Polyphenols content of selected medical plants and food supplements present at Bulgarian market

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Abstract

Background: Medicinal plants are a rich source of antioxidant polyphenols and in particular flavonoids.

Materials and methods: In the present study 5 Bulgarian medical plants and 5 food supplements, present at the Bulgarian market, are analyzed for their Total phenolic, Total flavonoids and selected individual flavonoids content. A HPLC method was developed and validated for simultaneous determination of (+)-catechin, (-)-epicatechin and rutin in plant and supplements, using PDA detection.

Results: The results show that polyphenols in the selected food supplements are lower than those of the medical plants analyzed. Mentha piperita and Melissa officinalis have the highest polyphenols content (67.38 and 65.17 mg GAE/g; 54.59 and 57.76 mg RE/g respectively). Rutin was detected in all samples analyzed, reaching highest levels in Mentha piperita (7332.5 µg/g), followed by fruits of Sambucus nigra (2818.7 µg/g).

Conclusion: The results of the study are a practical contribution to a more complete characterization of the polyphenolic composition in Bulgarian medicinal plants.

Keywords

Catechins, HPLC, Food Supplements, Medical plants, Rutin, Total phenolic, Total flavonoids

Introduction

Bulgarian medical herbs are known worldwide for their high content of biologically active substances. They are rich in various chemical compounds – alkaloids, glycosides, saponins, polysaccharides, polyphenols, coumarins, essential and fatty oils, vitamins, flavonoids and more. App. 500–600 medicinal plants are used in Bulgarian folk medicine. App. 300 species are most often used for the needs of the pharmaceutical industry, phytotherapy and for export. Around 12,000 species of medicinal plants are used worldwide, of the currently known about 500,000 species of higher plants or only 2.4% of the world’s flora are known and used as medicinal plants.

Polyphenols, including the flavonoids group, are secondary metabolites in higher plants, and more than 8,000 compounds are currently known. They are present in various parts of plants that are used for food, as well as in a number of medicinal plants. It is interesting to note that half of the polyphenolic compounds belong to the group of flavonoids and are found as aglycones, glycosylated forms
Content of polyphenolic antioxidants in Bulgarian medicinal plants and food supplements

It is known that fruits and vegetables are a very rich source of flavonoids from the group of catechins and the flavonol quercetin (Tsanova-Savova et al. 2003, 2005, 2013, 2017, 2018; Velcheva-Kuzmanova et al. 2007). At the same time, in the scientific literature, there are data on a number of medicinal plants that also contain flavonoids from the group of catechins and flavonol rutin, such as in Pentace burmanica (Duangyod et al. 2014); in Dandelion (Taraxacum officinale), in Hawthorn (Crataegus monogyna), in Chamomile (Matricaria chamomilla), etc. (Yoo et al. 2009; Sofic et al. 2010). However, quantitative data on the content of polyphenolic antioxidants in Bulgarian medicinal plants and their fruits are sporadic and unsystematic. Data for the content of flavonol rutin in medicinal plants were reported by Atanasova and Georgieva in Lemon balm (Melissa officinalis), Sage (Salvia officinalis) and Mint (Mentha piperita), determined by spectrophotometric method (Atanasova and Georgieva 2010). There is more data only on integrated parameters, such as Total phenolic content or assessment of antioxidant potential (Ivanova et al. 2005; Atanasova et al. 2011; Kiselova et al. 2011; Nikolova 2011; Georgieva and Mihaylova 2015).

The aim of the study is to determine the content of polyphenols in selected Bulgarian medicinal plants and food supplements, present at Bulgarian market, by developing a validated HPLC method for determination the individual representatives of catechins – (+)-catechin and (-)-epicatechin and of the flavonol – rutin. Furthermore for overall characterization of their antioxidant polyphenolic content, the selected samples are analyzed for Total phenolic and Total flavonoids with validated spectrophotometric methods.

Materials and methods

Plant material and Food supplements

Medical plant dry material (100 g) was purchased from herbal pharmacy, and food supplements were purchased from pharmacy net in Sofia, according their wide application at Bulgarian market. The selected samples are described in Table 1.

### Table 1. Description of samples.

| Plant material and Food supplements | Samples                                      |
|------------------------------------|----------------------------------------------|
| Elderberry, fruit                  | Elderberry, Syrup, containing extract of      |
| Cranberry, fruit                   | Sambucus nigra fruit                          |
| Hawthorn, fruit                    | Crataegus monogyna                            |
| Wild mint, leaves                  | Mentha piperita                               |
| Lemon balm, leaves                 | Melissa officinalis                           |
| Mint, Hawthorn, Valeriana          | Mint, Hawthorn, Valeriana, Tablets, containing |
|                                   | dry standardized extract of                    |
|                                   | Crataegus monogyna – 120 mg, Valeriana officinalis – 100 mg, Menta piperita – 80 mg, and Matricaria chamomilla – 80 mg |
| Hawthorn                           | Hawthorn, Tablets, containing 325 mg Crataegus monogyna |

Sample preparation

The powdered material of plant or food supplements (0.2500 g) were weighted into a 25 ml volumetric flask of and extracted with 80% methanol/water (w/w) in an ultrasonic bath for 30 min at 30 °C. The solutions were ultra centrifuged at 10000 rpm for 5 min and filtered through a membrane filter (0.45 µm) prior to analysis. The extraction solution was selected according our previous studies and literature data (Tsanova-Savova et al. 2003; Marinova et al. 2005)

Chemicals

(+)-Catechin and Gallic acid were purchased from Sigma (St. Louis, MO). (-)-Epicatechin and Rutin trihydrate, Folin – Ciocalteau reagent, was obtained from Alfa Aesar (Thermo Fisher Scientifics, Kandel, Germany). Acetone, methanol and water were HPLC grade and were obtained from Macron Fine Chemicals (Avantor, Gliwice, Poland), and all other chemicals and reagents were purchased from Alfa Aesar (Thermo Fisher Scientifics, Kandel, Germany).

Determination of total phenolic

Folin – Ciocalteau reagent was used to determine the total phenols, which oxidizes the phenolates to a blue complex, which is determined at λ = 750 nm. The absolute calibration method was used to calculate the amount of Total phenolic. The determination was performed according Marinova et al. 2005. In brief, an aliquot of 1 ml of suitably diluted extracts or standard gallic acid (GA) solutions, which were added to a 25 ml volumetric flask containing 9 ml of distilled water. 1 ml of Folin-Ciocaltuie is added to the mixture, and after 5 minutes 10 ml of 7% NaCO₃ are added. The solution was made up to the mark with distilled water stirred and allowed to stand for 90 minutes at room temperature. Then the absorbance against a reagent
The identification of chromatographic peaks was achieved by comparing the retention time of the eluted peaks and comparing peak shape at two channels with different wavelength (280 and 355 nm). For quantitative analysis, a calibration curve was obtained by injection of known concentration of standard solutions.

**Standard solutions**

Stock solutions (1 mg/ml) of analytes (+)-catechin, (-)-epicatechin, rutin, gallic acid were prepared from pure compounds by dissolving each compound in methanol. Working standard solutions were prepared daily from stock solutions by dilution with appropriate volume of 80% methanol. All solution were stored at 4 °C and used within five days.

**Limit of detection and limit of quantification**

The values of limit of detection (LOD) and limit of quantification (LOQ) were determined from Signal to Noise ratio, using progressively lower concentration of analytes for a S/N ratio of approximately 3 and of approximately 10, respectively (Eurachem 2014).

**Calibration curves**

The linearity of the response was evaluated by analysis of standard solutions of (+)-catechin, (-)-epicatechin and rutin for HPLC analysis; of gallic acid for Total phenolic determination and rutin for Total flavonoids determination. Triplicate measurement was made for each standard solution. Calibration curves, correlation coefficients and linearity range are presented in Table 2 and Table 3. The calibration curves were constructed by means of last-square linear regression analysis.

**Precision**

The precision of both HPLC method and spectrophotometric methods for determination of Total phenolic and Total flavonoids was assessed at two levels by multiple analysis of a standard solution (2 µg/ml). At first level the intra-day precision of repeatability was determined within the same day in a single analysis on a single instrument.

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**Table 2.** Linearity, LOD, LOQ of the HPLC method for quantitative evaluation of (+)-catechin, (-)-epicatechin and rutin.

| Compound   | Calibration curve | Correlation coefficient R² (n = 3) | Linear range (µg/ml) | RT, min | RSD, % (n = 6) | LOQ (µg/ml) | LOD (µg/ml) |
|------------|-------------------|-----------------------------------|----------------------|--------|----------------|-------------|-------------|
| (+)-Catechin | y = 11187x - 1240.8 | 0.9996                           | 0.20–30.00           | 4.41   | 0.20           | 0.12        | 0.40        |
| (-)-Epicatechin | y = 12925x - 2637 | 0.9993                           | 0.20–30.00           | 6.09   | 0.26           | 0.12        | 0.40        |
| Rutin      | y = 12925x - 2637 | 0.9997                           | 0.02–40.00           | 9.67   | 0.09           | 0.02        | 0.07        |

**Table 3.** Linearity, LOD, LOQ of the spectrophotometric methods for determination of Total phenolic and Total flavonoids.

| Method          | Calibration curve | Correlation coefficient R² (n = 3) | Linear range (µg/ml) | LOQ (µg/ml) | LOD (µg/ml) |
|-----------------|-------------------|-----------------------------------|----------------------|-------------|-------------|
| Total phenolic  | y = 0.1x + 0.0088  | 0.9991                           | 0.21–7.22            | 0.10        | 0.21        |
| Total flavonoids| y = 0.0131x + 0.0014 | 0.9959                           | 0.47–18.75           | 0.25        | 0.47        |
The second level was the inter-day precision or reproducibility and was determined over three days on the same instrument. The obtained results were expressed as relative standard deviation (RSD, n = 6). Furthermore the intra-and inter-day precision was determined of hole analytical procedure by analyzing a cranberry fruit sample Vaccinium vitis-ideae (RSD, n = 6) (see Table 4).

Recovery studies

The recovery was evaluated by adding measured amount of pure standards to a sample of Sambucus nigra (fruit) and Cranberry Food supplement, following the extraction procedure described above, by analyzing the fortified samples with HPLC and spectrophotometric methods. The recoveries were determined by subtracting the values obtained for the control matrix from those of the samples prepared with added standards. Each sample was analyzed three times. The calculated mean amount of recovery was reported in Table 4.

Table 4. Repeatability. Reproducibility and Accuracy of determination of (+)-catechin. (-)-epicatechin and rutin and of Total phenolic and Total flavonoids.

| HPLC Determination | RSD%1 | RSD%1 | RSD%2 | RSD%2 | Accuracy |
|--------------------|-------|-------|-------|-------|----------|
| (+)-Catechin        | 2.59  | 2.72  | 3.56  | 4.75  | Sambucus nigra. Fruit 99.53 |
| (-)-Epicatechin     | 2.00  | 2.35  | 1.94  | 3.53  | Cranberry. Supplement 97.41 |
| Rutin              | 2.17  | 1.39  | 1.24  | 2.28  | Sambucus nigra. Fruit 92.46 |
| Total phenolic      | 1.98  | 3.06  | 2.04  | 3.78  | Cranberry. Supplement 93.57 |
| Total flavonoids    | 1.51  | 2.72  | 2.40  | 4.12  | Sambucus nigra. Fruit 95.97 |

1 Repeatability and Reproducibility of Standard Solution (2 μg/ml).
2 Repeatability and Reproducibility of Vaccinium vitis-ideae fruit sample.

Results and discussion

Validation of the methods

The results of HPLC analysis enabled the identification of (+)-catechin, (-)-epicatechin, and rutin within 12 min. The HPLC chromatograms of standard solutions and Sambucus nigra fruit and food supplement are presented in Figures 1–3.

The chromatograms show a very good baseline resolution of analytes. The use of formic acid in the mobile phase suppresses the strong specific interactions between the sorbent (residual silanol groups) and the sorbate (phenolic groups in the analytes) due to orientational interactions between molecules with permanent dipoles. Furthermore, to eliminate such interactions, for HPLC separation of flavonoids, the use of octadecylsilane reverse phase columns, with no further endcapping of the residual silanol groups, results in a large tail of the picks. In the present study, the use of a Luna column, in combination with the reduced

Figure 1. Chromatogram of Standard mix of (+)-catechin, (-)-epicatechin and rutin at 275 nm (a) and 355 nm (b).
size of the sorbent (3 µm) and the suppression of the ionization of the analyte by proper selection of the mobile phase, provide symmetrical peaks in the chromatogram.

The limits of quantification were 0.4 µg/ml for (+)-catechin and (-)-epicatechin, and 0.07 µg/ml for rutin (Table 2). According to the scientific literature, in HPLC analysis of catechins, different detection methods have been applied. Several authors performed HPLC analysis of catechins using fluorescence detection (Arts et al. 2000; Tsanova-Savova et al. 2003), leading to lower LOQ. However a simultaneous determination of rutin and catechins is not applicable with fluorescence detection. Several authors performed HPLC analysis of catechins by using conventional UV detector at 280 nm (Zuo et al. 2002; Neilson et al. 2006), 205 nm (Lee and Ong 2000), 210 nm (Bronner et al. 1998; Dalluge et al. 1998; Mizukami et al. 2007) In the present study, using PDA detection the analytes were measured at their higher response, namely 275 nm for (+)-catechin and (-)-epicatecin, while rutin gives higher response at 355 nm. Santagati et al. 2008 have reported LOD values for catechins and rutin between 0.77 to 1.94 µg/ml range, using detection at 210 nm. Our results show that the HPLC method has a very good sensitivity, especially for rutin. The correlation coefficients of the standard curves linear regression were greater than 0.999. The retention times of chromatographic peaks vary no more than 0.26% (Table 2).
The results for limit of determination for Total phenolic and Total flavonoids measurements are 0.21 µg GAE/ml and 0.47 µg RE/ml (Table 3), and are in line with literature data (Marinova et al. 2005). The correlation coefficient of calibration curves was more than 0.995. The linear range for Total flavonoids method (0.47–18.75 µg RE/ml) is broader than the linearity for Total phenolic (0.21–7.22 µg GAE/ml).

The data for repeatability, reproducibility and accuracy of methods applied is presented in Table 4. The results show that higher variation was measured for intra-day reproducibility of (+)-catechin in real samples of *Vaccinium vitis-ideae* fruit (n = 6), but however the RSD% was lower than 5%. The Inter-day and Intra-day reproducibility and reproducibility of standards measurement were lower than 3% RSD. The analytical recoveries were within the range of 92.46% and 99.53%, resulting in very good accuracy of the methods, both for medical plants and food supplements.

These data support the suitability of the methods for its application to real samples.

### Polyphenols content in selected medical plants and food supplements

The results of analysis of the selected medical plants and food supplements in this study show that among medical plants studied *Mentha piperita* and *Melissa officinalis* have the highest values of Total phenolic and Total flavonoids (67.38 and 65.17 mg GAE/g; 54.59 and 57.76 mg RE/g, respectively. The lowest polyphenolic content was measured in *Crataegus monogyna* fruit – 12.49 mg GAE/g and 7.08 mg RE/g. Rutin was detected in all samples analysed, reaching the hinges level in *Mentha piperita* (7332.5 µg/g), followed by fruits of *Sambucus nigra* fruits (2818.7 µg/g). (+)-Catechin was detected only in *Sambucus nigra* and *Vaccinium vitis-ideae* fruit samples, while (-)-Epicatechin in *Vaccinium vitis-ideae* and *Crataegus monogyna*.

The results of the selected food supplements show that their polyphonic load is in general lower than those of individual medical plants analysed. It should be noted the great variability of results of Elderberry Syrup, containing unstandardized and standardized extract of *Sambucus nigra* fruit. For instance Total phenolics content in Elderberry food supplement (1) is 5.16 mg GAE/g, while in Elderberry (2) was 20.76 mg GAE/g. The same tendency was observed for Total flavonoids and for rutin values. The highest phenolics content was measured in Lemon balm food supplement – 32.30 mg GAE/g, but this value is about two times lower than in *Melissa officinalis* leaves. The highest amount of Total flavonoids was found in Cranberry fruit supplement – 20.25 mg RE/g. It is interesting to notice that Mint, Hawthorn, Valeriana food supplement contains all individual flavonoids studied. Since (+)-catechin was not found in *Mentha piperita* or *Crataegus monogyna* samples, it is possible to come from other constituents of the Tablets form – *Valeriana officinalis* or *Matricaria chamomilla*. This food supplement formulation is also the richest source of rutin – 1214.7 µg/g.

Our results for Total phenolic and Total flavonoids in Lemon balm (*Melissa officinalis*) and mint (*Mentha piperita*) are higher than reported from Marinova et al. 2005. Nevertheless, we should point out that the results for Total flavonoids is difficult to compare with literature others, since they could be expressed as Quercetin equivalent (Sulaiman and Balachandrin 2012), Rutin equivalent (Shulaila et al. 2013), or Catechin equivalent (Marinova et al. 2000). The comparison of our data for (-)-catechin and (+)-epicatechin in Cranberries fruit (*Vaccinium vitis-ideae*) with USDA Flavonoids Food Composition database (Bhagwat et al. 2014) show that although their results are far row samples (40.7 µg/g for (-)-epicatechin and 3.9 µg/g for (+)-catechin), our data for dry fruits are significantly higher. We could not find in the scientific literature data for polyphenols and flavonoids content in the selected food supplements.

### Conclusion

It is known that medicinal plants and fruits are widely used in folk medicine are a rich source of antioxidant polyphenols and in particular flavonoids. At the same time, a rich palette of food supplements is currently available, with claims to powerful antioxidant activity. Furthermore, quantitative data for their evaluation are sporadic and unsystematic. In this regard, we have of characterized the...
quantitative content of Total phenolic compounds, Total flavonoids and the individual representatives of catechins – (+)-catechin and (+)-epicatechin, as well as the flavonol rutin with a validated HPLC method in a 5 of Bulgarian medicinal plants and fruits, which are widely used for the preparation of herbal infusions and decoctions and in 5 food supplements. The results show that Mentha piperita leaves have a very high content of Total phenolic (67.38 mg GAE/g), Total flavonoids (54.59 RE/g) and of rutin (7.33 mg/g). In general, the food supplements studied have lower polyphenols values, in comparison with the selected medical herbs and fruits. The results of the study will be a practical contribution to a more complete knowledge and characterization of the polyphenolic composition in Bulgarian medicinal plants.

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