the compounds contained in medicinal plants.

Due to the free radical metabolism of oxygen, the existence of aerobic organisms in an oxygen environment is possible only due to the functioning of appropriate defense systems. Under physiological conditions, most of the damaging effects of free radicals are prevented by the action of substances with antioxidant properties, which form the body's antioxidant defenses [2-4].

Medicinal plants and fruits that are widely used in folk medicine are a rich source of antioxidant polyphenols and in particular flavonoids [5, 6]. Polyphenols, including the flavonoids group, are secondary metabolites in higher plants, and more than 8,000 compounds are currently known [7]. They are present in various parts of plants used for food, as well as in a number of medicinal plants. It is interesting to note that half of the polyphenol compounds belong to the group of flavonoids and are found as aglycones, glycosylated forms and methylated derivatives [8].

Pharmacological studies of flavonoids have shown that they have antioxidant, cardioprotective, antispasmodic, antihypertensive, antimicrobial, antiviral, antiulcer, antidiabetic, antitumor and other effects [9-13].

According to the WHO, today 80% of the world's population relies on the healing properties of plants in various forms [14]. Even today, 11% of the 252 drugs considered as basic and essential by the WHO were exclusively of flowering plant origin and up to 50% of the approved drugs during the last 30 years are directly or indirectly from natural products [15]. In Bulgarian folk medicine a wide variety of infusions and decoctions is used, reflected not only in the seasonal collection of herbs, but in the presence of a wide net of herbal pharmacies in the country. However, no sufficient data for their polyphenol profile are available.

The aim of the present study is to determine the total phenols, total flavonoids and individual representatives of flavonoids – catechin, epicatechin and rutin in decoctions of fruits of medicinal plants widespread in Bulgaria in order to provide new analytical data for their polyphenol content and, correspondingly, to their antioxidant potential.

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EXPERIMENTAL

Sample preparation

In the present study the following fruits of medicinal plants were studied: elderberry (*Sambucus nigra*), cranberry (*Vaccinum vitis-ideae*), hawthorn (*Crategus monogyna*); rosehip (*Rosa canina*), dogwood (*Cornus mas*), blackthorn (*Prunus spinosa*), black chokeberry (*Aronia melanocarpa*). For analytical determination of the content of total phenols, total flavonoids and individual flavonoids - catechin, epicatechin and rutin, an average fruit sample was prepared from three individual samples (100 g), with a dissipate degree, according to the pharmacopoeia requirements, purchased from the pharmacy net in Sofia.

Preparation of water decoctions

In order to obtain a quality aqueous extract, the decoctions preparation followed a pharmacopoeia technological regime, accordingly – the contact time was not less than 40 min and the ratio plant material/water was 5 g of dry fruit/100 g of distilled water. The fruit material was boiled in distilled water for 5-10 minutes, according to the manufacturer's instructions. Soak for 30-35 minutes, cool and strain. The samples obtained were stored at 4 °C without the addition of preservatives. The samples were analyzed no later than 24 hours.

Chemicals

Certified reference materials - catechin and gallic acid were purchased from Sigma (St. Louis, MO), epicatechin and rutin trihydrate from Alfa Aesar (Thermo Fisher Scientifics, Kandel, Germany). Folin- Ciocalteu reagent was purchased from Alfa Aesar. Solvents - acetonitrile, methanol and water are of HPLC purity (Macron Fine Chemicals, Avantor, Glivice, Poland). All other chemicals and reagents used have a degree of purity p.a. and were purchased from Alfa Aesar.

Determination of total phenols

Folin-Ciocalteu reagent is used to determine total phenols due to its ability to oxidize polyphenols and form a blue complex with them, which is determined spectrophotometrically at a wavelength $\lambda = 750$ nm. The determination was performed according to Marinova *et al.* [16]. Briefly, an aliquot of 1 ml of a suitably diluted aqueous extract or standard gallic acid (GA) solution was added to a 25 ml volumetric flask containing 9 ml of distilled water. 1 ml of Folin-Ciocalteu reagent was added to the solution and after 5 min 10 ml of 7% Na_2CO_3 was added. The solution was brought to the mark with distilled water, stirred and allowed to stay for 90 min at room temperature. The absorbance was measured against a reagent blank at $\lambda = 750$ nm. The method of external standard with gallic acid was used to calculate the amount of total phenols. Results are expressed as gallic acid equivalent (mg GAE/100 ml). The parameters of the method were as follows: LOQ 0.10 μg GAE/ml; LOD 0.21 μg GAE/ml; RSD = 3.8 %, no systematic error was found [17].

Determination of total flavonoids

Total flavonoids were determined according to Marinova *et al.* [16]. An aliquot of 1 ml of aqueous decoction or standard solution of rutin was placed in a 10 ml volumetric flask containing 4 ml of distilled water. 0.3 ml of 5% $NaNO_2$ was added to the solution and after 5 min 0.3 ml of 10% aqueous $AlCl_3$ was added. After 6 min, 2 ml of 1 M NaOH was added and the solution was made up to the mark with distilled water, stirred and allowed to stay for 30 min. The absorbance of the formed pink complex was compared to a reagent blank at a wavelength $\lambda = 510$ nm. The total flavonoids content was calculated by the external standard method and the results were expressed as rutin equivalent (mg RE/100 ml) for aqueous extract (decoction). The parameters of the method were as follows: LOQ = 0.25 μg RE/ml; LOD = 0.47 μg RE/ml; RSD = 4.1 %; no systematic error was found [17].

Spectrophotometric measurement of the absorption of total phenolic compounds and total flavonoids was performed with a Lambda 25 spectrophotometer, Perkin-Elmer.

Determination of catechin, epicatechin and rutin by HPLC method

The HPLC system consisted of a Perkin-Elmer (Norwalk CT) Flexar LC pump, Flexar Photo Diode Array Plus detector (PDA), autosampler, thermostat and built-in degasser. Chromatographic data were processed with Chromera HPLC PDA data software, version 4.1.1.6396.

Chromatographic separation of phenolic compounds - catechin, epicatechin and rutin was performed on a Luna C18 column (3 μm, 150 mm × 4.6 mm, Phenomenex, USA), equipped with a precolumn with the same stationary phase [17]. Elution was performed at a flow rate of 0.9 ml/min at a temperature of 30 °C. Mobile phase: 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) were used. A program with linear gradient elution was applied as follows: 0-2 min, 15% B; 2-4 min, 20% B; 4-6 min, 25% B; 6-8 min, 30% B; 8-10 min, 35% B; 10-12 min, 35% B; 5 min recalibration of the column by 15% B. The injection volume of the sample solution and standard solutions was 20 µl. The following wavelengths were used for detection of catechin, epicatechin and rutin: channel 1 - 275 nm for catechin and epicatechin and channel 2 - 355 nm for rutin, with the reference wavelength set to 620 nm at a fixed bandwidth of 19 nm.

The identification of the chromatographic peaks was performed by comparing the retention times of the eluted peaks and comparing the peak shape in the two channels with different wavelengths (-275 and 355 nm). For the quantification of catechin, epicatechin and rutin, the external standard method was used to construct a calibration curve by injecting a series of standard solutions. The parameters of the method were as follows: LOQ (catechin and epicatechin) = 0.40 µg/ml; LOQ (rutin) = 0.40 µg/ml; LOD = 0.02 µg/ml – 0.07 µg/ml; The repeatability and reproducibility of determination of catechin was – RSD = 4.8 %, of epicatechin – RSD = 3.5 %; of rutin – RSD = 2.3 %. All measurements were performed in triplicate and no systematic error was found [17].

RESULTS AND DISCUSSION

The results for total phenols and total flavonoids content of the studied fruit samples are presented in Table 1. The results are expressed as average value of triplicate analysis, along with confidence interval (CI) estimate at 95% confidence level, calculated with use of t-distribution. The basis of the action of biologically active substances from herbal drugs is the process of solid/liquid extraction with a suitable extraction solvent. Water is often used as an extraction agent due to its unique dissolving properties, which can change with temperature. The preparation of aqueous extracts of medicinal plants at home has a long tradition and is widespread in our country. For this reason, in the present study, water was selected as an extracting agent for the preparation of various plant decoctions, in compliance with the prescriptions provided by herbal pharmacies. The results show that the content of total phenols exceeds that of total flavonoids in all tested samples with exception of blackthorn, where equal amounts were found. The contents of total phenolic compounds among the fruits decoctions ranged from 2.45 to 167 mg GAE/100 ml. The highest content of total phenols was found in rosehip – 167 mg GAE/100 ml, which is about twice as high as the amount in the second- richest source of total polyphenols – elderberry (83 mg GAE/100 ml). The total flavonoids are highest again in rosehip (85 mg RE/100 ml), followed by elderberry (71 mg GAE/100 ml) and dogwood (12.7 mg/100 ml). The amount of total flavonoids in decoctions of cranberry, hawthorn, blackthorn and black chokeberry is low, ranging between 2.45 - 8.0 mg GAE/100 ml.

The results emphasize that the major sources of total phenols and total flavonoids in traditional teas used in Bulgarian traditional medicine are rosehip and elderberry.

Rosehip (*Rosa canina*) is a species of wild rose. The fruit is characterized by a rich content of vitamin C, carotene, lycopene, pectin, vitamins B_1 and B_2, as well as vitamins PP and K. Usually fresh ripe fruits contain up to 600 mg (per 100 grams of fruit) of vitamin C, according to some sources - up to 2 grams (for certain varieties) [18, 19]. The seeds contain vitamin E as well. Rosehip is often mentioned in traditional folklore and was used in ritual practices with a medicinal focus. The unique combination of vitamin C and other ingredients in rosehip (β-carotene, flavonoids, minerals) stimulates the formation of red blood cells, increases efficiency and strengthens the body's resistance. Therefore, it is recommended for patients with diabetes, anemia, mental and physical fatigue, colds due to its antioxidant polyphenols content as well [19-21].

Table 1. Total phenols and total flavonoids contents in decoctions of Bulgarian medicinal fruits

Latin name	Common name	Total phenols ± CI*, mg GAE/100 ml	Total flavonoids ± CI*, mg RE/100 ml
Sambucus nigra	Elderberry	83 ± 5	71 ± 5
Vaccinum vitis-ideae	Cranberry	5.4 ± 0.2	3.9 ± 0.2
Crategus monogyna	Hawthorn	36 ± 2	8.0 ± 0.7
Rosa canina	Rosehip	167 ± 12	86 ± 7.44
Cornus mas	Dogwood	44 ± 2	12.7 ± 1.7
Prunus spinosa	Blackthorn	2.45 ± 0.2	2.45 ± 0.25
Aronia melanocarpa	Black chokeberry	35 ± 2	3.25 ± 0.12

*CI – confidence interval at 95% confidence level.

Elderberry (*Sambucus nigra*) contains useful micronutrients including iron, potassium, phosphorus, copper, as well as vitamins A, B and C, organic compounds with anti-inflammatory and antioxidant activity [19-21]. Both the flowers and the fruits of the black elderberry are important for pharmaceutical practice. They are applied in the form of teas, juices, extracts and syrups. Fruits have a strong healing effect, whose pronounced antioxidant activity is due to their high polyphenols content, namely antocyanins and flavonols. Our results emphasize the high level of polyphenols in rosehip and elderberry, supporting their phytotherapeutic use as natural antioxidant sources.

The HPLC separation of catechin, epicatechin and rutin of standard solution and elderberry decoction is presented in Figures 1 and 2. The results of the HPLC method show that the elution of catechin, epicatechin and rutin takes place within 11 minutes. The identification of the chromatographic peaks was performed by comparing the retention times of the eluted peaks and comparing the peak shapes in detection at two different wavelengths (275 nm and 355 nm). The chromatograms given in Figures 1 and 2 show a very good baseline separation of the analytes. The use of formic acid in the mobile phase suppresses the strong specific interactions between the sorbent (residual silicol groups) and the sorbate (phenolic groups in the analyte) caused by orientational interactions between molecules with constant dipoles. In the present study, detection with a PDA photodiode detector was used, the analytical signal being measured at the wavelength of the highest sensitivity as follows - 275 nm for catechin and epicatechin, and 355 nm for rutin. Our results show that the HPLC method has a very good sensitivity and, correspondingly, a low limit of quantification (LOQ). The values of the limit of detection (LOD) and the limit of quantification (LOQ) were determined from the signal-to-noise ratio S/N, using a progressively lower concentration of analytes for the ratio S/N - 3 for LOD and 10 for LOQ. The limits of quantification were determined as follows: 0.40 μg/ml for catechin and epicatechin (275 nm) and 0.07 μg/ml for rutin (355 nm) [17].

The results for catechin, epicatechin and rutin contents of decoctions from the medicinal fruits are presented in Table 2. The results are expressed as mean values of triplicate analysis in μg/100 ml, along whit their confidence intervals.

The present results show for the first time original analytical data not only for the integral parameters for polyphenols contents such as total phenols and total flavonoids, but for individual flavonoids as well, obtained by HPLC analysis. It could be noted that in contrast to the results obtained by spectrophotometric methods, the HPLC analysis shows presence of catechin, epicatechin or rutin in aqueous decoctions only in 3 out of 7 samples studied.

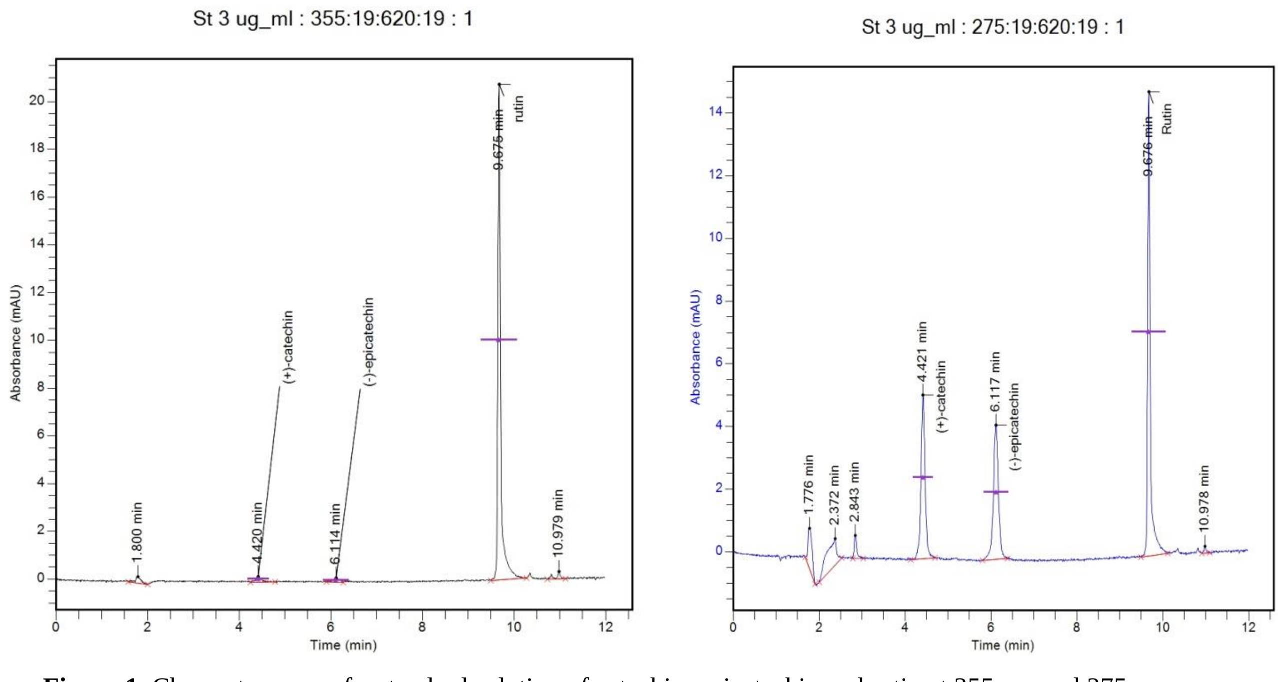

Figure 1. Chromatograms of a standard solution of catechin, epicatechin and rutin at 355 nm and 275 nm.

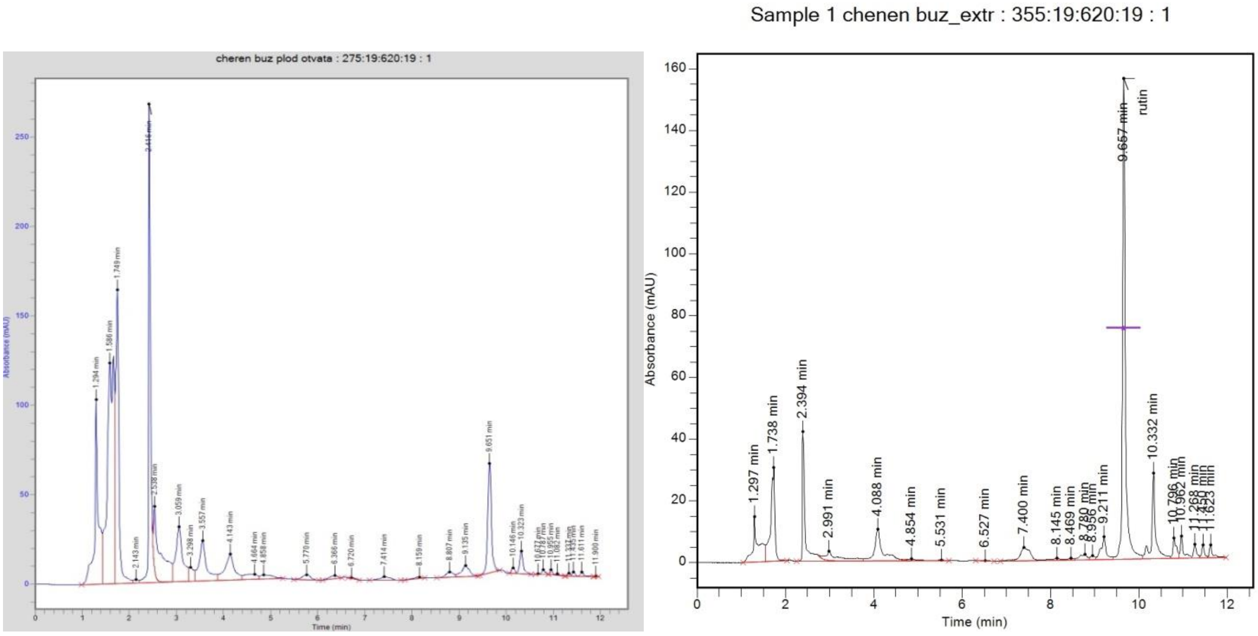

Figure 2. Chromatograms of catechin, epicatechin and rutin in elderberry fruits decoction at 275 nm and 355 nm.

Table 2. Catechin, epicatechin and rutin content in decoctions of Bulgarian medicinal fruits

Latin name	Common name	Catechin ± CI* µg/100 ml	Epicatechin ± CI* µg/100 ml	Rutin ± CI* µg/100 ml
Sambucus nigra	Elderberry	<LOD	<LOD	10700 ± 695
Vaccinum vitis-ideae	Cranberry	<LOD	<LOD	<LOD
Crategus monogyna	Hawthorn	<LOD	153 ± 7	174 ± 15
Rosa canina	Rosehip	<LOD	<LOD	<LOD
Cornus mas	Dogwood	<LOD	<LOD	<LOD
Prunus spinosa	Blackthorn	<LOD	<LOD	<LOD
Aronia melanocarpa	Black chokeberry	448 ± 12	<LOD	80 ± 2

*CI – confidence interval at 95% confidence level.

Catechin is found only in black chokeberry decoctions, and epicatechin only in hawthorn. With the exception of black elderberries, in other fruit samples the level of rutin is low or undetectable (cranberry, rosehip, dogwood, and blackthorn). In black chokeberry and hawthorn decoctions, the rutin content is 80 and 174 µg/100 ml, respectively. A comparison of these results with literature data and our previous studies shows that aqueous decoctions are not the most efficient extraction solvent for flavonoids compared to extraction systems with higher lipophilicity such as methanol/water [22]. For example, when 80% methanol/water solvent was applied, rutin was found in all tested fruits, and catechin in samples of elderberry and cranberry, and epicatechin in cranberry as well [23]. However, the present study aims to determine the values of the polyphenols in aqueous formulations according to the instruction of preparation and designed for direct consumption. We could note that the amount of rutin in black elderberry decoctions is extremely high and reaches 10.7 mg/100 ml. This is reflected in the widespread home-made formulas of syrups, infusions and others made from *Sambucus nigra* fruits in recent years.

CONCLUSION

In the present study seven traditional for Bulgarian folk medicine fruits were studied for their polyphenols content. The results show that decoctions of rosehip have higher contents of total phenols and total flavonoids but no catechin, epicatechin or rutin. The highest amount of rutin was detected in elderberry along with very high content of total phenols and total flavonoids, suggesting the promotion of *Sambucus nigra* to be used in antioxidant phytochemical formulations.

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