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Phytochemical Analysis of *Symphytum officinale* Root Culture Extract

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Abstract: Comfrey (*Symphytum officinale*) root preparations are known for their analgesic and anti-inflammatory properties. In this study, the phenolic profile of extract samples of *Symphytum officinale* callus root cultures (up to 70% of ethanol) was determined using various approaches. For the first time, a unique polymer of two types was isolated from samples of comfrey root cultures (the caffeic acid derivative is a new class of natural polyether with 3-(3,4-dihydroxyphenyl) glyceric acid as a repeating unit). M-methoxybenzoic acid (30.05 µg/mL extract) or rosmarinic acid (45.70 µL/mL extract) were identified as the main phenolic component. Rosmarinic, chlorogenic, and caffeic acids have anti-inflammatory, anti-apoptotic, antitumor, neuroprotective, antioxidant, and other properties, which also determine the characteristics of comfrey and preparations made from it. The studied phenolic profile and the analysis of published data showed that the extract samples of comfrey callus cultures had similar characteristics, which makes them an important source of phenolic compounds with pronounced antioxidant activity.

Keywords: *Symphytum officinale*; callus cultures; squalene; pyrrolizidine alkaloids; antioxidant activity

1. Introduction

The *Symphytum* genus (Boraginaceae) includes about 40 perennial species growing in Eurasia [1]. Researchers pay close attention to several species, including *Symphytum officinale* L., *Symphytum asperum* Lepech, *Symphytum caucasicum*, *Symphytum tuberosum* L., and the hybrid species *Symphytum uplandicum* Nyman. The literature also provides a description of the following species: *Symphytum cordatum* Willd., *Symphytum grandiflorum* DC., *Symphytum ibericum* Steven ex M. Bieb., *Symphytum orientale* L., *Symphytum popovii* Dobrocz, and *Symphytum tauricum* Willd., without a clear indication of the component composition of secondary metabolites [2].

Despite the fact that numerous clinical trials have demonstrated the effectiveness of *S. officinale* as an anti-inflammatory agent in muscle and joint inflammatory diseases, the key bioactive components, as well as their molecular mechanisms of action, remain poorly understood [3]. It is reported that comfrey has antibacterial, anti-inflammatory, antioxidant,

and antinociceptive properties, and it can be used for hepatoprotection and tissue regeneration [4–6]. Numerous chemical components of comfrey determine its pharmacological and therapeutic potential, including in neurology [7,8].

Symphytum officinale is reported to contain polysaccharides, allantoin, phenolic acids (chlorogenic, caffeic, and rosmarinic), phytosterols and triterpene saponins, glycosides, and pyrocatechol-type tannins, as well as pyrrolizidine alkaloids (intermedin, acetylintermedin, lycopsamine, acetyllycopsamine, and simfitin, and their corresponding N-oxides) [9–15]. At the same time, the details of the morphological localization of these components (shoots and roots) are not provided. The root and herb (*Radix cum Herba Symphyti*) in the form of a cold extract infusion, is used both internally and externally. In folk medicine, it is used for joint deformities, myalgia, and bone fractures [1]. In Europe, fresh roots and fresh flowering plants, collected from March to June and from September to October, are used. The internal use of *Symphytum officinale* is currently being disputed. Long-term use is not recommended due to the presence of pyrrolizidine alkaloids (PA). PA are esters formed by noncyclic acids and amino alcohols—necines. The necines are oxygenated derivatives of L-methylpyrrolizidine. Pyrrolizidine alkaloids are found in common foods. It is the potential long-term health risks associated with low-dose chronic exposure to this class of compounds from foods that is the source of recent concern. The European Medicines Agency (EMA) indicates that milk, eggs, honey, pollen products, grains, and meat, as well as herbal products, including comfrey, used in salads and brewed like tea, are sources of PA [15].

All these features of the *Symphytum* genus representatives create the prerequisites for a comprehensive, in-depth study of their component composition using modern physico-chemical methods.

This work aimed to study the main components of the *Symphytum officinale* root culture by a complex of chromatographic methods.

2. Materials and Methods

2.1. Research Objects

The objects of the study were the seed cultures of *Symphytum officinale* L. (*Boraginaceae* family) grown in vitro on liquid nutrient media obtained at the early stages of the study [1].

The seeds were collected at the maturity phase in the second half of August in the territory of the Topkinsky municipal district of the Kemerovo region—Kuzbass (Russia) near the village of Topki 55°20' N and 85°46' E. *Symphytum officinale* seeds were defatted, dried, sterilized, thoroughly washed with sterile distilled water, placed on AS medium, germinated in the dark, and then transferred to separate containers with a medium of the same composition and placed in the light until young leaves (plant explants) were formed. Then, the aerial part of the seedlings was separated from the roots; the leaves, mesocotyl, and hypocotyl were cut into 1.0–1.5 cm segments; the wild (not modified) strain of *Agrobacterium rhizogenes* (A4) was impaled with an insulin syringe along the leaf vein, along the epicotyl and hypocotyl, to the vascular system of the plant. These were grown on YEY nutrient medium for 48 h in the dark at 26 °C, or at 32 °C for 24 h on a shaker with a circular motion; prepared explants of plants were transferred to YEY medium with grown *Agrobacterium*, kept in a magnetic bath for 10–100 s and incubated for 12–24 h. Then they were washed with sterile nutrient medium MS N, transferred to nutrient agar medium Murashige and Skoog MS N supplemented with cefotaxime (250 mg/0.5 L) for the elimination of *A. Rhizogenes* (A4), and placed in the lightroom until the formation of transformed roots. The roots were transplanted onto fresh Gamborg's B-5 nutrient medium without hormones for the complete elimination of *A. rhizogenes* (A4) bacteria and were grown in a dark room under rocking conditions.

The 3,4-dimethoxybenzoic acid (3,4-Dimethoxybenzoic acid, veratric acid, ≥99%, D131806); caffeic acid (3,4-Dihydroxybenzoic acid, 3,4-Dihydroxycinnamic acid, 3-(3,4-Dihydroxyphenyl)-2-propenoic acid, ≥98.0%, C0625), 1-caffeoylquinic acid (certified reference material, CAS: 928005-87-2), 5-caffeoylquinic acid (IUPAC, 1,4,5-Trihydroxycyclo-

hexanecarboxylic acid 3-(3,4-dihydroxycinnamate), 3-(3,4-Dihydroxycinnamoyl)quinic acid, 5-CGA, $\geq 95\%$, C3878), coumarin (1,2-Benzopyrone, 1-Benzopyran-2-one, 2H-Chromen-2-one, $\geq 99\%$, C4261), 3-caffeoylquinic acid (certified reference material, CAS: 327-97-9), lithospermic acid (certified reference material, CAS: 28831-65-4), m-hydroxybenzoic acid (certified reference material, CAS: 99-06-9), m-methoxybenzoic acid (certified reference material, CAS: 579-75-9, 586-38-9, 100-09-4), rosmarinic acid ((R)-O-(3,4-Dihydroxycinnamoyl)-3-(3,4-dihydroxyphenyl)lactic acid, 3,4-Dihydroxycinnamic acid (R)-1-carboxy-2-(3,4-dihydroxyphenyl)ethyl ester, $\geq 98\%$, R4033), and salicylic acid (certified reference material, CAS: 69-72-7) were purchased from Fluka/Sigma-Aldrich (Sigma-Aldrich Rus, Moscow, Russia). All the other chemicals (analytical grade and above) used in this study were obtained from the Research Institute of Biotechnology, Kemerovo State University (Kemerovo, Russia).

2.2. Extraction of Biologically Active Components

UV spectra were recorded on an SF-2000 instrument; both pure components and those with the addition of reagents were scanned photometrically, which made it possible to reveal the location of aromatic hydroxyl groups.

The extraction of alkaloids was carried out according to the method [14] with modifications. Dried, crushed calli (40.0 g) were extracted with hot EtOH acidified to pH 2.6 with 0.1 M HCl in an extractant:raw material ratio (50:1). The extraction was carried out for 8 h with repeated replacement of the solvent. Then the filtered extract samples were combined and evaporated under reduced pressure on an RV 8V rotary evaporator (IKA, Staufen, Germany). The distillation was analyzed using gas chromatography with mass spectrometry, and the residue was dissolved in a 0.05 M NaOH solution and extracted three times with CHCl_3 , followed by combining the extract and evaporating it in a vacuum. The alkaline residue was neutralized to pH 7.0 by acidification with HCl and subjected to subsequent chromatographic analysis.

2.3. Determination of the Contents of Individual Phenolic Compounds by HPLC

Analytical and preparative HPLC was performed on a Prominence LC-20 liquid chromatograph (Shimadzu, Kyoto, Japan) with diode array detection. The chromatographic column Kromasil C18 had a size of 250×4.6 mm (sorbed particle size 5 μm). A mixture of deionized water with o-phosphoric acid (pH = 4.6) (A) and acetonitrile (B) was used as a mobile phase. The elution gradient (% c) was 0–20 min with a gradient change of 10–20% and 20–60 min 20–50%. The flow rate of the eluent was 1.0 mL/min, the temperature of the column thermostat was 40 °C. During preparative accumulation, no acid was added to the mobile phase. The elution of some compounds was calculated based on pre-built calibration curves. The lower limit of quantitation was 1.50 $\mu\text{g/mL}$; the signal-to-noise ratio was ≥ 10 .

2.4. Determination of the Contents of Individual Phenolic Compounds by Layer Chromatography

Thin layer chromatography was performed on Sorbfil PTS-AF-A plates. The extract sample was applied to the start line, dried, and placed in a chromatographic chamber filled with a mixture: n-butanol–acetic acid–water (60–15–25). Development with a solution of phosphoric-tungstic acid 25% was followed by heating at 95 °C for 10 min. Densitometric analysis of the plate was performed using a densitometer with a Sony Handycam HDR-CX405 photofixation system (Sony Corporation, Tokyo, Japan). Chromatographic zones were excised and further analyzed. GC-MS was performed on a GsBP-1MS column with an inner diameter of 0.25 mm and a length of 30 m. The carrier gas was helium and the flow rate was 1.4 mL/min. The injector temperature was 240 °C, the interface temperature was 280 °C, and the column temperature was programmed from 100 to 270 °C at a rate of 20 deg/min. Sample volume was 3 μL . Method of administration was without dividing the carrier gas flow. The electron impact mode at 70 eV was used, followed by scanning in the range from 50 to 550 m/z. After the study, the mass spectra taken from the tops of the chromatographic peaks were compared according to the standard method with the mass

spectra of the libraries. A substance was considered identified when its mass spectrum coincided with the library one by more than 95%.

2.5. Determination of Anti-Inflammatory Activity

Comfrey extract samples were tested for their potential anti-inflammatory activity using IL-1-induced expression of E-selectin and other pro-inflammatory genes in primary HUVECs. IL-1 is a well-described pro-inflammatory mediator that plays a central role in regulating immune and inflammatory responses, not only in response to infections but also to sterile injuries such as trauma and blunt injuries.

2.5.1. Cellular Enzyme-Linked Immunoassay

Post-confluent human umbilical vein endothelial cells (HUVEC) in 96-well plates were treated for 30 min with comfrey extract samples and then stimulated with IL-1 β (5 ng/mL) in the same medium. After 2 h, cells were fixed with 4% paraformaldehyde for 15 min after blocking with 2.5% bovine serum albumin (BSA) in Tris-buffered saline-1% tween (TBS-T) for 1 h. The cells were then incubated overnight at 4 °C with mouse anti-E-selectin antibody (R&D Systems) diluted 1:500 in 1% BSA/TBS-T. After washing with TBS-T for 1 h, goat anti-horseradish peroxidase (HRP) antibody (Sigma) diluted 1:10,000 in 1% BSA/TBS-T was added. HRP activity was assessed using tetramethylbenzidine (Sigma) as substrate. The addition of 2M H₂SO₄ stopped the reaction, and the optical density OD₄₅₀ was measured. E-selectin levels were normalized by crystal violet staining.

2.5.2. Cyclooxygenase Assays

To determine the total COX activity in HUVEC, a COX activity assay kit (Cayman Chemical, No. 760151) was used according to the manufacturer's recommendations. HUVEC was cultured in 10 cm dishes and incubated with comfrey for 30 min, followed by stimulation with IL-1 β for 1.5 h. After that, cells were scraped off in cold PBS and centrifuged at 2000 $\times$ g for 10 min. Cell precipitate was resuspended in 200 μ L cold PBS, sonicated, and centrifuged at 10,000 $\times$ g for 15 min. The supernatant was collected and used for analysis. Recombinant COX-1 from the same assay was used to analyze the effect of comfrey extract samples on the enzymatic activity of COX-1.

2.5.3. Immunofluorescence Microscopy

Post-confluent HUVECs cultured on fibronectin-coated glass coverslips were treated with comfrey or TAK inhibitor for 30 min before IL-1 β stimulation. At the indicated time points, cells were fixed for 15 min with 4% paraformaldehyde (Sigma), permeable for 30 min with 0.1% Triton X-100 (Sigma), washed with PBS, and finally blocked for 1 h with 3% BSA-TBS-T. For immunostaining, rabbit polyclonal antibodies against p65 (Santa Cruz) (1:500) were used with a secondary antibody conjugated to an Alexa-Fluor 488 goat antibody against rabbit IgG antibody (Invitrogen) in a ratio of 1:1000. Cells were contrasted for 15 min with Alexa Fluor 568 phalloidin (1:1000) and 5 min with 4',6-diamidino-2-phenylindole (DAPI, Life Technologies; 1:10,000). The samples were examined using an Olympus IX71 microscope with a UPlanSApo 20 $\times$ /0.75 objective. The images were processed using the ImageJ program.

2.6. Determination of Antioxidant Activity by Amperometric Method

The antioxidant activity of comfrey extract samples was determined using a Tsvet-Yauza-01-AA device (Khimavtomatika, Moscow, Russia) according to the measurement procedure (MP) for the content of antioxidants in drinks and food products and biologically active additives extract samples of medicinal plants by the amperometric method developed by Khimavtomatika (2007). The amperometric method for measuring the mass concentration of antioxidants is based on measuring the electric current arising from the oxidation of antioxidant molecules on the surface of the working electrode at a certain potential, which, after amplification, is converted into a digital signal.

2.7. Determination of Antioxidant Activity by Spectrophotometric Method (DPPH-Method)

The method is based on the ability of the reactive radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) molecules to bind with antioxidants in the samples under study. Each extract sample was mixed with 2.85 mL of a freshly prepared 0.1 mM solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in ethanol. The sample was incubated for 30 min at room temperature in the dark. The decrease in optical density at 515 nm (UV-3600, Shimadzu, Japan) was measured spectrophotometrically [16].

Solutions of precisely known concentrations of ascorbic acid, trolox (a water-soluble analog of vitamin E), and quercetin were used as standards for determining antioxidant activity by the methods described above.

Sample preparation included preparation of a saturated solution, filtration, and mass measurement. The study of antioxidant activity (AA) was carried out by spectrophotometric method based on inhibition of the stable chromogen radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). In the visible region of the spectrum, DPPH in organic solvents has a wide absorption maximum at wavelengths of $515 \div 520$ nm, which disappears when the radical interacts with substances—donors of hydrogen atoms or free radicals of a different structure.

The reaction of DPPH with antiradical antioxidants occurs in a serial-parallel mechanism. At the first stage (the limiting step of the reaction), the antioxidant molecule gives the radical the most mobile hydrogen atom:

At the second stage, the antioxidant radical formed in reaction (1) attacks a new DPPH molecule in the para-position of the phenyl substituent:

This also results in an uncolored reaction product. Reaction (1) can proceed by two independent mechanisms. The first one, based on the direct abstraction of a hydrogen atom from an AA molecule, proceeds at the highest rate in non-polar solvents. The second, based on the transfer of an electron by an ionized phenolic AA molecule to a DPPH molecule, prevails in solvents with a high affinity for the proton.

2.8. Statistical Analysis

All experiments were carried out in triplicate and results are given as a mean. The correspondence of the samples used to the normal distribution was assessed via t-test (mathematical expectations) for independent samples, and by Fisher's test (variance). Post hoc analysis (Duncan's test) was undertaken to identify samples that were significantly different from each other. The equality of the variances of the extracted samples was checked using the Levene test. These tests were performed in Statistica 10.0 (StatSoft Inc., 2007, Tulsa, OK, USA).

3. Results

It was found that the studied extract samples contained a unique polymer—a derivative of caffeic acid, which is a new class of natural polyether with 3-(3,4-dihydroxyphenyl) glyceric acid as a repeating unit. Figure 1 shows the UV spectrum (max absorption at 206; 285 nm with a 236 nm wing) of this polymer.

Table 1 shows the results of spectrophotometric studies in the UV range of the key fractions of biologically active substances of extract samples of *Symphytum officinale* root cultures obtained under various technological modes (4 h extraction).

Figure 1. UV spectrum of polymer ($\lambda_{\max} = 206; 285 \text{ nm}$).

Table 1. Conditions for the biotechnological process of extracting biologically active substances and the amount of the isolated polymer.

Sample No.	Temperature, °C	Volume Fraction of Ethanol in the Extractant, %	OD	m, mg/100 mL
1	30	30	0.0160 ± 0.0001^a	58 ± 0.1^a
2	30	40	0.0230 ± 0.0001^a	60 ± 0.2^a
3	30	50	0.0260 ± 0.0001^a	75 ± 0.1^a
4	30	60	0.3562 ± 0.0050^b	78 ± 0.2^a
5	30	70	0.2845 ± 0.0030^c	73 ± 0.1^a
6	40	30	0.2118 ± 0.0030^c	72 ± 0.2^a
7	40	40	0.2138 ± 0.0030^c	160 ± 0.1^a
8	40	50	0.2652 ± 0.0030^c	180 ± 0.1^a
9	40	60	0.1942 ± 0.0030^c	240 ± 0.1^b
10	40	70	0.3827 ± 0.0056^b	235 ± 0.2^b
11	50	30	0.2918 ± 0.0050^c	230 ± 0.1^b
12	50	40	0.3315 ± 0.0056^b	390 ± 0.2^b
13	50	50	0.2180 ± 0.0050^c	370 ± 0.1^b
14	50	60	0.4490 ± 0.0059^d	330 ± 0.1^b
15	50	70	0.3680 ± 0.0056^b	332 ± 0.1^b
16	60	30	0.1290 ± 0.0010^e	250 ± 0.2^b
17	60	40	0.0400 ± 0.0001^a	330 ± 0.1^b
18	60	50	0.1260 ± 0.0010^e	620 ± 0.1^c
19	60	60	0.2070 ± 0.0030^c	210 ± 0.2^b
20	60	70	0.2410 ± 0.0030^c	490 ± 0.3^c
21	70	30	0.2210 ± 0.0030^c	370 ± 0.1^b
22	70	40	0.3350 ± 0.0050^b	260 ± 0.1^b
23	70	50	0.3490 ± 0.0050^b	510 ± 0.3^c
24	70	60	0.2000 ± 0.0010^c	320 ± 0.1^b
25	70	70	0.0600 ± 0.0001^a	509 ± 0.1^c
26	30	30	0.0800 ± 0.0001^a	454 ± 0.3^c
27	30	40	0.0600 ± 0.0001^a	316 ± 0.1^b

Data presented as a mean $\pm$ SD ($n = 3$). Values in columns followed by the same letter do not differ significantly ($p > 0.05$).

Figure 2 presents an HPLC chromatogram of the *Symphytum officinale* extract samples. The components and their retention times, found under HPLC conditions, are presented in Tables 2 and 3.

Figure 2. HPLC chromatogram of the *Symphytum officinale* extract samples containing (a) type I and (b) type II of the recovered polymer: peak 1—m-methoxybenzoic acid; 2—5-caffeoylquinic acid; 3—caffeic acid; 4—3-caffeoylquinic acid; 5—rosmarinic acid; 6—1-caffeoylquinic acid; 7—coumarin; 8—lithospermic acid; 9—salicylic acid; 10—m-hydroxybenzoic acid; and 11—3,4-dimethoxybenzoic acid (unnumbered peaks of the chromatogram belong to unidentified substances).

Table 2. Component composition of the aqueous-alcoholic extract of *Symphytum officinale*, containing type I polymer, according to HPLC data.

Peak No.	Retention Time, min	Component	Quantitative Content *, µg/mL
1	6.164	m-methoxybenzoic acid	30.05 ± 0.49
2	6.502	5-caffeoylquinic acid	14.73 ± 0.72
3	7.300	caffeic acid	25.40 ± 0.60
4	7.811	3-caffeoylquinic acid	12.57 ± 0.62
5	8.528	rosmarinic acid	2.03 ± 0.05
6	9.620	1-caffeoylquinic acid	3.12 ± 0.20
7	11.350	coumarin	7.32 ± 0.41
8	13.426	lithospermic acid	11.27 ± 0.52
9	14.270	salicylic acid	9.25 ± 0.50
10	15.030	m-hydroxybenzoic acid	1.97 ± 0.20

* average of three measurements.

Table 3. Component composition of the aqueous-alcoholic extract of *Symphytum officinale*, containing type II polymer, according to HPLC data.

Peak No.	Retention Time, min	Component	Quantitative Content *, $\mu\text{g/mL}$
2	6.500	5-caffeoylquinic acid	6.50 ± 0.29
3	7.305	caffeic acid	35.70 ± 0.49
4	7.811	3-caffeoylquinic acid	25.10 ± 0.27
5	8.528	rosmarinic acid	45.70 ± 0.36
6	9.620	1-caffeoylquinic acid	17.30 ± 0.78
7	11.350	coumarin	2.45 ± 0.20
8	13.426	lithospermic acid	9.32 ± 0.32
9	14.270	salicylic acid	1.54 ± 0.20
10	15.030	m-hydroxybenzoic acid	8.92 ± 0.29
11	5.700	3,4-dimethoxybenzoic acid	7.35 ± 0.36

* average of three measurements.

The results of the chromatographic analysis are presented in Figure 3.

Figure 3. Densitogram of *Symphytum officinale* root extract.

9-Angeloylretronecin (IUPAC ((7R,8R)-7-hydroxy-5,6,7,8-tetrahydro-3H-pyrrolizin-1-yl)methyl (Z)-2-methylbut-2-enoate) and 7-Angeloylheliotridine (IUPAC ((1S,8R)-7-(hydroxymethyl)-2,3,5,8-tetrahydro-1H-pyrrolizin-1-yl) (Z)-2-methylbut-2-enoate) were found in comfrey extract samples. Their structural formulae as well as mass spectra are shown in Figures 4 and 5. The pyrrolizidine alkaloids (PA) found in the comfrey extract samples contained fragmentary ions m/z 125 ± 1 , and m/z 137 ± 1 , which are characteristic of the retronecin fragment [17].

When determining anti-inflammatory activity using enzyme immunoassay, cyclooxygenase, and immunofluorescence microscopy, IL-1-induced E-selectin mRNA levels were inhibited by comfrey extract samples containing type I polymer by about 60% at an initial concentration of $10 \mu\text{g/mL}$ and by 75% at $20 \mu\text{g/mL}$, which indicates a more than 10-fold increase in the ethyl acetate fraction activity. Comfrey extract samples containing type II polymer showed a similar result.

Figure 4. Mass spectrum at the top of the chromatographic peak at 5.547 min, corresponding to 7-Angeloylheliotridine extracted from *Symphytum officinale* (reference mass ions $[M + H]^+$ m/z 136 and m/z 124.03).

Figure 5. Mass spectrum at the top of the chromatographic peak 5.58 min, corresponding to 9-angeloylretronecin extracted from *Symphytum officinale* (reference mass ions $[M + H]^+$ m/z 138 and m/z 126).

Table 4 presents the results of determining anti-inflammatory activity of *Symphytum officinale* extract samples.

Table 4. The results of determining anti-inflammatory activity of *Symphytum officinale* extract samples.

Samples	Concentration, $\mu\text{g/mL}$	Anti-Inflammatory Activity, %
I	10.0 ± 0.5^a	60 ± 2^a
	20.0 ± 0.7^b	75 ± 3^b
	30.0 ± 0.8^c	75 ± 3^b
II	10.0 ± 0.6^a	63 ± 2^a
	20.0 ± 0.8^b	75 ± 3^b
	30.0 ± 0.8^c	75 ± 2^b

I-extract samples of *Symphytum officinale*, containing type I polymer; II-extract samples of *Symphytum officinale*, containing type II polymer. Data presented as a mean $\pm$ SD ($n = 3$). Values in columns followed by the same letter do not differ significantly ($p > 0.05$).

These results were also reflected at the protein level, where comfrey inhibited the expression of the E-selectin protein at the same concentrations as determined by a cellular ELISA. It is noteworthy that a pre-incubation with the extract for at least 30 min was necessary because no inhibition was observed when applied together with the stimulus.

To further determine the possible effects of comfrey on the kinetics of E-selectin expression, HUVECs were stimulated with IL-1 for 6 h in the presence of comfrey. E-selectin mRNA levels increased in response to IL-1 within 30 min, peaked after 3 h, and then decreased. Comfrey extract samples reduced the expression of E-selectin at all time points and confirmed the high effectiveness of comfrey. Since IL-1 induces many genes in HUVEC with very different kinetics ranging from early to late phase, other expressions related to vascular inflammation were tested using comfrey. In addition to E-selectin, cell adhesion molecules VCAM-1 and ICAM-1 and matrix metalloproteinase MMP10 were affected. In addition, the expression of $\text{I}\kappa\text{B}\alpha$ (NFKBIA), an inhibitor of the transcription factor NF- κB , and A20 (TNFAIP3), an anti-apoptotic gene, were also decreased. This indicated that comfrey extract samples had broader anti-inflammatory properties against IL-1-stimulated HUVEC.

Considering the importance of cyclooxygenases in the inflammatory process, the possibility of inhibiting COX-1 and/or COX-2 with comfrey extract samples was studied. First, HUVEC was stimulated with combinations of IL-1 and comfrey, and COX enzymatic activity was determined using a fluorescence-based assay that measures COX-1 and COX-2. Comfrey extract samples significantly reduced IL-1-induced but not constitutive COX activity; since it is COX-2 that is induced by IL-1, while COX-1 is expressed constitutively, it can be concluded that comfrey selectively affects the activity of COX-2. Western blot of the same samples using an antibody specific for COX-2 confirmed that COX-2 protein levels were induced by IL-1 and decreased by comfrey extract samples. In addition, when reanalyzing samples, COX-2 mRNA was lower in cells treated with comfrey extract samples compared to control. The potential COX-2 selectivity of comfrey was further tested using recombinant COX-1. While the nonspecific inhibitor diclofenac readily suppressed the enzymatic activity of COX-1, comfrey extract samples did not. Finally, unstimulated HUVECs (which express only constitutively present COX-1) were treated with either the nonspecific COX inhibitor diclofenac, the specific COX-1 inhibitor SC-560, or comfrey extract samples. This COX activity, which is attributed exclusively to COX-1, was inhibited by diclofenac and SC-560, but not by comfrey. Thus, we concluded that comfrey extract samples do not suppress the enzymatic activity of both COX isoforms but act on COX-2, preventing its mRNA and protein synthesis, and therefore, unlike diclofenac, act indirectly through inhibition of NF- κ B but specifically inhibit COX-2. It was found that comfrey extract samples were characterized by a high antioxidant status; the antioxidant activity of the samples was 235.00 mg AA/g. Both methods (amperometric and DPPH) showed similar results.

The results of determining the antioxidant activity of the *Symphytum officinale* extract samples are presented in Table 5.

Table 5. The results of determining the antioxidant activity of the *Symphytum officinale* extract samples.

Sample	Antioxidant Activity, mg AA/g
I	113.6 $\pm$ 6.56
II	112.8 $\pm$ 6.54

I-extract samples of *Symphytum officinale*, containing type I polymer; II-extract samples of *Symphytum officinale*, containing type II polymer. Data presented as a mean $\pm$ SD ($n = 3$). Values in columns followed by the same letter do not differ significantly ($p > 0.05$).

4. Discussion

This work established the presence of (5Z,9Z,12Z)-octadecatrienoic acid in the studied ethanol extract samples of *Symphytum officinale* callus cultures. Pinolenic acid was first detected in conifers of the pine family (*Pináceae*), its greater amount in seed lipids. In recent studies carried out in vitro, in animal models, and in humans, pinolenic acid exhibits anti-inflammatory and immunomodulatory activity, and is a potent dual agonist of free fatty acid receptors in vitro [18]. In addition, a significant amount of squalene was found in the comfrey sample. The detection of this biologically active component with pronounced antioxidant properties, in our opinion, is quite natural, considering the fact that comfrey produces significant amounts of triterpene saponins and other plant-steroid-like substances [12,14], which are synthesized from squalene [19]. A non-reducing sugar, xylitol, was found in the distillate. This carbohydrate is quite often a structural component of the glycosyl fragment of cardiotonic glycosides—cardenolides [20]. The presence of deoxysugar was confirmed by a positive Keller-Kiliani reaction with the formation of a cornflower blue ring.

Moreover, a unique polymer has been identified—a derivative of caffeic acid, which is a new class of natural polyether with 3-(3,4-dihydroxyphenyl) glyceric acid as a repeating unit. Two types of this polymer were identified depending on the samples. The extracted polymer samples were characterized by individual structural features. According to Fourier-transform infrared spectroscopy (FTIR) data, two types of polymer were isolated from the studied samples, differing according to the FTIR data (Appendix A). Type I

polymers are characterized by the presence of absorption bands: (KBr, ν , cm^{-1}): 3390–3410 (OH); 2928 (CH); 1740 C=O; 1617 (COO⁻); 1509, 1443 (C=C_B Ar); 1377, 1215 (ArOH); 1269, 1122, 1074, 1023 (R-O-R'); 867 (C-H in an aromatic ring with one isolated H atom); and 816 (C-H in an aromatic ring with neighboring H atoms). Type II polymers are characterized by the presence of absorption bands: (KBr, ν , cm^{-1}): 3417 (OH); 2922 (CH); 1707; 1632; 1509, 1455; 1404, 1263 (ArOH); 1263, 1221, 1095, 1017 (R-O-R'); 864 (C-H in an aromatic ring with one isolated H atom); and weak 816 (C-H in an aromatic ring with neighboring H atoms). According to preliminary data, the molecular weight of the isolated polymer was >1000 Da. The quantitative yield of the polymer depended on the characteristics of the extraction process (Table 1). Some extract samples contained a minimum amount of polymer (no more than 0.01 mg/mL). Phenolcarboxylic acids were found in all studied samples. In samples containing type I polymer, m-methoxybenzoic acid and m-hydroxybenzoic acid were found in amounts of 14.07 and 16.15 mg, which is 1.3–1.6 times higher than for extract samples obtained in other technological modes. Samples of extract containing type II polymer showed 3,4-dimethoxybenzoic acid (Figure 2) and a quantitatively higher content of rosmarinic acid by 1.6 times. Samples with a high polymer content showed a relatively lower content of low molecular weight phenol carboxylic acids. It was found that the quantitative (qualitative) content of phenylpropanoic acids was inversely proportional to the content of the polymer.

There is data on the identification of such polymers from water–ethanol extract samples of the roots of native comfrey plants. Trifan et al. [1] give a description of four oligomers of caffeic acid (globoidnan, rhabdosin, rosmarinic acid, and globoidnan) and isomers of salvianolic acids (polyphenolic acids, conjugates of 3,4-dihydroxyphenyl lactic acid and caffeic acid), indicating their total anti-inflammatory activity in extract samples. It was noted that rosmarinic acid was the main component of various alcoholic extract samples of comfrey [5,21], in our studies this is confirmed in samples of extract containing a polymer of the second type, while in samples with a polymer of type 1, m-methoxybenzoic acid was the main component.

Barbakadze et al. [22] demonstrate the isolation of a new biopolymer polyoxy-1-carboxy-2-(3,4-dihydroxyphenyl) ethylene from a water-soluble high molecular weight preparation of *Symphytum asperum* roots with strong anti-complementary and antioxidant activity. Later [23], using IR and NMR spectroscopy, two high molecular weight (>1000 kDa) water-soluble biopolymers with a similar main component (poly [3-(3,4-dihydroxyphenyl)glyceric acid] or poly [hydroxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene]. These polymers exhibited antioxidant activity, expressed in a decrease in reactive oxygen species (ROS), directly interfering with the process of their formation by polymorphonuclear neutrophils and directly binding ROS.

Comfrey contains polysaccharides, allantoin, phenolic acids, phytosterols, saponins, tannins, pyrrolizidine alkaloids, and other components. The pharmacological components include phenolic acids, which determine the therapeutic use of comfrey preparations [24,25]. Polyphenols demonstrate a neuroprotective effect when consuming foods rich in phenolic compounds [26,27]. Rosmarinic, caffeic, and caffeoylquinic acids were the leading components in our samples.

Rosmarinic acid is a water-soluble polyphenolic phytonutrient found in various plant families, e.g., in the Boraginaceae. It has antioxidant, anti-inflammatory, anti-apoptotic, antitumor, neuroprotective properties, and a wide range of therapeutic applications [28,29]. Moreover, rosmarinic acid has a potent neuroprotective effect against Parkinson's disease, Alzheimer's disease, epilepsy, and ischemic brain diseases [30–34]. Graffari et al. [35] showed a neuroprotective effect of rosmarinic acid against H₂O₂-induced neuronal cell damage. Cui et al. [36] reported that rosmarinic acid has antioxidant and anti-apoptotic properties that protect against ischemic stroke. In [29], it was noted that RA exhibited a neuroprotective effect in vivo and in vitro by inhibiting oxidative stress, inflammation, and apoptosis through the Nrf2, NF- κ B, and caspase-3 pathways, respectively. Rosemarinic acid has improved the locomotor function of rats with spinal cord injury, which unlocks

its potential as a target for developing therapeutic agents for this disease. Wang et al. [37] confirmed the neuroprotective effect of rosmarinic acid against nigrostriatal neurodegeneration (a model of Parkinson's disease induced by hydroxidopamine) after its intragastric administration for 21 days with a decrease in iron levels and regulation of Bcl-2/Bax gene expression (a regulator of apoptosis) as a mechanism of action. Gok et al. [38] demonstrated the neuroprotective effect of rosmarinic acid (intracerebral injection, for 14 days) by affecting antioxidant–oxidative imbalance and cholinergic disorders, increasing cholinergic tone, weakening peroxidation lipids, and potentiating antioxidant protection in an in vivo model of Alzheimer's disease induced by bilateral injection of A β 42 peptide. A study by Wang et al. [39] showed that rosmarinic acid has a potent protective effect against ischemia-reperfusion brain injury and ischemic depression.

Caffeic acid is widely available in vegetables, fruits, plants, and chemical synthesis [40]. It has antitumor and antioxidant properties. Caffeic acid improves cognitive function in animals with Alzheimer's disease and reduces damage caused by cerebral ischemia. Its potential in the treatment of complex neurological diseases has been noted [41–43].

Chlorogenic acid is the main polyphenolic compound in many plants and their fruits. It has antioxidant, anti-inflammatory, and other properties [44,45]. The study [46] provides evidence of the neuroprotective effect of chlorogenic acid in neurodegenerative diseases. Nabavi et al. [47] reported that chlorogenic acid could cross the blood–brain barrier and exert neuroprotective effects in brain tissue. Wang et al. [48] found that chlorogenic acid has a neuroprotective impact on cognitive impairment caused by AlCl₃ in mice.

Caffeoylquinic acid derivatives are natural functional compounds found in various plants, with a wide range of pharmacological properties (antioxidant, hepatoprotective, antibacterial, antihistamines, antitumor, and other biologically active effects). It was found that caffeoylquinic acid derivatives also have neuroprotective effects, including against cell death and neuronal damage caused by ischemia in vivo [49]. Cho et al. [50] concluded that regular consumption of Bidan kiwifruit, which contains antioxidant phenols such as caffeoylquinic acid, might be one way to prevent neurodegeneration, including Alzheimer's disease. Authors of [51] studied the characteristics of the ethanol extract of *A. glehni* leaves and stems, the main element of which was caffeoylquinic acid. They found that the extract, along with its caffeoylquinic acid, has antineurodegenerative properties due to the ability to induce neuroprotection and antineuroinflammation.

Seigner et al. [5] showed that the topical administration of comfrey extract samples is based on its analgesic and anti-inflammatory effects, confirmed by modern clinical trials. However, the molecular basis of its action remained unexplored. In studies [52,53] it is shown that the hydroalcoholic extract of comfrey slows down the development of the pro-inflammatory scenario in primary human endothelial cells, depending on the dose. The extract, and especially its mucus-depleted fraction, disrupted interleukin-1-induced expression of pro-inflammatory markers, including E-selectin, VCAM1, ICAM1, and COX-2. Both preparations inhibited the activation of NF- κ B, a transcription factor central to the expression of these and other pro-inflammatory genes. In addition, our biochemical studies provide evidence that comfrey inhibits NF- κ B signaling at two stages: it not only suppressed IKK1/2 activation and subsequent I κ B α degradation, but also interfered with NF- κ B p65 nucleocytoplasm transfer and transactivation. These results provide the first insight into the mechanism of action of popular herbal medicine. Research data [5,52] are in good agreement with our results on the study of anti-inflammatory activity of comfrey extract samples.

When studying the antioxidant activity of comfrey extract samples, it was found that our data are consistent with studies [53], in which polar extract samples of comfrey were studied. The highest antioxidant potential was shown by ethanol extract samples at a high concentration of polyphenols (6.93 mg of gallic acid/g of dry extract). The same extract had the highest antioxidant potential determined by amperometric and DPPH methods, as the extract showed a high degree of radical scavenging. Studies [53] have shown that the

antioxidant potential of polar comfrey extract samples with increased total polyphenols was evident.

All these results open up prospects for the use of extracts of our comfrey callus root cultures, the qualitative and quantitative composition of which requires further study.

5. Conclusions

The main types of secondary metabolites produced by *Symphytum officinale* L. calli were determined. The combination of various options for liquid and gas chromatography with mass spectrometric detection allowed us to determine the key components produced by the calli. To determine the properties and characteristics of the polymer isolated from our extract samples of callus cultures of the *Symphytum officinale* root, additional studies are required, especially for samples with type 1 polymer, but based on the results previously presented [5,21–23], it can be assumed that they have a pronounced antioxidant, anti-inflammatory, and neuroprotective properties. For this reason, extract samples of comfrey root callus cultures [54] can become a source of phenolic compounds or a pharmaceutical basis for drugs with general biological activity, including in products with dietary, therapeutic, and prophylactic, as well as geroprotective effects.

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Appendix A

Figure A1. FTIR spectra of type I (red line) and II (green line) polymers based on 3-(3,4-dihydroxyphenyl) glyceric acid.

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