Bulgarian Chemical Communications, Volume 57, Special Issue A (pp. 117-123) 2025 DOI: 10.34049/bcc.57.A.MU19

Comfrey (*Symphylus officinale* L.) roots – a source of polyphenols and fructans

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Received: September 03, 2024; Revised: April 04, 2025

The aim of this research was to evaluate the content of polyphenols, fructans and the antioxidant activity of water extracts of comfrey roots obtained using ultrasound-assisted extraction. Isolation of inulin from the roots using microwave-assisted extraction and chemical characterization was also performed. The total contents of phenols and flavonoids were evaluated using spectrophotometric methods. Antioxidant potential was evaluated using DPPH and FRAP methods. Sugars and fructans were determined by HPLC-RID method. Isolated inulin was characterized for yield, fructose content, degree of polymerization and molecular weight. Its structure was elucidated using FTIR spectroscopy. From the conducted research it was found that comfrey roots were rich sources of total flavonoids (64.86 mg quercetin equivalents/g dry weight) and fructans (13.43 g/100 g dry weight). Higher values of the antioxidant potential were found using FRAP method. Polysaccharide isolated by microwave-assisted extraction had fructose content of 64.1 %, high degree of polymerization (29–33) and molecular weight of 5.15-5.36 kDa. FTIR spectroscopy confirmed that isolated polysaccharide was inulin-type fructan with characteristic bands for $\beta(2\rightarrow1)$ bonds. To the best of our knowledge this is the first detailed analysis of inulin-type fructan isolated from comfrey (*Symphylus officinale* L.) roots. As sources of flavonoids and inulin, comfrey roots can find further application in cosmetics and pharmacy.

INTRODUCTION

Comfrey (*Symphylus officinale* L.) is a medicinal plant from *Boraginaceae* family that occurs mainly in Europe, Asia and South America. Its roots are traditionally used for curing wounds, joint disorders, and musculoskeletal injuries [1-3]. Due to the rich content of phytochemical compounds in comfrey roots as allantoin, rosmarinic acid, and other hydroxycinnamic acid derivatives, as well as muco-polysaccharides [4], inulin [5] A, B and C vitamins, triterpenoid saponins, tannins, calcium, potassium, and selenium [1,2] these roots find various applications in phytoterapy. Moreover, the phenolic acids (e.g., rosmarinic, p-hydroxybenzoic, caffeic, chlorogenic and p-coumaric) display remarkable antioxidant effects, besides their positive impact on human skin fibroblasts [6]. Extraction techniques using different solvents were the most important tools for obtaining biologically active compounds from comfrey roots. Many researches deal with different extraction approaches for obtaining mainly polyphenolic compounds [1, 3, 4] – conventional extraction [2, 5], pressure-liquid extraction [1], supercritical fluid extraction [1]. Less attention was paid to the extraction of carbohydrate and evaluation of the polysaccharide structure of comfrey roots. In our previous study the carbohydrate composition was partially revealed, especially inulin [5]. It was reported that polysaccharides from comfrey roots possess a strong antioxidant effect due to the presence of uronic acid group [7], as well as hypoglycemic activity [7-9]. Polysaccharides from comfrey roots were isolated by other researchers and the optimum extraction conditions were found. In most of the studies water was used as a sovent in combination with enzyme and or ultrasonic waves [8, 9]. In some studies, microwaves and ultrasonic irradiation were mentioned as prospective approaches for isolation of polysaccharides, especially inulin [8-10]. However, the analysis of bioactive compounds in water extracts, as well as the elucidation of the characteristics of inulin obtained from comfrey roots still remains a challenge. Little is known about the structure of inulin-type fructan from comfrey roots. The aim of this research was to determine the content of polyphenols and inulin-type fructans, as well as the antioxidant activity of water extracts of comfrey roots obtained using ultrasound-assisted extraction. As an additional task, inulin was isolated by microwave-assisted extraction and its chemical characterization was performed.

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EXPERIMENTAL

Plant material

Dried roots of *Symphylus officinale* (Radix Symphyti) were produced by Thalloderma (Bulgaria). The dry plant material was finely ground and passed through a 0.5 mm sieve. The ground samples were kept in a screwed capped container at room temperature for further analysis. For the isolation of fructan from comfrey roots, successive extraction with hexane, chloroform, and ethyl acetate was performed by maceration for 24 h at 25°C, at solid-to-liquid ratio (1:5 w/v), as previously described [10] to remove fatty and polyphenolic compounds.

Ultrasound-assisted extraction of comfrey roots

The extraction procedure was performed in 50 ml plastic tubes at solid-to-liquid ratio of 1:10 (w/v) in an ultrasonic bath SIEL UST 5.7-150 (Gabrovo, Bulgaria) operating at 36 kHz frequency and 240 W ultrasonic power. The extraction was done with distilled water at 75 °C for 20 min in duplicate [11]. The water extracts were filtered and combined for further analysis of total phenols, flavonoids, antioxidant activity and carbohydrate composition.

Microwave-assisted isolation of inulin from S. officinalis

The residual comfrey root sample after washing with hexane, chloroform, and ethyl acetate was dried at room temperature and then extracted with distilled water (30 g roots with 300 ml of boiling water) in duplicate using a microwave device Crown (2452 kW), with microwave power of 700 W for 5 min [10]. The hot water extract was filtered through nylon cloth. The cooled filtrate was precipitated with acetone (1:4 v/v). The precipitate was separated by centrifugation and then purified by recrystallization in boiling water and precipitated with acetone (1:5 v/v). The obtained polysaccharide was filtered and dried at 40 °C.

Evaluation of total phenols, flavonoids and antioxidant activity

The evaluation of total phenols was done using Folin–Ciocalteu's reagent. The total flavonoids were determined using $Al(NO_3)_3$ reagent at 415 nm [12]. The results were expressed as mg equivalents quercetin (QE)/ g sample [13].

DPPH radical scavenging assay was performed with DPPH (2,2-diphenyl-1-picrylhydrazyl radical) reagent as previously described [14]. Ferric-reducing antioxidant power (FRAP) assay was performed according to [14]. The absorbance of the reaction mixture was recorded at 593 nm. The results for the antioxidant activity were expressed as mM Trolox equivalent (TE)/g dw.

Carbohydrate analysis

The fructans content was determined by resorcinol-thiourea spectrophotometric method at 480 nm [10]. The individual sugars and inulin were determined by HPLC-RID method [5].

Characterization of fructan from comfrey roots obtained by microwave-assisted extraction

The melting point was measured with a Kofler apparatus. The reducing groups were determined by the PAHBAH method at 410 nm [10], while total fructose content - by resorcinol-thiourea reagent at 480 nm [11]. The purity of fructan was assessed on an HPLC instrument Elite Chrome Hitachi with a Shodex® Sugar SP0810 column (300mm × 8.0mm i.d.) (Shodex Co., Tokyo, Japan) at 85°C, flow rate of 1.0 ml/min and injection volume of 20 µl [10]. High-performance size-exclusion chromatography (HPLC-SEC) was used for the determination of number average molecular weight (Mn), and weight average molecular weight (Mw). The analysis of comfrey inulin was performed on HPLC chromatograph ELITE LaChrome (VWR Hitachi, Japan) using a column Shodex OH-pack 806 M (300mm × 8.0mm) at 30°C and an RI detector (VWR Hitachi Chromaster, 5450, Japan) with 0.1M $NaNO_3$ [15]. Polydispersity index (X) of inulin was calculated as the ratio of the two molecular weights (Mw/Mn).

FTIR spectroscopy

The isolated fructan (2 mg) was pressed into a KBr tablet and FTIR spectrum was collected on a FTIR Avatar Nicolet (Thermo Scientific, USA) spectrometer in the wavelength range of 4000–400 cm^{-1} after 128 scans at a resolution of 2 cm^{-1}.

Statistical analysis

All experimental measurements were carried out in triplicate and are expressed as average of three analyses ± standard deviation.

RESULTS AND DISCUSSION

Biologically active compounds and antioxidant activity in comfrey root water extracts

Most studies of comfrey roots using the acoustic cavitation effect induced by the ultrasound waves lead to sonolysis facilitating solvent migration into the cell. The extraction of intracellular compounds was connected with extraction of polysaccharides, polyphenols, allantoin and pyrrolizidine alkaloid

[16]. The results for total polyphenols, flavonoids, antioxidant activity and fructan content in water extracts obtained by ultrasound-assisted extraction of comfrey roots are presented in Table 1.

Table 1. Total polyphenols, flavonoids, fructans and antioxidant activity in water extracts of comfrey roots

Characteristics	Comfrey root water extract
Total polyphenols, mg GAE/g dw	0.72±0.07
Total flavonoids, mg QE/g dw	64.86±0.53
Antioxidant activity, mM TE/g dw	
DPPH	2.60±0.32
FRAP	4.31±0.35
Total fructans, g/100 g dw	13.41±0.53
Inulin, g/100 g dw	10.10±0.53
Nystose, g/100 g dw	Not detected
1-kestose , g/100 g dw	Not detected
Sucrose, g/100 g dw	0.80±0.01
Glucose, g/100 g dw	1.21±0.03
Fructose, g/100 g dw	1.32±0.05

It was found that the highest amount of total flavonoids was 64.86 mg QE/g dw. This confirmed our earlier finding that the comfrey root extracts showed higher content of flavonoids than phenolic acids [5]. The total phenolic content (0.72 mg GAE/g dw) in this study was between 10-100 times lower than the values for other extracts reported previously [5, 17, 18]. The antioxidant potential was evaluated by two methods based on different mechanisms, and FRAP showed the highest values 4.31 mM TE/g dw. The radical scavenging ability of the water extract obtained by ultrasonic extraction evaluated by DPPH method was 2.60 mM TE/g dw. Our results were higher than the literature data for the antioxidant potential of *S. officinale* root ethanol/water extracts evaluated by DPPH method (0.985 mM TE/g [2]. The antioxidant potential of comfrey water extracts was comparable with previous reports for comfrey extracts [5, 17]. In previous studies comfrey roots hydroalcoholic extracts demonstrated antioxidant activity by FRAP method between 14.42–82.42 mg TE/g extract [3,16], while that of the dichloromethane fraction was 6.67 mg TE/g extract [16].

The presence of fructans (or polyfructans) in *Symphylus* sp., especially comfrey *Symphylus officinale* was reported previously [19-21], as fructan content varied between 27-47% dw depending of time of harvest and development of plant [21]. Our previous study clearly demonstrated that comfrey roots contained fructans (27-32 g/100 g dw) [5]. In the present research the HPLC-RID chromatogram of the carbohydrate profile of comfrey root water extracts obtained by ultrasound-assisted extraction is shown (Fig. 1). In the water extracts inulin, sucrose and two monosacharides - glucose and fructose are clearly seen. The detected total fructans in the water extract of comfrey root obtained by ultrasound-assisted extraction were 13.43 g/100 g, while inulin was 10.10 g/100 g dw. However, fructooligosacchrides nystose and 1-kestose were not detected. Only small amounts of sucrose, glucose and fructose were found (Table 1). These results were almost two times lower in comparison with our previous report for fructan and inulin (27.6 and 24.9 g/100 g dw) in comfrey roots obtained by classical sequential ethanol and water extraction [5]. The levels of sucrose and glucose were also two times lower, while fructose was more than 4 times lower. The difference in composition of sugars and inulin can be related to harvest time of roots, as well as used extraction technique.

Isolation and characterization of inulin-type fructan from comfrey roots

The physicochemical characteristics of inulin from comfrey roots obtained after microwave-assisted extraction are summarized in Table 2.

Table 2. Physicochemical characteristics of inulin isolated form comfrey roots obtained by microwave-assisted extraction

Characteristics	Comfrey inulin	Inulin raftiline HP
Yield, %	15.4±1.4	-
Purity, %	36.6±0.5	85.5±0.4
Melting point, °C	183-184	175-179
Fructose content, %	64.1±0.5	75.3±0.4
Reducing groups, %	2.3±0.5	2.3±0.4
DP by spectrophotometric method	29	34
DP by HPLC-SEC	33	26
Mw, kDa	5.36	4.02
Mn, kDa	5.15	4.19
Polydispersity index	1.04	1.03

The results for the obtained comfrey inulin-type fructan were compared with those for commercial chicory high-molecular inulin with degree of polymerization 26. The yield of isolated comfrey polysaccharide was 15.4 % with low purity (36.6%). In this fructan the fructose content was 64.1 % and that of reducing groups was 2.3 %.

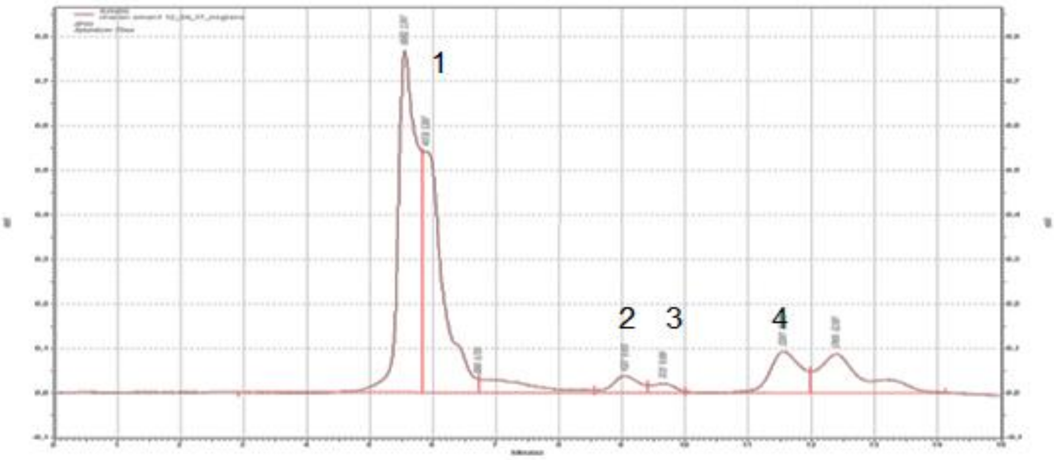

Fig. 1. HPLC-RID chromatogram of water extract from comfrey roots, where 1-inulin, 2-sucrose, 3-glucose and 4-fructose

It was the first detailed study about inulin isolated from the comfrey roots. Inulin isolated from its roots possessed high degree of polymerization (29–33) and molecular weight of 5.15-5.36 kDa. Vasfilova and Vorob'eva [21] reported on the presence of glucofructan in *Symphylus officinale* roots with low molecular weight at the beginning of the vegetation period and high molecular weight at the end of the vegetation - 45-47%. According to these authors the low-molecular fructans reached 11%, while high molecular ones - 29.5% with index of polymerization 0.7-1.0. It was reported that comfrey polysaccharide fractions (comprising 78.01% to 85.70% of total polysaccharides) were recovered with yields varying from 7.39 to 24.51% [8, 25]. Moreover, it was reported that polyfructans in roots and rhizomes of *Symphytum caucasicum* can reach 22-25%, as fructose containing carbohydrates are 44-51% with degree of polymerization of fructans between 0.49-0.50 [22]. It was also found that in a water-soluble high-molecular preparation of *Symphytum grandiflorum* the main components of mucilage are glucofructans (67 %) [24]. In addition, Haaβ *et al*. [25] reported for inulin type fructan from root and leaves of *Symphylus officinale* with degree of polymerization 33, that coincided with the degree of polymerization of inulin from comfrey roots obtained by microwave-assisted extraction found in this study. In general, the isolated inulin-type fructan is characterized with high molecular mass and degree of polymerization higher than chicory, dahlia and burdock inulin [10, 15]. The degree of polymerization is an important feature that brings about functional and biological activity of fructans [25]. The isolated comfrey inulin with DP 29-33 could be a good immunomodualtor and taste enhancer, but additional studies are required.

HPLC-SEC chromatogram of polysaccharide from comfrey isolated by microwave-assisted extraction is presented (Fig. 2). Four peaks are seen, the first broad peak at 9.6 min due to polysaccharide with high molecular mass (Mw 1158 kDa and Mn 955 kDa) and polydispersity index of 1.21. The second symmetric peak at 12.7 min is fructan with high molecular weight (Mw 5.1 kDa and Mn 5.3 kDa). The polydispersity index of the isolated comfrey fructan was 1.04, that was near to chicory inulin, as well as burdock, echinacea and dahlia inulin-type fructans [10, 15-23]. In addition, comfrey polysacchrides with molecular weight distribution from 2.8 to 3420 kDa have been reported [8, 9]. It was explained that the size distribution depended on the drying conditions or extraction procedures [26, 27]. Acording to Chen *et al.* [8] when comfrey root polysaccharides were extracted with hot water, there was a distinct group with a molecular weight of 5811.42 Da, that was close to our finding for fructan from comfrey with molecular weight of 5.3 kDa.

It was previously reported that comfrey polysaccharides are non-starch polysaccharides and are primarily composed of galactose, arabinose, glucose, and galacturonic acid, indicating that they may pass to the end of the intestinal tract of monogastric animals and be fermented by intestinal microflora [26].

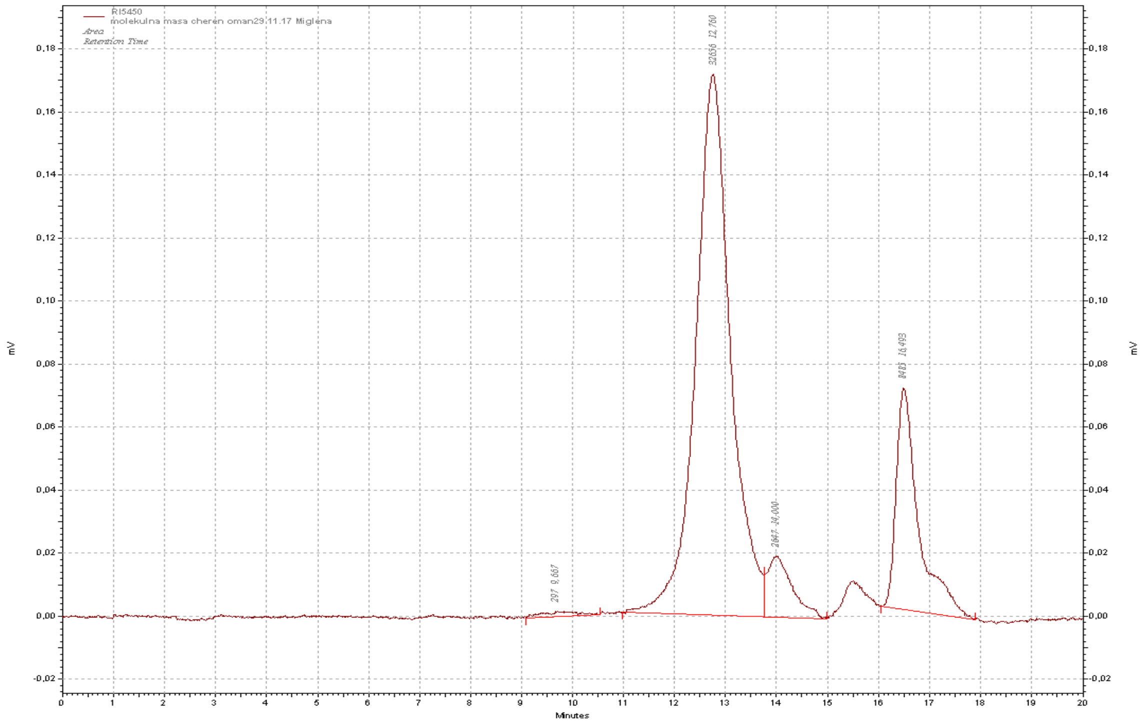

Fig. 2. HPLC-SEC chromatogram of inulin from comfrey isolated by microwave–assisted extraction

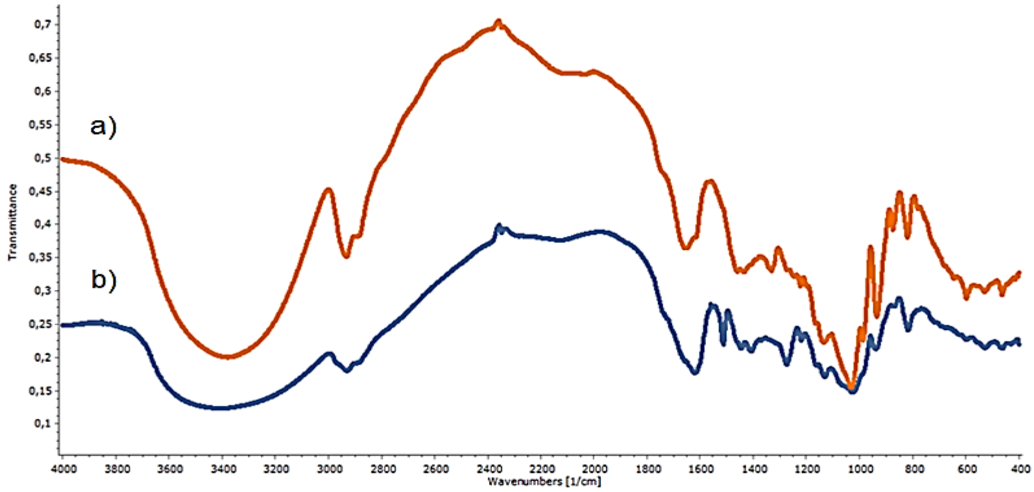

Fig. 3. FTIR spectra of inulin: a) chicory inulin Raftiline HPX DP=25, b) isolated inulin from comfrey roots by microwave-assisted extraction with DP 29-33

FTIR specroscopy

The structure of comfrey inulin isolated by microwave-assisted water extraction was confirmed by FTIR spectroscopy (Fig. 3). The comfrey inulin spectrum was compared with the spectrum of chicory inulin raftiline HPX DP=25. The assigned bands are summarized in Table 3. These spectra demostrated all typical bands for inulin-type fructans [28]. It was found that a broad band at 3396 cm^{-1} was due to O–H stretching vibrations associated with inter- and intramolecular hydrogen bonds in the inulin structure. The bands at 2930–2932 cm^{-1} were due to C–H asymmetric stretching vibrations. The bands at 2889 cm^{-1} were characteristic of symmetric stretching vibrations of C–H from CH_2. The bands at 1445 cm^{-1} were due to symmetric stretching vibrations of C–H in pyranosyl ring and β_{O-H} (OH). The bands at 1129 cm^{-1} were assigned to C–O–C ring stretching vibrations from glycoside linkage. The bands at 1024 cm^{-1} were assigned to C–O stretching vibrations. Similar bands at 3377 cm^{-1}, 2937 cm^{-1}, 1419 cm^{-1} and 1140 cm^{-1} were reported in the FTIR spectrum of comfrey root polysaccharide with molecular weight of 3812.39 Da and monosaccharide composition including galacturonic acid (GalA), arabinose (Ara), glucose

(Glc), and galactose (Gal) in a molar ratio of GalA, Ara, Glc, and Gal of 1.00:0.88:2.28:1.13, respectively [9]. The band at 937 cm^{-1} showed the presence of α-D-glucopyranosyl residue in the carbohydrate chain. The band for 2-ketofuranose and β-anomer bendings in C1–H was detected at 873 cm^{-1} and the occurence of a typical band at 816 cm^{-1} confirmed the presence of 2-ketose in a pyranosyl or furanosyl ring (Table 3).

Table 3. Assignment of bands in the FTIR spectrum of inulin isolated from comfrey roots

Band intervals	Observed bands	Assignment
3200 - 3400	3396	O–H stretching vibrations, intramolecular H-bonds
2933 - 2981	2930	νC–Has(CH_2)
2850 - 2904	2889	νC–Hs(CH_2)
1455 - 1470	1445	νC–Hs(CH_2) in pyranosyl ring, βo–н (OH)
1225 - 1235	1218	βo–н (OH)
1125 - 1162	1129	νC-O-Cas (C–O–C), glycoside linkage
1015 - 1060	1024	νC–O (C–O)
925 - 930	937	α-D- Glcp residue in polymer chain
867	873	ρ CH_2 in ring, β-anomer bendings C1-H, β-anomer, 2-ketofuranose
817	816	2-ketose in pyranosyl or furanosyl ring

These bands are in accordance with a previous report [27]. The last bands in the fingerprint region are typical for inulin and inulin-type fructans. Similar bands in the FTIR spectra were reported earlier for inulin-type fructan, especially the bands at 935, 873, and 818 cm^{-1} were typical for inulin from different plant sources such as burdock, echinacea, dahlia, and chicory [10, 15, 23, 27].

CONCLUSION

The current research enlarges the knowledge about the phytochemical composition of comfrey roots emphasizing on carbohydrate profile and flavonoids content in the extracts obtained after ulrasonic irradiation. This is the first detailed report on the isolation and chemical characterization of high- molecular long-chain inulin-type fructan with degree of polymerisation of 29-33 from defatted comfrey (*Symphylus officinale* L.) roots by microwave-assisted extraction. The comfrey roots were evaluated as a promising source of inulin – 10-15 g/100 g dw, depending on the applied extraction technique. Further studies about the health impract of comfrey fructan are needed. In general, it may be concluded that comfrey roots are sources of flavonoids, antioxidants and inulin, and may thus find application in cosmetics and pharmacy.

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