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Research article

# *Cichorium intybus* L. "hairy" roots as a rich source of antioxidants and anti-inflammatory compounds

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ABSTRACT

The present study aimed to determine the bioactive profile of various extracts of Cichorium intybus L. "hairy" roots. In particular, the total content of flavonoids as well as the reducing power, antioxidant and anti-inflammatory activity of the aqueous and ethanolic (70%) extracts were evaluated. The total content of flavonoids the ethanolic extract of the dry "hairy" root reached up to 121.3 mg (RE)/g, which was twofold greater than in the aqueous one. A total of 33 diverse polyphenols were identified by the LC-HRMS method. The experimental results showed a high amount of gallic (6.103 ± 0.008 mg/g) and caffeic (7.001 ± 0.068 mg/g) acids. In the "hairy" roots, the presence of rutin, apigenin, kaempferol, quercetin, and its derivatives was found in concentrations of 0.201±0.003 – 6.710±0.052 mg/g. The broad spectrum of pharmacological activities (antioxidant, anti-inflammatory, antimutagenic, anticarcinogenic, etc.) of the key flavonoids identified in the chicory "hairy" root extract was predicted by the General Unrestricted Structure-Activity Relationships algorithm based on in the substances detected in the extract. The evaluation of the antioxidant activity showed that the $EC_{50}$ values of the ethanol and the aqueous extracts were 0.174 and 0.346 mg, respectively. Thus, the higher ability of the ethanol extract to scavenge the DPPH radical was observed. The calculated Michaelis and inhibition constants indicated that the ethanolic extract of C. intybus "hairy" roots is an efficient inhibitor of soybean 15-Lipoxygenase activity ($IC_{50}$ = 84.13 ± 7.22 μM) in a mixed mechanism. Therefore, the obtained extracts could be the basis of herbal pharmaceuticals for the therapy of human diseases accompanied by oxidative stress and inflammation, including the pandemic coronavirus disease COVID-19.

## 1. Introduction

*Cichorium intybus* L. plants belong to the Asteraceae family [1]. They are cultivated in European countries, the USA, South America, India, and Turkey. In general, chicory is known as a popular coffee substitute. It is also used in medicine to treat various diseases, such

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as diabetes, tumors, tachycardia, hepatitis, atherosclerosis and others (Zhang et al., 2014), [2, 3]. To date, inulin, coumarins, flavonoids, isoflavones, flavones, anthocyanins, catechins, sesquiterpenoids, triterpenoids, steroids, organic acids, and other compounds have been found in *Cichorium intybus* L. plants [4]. Chicory leaves usually contain polyphenols of different chemical structures and activities [5,6]. The presence of these compounds attracted great interest due to their wide range of bioactivities. For example, C. intybus was demonstrated to possess hepatoprotective, antibacterial, and anti-inflammatory activities [6–9].

The increasing application of naturally occurring bioactive compounds in industry requires reliable methods for their production. Improvement of chicory cultivars as producers of bioactive compounds can be achieved by genetic transformation using *Agrobacterium rhizogenes* bacteria. The biofortification of chicory plants and the production of "hairy" root culture allow to select the samples – superproducers of different chemicals for medical application. This biotechnological approach allows to increase the content of valuable compounds in the root crop due to the transfer of bacterial *rol* genes to the plant genome [10]. Furthermore, *via* this method, it is possible to obtain the "hairy" root clones synthesizing compounds extrinsic to the mother plants, for example, in our previous study, a significant increase in flavonoid content was observed in *Artemisia tilesii* and *A. annua* "hairy" roots [11]. In addition, a strong positive correlation was observed between the flavonoid content in the root extracts, the antioxidant activity and the reducing power of these extracts [12].

The stimulation of the synthesis of flavonoids in plant tissues due to the peculiarities of the effect of bacterial *rol* genes on the secondary metabolism of the plant draws attention because the wide spectrum of activities of flavonoids and their usage for the treatment of human diseases. For instance, epicatechin is a powerful antioxidant that can neutralize reactive oxygen species [13,14].

Development of a functional and economically suitable extraction procedure is necessary to efficiently obtain plant-derived polyphenols. Firstly, the extraction of solutes depends on the solubility of the compounds synthesized in plants. Ethanol and methanol are commonly used for the extraction of most flavonoids from plant material [15], while water is often used to extract sugars and highly polar compounds [16]. Ethanol is often preferred over methanol because of its availability, efficacy, and safety. Secondly, numerical articles have been published about separation, purification, and identification of sugars and other bioactive compounds for different plants but only a few reports have focused on the chemical investigation of the *Cichorium* genus plants [1]. At the same time, understanding the polyphenolic and bioactive profiles of chicory is a prerequisite for its practical applications as a source of valuable chemicals.

Various liquid chromatography techniques carried out on C8- or C18-bonded silica columns [17] coupled with UV–visible, diode array (DAD) or electrochemical detection remain the most widely used methods for the routine determination of polyphenols contained in plants and biological samples [18,19]. Furthermore, detailed structural information is required to detect and identify unknown compounds in a multicomponent solution, multiple liquid chromatography techniques can be coupled to mass spectrometry (MS) with electrospray ionization to take advantage of their high sensitivity and selectivity [20]. Low-resolution MS instruments, usually single quadrupole or tandem mass spectrometers (MS/MS) setups are excellent in terms of adjustable $m/z$ range and reproducibility of analysis. This makes them particularly suitable for quantitative analysis and other similar applications, e.g., the evaluation of precise ratios of molecular species of analyzed various polyphenolic compounds [21,22]. The development of one of the latest techniques, high-resolution mass spectrometry (HRMS) has enabled researchers to elucidate complex polyphenolic structures and secondary metabolites by easy determination of precise molecular weights and limited fragmentation patterns [23]. Examples of HRMS instruments are time-of-flight, Orbitrap and Fourier transform ion cyclotron resonance. HRMS instruments are leaders in resolution and accuracy of $m/z$ determination of analytes. This allows them to separate peaks of compounds with close $m/z$ value and measure their $m/z$ with a standard accuracy within the fourth decimal place These instruments generally measure the exact mass of analytes without fragmentation. However, they can be combined with a quadrupole in which case fragmentation is also possible and can add more selectivity to the method. Recently, liquid chromatography with high resolution mass spectrometry (LC-HRMS) has emerged as a leading tool for detection and identification of complex polyphenolic compounds and their secondary metabolites with pharmacologically active properties [23,24]. The LC-HRMS method provides several advantages when compared to current methods because this technique allows direct identification target compounds and metabolites without the need for separation and purification, thus reducing the time of analysis, eliminating predictable sources of losses. At the same time, up to our best knowledge, there are no techniques applicable for the separation and determination of main target analytes at different levels of concentrations in one sample, e.g., extract of plants. Also, a complete characterization of the polyphenols found in *C. intybus* "hairy" roots extracts is not yet available.

Therefore, the aim of this work was to study the polyphenolic profile of the extracts of *C. intybus* "hairy" root cultures. The efficiency of the solvents (water and ethanol) was estimated in terms of concentrations of extracted bioactive compounds. Efficient approaches for the isolation, identification, and quantification of bioactive compounds in aqueous and ethanolic extracts of chicory "hairy" roots were developed. The ultra-high-performance liquid chromatography coupled with high-resolution quadrupole time-of-flight mass spectrometry were used for simultaneous analysis of polyphenols in the extracts. The screening step was achieved by identification with the retention time, accurate mass, and the candidate compounds were further analyzed in a mixed modes to compare their fragment mass spectra with the tunning and library data to confirm the candidates' results using the same analytical platform. The validated high-performance liquid chromatography with diode array method was applied to quantified extracts. In addition, the total flavonoid content, DPPH and reducing power analyses were determined by spectrophotometry. Finally, bioactive compounds from the *C. intybus* "hairy" roots were evaluated as potential useful active pharmaceutical ingredients for the development of new drugs based on their antioxidant and anti-inflammatory properties.

## 2. Materials and methods

### 2.1. Reagents

The initial standards of phenolic acids (gallic, caffeic, and hydroxybenzoic), flavonoids (epicatechin, apigenin, luteolin, rutin, quercetin, and kaempferol), and HPLC-grade solvents, such as methanol and acetonitrile, were purchased from Sigma-Aldrich (USA). Soybean 15-lipoxygenase (15-sLOX) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) from Sigma-Aldrich (Germany), aluminium chloride ($AlCl_3$ reagent grade 98%, Sigma-Aldrich), potassium ferricyanide, formic acid, and trichloroacetic acid (99%, Sigma-Aldrich) were also used in the experiments.

### 2.2. Plant materials

"Hairy" root clones of Chicory Palla Rossa from the collection of the Laboratory of Adaptational Biotechnology of the Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine were used in the study. "Hairy" roots were obtained by transformation of chicory cotyledons with *Agrobacterium rhizogenes* wild-type A4 strain. The transgenic nature of the roots was confirmed by PCR analysis [12]. Root clones were subcultured every four weeks for 12 years at 24 °C in twice reduced and solidified Murashige and Skoog (1/2MS) nutrient medium [25] (Duchefa Biochemie, Netherlands) supplemented with sucrose (Fig. 1). Control PCR analysis with the primers specific to the *rol* B gene was performed annually.

To study the effect of sugar, the "hairy" roots were cultivated in 100 mL of 1/2MS in 500 mL flasks at 24 °C. Each flask had 0.5 g of "hairy" roots as the initial inoculum. Various variants of the liquid medium with the addition of sucrose (20 or 30 g/L) and sucrose (20 g/L) with fructose (10 g/L) were used to optimize growth conditions as carbon sources. All roots were cultured for 3 weeks.

### 2.3. Extraction procedure

The 'hairy' roots were collected, washed with distilled water, lyophilized, and powdered by a mixer mill (Retsch MM400, Germany) before the extraction procedure. EtOH (70 vol %) or deionized water was added to the powdered roots (8 g/240 mL) and extracted for 3 days on a rotary shaker (Clim-*O*-Shake system Kuhner IRC-1-U) at 28 °C. The resulting extracts were filtered twice through filter paper and dried in a rotary evaporator to obtain the dry lyophilized extract (DLE).

### 2.4. Total flavonoid content assay

The determination of the total content of flavonoids in the extracts was performed according to the aluminium chloride method, based on the formation of a complex between $Al^{3+}$ ions and flavonoids [26]. Shortly, 0.25 mL of extracts were mixed with 1 mL of water and 0.075 mL of 5% $NaNO_2$ solution and allowed to react for 5 min at room temperature. Then, 0.075 mL of $AlCl_3$ solution (10%) were added. After 5 min of incubation, 0.5 mL of NaOH solution (1 M) and 0.6 mL of water were added to the reaction mixture. The absorbance of the sample was measured by a spectrophotometer (Fluorat-02 Panorama, Russia) at 510 nm. The total content of flavonoids expressed in rutin equivalent ($C_{rutin}$, mg (RE)/g) in the DLE was calculated using the calibration plot: $C_{rutin} = 0.7384 \cdot A_{510}$ ($R^2 = 0.9975$).

**Fig. 1.** *Cichorium intybus* L. "hairy" root culture.

### 2.5. DPPH assay

The determination of the antioxidant activity was based on the ability of the extracts to scavenge the DPPH radical [27]. In brief, 1 mL of DPPH solution in ethanol (0.0001 M) was added to 3 mL of extracts at different concentration (5, 10, 15, 20, 25, 30 μg/mL). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then, the absorbance was measured by a spectrophotometer (Fluorat-02 Panorama, Russia) at 515 nm. The results were given as an effective content ($EC_{50}$) presenting the amount of dry lyophilized extract required to scavenge the DPPH radical in the reaction mixture by 50%. The lower $EC_{50}$ values suggest that there is a better scavenging effect of DPPH.

### 2.6. Reducing power assay

The ability of root extracts to reduce $Fe^{3+}$ to $Fe^{2+}$ ions was determined by the spectrophotometry method [28]. The extracts with reducing capacity reacted with potassium ferricyanide ($K_3$ [$Fe(CN)_6$]) to form potassium ferrocyanide $K_4$ [$Fe(CN)_6$]. The reaction mixture contained 0.30 mL of phosphate buffer (pH 6.6), 0.30 mL of 1% $K_3$ [$Fe(CN)_6$], and 0.02–0.10 mL "hairy" root extract. The cuvettes with the mixture were incubated in a water bath at 50 °C for 30 min. Then, 0.30 mL of 10% trichloroacetic acid, 1.25 mL of deionized water and 0.25 ml of 0.1% $FeCl_3$ were added to the reaction mixture. The absorbance of the obtained mixtures was measured at 700 nm. The linear regression method was used to determine effective concentrations corresponding to the amount of DLE (mg) required to obtain an absorbance of 0.5 ($EC_{0.5}$). Ascorbic acid solution (1 mg/mL) was used as a positive control.

### 2.7. Chromatographic analysis

The extracts were mixed with ethanol (96%) for the separation of sugars *prior to* chromatographic analysis. The obtained suspension was stored at 20–25 °C for 2 days for the total precipitation of the sugar. Supernatants were analyzed by a combination of chromatographic methods.

*LC-HRMS analysis*. For screening analysis and identification of multiple compositions in the ethanolic extract of 'hairy' roots, the ultra-high-performance liquid chromatography (UHPLC) system (Dionex Ultimate 3000, Thermo Fisher Scientific) was coupled with high-resolution quadrupole time-of-flight (Q-ToF) mass spectrometry detector (Bruker Impact II) equipped with electrospray ionization source (ESI). The negative and positive parameters of the ion mode ESI source had previously been optimized by flow injection analysis. Finally, the mass spectrometer was operated in the ESI negative mode with a Duo-Spray source, and the mass scan range was set at *m/z* 50–2500 for the Q-ToF MS scanning using resolution of 2700 *m/z*. The following settings were used: 3500 V ion spray voltage; 500 °C ion source heater; 25 PSI collision gas; 10 eV collision energy; declustering potential 100. The presence of polyphenolic compounds in the extracts was determined based on their mass fragmentation pattern, low mass error within the acceptance range of ±5 mDa, and ionic response. Chromatography separation was accomplished with a thermostated (40 °C) UHPLC Kinetex® F5 (50 mm × 2.1 mm, 1.7 μm) core-shell column (Phenomenex).

The mobile phase was composed of 0.1% formic acid in water (*elution A*) and methanol (*elution B*) using an elution gradient (0.1 mL/min) of 30% elution B (0–5 min), from 30% to 50% of elution B (5–20 min), from 50% to 90% elution B (20–40 min), and from 90% to 100% of elution B (40–45 min).

Mefrucide was used as an internal standard (IS) for LC-HRMS measurements. Data acquisition and processing were performed with the Xcalibur 2.1 workstation.

*HPLC-DAD analysis.* The HPLC-DAD analysis of the extracts was performed on Shimadzu LC-20 (Japan). Polyphenol compounds were quantified using external standard mode with calibration plots of six representative compounds of these classes.

The mobile phase mixture consisted of acetonitrile (*elution* A) and 1.0% formic acid solution (*elution B*). The HPLC gradient was programmed as follows: 10% elution A (0–15.0 min); from 10 to 60% elution A (15.0–50.0 min) and returning to the starting conditions in the following 10 min. Separation was achieved by Zorbax Eclipse Plus C18 column (5 μm, 4.6 × 250 mm). Other chromatographic conditions were as follows: 0.8 mL/min flow rate and oven column set at 40 °C. The absorbance of the eluate was monitored at 250 and 295 nm.

Stock solutions of standards were prepared in methanol. Working solutions containing polyphenols mixture were prepared in 1.0 (v/v) % formic acid in methanol/water (2/3 v/v) and analyzed in three repetitions.

### 2.8. In vitro enzyme inhibitory assay

The 15-sLOX inhibition was performed using the traditional procedure [29] with some modifications. The activities were determined by recording the formation of the conjugate at 234 nm using the spectrophotometric method (SPECORD 200 Plus, Analytik Jena, Germany). Linoleic acid was used as a substrate. The DLE was dissolved in DMSO and filtered through a membrane filter with a pore diameter of 0.45 μm (*test solution*). Test solution at concentrations of 0, 25, 50, and 100 μM (RE) was added to the reaction mixture (total volume 2.5 mL), which consisted of phosphate buffer (pH 7.0) and 2.5 mM of linoleic acid. The reaction was initiated by adding 15-sLOX solution with a concentration of 0.65 mg/mL. The mixture was incubated at 25.0 ± 0.1 °C for 1.5 min. The buffer was used as a blank solution. Seven measurements were performed using different concentrations of the substrate in the incubation mixture for each of the test solution concentrations. Each measurement was repeated three times.

### 2.9. Statistical analysis

The results were expressed as average ± standard deviation evaluated in three independent replications (P = 0.95). Data were analyzed for statistical significance using one-way ANOVA with the Tukey HSD post-hoc test. Values of $p < 0.05$ were considered as significant.

## 3. Results and discussion

### 3.1. Optimization of C. intybus "hairy" root growth

The composition of the media has a great influence on the growth intensity of the culture *in vitro*. The addition of sugars to the culture is required for plant growth *in vitro* as a carbon source due to low photosynthetic activity under such growth conditions. Besides this, sugars play a significant role in regulating the osmotic potential of plant cells by affecting their growth and survival (Cui et al., 2010). At the same time, the high level of sugar concentration could inhibit plant growth (Tabatabaee et al., 2021). The authors reported that 3% sucrose was optimum for roots biomass production. Fathi and coauthors showed that the addition of 3% sucrose with 0.5 mg/L supplements of 1-naphthaleneacetic acid (NAA) had the best effect on chicory "hairy" root growth (Fathi et al., 2018). Basing on these results, the influence of the type and concentration of sugars in the nutrient medium on the weight of Chicory "hairy" roots were studied (Fig. S1). The impact of cultivation with nutrient media on the weight gain by the roots is shown in Fig. 2.

The weight gain by the "hairy" roots after three weeks of cultivation in the medium with 20 g/L of sucrose was 8.58 ± 0.75 g (Fig. 2). The addition of fructose to the medium containing 20 g/L sucrose did not affect the growth of the 'hairy' roots. Growth of "hairy" roots in the medium with the highest sucrose content resulted in a weight gain of ~17.0%. Thus, the most significant weight gain was observed for roots growing in 1/2MS medium supplemented with 30 g/L of sucrose. In this nutrient medium, "hairy" roots were grown to obtain material for further studies.

### 3.2. Properties of extracts from the "hairy" roots of C. intybus

To determine the effect of the extraction procedure on the yield of bioactive compounds in the *Cichorium intybus* "hairy" roots, two extraction solvents water and ethanol (70%) were used. Antioxidant ability, total flavonoid content and reducing power of the obtained extracts were estimated by DPPH scavenging, metal chelating and ferric reducing assays, respectively. The obtained results are summarized in Fig. 3.

The total flavonoid content in the aqueous and ethanolic extracts was found to be 121.3 mg (RE)/g and 60.3 mg (RE)/g, respectively (Fig. 3). Thus, the extraction conditions have a significant effect on the total amount of biologically active compounds in the obtained extracts. The ethanolic extract was a richer source of flavonoids than the aqueous one. The obtained results can be compared with the data of the total flavonoid content in red chicory leaves, grown *in vivo* [5]. They reported the total flavonoid content in the extracts in the range 48.75–92.95 mg (QE)/100 g depending on the extraction conditions. Denev et al. studied the content of flavonoids in dried extracts of chicory which ranged from 1.0 ± 0.1 to 2.8 ± 0.2 mg QE/g DW [30]. A comparison of our results with data from the studies cited above indicates that the "hairy" roots are indeed a promising source of flavonoids.

The highest total flavonoid content, as well as the highest antioxidant activity, and reducing power were found in the ethanolic extract compared to the aqueous one. Thus, the antioxidant and reducing activities of the aqueous extract was four and two-fold lower, respectively (higher values of $EC_{50}$ and $EC_{0.5}$), than the same activities of the ethanolic extract. This strong activity of ethanolic extract of chicory "hairy" root is of a great interest because previously reported extracts with high antioxidant activity were pointed out as promising sources for preparation of effective pharmaceuticals [31]. Based on these expectations, we tested the ethanolic extract for its anti-inflammatory activity.

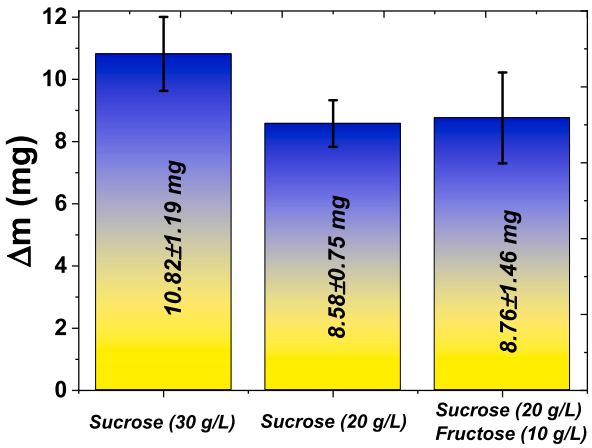


**Fig. 2.** Weight gain of *Chicory* "hairy" roots (Δm) after cultivation in the various nutrient medium.

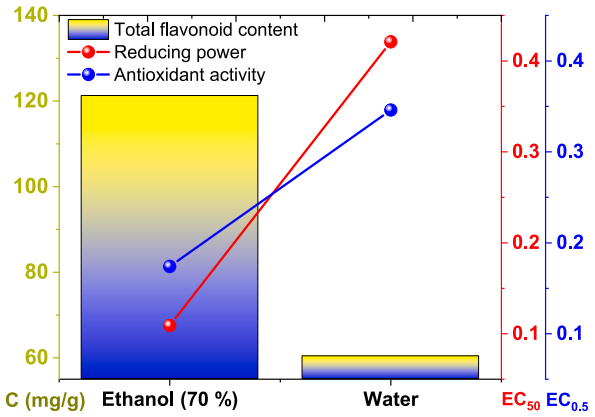


**Fig. 3.** Effect of the extraction condition on the total flavonoid content, the antioxidant activity and the reducing power of the extracts of *Cichorium intybus* "hairy" roots.

### *3.3. Screening of polyphenolic compounds in the ethanolic extract of "hairy" roots of C. intybus*

We used the ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry for fast analysis of the chemical components of the extract (in order to identify active components among them at the next step). This method screens target compounds in full scan mode and successfully separates interfering matrix compounds. Furthermore, the method has lower detection limits even upon scanning in the full range of masses if compared with UPLC-MS/MS and HPLC-MS. The major organic constituents of these extracts were studied after separation of the polysaccharides.

Chromatographic separation by LC-HRMS usually results in fractionation and visualization of a complex mixture of compounds present in crude extracts, and that is what we attempted to get. To identify the main polyphenolic compounds, the first step of this work was to study the tuning of standard analytes or corresponding derivates for MS fragmentation patterns. Mefruside was selected as the IS. The choice was based on the fact that this compound can be detected in positive, negative, and dual ionization modes, which allows monitoring performance of a LC-HRMS instrument in both polarities. A representative chromatogram of the ethanolic extract is shown in Fig. 4.

Organic compounds attribution to each class based on the retention time of standard compounds and HRMS data of Drug Application and Research Center library, and comparisons with the literature data is summarized below (Table 1). The table also lists identified compounds with the parent ion *m/z* values and daughter ions found in MS spectra. Although the determination of the positioning at 6-O (6-C) and 8-O (8-C) of sugar moieties in flavonoids often is problematic, it has been established based on differences in the intensity of product ions in the MS spectra according to tuning results and library. The MS data of the ethanolic extract show the accumulation of flavonoids and phenolic acids in the studied sample (**Table 1**).

*Phenolic acids.* Besides the simple phenolic acids (gallic, caffeic, chlorogenic, and caftaric acids), the corresponding glycosylated or ester forms were also identified. Compounds ***5*** and ***8*** were identified as caffeic and chlorogenic acids, respectively, using standards. Caffeic acid-3-glucoside (***16***) and chlorogenic acid-3-glycosides (***13***) were identified based on comparison of their profile data, including elution order and MS spectra, with those reported in the literature [32]. Compound ***2*** with $[M-H]^-$ at *m/z* 137.0285 a fragment at *m/z* 93.0255 $[M\text{-}H\text{-}COH]^-$ formed due to loss of the COH moiety was identified as hydroxybenzoic acid. Compound ***9***, with $[M-H]^-$ at *m/z* 153.0182 and fragments at *m/z* 83.0128 $[M\text{-}H\text{-}C_3H_2O_2]^-$ and 107.0138 $[M\text{-}H\text{-}COOH_2]^-$, was recognized as 2,5-dihydroxybenzoic acid. Compound ***39***, with $[M-H]$ at *m/z* 473, was identified as chicoric acid (known as hydroxycinnamic acid). This component is the derivative of both caffeic and tartaric acids.

*Flavonoids.* Free flavonoids and mainly conjugated with glucoside/glucuronide were found in the extract of the "hairy" root of the chicory studied. Compounds ***4***, ***6***, ***21***, ***31 36,*** and ***45*** were identified as epicatechin, apigenin, luteolin, kaempferol, quercetin, and rutin by comparison to standards. 3′-*O*-methyl-(−)-epicatechin-7-*O*-glucuronide (***15***), apigenin-7-*O*-apiosyl-glucoside (***25***), isoquercetin (***41***), kaempferol-7-*O*-(6-*O*-malonyl)-glucoside (***42***), luteolin-6-C-glucoside-8-C-arabinoside (***52***) were determined by comparing their profile data with MS library and literature data [21,32]. The glycosylated flavonoid derivatives were readily

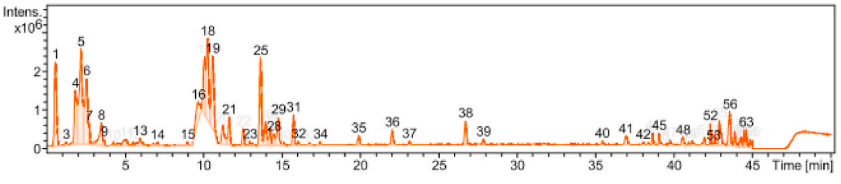


**Fig. 4.** A representative TIC chromatogram of the ethanolic extracts of "hairy" roots of *Cichorium intybus* obtained in the negative ion mode. Peak numbers correspond to the labeling adopted in Table 1.

**Table 1**
Identification of polyphenols, phenolic acids, and their derivatives in the ethanolic extract of "hairy" roots of *Cichorium intybus*.

| Entry | Retention time, min | Proposed structure | Precursor ion [M − H] (*m/z*) | Detectable mass |
|---|---|---|---|---|
| *1* | 0.68 | Gallic acid [$C_7H_6O_5$] | *169.0142* | *125.0125;153.0125; 108.01332* |
| *2* | 1.12 | Hydroxybenzoic acid [$C_7H_6O_3$] | *137.0285* | 93.0255; *65.1249* |
| *3* | 1.33 | Baicalein [$C_{15}H_{10}O_5$] | *269.0254* | *269.2357; 113.1956* |
| *4* | 2.04 | Epicatechin [$C_{15}H_{11}O_5$]$^-$ | *271.0603* | *245.0811; 205.0812; 179.0346* |
| *5* | 2.51 | Caffeic acid [$C_9H_8O$] | *179.1574* | *135.1523; 107.1423; 79.1286* |
| *6* | 2.95 | Apigenin [$C_{15}H_{10}O_5$] | *315.1124* | *284.1125; 117.0124; 151.0127* |
| *8* | 3.54 | Chlorogenic Acid [$C_{16}H_{18}O_9$] | *353.3112* | *191.0124; 179.0114; 135.0235* |
| *9* | 3.65 | 2,5-dihydroxybenzoic acid [$C_7H_6O_4$] | *153.0182* | *107.0138; 83.0128* |
| *13* | 5.98 | Chlorogenic acid-glycosides | *341.0024* | *180.0125, 134.0235* |
| *14* | 6.98 | *trans*-Caftaric acid [$C_{13}H_{12}O_9$] | *311.2457* | *179.0025; 149.0120; 135.1255* |
| *15* | 9.01 | 3′-*O*-methyl-epicatechin-7-*O*-glucuronide [$C_{22}H_{24}O_{12}$] | *479.0235* | *149.0256; 171.0235* |
| *16* | 9.56 | Caffeic acid 3-glucoside [$C_{15}H_{18}O_9$] | *515.2501* | *353.1423; 191.1452; 179.0423* |
| *18* | 10.05 | Apigenin-7-*O*-glucoside [$C_{21}H_{20}O_{10}$] | *431.0424* | *269.0128; 268.0113; 117.0188* |
| *19* | 10.85 | Quercetin-7-*O*-glucoside [$C_{21}H_{20}O_{12}$] | *463.0124* | *303.1001; 302.0117; 255.0447* |
| *20* | 11.34 | Baicalein-7-*O*-glucuronide [$C_{21}H_{18}O_{11}$] | *446.0233* | *445.1220; 269.0235; 113.0112* |
| *21* | 11.64 | Luteolin [$C_{15}H_{10}O_6$] | *315.0332* | *257.0112;133.0012* |
| *23* | 12.54 | Petunidin-3-*O*-(6-*O*-malonyl)-glucoside [$C_{25}H_{25}O_{14}$] | *563.1235* | *317.0777;* |
| *25* | 13.63 | Apigenin-7-*O*-apiosyl-glucoside (Apiin) [$C_{26}H_{28}O_{14}$] | *563.2548* | *444.2536;* |
| *29* | 14.86 | Quercetin 3-*O*-galactoside (Hyperoside) [$C_{21}H_{20}O_{12}$] | *463.4224* | *299.4257;* |
| *31* | 15.86 | Kaempferol [$C_{15}H_{10}O_6$] | *285.2415* | *255.2536; 151.1235; 133.2458* |
| *34* | 17.34 | Esculetin [$C_9H_6O_4$] | *177.1400* | *133.1233* |
| *35* | 19.61 | Kaempferol-7-*O*-glucuronide [$C_{21}H_{18}O_{12}$] | *461.0288* | *286.1201; 287.1008* |
| *36* | 22.01 | Quercetin [$C_{15}H_{10}O_7$] | *301.0499* | *178.0127; 151.0159; 121.0253* |
| *37* | 23.04 | 6-*O*-Methyl-baicalin-7-*O*-β-glucopyranuronoside | *459.1125* | *283.1278; 268.0153* |
| *38* | 26.56 | Kaempferol-3-*O*-glucoside [$C_{21}H_{19}O_{11}$] | *447.0057* | *285.0024; 257.0036; 151.005* |
| *39* | 27.76 | Chicoric acid [$C_{22}H_{18}O_{12}$] | *473.0012* | *311.0021* |
| *41* | 36.98 | Quercetin 3-*O*-glucoside (isoquercetin) [$C_{21}H_{20}O_{12}$] | *463.0125* | *301.0045; 155.0145* |
| *42* | 37.99 | Kaempferol-7-*O*-(6-*O*-malonyl)-glucoside [$C_{24}H_{20}O_{14}$] | *533.0023* | *449.0010; 287.0100; 286.0038* |
| *45* | 39.12 | Quercetin-3-*O*-rhamnosylglucoside (rutin) [$C_{27}H_{30}O_{16}$] | *609.0078* | *463.0018; 301.0126; 274.0147* |
| *48* | 40.54 | Kaempferol-3-*O*-glucosyl-7-*O*-(6-*O*-malonyl)-glucoside [$C_{30}H_{32}O_{18}$] | *679.0128* | *535.0065; 449.0100; 287.0030* |
| *52* | 42.34 | Luteolin-6-C-glucoside-8-C-arabinoside [$C_{27}H_{30}O_{16}$] | *609.0157* | *449.0109; 564.0164* |
| *56* | 43.64 | Quercetin-3-*O*-diglucoside-7-*O*-glucside | *787.0249* | *609.1488; 462.0281; 301.0277* |
| *63* | 44.86 | Kaempferol-3-*O*-glucosyl-7-*O*-(6-*O*-malonyl)-glucoside (robinin) [$C_{24}H_{20}O_{14}$] | *739.0127* | *593.0258; 430.0268; 285.0357* |

recognized by the intense peak in the MS/MS spectra with the typical neutral loss of the glycosyl part. Compound ***23***, with [M − H]$^-$ at *m/z 563.1235* and fragments at *m/z* 317.0777 was suggested as petunidin-3-*O*-(6-*O*-malonyl)-glucoside. In addition, mass peaks were detected in the spectra corresponding to sugar ring cleavage as well as malonyl group cleavage (i.e., fragmentation transitions for petunidin-3-*O*-(6-*O*-malonyl)-glucoside, kaempferol-7-*O*-(6-*O*-malonyl)-glucoside, kaempferol-3-*O*-glucosyl-7-*O*-(6-*O*-malonyl)-glucoside, etc.). Compounds reported in entries ***3***, ***20,*** and ***37*** were identified as baicalein, baicalein with the corresponding O-glucuronidyl moiety and its methyl flavonoid derivative (methylbaicalein). Compound ***25*** with [M − H]$^-$ at 563.2548 and a fragment at *m/z* 444.2536 [M-H-$C_4H_7O_4$]$^-$ was annotated as apigenin-7+-*O*-apiosyl-glucoside. Compound ***26*** with a similar retention time and [M − H]$^-$ was suggested as its isomer. Compound ***56*** was identified as luteolin 6-C-glucoside 8-C-arabinoside based on the fragment *m/z* equal to 609.1488.

*Distribution of bioactive compounds in ethanolic extract of "hairy" roots*. Thus, a total of **33** compounds including ***9*** phenolic acids and ***24*** flavonoids, belonging to various phenolic subclasses were identified or tentatively identified, as previously discussed [4]. Apigenin, quercetin, kaempferol, luteolin and their conjugates (glucosides, galactosides, glucuronides, etc.) were the main flavones in this extract. The flavonoids and flavones were mainly in the form of easy hydrolysable O-glucoside with high bioavailability [21].

Generally, phytochemicals such as polyphenolic and flavonoid isolated from various parts of the plants are used as antioxidant,

antimicrobial, antiulcer, antidiabetic, anticancer, antihyperlipidemic, antidiabetic, and hepatoprotective candidates [19]. Rutin [33], quercetin [34], luteolin [35], apigenin [36] possess antioxidant properties and also demonstrate antioxidant and anti-inflammatory activity [37]. Thus, polyphenols can be used in the therapy of inflammation of different origins due to these activities. Flavonoids were studied also as inhibitors of regulatory enzymes or transcription factors involved in the control process of inflammatory mediators [38]. Catechins, including epicatechin and its derivatives, are known as antioxidant, anticancer, antiallergic, and DNA-protective chemicals [39,40]. It has been reported that the combination of polyphenols can increase the bioactivity of the individual components of the mixture, having a synergistic effect [36]. For example, a mixture containing quercetin, curcumin, green tea, Cruciferex®, and resveratrol dose-dependent inhibited cancer cells (fibrosarcoma HT-1080 and melanoma A2058), proliferation and induced apoptosis [41]. Therefore, the combination of flavonoids and phenolic acids found in the chicory "hairy" root extract is potentially bioactive. It is obvious that further research can be of a practical interest for medical applications.

### 3.4. Quantitative analysis of phenolic and polyphenolic compounds

Quantitative analysis of the individual bioactive compounds revealed more information about the future application of the obtained *C. intybus* "hairy" root extracts. DAD and UV remains the gold standard detection modes for routine polyphenols determination, even if more selective detector could be use in some instance [19]. The HPLC-DAD assay was used to determine the concentration of individual components of the extracts, including flavonoids as the principal components of these extracts. The metrological parameters of the applied HPLC-DAD assay are given in Table S1. Studies of method validation parameters confirmed the applicability of the quantitative analysis of the detected polyphenolic compounds (Fig. S2). Thus, the main phenolic and polyphenolic components of "hairy" root extracts of *C. intybus* were quantified by comparing the chromatogram of extract with that of standards in water-methanol solution (Table 2).

Twenty-six main components, including flavonoids and phenolic acids, were quantified in the extracts. Phenolic acids were represented by gallic, hydroxybenzoic, 2,5-dihydroxybenzoic, chlorogenic, caffeic acids and their glycosides in this list. Concentrations of luteolin, kaempferol, quercetin, apigenin, and epicatechin, as well as their conjugates, were also detected in both ethanolic and aqueous extracts. The results show that the polyphenol content was significantly higher in the ethanolic extract than in the aqueous one, which depended on the properties of extractable compounds [15]. For instance, the content of caffeic acid in the ethanolic extract was 7.001 ± 0.068 mg/g compared to 1.013 ± 0.007 mg/g in the aqueous one. The concentration of rutin, luteolin, and apigenin was 38.8, 13.9, and 77.6 times higher in the ethanolic than in the aqueous extract. It is worth emphasizing that the differences in the concentrations in the ethanolic and aqueous extracts were characteristic for all the identified compounds.

Gallic and caffeic acids were the most abundant phenolic compounds (7.61% and 8.74%, respectively) in the ethanolic extract of "hairy" roots of *C. intybus* studied in these experiments (Table 2). Hydroxybenzoic acid was detected in low concentration in the ethanolic extract (0.042 ± 0.010 mg/g). Caffeic acid was found in the extract mainly in the free form. Its content was comparable with

**Table 2**
Quantification of the main components of *Cichorium intybus* "hairy" root extracts by the HPLC-DAD method.

| N⁰ | Compounds | Standard* | Concentration (mg/g) | |
|---|---|---|---|---|
| | | | Ethanolic extract | Aqueous extract |
| 1 | Gallic acid | *Gallic acid* | 6.103 ± 0.008 | 1.165 ± 0.019 |
| 2 | Hydroxybenzoic acid | *Hydroxybenzoic acid* | 0.042 ± 0.010 | 0.011 ± 0.011 |
| 3 | Epicatechin | *Epicatechin* | 3.090 ± 0.047 | 0.052 ± 0.012 |
| 4 | Caffeic acid | *Caffeic acid* | 7.001 ± 0.068 | 1.013 ± 0.007 |
| 5 | Apigenin | *Apigenin* | 3.960 ± 0.059 | 0.051 ± 0.014 |
| 6 | Chlorogenic acid | *Chlorogenic acid* | 1.804 ± 0.038 | 0.032 ± 0.011 |
| 7 | 2,5-dihydroxybenzoic acid | *Hydroxybenzoic acid* | 0.406 ± 0.052 | 0.122 ± 0.059 |
| 8 | Chlorogenic acid glycosides | *Chlorogenic Acid* | 1.514 ± 0.069 | 0.064 ± 0.063 |
| 9 | 3′-*O*-methyl-epicatechin-7-*O*-glucuronide | *Epicatechin* | 0.410 ± 0.058 | 0.145 ± 0.054 |
| 10 | Caffeic acid 3-glucoside | *Caffeic acid* | 2.911 ± 0.076 | 1.052 ± 0.055 |
| 11 | Apigenin-7-*O*-glucoside | *Apigenin* | 5.109 ± 0.064 | 0.310 ± 0.057 |
| 12 | Quercetin-7-*O*-glucoside | *Quercetin* | 6.710 ± 0.052 | 0.401 ± 0.011 |
| 13 | Luteolin | *Luteolin* | 4.320 ± 0.085 | 0.31 ± 0.049 |
| 14 | Apigenin-7-*O*-apiosyl-glucoside | *Apigenin* | 6.401 ± 0.066 | 0.81 ± 0.025 |
| 15 | Quercetin 3-*O*-galactoside | *Quercetin* | 5.011 ± 0.045 | 0.607 ± 0.016 |
| 16 | Kaempferol | *Kaempferol* | 4.401 ± 0.048 | 1.531 ± 0.044 |
| 17 | Kaempferol-7-*O*-glucuronide | *Kaempferol* | 1.303 ± 0.072 | 0.474 ± 0.005 |
| 18 | Quercetin | *Quercetin* | 4.307 ± 0.016 | 0.068 ± 0.004 |
| 19 | Kaempferol-3-*O*-glucoside | *Kaempferol* | 4.030 ± 0.005 | 0.434 ± 0.006 |
| 20 | Quercetin 3-*O*-glucoside (isoquercetrin) | *Quercetin* | 1.707 ± 0.004 | 0.206 ± 0.005 |
| 21 | Kaempferol-7-*O*-(6-*O*-malonyl)-glucoside | *Kaempferol* | 0.201 ± 0.003 | 0.035 ± 0.005 |
| 22 | Quercetin-3-*O*-glucoside (rutin) | *Rutin* | 1.203 ± 0.005 | 0.031 ± 0.004 |
| 23 | Kaempferol-3-*O*-glucosyl-7-*O*-(6-*O*-malonyl)-glucoside | *Kaempferol* | 1.308 ± 0.004 | 0.482 ± 0.008 |
| 24 | Luteolin-6-C-glucoside-8-C-arabinoside | *Luteolin* | 1.703 ± 0.008 | 0.515 ± 0.007 |
| 25 | Quercetin-3-*O*-diglucoside-7-*O*-glucoside | *Quercetin* | 1.012 ± 0.005 | 0.025 ± 0.005 |
| 26 | Kaempferol-3-*O*-glucosyl-7-*O*-(6-*O*-malonyl)-glucoside | *Kaempferol* | 4.375 ± 0.006 | 0.454 ± 0.004 |

Note. * Standard used for quantification.

the same in cauliflower (0.058 mg/g), carrot (0.09 mg/g), lettuce (1.57 mg/g), potato (2.80 mg/g) [42], and leaves of red chicory (11 ± 1 mg/100 g) [5]. This compound is one of the main hydroxycinnamic acids and is a well-known antioxidant that boosts immunity, controls lipid levels in blood, and has anti-cancer properties [43].

The rutin content in the ethanolic extract (1.203 ± 0.005 mg/g) of the "hairy" roots was higher than in the leaves of anteriorly studied vegetables such as common buckwheat (0.12 mg/g), lemon balm (0.30 mg/g), celery (0.046 mg/g), cabbage (0.0001 mg/g), red chicory leaves (5.4 ± 0.5 mg/100 g) [5], and sweet potato (0.0012 mg/g) [20]. So, the "hairy" roots of chicory might be considered a valuable and promising source of rutin.

Quercetin was present in the extracts not only in the free form but also as a glucoside (Table 2). The ethanolic extract of "hairy" roots was found to contain a high concentration of quercetin – 4.307 ± 0.016 mg/g. For example, an onion known to be a natural source of quercetin accumulates this flavonoid in a lower concentration (2.60 mg/g) than in the extract of the chicory "hairy" roots [44]. Usually [44], dietary sources of quercetin include apples (0.021 mg/g), lettuce (0.011 mg/g), tomato (0.055 mg/g) [13]. Quercetin demonstrated antihistamine, anticancer, and anti-inflammatory activities, which mostly follow its antioxidant traits [45].

It should be noted that so far, no research has been conducted on the quantitative content of flavonoids and phenolic acids in biotechnological chicory roots. In addition, data on such content in chicory plants grown in the soil is quite limited. The obtained information, which demonstrates a wide range of phenolic compounds in the "hairy" roots of chicory, is of great interest. Since the ethanolic extract contained a rather high concentration of phenolic acids and flavonoids, it was used in further experiments to study its anti-inflammatory activity.

### 3.5. Pharmacological prospective of the extracts

Prediction of biological activities of the main flavonoids identified in the chicory "hairy" root extract was performed using the online service Way2Drug (http://www.way2drug.com/) [46]. The study was carried out by the *in silico* method using virtual screening in the Prediction of Activity Spectra for Substances (PASS) service, which is based on the General Unrestricted Structure-Activity Relationships (GUSAR) algorithm. The data obtained during the virtual screening estimated the probability of presence ($P_a$) and absence ($P_i$) of an activity with values from 0 to 1. Flavonoids with $P_a > 0.5$ and $P_i < 0.03$ were selected as potentially biologically active agents (Table 3).

The data of this analysis demonstrate the possible high free radical scavenger (0.711–0.991), antioxidant (0.732–0.936), antimutagenic (0.435–0.94), anticarcinogenic (0.641–0.988), anti-inflammatory (0.548–0.767), antineoplastic (0.67–0.849), and chemopreventive activities (0.593–0.976) of all flavonoids found in the highest concentrations in the chicory "hairy" root extract. The extract components are also agonists of membrane integrity (0.772–0.978) and membrane permeability inhibitors (0.566–0.984). Rutin was identified as the most bioactive compound possessing anti-influenza (0.653), antifungal (0.786), and cardioprotective (0.975) activities. The results of this analysis suggest a possible high bioactivity of the extract with a wide spectrum of activities.

**Table 3**
Prediction of biological activities of the main flavonoids identified in the *Chicory* "hairy" roots.

| Activity | Apigenin | | (−)-Epicatechin | | Kaempferol | | Luteolin | | Quercetin | | Rutin | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $P_a$ | $P_i$ | $P_a$ | $P_i$ | $P_a$ | $P_i$ | $P_a$ | $P_i$ | $P_a$ | $P_i$ | $P_a$ | $P_i$ |
| LogBB | −0.472 | | −0.682 | | −0.472 | | −0.364 | | −0.52 | | −0.392 | |
| Free radical scavenger | 0.711 | 0.004 | 0.842 | 0.002 | 0.771 | 0.003 | 0.749 | 0.003 | 0.811 | 0.003 | 0.991 | 0.001 |
| Antioxidant | 0.732 | 0.004 | 0.81 | 0.003 | 0.856 | 0.003 | 0.775 | 0.004 | 0.872 | 0.003 | 0.936 | 0.002 |
| Reductant | 0.521 | 0.021 | 0.799 | 0.004 | 0.452 | 0.035 | 0.546 | 0.017 | 0.475 | 0.030 | 0.363 | 0.062 |
| Antiviral (Herpes) | 0.440 | 0.021 | 0.447 | 0.019 | 0.436 | 0.022 | 0.438 | 0.021 | 0.435 | 0.022 | 0.544 | 0.006 |
| Antiviral (Influenza) | 0.214 | 0.176 | 0.343 | 0.067 | 0.219 | 0.017 | 0.212 | 0.179 | 0.216 | 0.174 | 0.653 | 0.009 |
| Antibacterial | 0.388 | 0.033 | 0.32 | 0.053 | 0.389 | 0.033 | 0.386 | 0.034 | 0.387 | 0.033 | 0.648 | 0.006 |
| Antifungal | 0.518 | 0.026 | 0.552 | 0.023 | 0.486 | 0.033 | 0.513 | 0.028 | 0.481 | 0.034 | 0.786 | 0.006 |
| Radioprotector | 0.383 | 0.050 | 0.335 | 0.070 | 0.320 | 0.076 | 0.392 | 0.047 | 0.33 | 0.072 | 0.799 | 0.005 |
| Antidote | 0.312 | 0.029 | 0.287 | 0.038 | 0.303 | 0.108 | 0.326 | 0.024 | 0.317 | 0.027 | 0.716 | 0.003 |
| Antimutagenic | 0.921 | 0.002 | 0.571 | 0.011 | 0.919 | 0.002 | 0.940 | 0.001 | 0.940 | 0.001 | 0.435 | 0.019 |
| Anticarcinogenic | 0.641 | 0.011 | 0.795 | 0.005 | 0.715 | 0.008 | 0.690 | 0.009 | 0.757 | 0.007 | 0.988 | 0.001 |
| Antineoplastic | 0.774 | .015 | 0.675 | 0.030 | 0.791 | 0.013 | 0.783 | 0.014 | 0.797 | 0.012 | 0.849 | 0.007 |
| Chemopreventive | 0.593 | 0.010 | 0.788 | 0.004 | 0.669 | 0.008 | 0.648 | 0.008 | 0.712 | 0.006 | 0.976 | 0.001 |
| Proliferative diseases treatment | 0.541 | 0.015 | 0.681 | 0.007 | 0.588 | 0.011 | 0.566 | 0.013 | 0.614 | 0.010 | 0.959 | 0.001 |
| Hepatoprotectant | 0.612 | 0.011 | 0.679 | 0.008 | 0.589 | 0.013 | 0.644 | 0.009 | 0.623 | 0.011 | 0.980 | 0.001 |
| Apoptosis agonist | 0.847 | 0.005 | 0.649 | 0.021 | 0.881 | 0.005 | 0.860 | 0.005 | 0.887 | 0.005 | 0.722 | 0.013 |
| Antihemorrhagic | 0.521 | 0.003 | 0.537 | 0.003 | 0.480 | 0.003 | 0.598 | 0.003 | 0.601 | 0.003 | 0.888 | 0.001 |
| Anti-inflammatory | 0.644 | 0.024 | 0.548 | 0.044 | 0.676 | 0.019 | 0.661 | 0.021 | 0.689 | 0.017 | 0.767 | 0.009 |
| Non-steroidal anti-inflammatory agent | 0.311 | 0.040 | – | – | 0.375 | 0.025 | 0.319 | 0.037 | 0.385 | 0.023 | 0.674 | 0.005 |
| Hepatic disorders treatment | 0.509 | 0.011 | 0.495 | 0.012 | 0.391 | 0.016 | 0.515 | 0.011 | 0.407 | 0.021 | 0.734 | 0.004 |
| Cardioprotectant | 0.669 | 0.004 | 0.421 | 0.021 | 0.795 | 0.004 | 0.717 | 0.004 | 0.833 | 0.003 | 0.975 | 0.001 |
| Hemostatic | 0.622 | 0.004 | 0.334 | 0.019 | 0.701 | 0.003 | 0.665 | 0.003 | 0.771 | 0.003 | 0.982 | 0.001 |
| Membrane integrity agonist | 0.868 | 0.019 | 0.772 | 0.042 | 0.911 | 0.009 | 0.863 | 0.021 | 0.902 | 0.011 | 0.978 | 0.002 |
| Membrane permeability inhibitor | 0.924 | 0.003 | 0.739 | 0.924 | 0.924 | 0.003 | 0.935 | 0.003 | 0.938 | 0.003 | 0.984 | 0.001 |

### 3.6. Inhibition of 15-sLOX by ethanolic extract of "hairy" roots of C. intybus

The interaction of bioactive compounds with 15-lipoxygenase merits particular attention, since this nonheme iron-containing enzyme is a potential target for testing the health-preserving effect of the ethanolic extract in humans. 15-Lipoxygenase inhibitory effects are predominantly assessed by using soybean 15-lipoxygenase or 15-lipoxygenase from rabbit reticulocytes for the *in vitro* assay. The 15-sLOX was usually applied as a model for human 15-lipoxygenase due to its similarity in structure and mechanism of interaction [29]. In our study, the ethanolic extract of the "hairy" roots of *C. intybus* was tested as a possible inhibitor of 15-sLOX using linoleic acid as a substrate (S). The results are presented in Fig. 5a.

The inhibitory effect in the system was observed in the range of the extract concentrations from 25 to 100 μM (RE) in terms of the total flavonoid content (Fig. 5a). This effect was greater when the concentration of the flavonoids in the extract increased up to 100 μM (RE). To characterize the inhibitory effect of the ethanolic extract, the kinetic parameters should be considered. The Michaelis–Menten equation was proposed to evaluate the kinetic mechanism of 15-sLOX inhibition [47]. The Michaelis constant ($K_m$) and the maximum rate ($V_m$) of the reaction were determined using inverse Lineweaver-Burk coordinates (Fig. 5b). The values of $K_m$ and $V_m$ were calculated as 57.44 ± 7.52 μM and 0.9915 μM/s, respectively. The ethanolic extract of "hairy" roots of chicory as an inhibitor of 15-sLOX increased the Michaelis constant and reduced the maximum rate of the enzymatic reaction. These results are fully consistent with the effect of mixed (partial) inhibition [47]. Such type of inhibition occurs when the inhibitor binds to a other site on an enzyme (allosteric site), i.e., a site different from the active site where the substrate binds. The inhibitor binds to this allosteric site and changes the conformation of the enzyme (i.e., 3D shape). So, the affinity of the substrate for the active site of the enzyme gets reduced compared to the native enzyme.

Overall, the 15-sLOX inhibitors is classified related to several types.

a) redox inhibitors, which reduce the active site iron or trap the radical intermediates;
b) iron-chelating inhibitors; and
c) non-redox inhibitors, which compete with fatty acid substrate (linoleic acid) for binding to enzyme active sites.

Redox-active inhibitors include reducing agents that alter the oxidation state of the catalytic iron, turning its active ferric state (Fe3+) into inactive ferrous (Fe2+) state. In this extract, there are many lipophilic compounds in an original state (phenolics and polyphenolic compounds) and other compounds resulted from iron-catalyzed auto-oxidation of the flavonoids according to reduction-oxidation reactions (Table 1). Besides that oxidation products of certain polyphenols can be exhibit higher inhibitory potencies towards 15-sLOX than initial polyphenol compounds [48].

Iron ligand inhibitors include compounds that act as iron-chelators without altering its oxidation state. This class comprises derivatives of hydroxybenzoic acid, functional groups that are high iron-chelating effects with weak reducing properties. Inhibitions are achieved by replacing one of the Fe-coordination sphere ligands (probably the hydroxyl groups or water molecules) by the inhibitor molecule itself. Since flavonoids, in particular those containing a catechol group in the chemical structure, are known to chelate iron and other transition metal ions, e.g., the inhibitory effect of quercetin and other flavonoids might be due to chelating of enzyme iron [49]. Finally, non-redox inhibitors are competed with fatty acid substrates for binding to 15-LOX active sites.

The complete characterization of an inhibitor includes the determination of its inhibition constant ($K_i$) and $IC_{50}$. The latter value is calculated as the concentration of competitive inhibitor that is necessary to inhibit 50% of the enzyme at a particular substrate concentration. The binding affinities based on $K_i$ of all the inhibitors were calculated using the Cheng-Prusoff equation [50]. For the tested extract, the concentration required to achieve 50% inhibition of 15-sLOX was 84.13 ± 7.22 μM. Their $K_i$ and **α** values were found to be 23.29 ± 5.42 μM and 1.6, respectively. Mathematically, mixed inhibition occurs when the factor **α** in the Michaelis-Menten equation is greater than 1. In our study, this value was 1.6. Thus, analysis of the kinetics of inhibition of 15-sLOX by the ethanolic

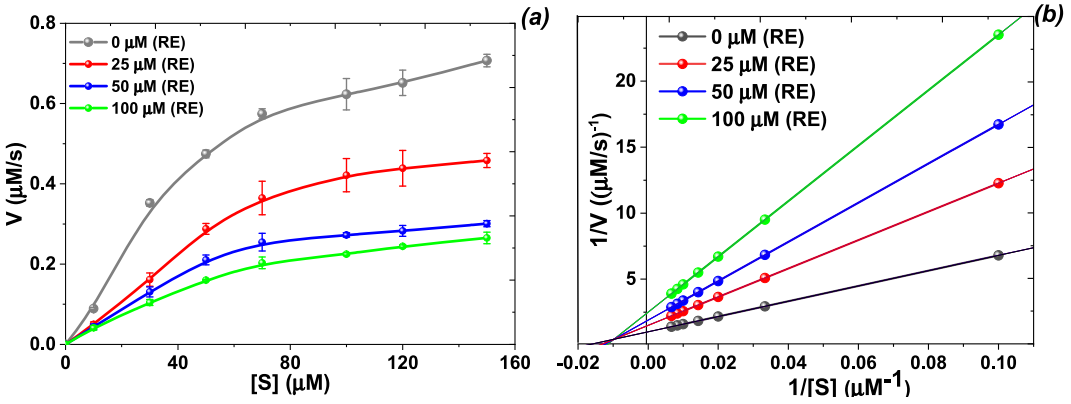


**Fig. 5.** Kinetic curves of inhibition of 15-sLOX by the ethanolic extract of "hairy" roots of *Chicorium intybus* at various dilutions (*a*) and corresponding Lineweaver–Burk plots (*b*).

extract (70%) of "hairy" roots of *C. intybus* show that the enzyme was inhibited by a mixed mechanism [51]. In this case, the extract components are said to be a mixed inhibitors with greater affinity for the native enzyme than the enzyme-substrate complex which retains of the activity.

Heterocyclic (pyranes, indoles, pyrazoles, etc.), phenolic/polyphenolic (catechols, flavonoids, etc) compounds and allyl derivatives have been used earlier in numerous studies as inhibitors of low-density lipoprotein oxidation induced by 15-lipoxygenase [52,53]. The chemical structure of the organic compounds (number of hydroxyl groups, 2,3-double bond present in the benzene ring, –NHCH2R, –C≡CR and –CH=CHR groups) is essential in this process [53]. For instance, a series of polymethoxylated flavonoids were isolated from orange peel, and inhibitory activity toward 15-sLOX was confirmed [49]. The best inhibition was shown by hesperidin ($IC_{50}$ = 180 μM), sinensetin ($IC_{50}$ = 74 μM), nobiletin ($IC_{50}$ = 86 μM), tangeretin, tetramethylscutellarein, and 3,5,6,7,8,3′,4′-heptamethoxyflavone were somewhat less active, comparable to the comparable to the positive control quercetin ($IC_{50}$ = 68 μM). Usually, published data indicate that quercetin is one of the most potent inhibitors of different 15-Lipoxygenase (mammalian and soybean) [52,54]. The polymethoxylated flavonoids were virtually inactive as scavengers of the DPPH radical. Hesperidin was only slightly, and ferulic acid showed good activity (IC50 = 111 μM). Although most of the 15-sLOX inhibitors had IC50 values between 80 and 120 μM [53]. Thus, as determined by the value of IC50 (84.13 ± 7.22 μM) the ethanolic extract of "hairy" roots showed a high level of 15-sLOX inhibition. Accordingly, the ability of the extract to inhibit the activity of 15-sLOX and a high level of antioxidant activity may be due to the presence of individual components, including identified flavonoids and phenolic acids [48], as well as the presence of a combination of these compounds and their synergistic action. Several chemicals identified in the studied "hairy" root extract, for example, gallic acid [55], kaempferol [56], quercetin [49] and other flavonoids [37], which are known as anti-inflammatory agents acting *via* 15-sLOX the inhibition of 15-sLOX.

Thus, the ethanolic extract from "hairy" roots of Chicory contains inhibitors of the activity of the enzyme associated with the initiation of inflammation and, at the same time, chemicals with high radical-scavenging properties. The combination of the named activities is the basis of the possible prospective use of this extract for develop new pharmaceuticals. Thus, the ethanolic extract of "hairy" roots of C. intybus L. "hairy" roots can be considered a potential active pharmaceutical ingredient of antioxidant and anti-inflammatory drugs [14,57,58]. Based on these data, the ongoing study of the anti-inflammatory activity of chicory "hairy" root extract, including that induced by SARS-CoV-2, which is accompanied by inflammation and oxidative stress, is of special interest.

## 4. Conclusions

The bioactive profile of *Cichorium intybus* L. "hairy" root extracts was determined for the first time. The powerful separation equipment, such as liquid chromatography coupled with high-resolution mass spectrometry and diode array detectors, was proved effective for the identification and quantification of bioactive compounds in the extracts. We found that the extraction solvent (ethanol or water) had a significant effect on the amount of polyphenolic compounds in the resulting extract. Overall, 33 polyphenolic compounds, including phenolic acids, flavones and flavonoids were identified in the Cichory "hairy" root ethanolic extract. Flavonoids were the most important group of polyphenolic compounds represented in Cichory "hairy" root extract. Quercetin, baicalein, epicatechin, apigenin, esculetin, and kaempferol were detected in the free state without carbohydrate moieties. The structural diversity of the metabolites identified in the "hairy" root ethanolic extract were discussed, as well as their biosynthesis. This includes rutinoside, glucoside, glucuronide, and rabinoside which were also extracted. Likewise, the ethanolic extract was characterized by a higher level of antioxidant and reducing activity. This extract demonstrated dose-dependent anti-inflammatory activity, determined by the ability to inhibit the enzyme 15-lipoxygenase ($IC_{50}$ = 84.13 ± 7.22 μM) by a mixed (partial) mechanism. The obtained results indicate that chicory "hairy" roots are a rich source of compounds with high antioxidant and anti-inflammatory activities. The results of the study show that the flavonoid-containing extract of chicory "hairy" roots can be a useful source of bioactive components. Such extract is also likely to be the basis for the development of herbal medicines for the treatment of the pandemic coronavirus disease COVID-19, which is often accompanied by oxidative stress and intense inflammation.

## Author contribution statement

Nadiia Matvieieva: Analyzed and interpreted the data; Wrote the paper.
Volodymyr Bessarabov: Analyzed and interpreted the data.
Olena Khaynakova; Vadym Lisovyi; Taisa Bohdanovych; Nazar Zderko: Contributed reagents, materials, analysis tools or data.
Volodymyr Duplij: Analyzed and interpreted the data.
Yakiv Ratushnyak; Galina Kuzmina: Performed the experiments.
Natalia Kobylinska: Conceived and designed the experiments; Wrote the paper.

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## Data availability statement

No data was used for the research described in the article.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e14516.

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