

Phytochemical Study of Comfrey (*Symphylus officinale* L.) Root Extracts

Nadezhda Petkova

Department of Organic Chemistry
and Inorganic Chemistry
University of Food Technologies 26
Maritza Blvd, 4002, Plovdiv
Plovdiv, Bulgaria
petkovanadejda@abv.bg

Kristiyan Stefanov

Department of Analytical Chemistry
and Physical Chemistry
University of Food Technologies 26
Maritza Blvd, 4002, Plovdiv
Plovdiv, Bulgaria
kristiqnst@gmail.com

Manol Ognyanov

Laboratory of Biologically Active
Substances, Institute of Organic
Chemistry with Centre of
Phytochemistry)
Bulgarian Academy of Sciences
Plovdiv, Bulgaria
mogn@orgchm.bas.bg

Dasha Mihaylova

Department of Biotechnology
University of Food Technologies, 26
Maritza Blvd, 4002, Plovdiv
Plovdiv, Bulgaria
d_mihaylova@uft-plovdiv.bg

Dragomir Vassilev

Department Mathematics,
Informatics, and Natural Sciences,
Technical University of Gabrovo)
Gabrovo, Bulgaria
dvasilev@tugab.bg

Abstract. The purpose of the present study was to determinate the biologically active substance in 95% ethanol and subsequent water extracts from Bulgarian comfrey (*Symphylus officinale* L.) roots and to evaluate their antioxidant potential. The antioxidant activity was evaluated by several reliable methods such as DPPH-, ABTS-, FRAP-, CUPRAC-, ORAC and HORAC-assays, as well as the total phenolic content. In addition, the total organic, fructose and sugar content were determined by spectrophotometric and HPLC-RID methods. The level of fructans in ethanol extracts was 25.2 g/100g dry weight, as nystose and 1-kestose were only 0.1g/100g dry weight, and 0.3g/100g dry weight, respectively. The absence of fructooligosacharides in water extracts after the ethanol pretreatment was established. Inulin content was evaluated to be 25.2 g/100 g dry weight. In addition, total uronic content was established to be 2.0 g/100 g dw as its level dominated in water extracts – 1.7 g/100 g dw, respectively. The metabolite profile of roots revealed their potential application as radical scavengers due to the presence of polyphenols. Phenolic acids (neochlorogenic, p-coumaric and gallic acids) and flavonoids (quercetin, myricetin and naringin) were the dominant polyphenols in comfrey extracts. Therefore, the root extracts of *Symphylus officinale* L. could be assumed as a rich source of biologically active substance, in particular dietary fiber with potential prebiotic effect, due to the presence of polysaccharide inulin and fructooligosacharides.

Keywords: antioxidant, comfrey, inulin, roots

I. INTRODUCTION

Comfrey (*Symphytum officinale* L.) is a medicinal plant that belongs to Boraginaceae family. It is perennial herb widely spread across Europe, but it can also be found

in some parts of Asia and South America and North America [1], [2]. It can grow as weed in moist, low meadows, or along ponds and river banks where it may reach to a height of 20±150 cm, (usually 0.3-1.2 m) with long, hairy leaves with narrowing ends, and yellowish to red-violet flowers [3]–[5]. In Bulgaria is distributed from 0 to 1500 [5] and can be also found in forest throughout the country. Comfrey root is large up to 30 cm (typically 8-12 cm), branching, and black on the outside with a creamy white interior containing slimy mucilage [3, 5]. It is collected during spring (March), at the end of summer (August) and during autumn (November) [3]. In traditional medicine, comfrey roots are used from century for the treatment of wounds, joint disorders, and musculoskeletal injuries [1]-[3]. In Bulgaria tea has an expectorant, diuretic and anti-inflammatory effect. It is recommended as an aid in the treatment of duodenal and stomach ulcers, gastric bleeding, cough, inflammation of the upper respiratory tract, periodontitis, sprains, pleurisy, contusions, bone inflammations, difficult-to-heal wounds, conditions after amputation and purulent processes.

Compounds that were identified in comfrey root as active in the treatment of sprains, arthritis, fractures, and hematoma include allantoin, rosmarinic acid, and other hydroxycinnamic acid derivatives, as well as mucopolysaccharides, A, B and C vitamins, triterpenoid saponins, tannins, calcium, potassium, and selenium [1]. Other compounds found in comfrey root include abundant mucilage polysaccharides (about 29%) composed of fructose and glucose units [2], starch, inulin, resins, asparagine (1-3%), choline [3], phenolic acids such as rosmarinic acid (up to 0.2%), chlorogenic acid (0.012%)

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as well as caffeic acid (0.004%) and α -hydroxy caffeic acid, glycopeptides and amino acids and triterpene saponins in the form of monodesmosidic and bidesmosidic glycosides based on the aglycones hederagenin (e.g. symphytoxide A), oleanolic and lithospermic acids [2].

Comfrey roots or their extracts is scarcely investigated as a source of bioactive compounds with potentially beneficial biological effects. The information about the presence of carbohydrate content as a potential source of dietary fibers is limited. No detailed information about carbohydrate composition, especially sugars, fructan and uronic acid content in the comfrey roots growth in Bulgaria were found.

Therefore, the objective of the present study was to evaluate carbohydrate composition, the total phenolic content and antioxidant potential of comfrey roots extracts and to enrich the knowledge about this medicinal plant.

II. MATERIALS AND METHODS

All reagents and solvents were of analytical grade scale. Dried roots of comfrey (*Radix Symphyti*) were produced by Bilki.bg (Bulgaria). The plant material was finely ground and passed through a 0.5 mm sieve. The moisture content analyzed by AOAC 945.32 [6] was established to be 8.8%. The ground roots were kept in a screwed capped container at room temperature for further analysis.

A. Preparation of root extracts

The extraction was performed by previously described method by Olennikov et al. [7] with slight modification [8]. Comfrey roots (0.8 g) were extracted with 20 mL boiling 95% ethanol under a reflux for duration 60 min. The extraction process was repeated twice with 20 mL and 10 mL solvents, respectively. The residue was dried and then it was extracted successively three times with distilled water - 20 mL, 20 mL and 10 mL under reflux for 60 min. The obtained extracts were analysed in terms of antioxidant activity, total phenolic and carbohydrate content. Each sample was extracted in duplicate.

B. Carbohydrate analysis

The content of low molecules and high molecules fractions of fructans were presented as fructose equivalent was determined by spectrophotometric method at 480 nm. The content of mono-, di-, oligosaccharides and inulin was analysed by HPLC-RID method [9]. The uronic acid content in the extracts was assayed by m-hydroxybiphenyl method [10]. Galacturonic acid (5-100 $\mu\text{g/mL}$) was used for the calibration curve.

C. Determination of total saponin content

Total saponin content was determined colorimetrically by vanillin-sulfuric acid method [11]. The analysis was performed as described by Pasaribu et al. [12]. Diosgenin (200-500 $\mu\text{g/mL}$) was used as a standard and the analysis was performed in duplicate.

D. Determination of total phenolic content (TPC)

A modified Kujala et al. [13] method with Folin – Ciocalteu's reagent was used for the determination of the total polyphenolic content (TPC). Gallic acid was employed as a calibration standard and the results were

expressed as mg gallic acid equivalents (mg GAE) per gram of plant dry weight (dw).

E. Determination of antioxidant activity (AOA)

DPPH radical scavenging assay

Antioxidant activity was established toward the stable form of the synthetic product DPPH (2,2-diphenyl-1-picrylhydrazil radical) by the method of Brand-Williams et al. [14] with slight modifications. A freshly prepared 4.10⁻⁴ M solution of DPPH (in methanol) was mixed with the sample in a ratio of 2:0.5. The unit of Trolox equivalent antioxidant capacity (TEAC) defined the concentration of Trolox having equivalent antioxidant activity expressed as $\mu\text{M TE/g dw}$.

ABTS radical scavenging assay

The radicals scavenging activity of the studied extracts against radical cation (ABTS^{•+}) was estimated according to a previously reported procedure with some modifications [15]. The antioxidant value was defined as the concentration of Trolox having equivalent antioxidant activity expressed as $\mu\text{M TE per gram dry weight}$ ($\mu\text{M TE/g dw}$).

Ferric-reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to [16] Benzie Ferric-reducing antioxidant power (FRAP) assay. The absorbance of the reaction mixture was recorded at 593 nm. The results were expressed as $\mu\text{M TE/g dw}$.

CUPRAC assay

The CUPRAC assay was carried out according to the procedure [17]. Absorbance against a reagent blank was measured at 450 nm after 30 min. Trolox was used as standard and total antioxidant capacity of herbal extracts was measured as $\mu\text{M TE/g dw}$.

Oxygen Radical Absorbance Capacity (ORAC)

This assay was measured according to the method [18]. The method measures the antioxidant scavenging activity against peroxy radical induced by 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) at 37°C. Fluorescein (FL) was used as the fluorescent probe. The final ORAC values were calculated using a regression equation between the Trolox concentration and the net area under the curve. Results were expressed as micromole Trolox equivalents per litre.

Hydroxyl Radical Averting Capacity (HORAC) assay

HORAC measures the metal-chelating activity of antioxidants under the conditions of Fenton-like reactions employing a Co(II) complex and hence the protecting ability against formation of hydroxyl radical [18]. HORAC values were calculated using a regression equation between gallic acid concentration and the net area under the curve. One HORAC unit was assigned to the net protection area provided by 1 $\mu\text{mol/l}$ gallic acid and the activity of the sample is expressed as $\mu\text{mol gallic acid equivalents (GAE) per litre}$. ORAC and HORAC analyses were carried out using a FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany), excitation wavelength of 485 nm and emission wavelength of 520 nm were used.

F. HPLC analysis of phenolic compounds

High Performance Liquid Chromatography (HPLC) analyses of phenolic components was performed on an Agilent 1220 HPLC system (Agilent Technology, USA), equipped with a binary pump and UV-vis detector. A wavelength of $k = 280$ nm was used. Separation of phenolic compounds was performed using an Agilent TC-C18 column (5 μ m, 4.6 \times 250 mm) at 25 C. Mobile phases constitute of 0.5% acetic acid (A) and 100% acetonitrile (B) at a flow rate of 0.8 ml/min. A gradient was used with 14% B, between 6 min and linearly increased to 25% B and then 50% B at 40 min. The standard compounds gallic acid, 3,4-dihydroxy benzoic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, ellagic acid, catechin, epicatechin, quercetin, quercetin 3- β -glucoside, myricetin, kaemferol and naringin were used [19].

G. Statistical analysis.

All analyses were performed in triplicate ($n=3$). The data were presented as mean values \pm standard deviation (SD). Statistical analysis was performed using ANOVA, with the Tukey's range. A difference was considered statistically significant, when $p < 0.05$.

III. RESULTS AND DISCUSSION

A. Carbohydrate content

The carbohydrate content in 95% ethanol and subsequently aqueous extract of comfrey root was presented (Table 1). Individual sugars and inulin content were detected on HPLC-RID (Fig.1). It was found that in 95% ethanol fraction dominated sugars and fructooligosacchrides (Fig.1a), while in subsequent water fraction only high content of inulin and minor content of sucrose and fructose were found (Fig.1b). It was the first detailed about presence of inulin and fructooligosaccharides in comfrey roots. Inulin content reached 24.9 g/100 g dry weight. The total fructan content reached 32.5 g/100 g dw. Nystose and 1-kestose were detected only in 95% fraction in small amount (0.1 and 0.3 g/100 g dw, respectively). Sucrose and fructose were found in both fraction, while glucose were found only in 95% ethanol comfrey root extract. In addition small amount uronic acid content found mainly in water fraction -1.7 g/100 g dw. This study demonstrate inulin as the main reserved carbohydrate in comfrey roots. In previous study Van Laere and Van Den Ende [20] only mentioned *Symphyllum officinale* L. as source of inulin. Vasfilova and Vorob'eva [21] reported for presence of glucofructan in roots of *Symphyllum officinale* with low molecular weight in the beginning of vegetation period and for high molecular in the end of vegetation in the roots-45-47%. In their study low molecular fructans were detected to be 11%, while high molecular 29.5%. In our case the results for high molecular fructan fraction 27.6 g/100 g was close to their findings, while low molecular fraction (in ethanol extract) was more than 3 times lower. This could be explained with harvest and climate conditions. Moreover, the total fructan content was higher in subsequent water extract, where their content reached to 27 g/100g, which was in accordance with previous reported values of 15-30% [22].

TABLE I. CARBOHYDRATES CONTENT IN EXTRACTS OF COMFREY ROOTS, G/100 G DW (MEAN \pm SD, N=3)

Carbohydrates	Comfrey Extracts		
	95% Ethanol	Water	Total
Uronic acid content	0.3 \pm 0.1b	1.7 \pm 0.2a	2.0 \pm 0.2a
Total fructans	4.9 \pm 0.5b	27.6 \pm 1.0a	32.5 \pm 0.5a
Inulin	0.3 \pm 0.1b	24.9 \pm 2.2a	25.2 \pm 2.2a
Nystose	0.1 \pm 0.0a	n.d.	0.1 \pm 0.0a
1-Kestose	0.3 \pm 0.1a	n.d.	0.3 \pm 0.1a
Sucrose	1.8 \pm 0.5a,b	1.5 \pm 0.5a	3.3 \pm 0.5a
Glucose	2.3 \pm 0.5a	n.d.	2.3 \pm 0.5a
Fructose	5.1 \pm 0.2a	1.0 \pm 0.4b	6.1 \pm 0.3a

Values are mean \pm standard deviation of three separate experiments. Different letters within each column indicate significant differences between treatments according to Tukey's test at $p < 0.05$; n.d. - not detected, ns - not significant

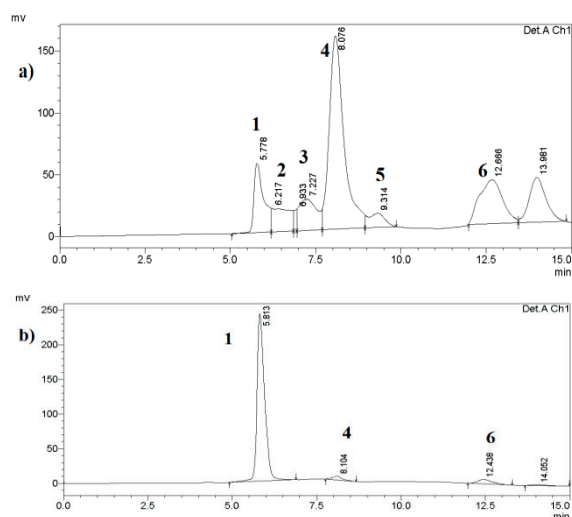


Fig. 1. HPLC chromatograms of the extracts obtained from comfrey (*Symphyllum officinale* L.) root, a) 95 % ethanol and b) water extracts, where 1. inulin; 2. nystose, 3. 1-kestose 4. sucrose, 5. glucose and 6. fructose.

B. Total saponins, total phenolic content and antioxidant activities

Total saponins, total phenolic content (TPC) and antioxidant activities (μ m Trolox equivalent/g dw) in root extracts of comfrey (*Symphyllum officinale*) were summarized in Table 2. It was found that saponins dominated in water fraction - 17.4 μ g/g dw, while its content in 95% ethanol fraction was more than half times lower. Total phenolic content in both fraction was approximately equal - 4 mg GAE/g dw. Six methods based on different mechanisms were used to evaluate antioxidant potential of the comfrey root extracts (Table 2). Water extracts demonstrated higher results for antioxidant potential by methods based on electron transfer - FRAP and CUPRAC methods. The methods based on hydrogen transfer or mixed mechanism (DPPH and ABTS) demonstrated higher antioxidant potential of the subsequent water extract. The highest antioxidant potential was observed by ORAC method for 95% ethanol extract -355.5 μ mol TE/g dw. The highest antioxidant

potential for the subsequent water extract was found by CUPRAC method – 129.0 $\mu\text{mol TE/g dw}$. The lowest values was observed by HORAC method for the water extract and DPPH assay for 95% ethanol fraction. In general the subsequent water extracts from comfrey roots exhibited stronger radical scavenging activity, methal reducing ability in comparison with ethanol extract. Ethanol fraction showed the highest potential for oxygen radical absorbance capacity.

Our results for antioxidant potential DPPH and ABTS methods were closed to these reported by Neagu, et al. [23]. Anlas et al. [24] reported higher than our results for the total phenolic contents of ethanolic and aqueous extracts of *S. officinale* - 116.93 mg GAE/g and 99.49 mg GAE/g, respectively and they also explained that the greater amount of phenolic compounds leads to more potent radical scavenging effect.

TABLE 2. TOTAL SAPONINS, TOTAL PHENOLIC CONTENT (TPC) AND ANTIOXIDANT ACTIVITIES IN ROOT EXTRACTS OF COMFREY (*SYMPHYTUM OFFICINALE L.*)

Analysis	Extracts	
	95% ethanol	Water
Total saponins, $\mu\text{g/g dw}$	7.2 \pm 1.6b	17.4 \pm 3.9a
TPC, mg GAE/g dw	4.4 \pm 0.1a	3.9 \pm 0.1ns
FRAP, $\mu\text{mol TE/g dw}$	62.2 \pm 0.6b	99.9 \pm 1.0a
CUPRAC, $\mu\text{mol TE}$	122.0 \pm 2.4a	129.0 \pm 1.5ns
ABTS, $\mu\text{mol TE/g dw}$	71.4 \pm 0.6b	84.6 \pm 0.6a
DPPH, $\mu\text{mol TE/g dw}$	60.9 \pm 0.6b	79.5 \pm 0.4a
ORAC, $\mu\text{mol TE/g dw}$	355.5 \pm 7.1a	78.1 \pm 0.6b
HORAC, $\mu\text{mol GAE/g dw}$	67.6 \pm 1.0a	41.8 \pm 3.3b

Values are mean \pm standard deviation of three separate experiments. Different letters within each column indicate significant differences between treatments according to Tukey's test at $p < 0.05$; ns - not significant

Therefore, water extracts contained much more active biocompounds than an ethanol extract that were relatively strong scavengers of free radicals, as it was shown in Table 3.

C. Phenolic acids and flavonoids

The contents of phenolic acids (mg/100 g) and flavonoids extracts in comfrey root were summerized in Table 3. Ten phenolic acids and eight flavonoids were detected mainly in the subsequent water fraction, whereas 95% ethanol contained only eight phenolic acids and six flavonoids. In general neochlorogenic acid dominated in 95% ethanol comfrey extract (26.0 mg/100 g), while in the subsequent water extract *p*-coumaric acid was in the highest amount (38.3 mg/100 g). Chlorogenic and vanilic acids, as well as flavonoids quercetin 3- β -glucoside and epicatechin were not detected in 95% ethanol, while these four phenolic compounds presented in the subsequent water extract of comfrey roots. Quercetin and catechin dominated in 95% ethanol comfrey root extract, while there content in the subsequent water extract was two times lower. Myricetin, kaemferol, naringin and naringenin dominated in water fraction.

In agreement with some previous reports [24]-[27] the phenolic acid and flavonoids in comfrey roots including chlorogenic acid, cafeic acid, ferulic acid, coumaric acid, ellagic acid, epicatechin, myricetin, quercetin, kaempferol were detected. Besides these bioactive compounds, the presence of neochlorogenic acid was also detected. In the water extract of comfrey root, the content of ellagic acid is 1.5 g/100 g dw.

TABLE 3. CONTENTS OF PHENOLIC ACIDS (MG/100 G) AND FLAVONOIDS IN *S. OFFICINALE* EXTRACTS

Phenolic compounds		Comfrey Extracts			
		95% Ethanol	Water	Total	
Phenolic acids	Chlorogenic acid	n.d.	0.7 \pm 0.4a	0.7 \pm 0.4a	
	Neochlorogenic acid	26.0 \pm 3.1a	6.8 \pm 1.4b	32.8 \pm 2.5a	
	Vanilic acid	n.d.	1.5 \pm 0.2a	1.5 \pm 0.2a	
	Cafeic acid	2.5 \pm 0.3b	3.6 \pm 0.5a	6.0 \pm 0.3b	
	<i>p</i> -Coumaric acid	18.2 \pm 2.1b	38.3 \pm 5.8a	56.5 \pm 3.7a	
	Ferulic acid	0.4 \pm 0.1b	1.5 \pm 0.2a	1.9 \pm 0.3a	
Phenolic acids	Ellagic acid	0.5 \pm 0.1b	1.5 \pm 0.2a	2.0 \pm 0.3a	
	Cinnamic acid	4.0 \pm 1.0b	5.7 \pm 0.5a	9.7 \pm 0.6a	
	3,4-dihydroxy benzoic acid	1.2 \pm 0.1b	2.5 \pm 0.1a	3.7 \pm 0.1a	
	Gallic acid	7.3 \pm 3.0a	4.5 \pm 0.4b	11.8 \pm 2.6a, b	
	Flavonoids	Quercetin	73.4 \pm 1.4a	42.7 \pm 0.2b	116.0 \pm 0.8a, b
		Quercetin 3- β -glucoside	n.d.	19.8 \pm 0.1a	19.8 \pm 0.1a
Myricetin		21.5 \pm 1.1a, b	39.0 \pm 0.1a, b	60.5 \pm 5.3a, b	
Kaemferol		8.6 \pm 1.1b	10.4 \pm 3.0a	18.9 \pm 0.9a	
Naringin		21.4 \pm 3.0b	30.0 \pm 3.1a	51.3 \pm 3.1a, b	
Naringenin		6.6 \pm 1.1b	8.2 \pm 2.6a	14.8 \pm 3.9a	
Catechin		26.8 \pm 0.7a	13.3 \pm 3.1b	40.1 \pm 1.5a	
Epicatechin		n.d.	4.2 \pm 0.3a	4.0 \pm 0.3a	

Values are mean \pm standard deviation of three separate experiments. Different letters within each column indicate significant differences between treatments according to Tukey's test at $p < 0.05$; n.d. – not detected.

The high content of ellagic acid in comfrey root can be responsible for its biological and antioxidant activity. In general, the roots of comfrey contained in the highest amount *p*-coumaric, neochlorogenic and gallic acids and quercetin, myricetin, catechin and naringin. The comfrey root extracts showed higher content of flavonoids in comparison the phenolic acids.

IV. CONCLUSIONS

The current research revealed the main carbohydrate content in 95% ethanol and subsequent water extracts from comfrey roots. Therefore, the obtained results demonstrated that the plant material is potential source of fructans, especially the prebiotic inulin. Due to the antioxidant potentials, the polyphenol and flavonoids content, both extracts of comfrey root could be considered as a source of bioactive components with potential application in pharmacy and cosmetics.

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