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Phytochemical profile and *in vitro* antioxidant activity of *Centaurium erythraea* Rafn.

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Centaurium erythraea Rafn. (small centaury) is an important medicinal plant from Gentianaceae family, which is used traditionally in the folk medicine as a digestive, stomachic, tonic, depurative, sedative, and antipyretic. It is known with anti-inflammatory and antipyretic effects. The aim of current study was to evaluate and compare the total polyphenol content and antioxidant activity of water (infusion, decoction and microwave) and alcohol (tincture) extracts of *Centaurium erythraea*. The total flavonoid content, total monomeric anthocyanin content and detailed phenolic acids profile were assessed additionally. The polyphenol content was established to be in range from 1.23 to 12.46 mg GAE/g dw, the total flavonoids from 1.18 to 3.35 mg QE/g dw and total monomeric anthocyanins from 1.70 to 6.15 mg/L. *In vitro* antioxidant activity was evaluated by four common procedures and the highest results were established for the decoction and tincture extracts. The highest phenolic acids profile was disclosed in the small centaury infusion - 2208 μg/g dw. As a result the consumption of the studied *C. erythraea* extracts could be recommended as a good source of biologically active substances and bio-antioxidants in particular with potential benefit effects.

Keywords: *Centaurium erythraea*, water extract, ethanol extract, antioxidant activity

INTRODUCTION

Plants are an overall source of antioxidant activity compounds, such as phenolic acids, flavonoids, vitamins and carotenoids that may be used as pharmacologically active constituents [1]. In particular, the antioxidant activity is one of the most important properties of plant extracts, due to scientists have looked for sources of natural antioxidants to be introduced in many cosmetic, pharmaceutical and food formulations. The research for the new sources of antioxidants in the past resulted in the extensive studies on medicinal plants [2]. The use of herbal drugs and phytonutrients or nutraceuticals continues to expand rapidly across the world with many people now resorting to these products for treatment of various health challenges in different national healthcare settings [3].

Small centaury (*Centaurium erythraea* Rafin.) (Gentianaceae) is a medicinal plant with a long tradition, being included in the pharmacopoeias of many European and American countries. Phytochemically it is characterized by the presence of terpenoids [4], xanthones [5, 6], and phenolic acids and their derivatives [4, 7]. It has been used in human traditional medicine as a digestive, stomachic, tonic, depurative, sedative, and antipyretic [4]. The anti-inflammatory and antipyretic effects of an aqueous extract of the plant have already been observed experimentally in rats [8]. Many research articles deal with the phytochemical exploration of this plant species [9, 10]. Thus, the present study aimed at evaluating and comparing the total polyphenol content and antioxidant activity of water (infusion, decoction and microwave treatment) and alcohol (tincture) extracts of *Centaurium erythraea*, relying on widespread, simple for conduction and consumer-familiar extraction methods.

Many researchers have studied the influence of different extraction solvents and techniques on the content of natural antioxidants in extracts. Phytochemical compounds, such as phenolic acids, flavonoids, tannins and saponins are considered as major secondary metabolites in plants. Phenolic compounds, which possess a broad spectrum of biochemical activities, represent the largest group [11-15]. Interest in these classes of compounds are due to their pharmacological activities as radical scavengers [16]. Recently, different studies have shown that phenols and non-phenolic compounds are of great interest to the pharmaceutical industry for their anti-inflammatory, anti-aging, and antimicrobial benefits, which make them an important source of molecules for new drug discovery [17]. One very important step for utilizing bioactive compounds from plant resources is the extraction process. Selection of the extraction process itself is an important step for the standardization of herbal products, as they can be utilized in the removal of desirable soluble

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constituents. Due to the wide variations in the structures and polarities of chemical compounds, extraction from plant products is complex and challenging. The critical extraction parameters include solvent, time, solid-to-solvent ratio, number of extractions, temperature, and partial size of the sample material [17]. Selection of the extraction solvent depends on the specific nature of the bioactive compound being targeted. The extraction yield and, consequently, the biological activity of vegetal extracts can be strongly affected by the solvent applied [18]. For bioactive compound extraction, different solvents, including organic and/or aqueous solutions, have been reported [19]. Water and ethanol are often recommended for extract preparation because of their differences in polarity. The use of organic solvents for industrial extractions has several disadvantages, such as: (a) solvent residue in the product; (b) worker exposure; (c) disposal of waste solvents; and (d) environmental pollution [20]. Several studies have shown that ethanol and boiling water are effective for polyphenol extraction [21, 22].

In this regard, the aim of the present study was to explore and compare antioxidant properties, total polyphenolic contents and phytochemical profile in respect of phenolic acids composition of several extracts of *Centaurium erythraea* obtained by water and ethanol (tincture). The main purpose was to highlight the potential biological activity of the extracts and to recommend the most appropriate technique of extraction among studied.

.MATERIALS AND METHODS

Plant Material

The samples of *Centaurium erythraea Rafn.* Pers (stems) were obtained from local pharmacy (Plovdiv, Bulgaria) in 2016. The plant parts were dried, ground and stored at ambient temperature in air-tight containers prior to extraction.

Extraction preparation

Four extraction procedures were performed as follow: with water (infusion, decoction and microwave extraction) and with 70 % ethanol (v/v, tincture).

The infusion was obtained by pouring 2.5 g of plant material with 50 mL water and then, left for 30 min to cool. Water decoction was retrieved by boiling 2.5 g of plant material in 50 mL solvent for 30 min; Microwave-assisted extract (MAE) experiment was carried out in a domestic microwave oven (LG MB4047C) where 2.5 g of plant material was subjected to 2450 MHz frequency waves for 30 seconds with 50 mL water, at 800 W output power.

Ethanol (70 %, v/v) was used to obtain the tincture, which was prepared with manual agitation for a period of seven days, left in the dark, at room temperature. The material/solvent ratio used was 1:10 (w/v). All extracts were filtered after preparation and stored at 4 °C without adding any preservatives until analyses.

Phytochemical screening

Total Phenolic Content (TPC). The TPC was analyzed following the method of Kujala et al. [23] with some modifications. Each extract (0.1 mL) was mixed with 0.5 mL Folin-Ciocalteu phenol reagent and 0.4 mL 7.5% Na_2CO_3. The mixture was vortexed and left for 5 min at 50 ºC. After incubation, the absorbance was measured at 765 nm. The TPC was expressed as mg gallic acid equivalents (GAE) per g dry weight (dw).

Total flavonoid content. The total flavonoid content was evaluated according to the method described by Kivrac et al. [24]. An aliquot of 0.5 mL of the sample was added to 0.1 mL of 10 % $Al(NO_3)_3$, 0.1 mL of 1 mol CH_3COOK and 3.8 mL of ethanol. After incubation at room temperature for 40 min, the absorbance was measured at 415 nm. Quercetin was used as a standard and the results were expressed as mg QE/g dw.

Total monomeric anthocyanin content. The total monomeric anthocyanin content was determined using the pH- differential method [25]. Properly diluted samples were mixed with KCl (0.025 mol, pH 1.0) and CH_3COONa (0.4 mol, pH 4.5) with an appropriate dilution factor. Absorbance (A) was measured using UV-Vis spectrophotometer at 520 and 700 nm after 15 min incubation at room temperature, and the results were calculated as follows:

$$A = (A_{520} - A_{700})\text{pH } 1.0 - (A_{520} - A_{700})\text{pH } 4.5$$

The monomeric anthocyanin (MA) pigment concentration in the samples was calculated as:

$$\text{Monomeric anthocyanin pigment (mg/liter)} = (A \times MW \times DF \times 1000)/(\varepsilon \times 1)$$

where M represents the molar mass of cyanidin-3-glycoside (449.2 g/mol), DF is the dilution factor, ε is molar extinction coefficient (26,900 L/mol x cm), and 1 is the cuvette optical path length (10 mm). The final anthocyanin concentration is expressed as milligram per 1000 mL of sample of cyanidin-3-glycoside.

Determination of antioxidant activity:

DPPH assay: The ability of the extracts to donate an electron and scavenge 2,2-diphenil-1-picrylhydrazyl (DPPH) radical was determined by the slightly modified method of Brand-Williams et al. [26] as described by Mihaylova et al. [27]. Freshly prepared

4×10^{-4} mol solution of DPPH radical was mixed with the samples in a ratio of 2:0.5 (v/v). The light absorption was measured at 517 nm after 30 min incubation. The DPPH radical scavenging activity was presented as a function of the concentration of Trolox - Trolox equivalent antioxidant capacity (TEAC) and was defined as the concentration Trolox having equivalent antioxidant activity expressed as the μmol per g dw (μmol TE/g dw).

ABTS assay: The radical scavenging activity of the extracts against 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) was estimated according to Re et al. [28]. Briefly, ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution (7 mmol) with 2.45 mmol potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. Afterward, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.7 ± 0.02 at 734 nm and equilibrated at 30°C. After the addition of 1.0 mL of the extract against ABTS radical cation ($ABTS^{\bullet+}$) ~~diluted $ABTS^{\bullet+}$ solution~~ to 0.01 mL of samples, the absorbance reading was taken at 30 °C after 6 min. The results were expressed as TEAC value (μmol TE/g dw).

Ferric-reducing antioxidant power (FRAP) assay: The FRAP assay was carried out according to the procedure of Benzie and Strain [29] with slight modification. The FRAP reagent was prepared fresh daily and was warmed to 37°C prior to use. One-hundred and fifty microliters of plant extracts were allowed to react with 2850 μL of the FRAP reagent for 4 min at 37°C, and the absorbance was recorded at 593 nm. The results were expressed as μmol TE/g dw.

Cupric ion reducing antioxidant capacity (CUPRAC) assay: The CUPRAC assay was carried out according to the procedure of Apak et al. [30]. One mL of $CuCl_2$ solution (1.0×10^{-2} mol) was mixed with 1 mL of neocuproine methanolic solution (7.5×10^{-3} mol), 1 mL NH_4Ac buffer solution (pH 7.0), and 0.1 mL of herbal extract (sample) followed by addition of 1 mL water (total volume = 4.1 mL) and mixed well. Absorbance against a reagent blank was measured at 450 nm after 30 min. The results were expressed as μmol TE/g dw.

Identification and quantification of phenolic acids. Qualitative and quantitative determination of phenolic acids was performed by using Elite LaChrome (Hitachi) HPLC system equipped with DAD and ELITE LaCHrome (Hitachi) software. Separation of the phenolic acids was performed by Supelco Discovery HS C18 column (5 μm, 25 cm × 4.6 mm), operated at 30 ºC under gradient conditions with mobile phase consist of 2 % (v/v) acetic acid (solvent A) and acetonitrile (solvent B) as reported by Mihaylova et al. [31]. The gradient program used was: 0-1 min – 95 % A and 5 % B; 1-40 min: 50 % A and 50 % B; 40-45 min: 100 % B; 46-50 min: 95 % A and 5 % B. The detection of phenolic acids was carried out at 280 nm for gallic, protocatechuic and cinnamic acids and at 320 nm for chlorogenic, caffeic, ferulic, p-coumaric, sinapic, rosmarinic and chicoric acids at flow rate 0.8 mL/min.

RESULTS AND DISCUSSION

Phenolic compounds such as phenolic acids, flavonoids, tannins and are plant secondary metabolites and they are very important in plants. These compounds contain hydroxyl groups which are responsible for the radical scavenging effect [32, 33] which provoke our interest in total polyphenolic, total flavonoid and total monomeric anthocyanins contents establishment in *C. erythraea* various extracts. The results are presented in Table 1. The total phenolic content established varied between 1.23 ± 0.02 and 12.46 ± 0.15 mg GAE/g dw. The maximum polyphenolic extraction yield was obtained in the tincture extract. The same tendency was observed regarding the total flavonoid content (1.18 ± 0.07 – 3.35 ± 0.03 mg QE/g dw) and total monomeric anthocyanins, where the presence of these compounds in infusion and microvawe extracts was even not determined. The phytochemical profile results revealed the tincture of *C. erythraea* as most active one among the investigated extracts. This is possible due to the solvent used.

Despite water as extragent is a cheap, safe and abundant *solvent* [34] total phenolic and total flavonoid contents and total monomeric anthocyanins were assessed with higher results in 70 % ethanol extracts. However, it is well known that solvent polarity will play a key role in increasing phenolic solubility, which probably influences the extraction of the target compounds.

Tusevski et al. [35] established TPC of 22.28 ± 1.07 mg GAE/g dw in common centaury 80% methanol extract obtained with ultrasonic bath and Bentahar et al [36] 49.629 ± 0.279 mg GAE/g of dry extract in decoct of the plant and 0.159 ± 0.001 mg QE/g of dry extract.

Table 1. Total phenolic contents (mg GAE/g dw), total flavonoid content (mg QE/g dw) and total monomeric anthocyanins content (mg/L) of *C. erythraea* extracts

Extraction technique	Total polyphenolic content	Total flavonoid content	Total monomeric anthocyanins
Infusion	3.41 ± 0.04	1.89 ± 0.04	-
Decoction	3.11 ± 0.10	2.59 ± 0.05	1.70 ± 0.14
Microwave	1.23 ± 0.02	1.18 ± 0.07	-
Tincture	12.46 ± 0.15	3.35 ± 0.03	6.15 ± 0.60

The antioxidant potential of the studied extracts of *C. erythraea* was evaluated by four different *in vitro* assays (DPPH and ABTS antiradical activities; FRAP and CUPRAC methods) in order to accomplish many authors recommendation for using several methods for antioxidant activity assessment in plants [37]. The results are presented in Table 2. The values ranged from 22.0 ± 0.05to 96.0 ± 1.0 μmol TE/g dw among the different assays. However, the highest results were established in the decoction and tincture with prevalence of one of the both sample in the different assays. Interestingly in respect of ABTS assay the highest result was established for common centaury decoction (88.3 ± 1.5 μmol TE/g dw) followed by the tincture (72.5 ± 1.8 μmol TE/g dw), whereas the other three conducted assays (DPPH, FRAP and CUPRAC) show the dominant activity for the tincture. These findings could be due to the different mechanism of action of the methods applied and of the various contributions of the compounds extracted [38]. However, the established antioxidant activity could be attributed to the extracted compounds such as flavonoids which showed strong antioxidant activity according some researchers [39].

Table 2. *In vitro* antioxidant activity of *C. erythraea* extracts (μmol TE/g dw)

Extraction technique	$TEAC_{ABTS}$	$TEAC_{DPPH}$	$TEAC_{FRAP}$	$TEAC_{CUPRAC}$
Infusion	33.2 ± 0.1	22.0 ± 0.05	44.7 ± 0.7	65.7 ± 1.0
Decoction	88.3 ± 1.5	41.3 ± 0.6	70.1 ± 0.3	94.7 ± 0.3
Microwave	33.9 ± 0.2	32.2 ± 0.1	33.7 ± 0.3	40.4 ± 0.2
Tincture	72.5 ± 1.8	48.6 ± 0.4	79.3 ± 1.8	96.0 ± 1.0

Tusevski et al. [40] evaluated higher antioxidant activity for aerial parts of *C. erythraea* toward $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ (79.29 ± 1.22 and 152.53 ± 9.64 μmol TE/g DW, resp.) and according CUPRAC assay (105.64 ± 4.61 μmol TE/g DW). These significant differences could be explained by type of extraction explored - ultrasonic extraction conducted with 80% methanol.

Table 3. Correlation coefficients (r) between the assays

	TFC	$TEAC_{ABTS}$	$TEAC_{DPPH}$	$TEAC_{FRAP}$	$TEAC_{CUPRAC}$
TPC	0.8660	0.4326	0.6947	0.7745	0.6622
TFC		0.7853	0.7706	0.9803	0.9477
$TEAC_{ABTS}$			0.8144	0.8919	0.8870
$TEAC_{DPPH}$				0.8301	0.7573
$TEAC_{FRAP}$					0.9712

The established correlations between the content of total phenolic, total flavonoid compounds and the antioxidant activity of the investigated extracts, assessed by four different methods are presented in Table 3. Correlation coefficients (r) showed a moderate relationship between the conducted methods. R-values ranged from 0.4326 to 0.9803. A very good correlation was observed between the applied antioxidant assays. In accordance with Stojiljković et al. [41] a lower existing correlation

was established in the antioxidant capacity and the total phenolic content.

Phenolic acids constitute a group of potentially immunostimulating compounds. They occur in all medicinal plants and are widely used in phytotherapy and foods of plant origin. In recent years, phenolic acids have attracted much interest owing to their biological functions [42].

The detailed phytochemical profile in respect of phenolic acids of the investigated extracts of *C. erythraea* is presented on Table 4. The total amount established ranged between 595.17 and 2208.6 μg/g dw in prevalence for the infusion. The lowest values were detected in the microwave extract. The *C. erythraea* decoction resulted in 1009 μg/g total phenolic acids, which could be due to the aggressive thermal heating during the extraction. However, a detailed phytocompound research is needed in order to evaluate the most suitable extraction approach in respect of total phenolic acids. The predominant established compounds were rosmarinic and p-coumaric acids, which were detected to be present in all samples. These findings are in accordance with the reported by HMPC in 2015 [43] presence of p-coumaric, O-hydroxyphenylacetic, ferulic, protocatechuic, sinapic, vanillic and syringic acids in *C. erythraea*.

Table 4. Phenolic acids composition of *C. erythraea* extracts (μg/g dw)

Extraction technique	Gallic acid	Proto-catechuic acid	Chlorogenic acid	Caffeic acid	Ferulic acid	p-Coumaric acid	Sinapic acid	Rosmarinic acid	Cichoric acid	Cinnamic acid	Total phenolic acids
Infusion	-	-	-	-	1665.42	6.	144.	391.	-	-	2208.
Decoction	168.	-	297.05	72	Traces *	27.7	-	443.	-	-	1009.
Microwave	-	-	200.69	49.69	-	16.	-	290.	-	36.87	595.
Tincture	-	436.	405.	44.	-	60.	60.	985.	68.	-	2061.

Traces - the values are below limit of detection.

CONCLUSIONS

The present study explore the total phenolic content, total flavonoid content and total monomeric anthocyanins present in various *Centaurium erythraea* extracts obtained by water and 70 % ethanol. The phytochemical profile in respect of phenolic acids and the antioxidant potential were assessed as well. Antioxidant properties and total phenolic amounts differed significantly among the evaluated extracts revealing the decoction and tincture as the most suitable approach for extraction when it comes to home preparing. The lowest values were detected in the microvawe extract. As a result, the consumption of the studied *C. erythraea* extracts could be recommended as a good source of biologically active substances and bio-antioxidants in particular with potential benefit effects.

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