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Antioxidant and chemical properties of *Inula helenium* root extracts

Research Article

Iuliana Spiridon[1], Constantin Bogdan Nechita[2],
Marius Niculaua[3], Mihaela Silion[1], Alice Armatu[4],
Carmen-Alice Teacă[1*], Ruxanda Bodîrlău[1]

[1]*Department of Natural Polymers, "Petru Poni"
Institute of Macromolecular Chemistry, Romanian Academy,
Iasi 700487, Romania*

[2]*Agricultural Sciences and Veterinary Medicine University,
Iasi 700490 Romania*

[3]*Oenological Research Center, Romanian Academy,
Iasi 700490, Romania*

[4]*National Institute for Chemical Pharmaceutical Research and Development,
Bucuresti 031299, Romania*

Abstract: The objective of the paper was to investigate the chemical composition of *Inula helenium* roots extracts and to evaluate the antioxidant potential conferred by the chemical constituents. GC/MS and HPLC/MS techniques were used to characterize two extracts separated from *Inula helenium* roots by extraction with chloroform and ethyl acetate, respectively. Volatile compounds have been identified by GC from their mass spectra and retention time values, while HPLC identification of phenolic compounds was realized by comparing their retention times, UV and MS spectra with those of standards or literature data. Measurements of antioxidant activity of *Inula helenium* root extracts showed a variation between them, which can be correlated with the flavonoid and total phenolic contents. Both *Inula helenium* root extracts contain phenolic acids (caffeic, chlorogenic, dicaffeoyl quinic, hydroxibenzoic), terpenes (alantolactone) and different flavonoids (epicatechin, catechin gallate, ferulic acid-4-O-glucoside, dihydroquercetin pentosyl rutinoside, kaempherol-7-O-dipentoside, quercetin-3-O-β-glucopyranoside). In addition, the study provides preliminary data on the anti-inflammatory activity of *Inula helenium* root extracts, this being evaluated using the fresh egg albumin as phlogistic agent, and aspirin as reference compound. Root extracts of *I. helenium* did not exert any significant anti-inflammatory effect on egg albumin-induced rat paw edema.

Keywords: *Inula helenium* • Phenolics • Flavonoids • Antioxidant activity • Anti-inflammatory activity
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1. Introduction

Plant-derived phenolics receive considerable interest because of their potential antioxidant and antimicrobial properties. These bioactive compounds naturally available from plants have lower potency than 'allopathic' drugs, however, as they are traditionally consumed in significant amounts through the diet, they may have a long-term physiological benefits without any harmful side effects. This is why the role of the phenolics and flavonoids as natural antioxidants and free radical scavengers has attracted considerable interest in the last years due to their pharmacological behavior.

The genus *Inula* (Asteraceae; tribe Inuleae) comprises several species of reputed medicinal value (*Inula helenium* L., *I. racemosa*, *I. viscosa* L., and *I. britannica* L.). *Inula helenium*, a perennial plant widely occurring in Europe and East Asia, belongs to the *Compositae* family. Its oil consists mainly of sesquiterpenoid lactones [1], recognized for the strong anti-helminthic activity.

It was reported that extracts from the root possess anti-inflammatory, anti-microbial and anti-helminthic activities [2-4]. Moreover, the compounds show cytotoxic and antiproliferative activities against human cancer cell lines [3,5,6]. This biological significance has prompted phytochemists to investigate the chemical constituents of *I. helenium* plants, which has led to the identification of many bioactive compounds.

Gas chromatography/mass spectrometry (GC/MS) has been demonstrated to be an useful technique for the analysis of non-polar components and volatile essential oils, while liquid chromatography/mass spectrometry (LC/MS) allows the characterization of active components ranging from small polar molecules to macromolecules such as peptides/protein, carbohydrates and nucleic acids [7-10].

Several studies have been reported the chemical composition of *Inula sp* obtained by use of GC [11,12] and LC techniques [13]. It is well known that *Inula* root contains an essential oil with eudesmane-type sesquiterpene lactones (mainly alantolactone and isoalantolactone), thymol derivatives [14-16], terpenes, sterols, and inulin [17].

Current interest in the investigation of *I. helenium* root as a source of new pharmaceuticals has stemmed from compelling evidence of the components with biological activities of these compounds. Study of *I. helenium* L. plant growing in Romania constituted one of the objectives of a research project aimed to develop a formulation of active drug based on plants, practically non-toxic, with extremely effective anti-inflammatory action due to components that act synergistically focusing key targets of intracellular signalling pathways to induce an efficient inflammatory response. Some herbal plants from Romania widely used as natural food additives or for health promotion in traditional medicine (including *I. helenium* plant) were previously investigated only for their antioxidant activity [18].

In order to contribute for understanding the mechanisms underlying the beneficial effects of *I. helenium* root extracts, we have attempted to perform a more in-depth analysis of the chemical composition for this plant and the antioxidant potential conferred by the hydrophilic compounds (mainly phenolic compounds). The extraction is usually carried out by using polar solvents and, like flavonoids, the yield of phenolic acids from plants can be varied by using different proportion mixtures of extraction solvents [19]. In the present study, GC/MS and LC/MS (using ESI positive mode) were applied for the characterization of two extracts separated from the roots of *I. helenium*. We have also screened the anti-inflammatory activity of these extracts using the induced rat hind paw edema test as a model of acute inflammation. The fresh egg albumin was injected as phlogistic agent, and aspirin was used as reference compound.

2. Experimental procedures

2.1. Chemicals

Reference compounds HPLC grade (chlorogenic acid, catechin gallate, caffeic acid, quercetin-3-O-beta-glucopyranoside, epicatechin, p-hydroxybenzoic acid, quercetin-7-*O*-galactoside, quercetin-rhamnosyl-glucoside, alantolactone), gallic acid, rutin, ascorbic acid, DPPH (2, 2-diphenyl-1-picryl-hydrazyl), and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich Ldt (Germany). Acetylsalicylic acid (aspirin) from Zentiva, Romania was used as reference compound in acute inflammation tests. Acetonitrile was HPLC gradient grade (Sigma-Aldrich Ldt., Germany) and water was purified in Ultra Clear TWF PLUS purification system (SG Water – Germany). All solvents, analytical grade, were used as received.

2.2. Plant samples and extraction

Dried roots of *I. helenium* were purchased from the herbal drugstore. The plant material was ground and kept in a dark place. The dry material (200 g) was ground into powder and extracted with 2000 mL methanol (MeOH) at room temperature, for 7 days. After filtration, the obtained yellow methanol extract (1700 mL) was concentrated under reduced pressure ([9.59-9.87]×10^{-3} MPa), being further suspended into 100 mL distilled water. The resulted aqueous extract was twice extracted with 200 mL chloroform, resulting two liquid fractions: a chloroformic solution (390 mL) was concentrated at 40°C and reduced pressure to obtain 11.38 g brown powder, aromatized, coded as fraction 1 (F1). The second fraction was further subjected to liquid-liquid extraction with 200 mL ethyl acetate. After concentration under reduced pressure at 40°C, a yellow residue (0.1 g) was obtained coded as fraction 2 (F2).

2.3. Determination of total phenolic content

The total phenolic contents were estimated as gallic acid equivalents per gram of dried plant extract, according to Folin-Ciocalteu phenol reagent method [20]. First, a standard curve was plotted using gallic acid as a standard. Different concentrations of gallic acid were prepared in 80% of methanol, and their absorbance values were recorded at 765 nm. To 100 µL of the extract was added 0.5 mL (1/10 dilution) of Folin-Ciocalteu phenol reagent and 1000 mL of distilled water. The solutions were mixed and incubated

at room temperature for 1 min. After 1 min, 1500 µL of 20% sodium carbonate (Na_2CO_3) solution were added, the components being mixed and allowed to stand for 120 minutes. Absorbance at 765 nm was measured. All absorbance measurements for determination of total phenolic and flavonoid contents were carried out using a JENWAY 6405 UV–VIS spectrophotometer. A 1.0 cm optical path length glass cell was used in all measurements. The total phenolic content was expressed as gallic acid equivalents using a standard curve of gallic acid. Data presented are average of four measurements.

2.4. Determination of total flavonoid content

Total flavonoid contents were estimated following the aluminium chloride colorimetric method [21]. Briefly, aliquots of 2 mL (200 µg mL^{-1}) of the extracts were added to 2 mL of a 3% $AlCl_3$ solution in methanol and after incubation for 10 min at room temperature, the absorbance was measured at 430 nm. Total flavonoid contents were calculated from a calibration curve of rutin analyzed under the same conditions, being expressed in rutin equivalents per gram of dried plant extract. The determination was conducted in triplicate and values are expressed in mean ± SD.

2.5. Determination of DPPH radical scavenging capacity

Free radical scavenging potentials of the extracts were tested against a methanol solution of 1, 1-diphenyl-2-picryl hydrazyl (DPPH) [22]. 80 to 800 µg mL^{-1} of extract, 160 to 800 µg mL^{-1} of ascorbic acid, as standard in 500 µL methanol, were taken and added to 5 mL of 100 µM DPPH in methanol. The control was prepared as above without extract. The readings were taken at 517 nm using methanol as blank. The change in absorbance of the samples was measured for 20 minutes. Scavenging activity is expressed as the inhibition percentage calculated using the following equation:

$$Anti\text{-}radical\ activity\ (\%) = \{(control\ absorbance - sample\ absorbance) / control\ absorbance\} \times 100 \quad (1)$$

Each determination was carried out in triplicate.

2.6. Determination of total antioxidant capacity

For total antioxidant capacity assay [23], 2 mL of the extract (100 µg mL^{-1}) dissolved in methanol was combined in a tube with 2 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 minutes. After cooling to room temperature, the absorbance of the solution of each extract was measured at 695 nm against a blank. Ascorbic acid was used as standard and the total antioxidant capacity is expressed as equivalents of ascorbic acid. The experiment was performed in triplicate and values are expressed in mean ± SD.

2.7. Gas chromatography–mass spectrometry (GC/MS) analysis

I. helenium root extracts were analyzed without derivatization using gas chromatography–mass spectrometry. GC/MS analyses were performed on a Shimadzu GC apparatus coupled with a mass detector and a Thermo Scientific column (60 m, 0.25 µm, 0.25 mm). Working conditions were: static phase methylsilicone SE-30; injector 220°C, oven temperature: start 35ºC hold 5 min; programmed from 35 to 150°C at 2ºC min^{-1}, maintain for 8 minutes, then increase to 220ºC at 5ºC min^{-1} and finally hold to 220ºC for 40.5 minutes; carrier gas helium (at linear velocity 24 cm s^{-1}); a split/splitless injector was used at 220°C in split mode. MS conditions: detector voltage: 1 kV; ion source temperature: 250°C; mass range: 35–1000 m/z. Identification of the components was based on GC retention time values and computer matching with the Wiley and NIST libraries, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature and, whenever possible, with those for authentic compounds.

2.8. Liquid chromatography–mass spectrometry (HPLC/MS) analysis

The HPLC conditions for HPLC–MS and HPLC–UV analyses were identical. Chromatographic analysis of *I. helenium* root extracts was carried out on an Agilent 6500 Series Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS system with Agilent 1200 Series Diode Array Detector (module G1315B; detection type: 1024-element photodiode array; light source: deuterium and tungsten lamps; wavelength range 190 – 950 nm) by reverse phase elution (Waters Symmetry LC-18 column 250×4.6 mm, 5 µm). The mobile phase consisted of 2% acetic acid in water (solvent A) and acetonitrile (solvent B) with the following gradient: initially 95% A for 10 min; to 90% A in 1 min; to 60% A in 9 min; to 80% A in 10 min; to 60% A in 10 min; to 0% A in 5 min; and continuing at 0% A until completion of the run. The mobile phase was delivered with a flow rate of 1 mL min^{-1}, and the injection volume was 5 µL. Peaks were detected with the DAD at 280 nm. Mass spectra in the positive and negative mode were generated under the following conditions: fragmenter voltage 200 V; capillary voltage 4000 V; nebulizer pressure 15 psi; drying gas temperature

350°C; and mass range 100-1000 D. Comparing their UV–VIS absorption spectra, ESI–MS spectra, and chromatographic characteristics with the literature data reported, or with available reference standards identified the phenolic compounds of plant extracts.

Samples (0.1 g of F1 and 0.01 g of F2) were dissolved in 25 mL MeOH by ultrasonication for 40 min. The extracts were filtered and then an aliquot of 1mL of each one sample was diluted with deionized water of three-fold volume. The diluted solutions were mixed and filtered through 4.5 mm membrane. Aliquots of 10 mL were further injected for HPLC-DAD-ESI-MS analysis. The method was validated for the simultaneous analysis of 3 phenolic acids (caffeic acid, chlorogenic acid, p-hydroxybenzoic acid) and one flavonoid glycoside (quercetin-rhamnosyl-glucoside).

The calibration curves were obtained by the external standard method on six levels of concentration of standard mixtures, with three injections per level. Calibration curves were obtained by plotting the peak area *versus* compound content for a set of standard solutions. All the calibration curves showed a good linearity with correlation coefficients (r^2) no less than 0.976. Three different concentrations of standard mixtures (0.005; 0.015 and 0.030 mg mL^{-1}) were used for precision testing. The areas under curves and retention times of the three consecutive injections, performed at each concentration were used to calculate % RSD (relative standard deviation).

2.9. Determination of anti-inflammatory activity

The anti-inflammatory assay was carried out through the induced rat hind paw edema as a model of acute inflammation, using the fresh egg albumin as phlogistic agent. This is vehicle that provides a skin inflammation model suitable for analyzing topical anti-inflammatory agents.

The experiment was carried out using adult Wistar rats (150-200 g) of either sex, provided by the "Cantacuzino" National Institute for Microbiology and Immunology Research and Development, Bucharest, Romania. Animals were maintained on 12 h light/dark cycle at approximately 22±2°C, relative humidity 50–60% and allowed food and water *ad libitum*. All treatments were conducted between 9:00 and 10:00 a.m. to minimize variations in animal response due to circadian rhythm. The animals were deprived of food for 12 h before experimentation but allowed free access to tap water throughout. All studies were carried out by using six rats in each group and all the animals were used only once. Throughout the experiments, animals were processed according to the suggested ethical guidelines for the care of laboratory animals.

The fresh egg albumin-induced rat paw edema method was used [24]. Acute inflammation was measured in terms of change in volume of the rat hind paw induced by sub-plantar injection of egg albumin. Animals of six per group received 250 mg mL^{-1} per 100 g b.w. of the methanol extracts of *Inula helenium* roots by gastric gavage. Sixty minutes later, edema was induced with 0.1 mL of fresh undiluted egg albumin injected into the sub-plantar region of the right hind paw of the rats. Control groups received equivalent volume of vehicle (normal saline solution) or 100 mg kg^{-1} p.o. acetylsalicylic acid (aspirin).

The paw volume was measured at 30, 60, 120 and 180 min by the mercury displacement method using a plethysmometer (Ugo-Basile, Italy). Thus, inflammation was assessed as the difference between zero time paw diameter and that 30, 60, 120 and 180 minutes after administration of phlogistic agent. The percentage inhibition of paw volume in drug treated group was compared with the fresh egg albumin control group. Anti-inflammatory activity was calculated as percent of paw edema inhibition according to the following equation:

Anti inflammatory activity (%) = 100x [1- (a-x) / (b-y)],

where: a = mean paw volume of treated group (mL)
b = mean paw volume of untreated group (mL)
x = mean initial paw volume of treated group (mL)
y = mean initial paw volume of untreated group (mL)

All the results were expressed as mean ± standard error for each measurement time. Data were analyzed using one-way analysis of variance test (ANOVA) followed up with Tukey's range test. P-values < 0.01 were considered as being statistically significant.

3. Results and discussion

In this study, HPLC- and GC-MS techniques were used in order to obtain preliminary information concerning the composition of two different extracts of *I. helenium* root. It is well known that the solvent polarity and that of the different antioxidant compounds affects the efficiency of the extraction and the activity of the obtained extracts. Water, methanol, ethanol, acetone, aqueous solutions of the aforementioned solvents and ethyl acetate are commonly used as extraction solvents [25]. Thus, qualitative differences have been found in the chemical composition of analyzed samples, depending of the extraction method.

The components identified in *I. helenium* extracts by GC/MS technique, as well as their retention time values
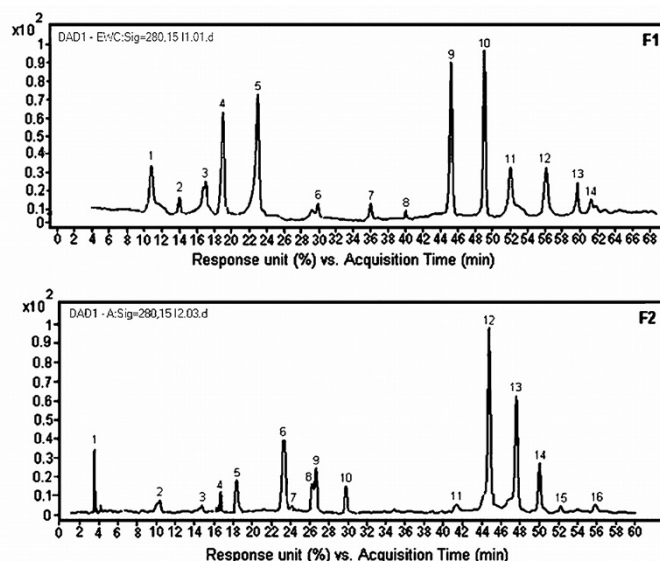
Figure 1. HPLC chromatograms recorded for F1 and F2 extracts separated from *Inula helenium* roots. **F1**: **1** -3,5-dihydroxybenzoic acid-*O*-glucosyl-xyloside; **2**- catechin gallate; **3**- chlorogenic acid; **4**- alantolactone; **5**- caffeic acid; **6**- unknown; **7**- galloyl-caffeoylhexose; **8**- kaempherol-7-O-dipentoside; **9**- dicaffeoyl quinic acid; **10**- quercetin-3-O-beta-glucopyranoside; **11**-5-O-feruloylquinic acid; **12**-9-O-beta-D-glucopyranosyl-9-hydroxythymol; **13**- dihydrocaffeic acid derivative; **14**-6"-O-malonyl genistein **F2**: **1**- unknown; **2**- epicatechin; **3**- p-hydroxybenzoic acid; **4**- chlorogenic acid; **5**- alantolactone; **6**- caffeic acid; **7**- 9,10-dihydroxy-8-methoxythymol; **8**- ferulic acid-4-*O*-glucoside; **9**- dehydro-9,10-isobutyryloxythymol; **10**- caffeic acid hexose; **11**- quercetin-7-*O*-galactoside; **12**- dicaffeoyl quinic acid; **13**- quercetin-rhamnosyl-glucoside; **14**- dihydroquercetin pentosyl rutinoside; **15**-3-feruloyl-4-caffeoyl-quinic acid; **16**-3,4,5-tricaffeoyl quinic acid.

and their percentage composition are summarized in Table 1, where all the compounds are arranged in order of their elution on the column. Twenty-five compounds and twenty-eight compounds were observed in F1 and F2 extract, respectively. Most of the constituents have been identified from their mass spectra and retention time values.

An essential oil is a volatile mixture of organic compounds derived from odorous plant material by physical means. The constituents of an essential oil may be classified into two main groups: (a) hydrocarbons (terpenes, sesquiterpenes and diterpenes); (b) oxygenated compounds derived from these hydrocarbons including alcohols, aldehydes, esters, kethons, phenols, oxides, *etc.*

Volatile compounds identified in both extracts were alcohols, aldehydes, organic acids, and esters. Aldehydes, especially C6-compounds, are very volatile substances giving the aroma of the extracts. The main aroma volatile in our extracts was comprised of benzaldehyde. Among the terpenoids, fraction F1 contains beta-elemene, alpha-muurolene, beta-selinene and alpha–selinene, while in the fraction F2 beta-elemene, alpha-amorphene, beta-selinene and alpha–selinene were identified. This finding indicates that the composition of any plant extract studied is influenced by several factors, such as local, climatic and experimental conditions, a fact which influences its antioxidant activity.

High performance liquid chromatography coupled with photodiode-array detector and mass spectrometer (HPLC-DAD-MS) provides powerful and economical tool for phenolic compounds analysis. Also, extensive information on compounds structures could be obtained [8,9,26,27]. The HPLC chromatograms of the extracts are shown in Fig. 1.

The content of chemical constituents was expressed on the dry root weight basis. Thus, it was found that F1 extract contains 3,5-dihydroxybenzoic acid-*O*-glucosyl-xyloside (14.2 mg per 100 g), catechin gallate (24.5 m per 100 g), chlorogenic acid (35.3 mg per 100 g), alantolactone (44.9 mg per 100 g), caffeic acid (96.7 mg per 100 g), galloyl-caffeoylhexose (10.8 mg per 100 g), kaempherol-7-O-dipentoside (41.2 mg per 100 g), dicaffeoyl quinic acid (73.4 mg per 100 g), quercetin-3-O-beta-glucopyranoside (49.5 mg per 100 g), 5-O-feruloylquinic acid (30.7 mg per 100 g), 9-O-beta-D-glucopyranosyl-9-hydroxythymol (20.4 mg per 100 g), dihydrocaffeic acid derivative (14.8 mg per 100 g); 6"-O-malonyl genistein (1.9 mg per 100 g), while in F2 extract 15 compounds were identified and quantified, as follows: epicatechin (23 mg per 100 g); hydroxybenzoic acid (35 mg per 100 g), chlorogenic acid (47 mg per 100 g) alantolactone (53 mg per 100 g), caffeic acid (72 mg per 100 g), 9,10-dihydroxy-8-methoxythymol (23 mg per 100 g), ferulic acid-4-*O*-glucoside (76 mg per 100 g), dehydro-9,10-isobutyryloxythymol (40 mg per 100 g), caffeic acid

Table 1. Retention time (Rt) of compounds identified in the fractions 1 and 2 of *Inula helenium* L. root extract.

Constituent	Retention time (min)	Extract fraction	Identification
2-methoxyethanol	16.459	F2	Ref, Lib
nonane, 3-methyl-5-propyl-	17.017	F2	Ref, Lib
methyl benzoate	17.791	F2	Ref, Lib
alpha-muurolene	20.425	F2	Ref, Lib
2-phenyl-2-propanol	20.588	F2	Ref, Lib
benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	23.584	F2	Ref, Lib
1-butanol	24.342	F1, F2	Ref, Lib
hexanal dimethyl acetal	24.684	F2	Ref, Lib
2-methoxyethanol	25.841	F1	Ref, Lib
1-pentanol	27.884	F2	Ref, Lib
propionitrile, 2-(3-fluorophenylhydrazono)-3-imino-3-(4-morpholyl)-	38.982	F2	Ref, Lib
tetradecane	40.214	F2	Ref, Lib
1-hexanol, 2-ethyl	46.093	F1, F2	Ref, Lib
2-propenoic acid, 2-methyl-cyclohexyl ester	46.724	F1, F2	Ref, Lib
benzaldehyde	48.124	F1, F2	Ref, Lib
beta elemene	52.212	F1, F2	Ref, Lib
nonane, 3-methyl-5-propyl-	52.876	F1	Ref, Lib
benzoic acid	54.106	F2	Ref, Lib
methyl benzoate	54.124	F1	Ref, Lib
acetophenone	55.774	F1, F2	Ref, Lib
alpha-muurolene	57.484	F1	Ref, Lib
alpha-amorphene	57.49	F2	Ref, Lib
naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7- (1-methylethenyl)-, (1R,7R,8aS)-	59.229	F2	Ref, Lib
2-phenyl-2-propanol	59.238	F1	Ref, Lib
beta-selinene	59.703	F1, F2	Ref, Lib
alpha – selinene	60.004	F1, F2	Ref, Lib
azulene	60.825	F1, F2	Ref, Lib
benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	62.959	F1	Ref, Lib
neopentanediol	64.688	F2	Ref, Lib
1,3-propanediol, 2,2-dimethyl-	64.758	F1	Ref, Lib
benzyl alcohol	68.276	F1, F2	Ref, Lib
hexanoic acid, 2-ethyl-	70.799	F1	Ref, Lib
1-dodecanol	71.08	F1	Ref, Lib
phenol	71.991	F1, F2	Ref, Lib
1-tetradecanol	75.315	F1	Ref, Lib
hexadecanoic acid, methyl ester	76.338	F1	Ref, Lib
2,4-di-tert-butylphenol	78.252	F1, F2	Ref, Lib
methyl oleate	82.088	F1	Ref, Lib
1H-indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl	83.99	F2	Ref, Lib
palmitic acid	84.22	F2	Ref, Lib
1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester	86.74	F1	Ref, Lib

GC/MS = comparison with the GC/MS of standard
Ref = comparison with GC and MS literature values
Lib = Computer Library Identification (NIST 08 Mass Spectral Library -NIST GC Library; NIST MS/MS Library; Wiley Registry™ of Mass Spectral Data, 8th Edition)

hexose (24.5 mg per 100 g), quercetin-7-*O*-galactoside (14.2 mg per 100 g), dicaffeoyl quinic acid (60.8 mg per 100 g), quercetin-rhamnosyl-glucoside (91.7 mg per 100 g), dihydroquercetin pentosyl rutinoside (72.9 mg per 100 g), 3-feruloyl-4-caffeoyl-quinic acid (16 mg per 100 g) and 3,4,5-tricaffeoyl quinic acid (17.2 mg per 100 g). Peaks were assigned based on the retention time, UV spectra and MS spectra with those of standard compounds or data from the literature.

The identification of each compound (Table 2) was based on a combination of retention time and spectral matching, since polyphenols absorb in the ultraviolet (UV) region. According to the literature [28], most benzoic acid derivatives show a maximum absorption at 246–262 nm with a shoulder at 290–315 nm (except gallic acid that shows a maximum at 271 nm), while two absorption bands are characteristic of flavonoids (band II with a maximum in the 240–285 nm range, is believed to arise from the A-ring and band I, with a maximum in the 300–550 nm range is attributed to the B-ring). Also mass spectra were compared with those of reference standards or literature data. The identification of some of the above mentioned compounds were confirmed by the addition of internal standards.

The compounds identified in both extracts belong to flavonols, benzoic acid derivatives, and cinnamic acid derivatives, the differences being related to the extraction method. Thus, the major components present in the F1 extract were identified as caffeic acid showing a [M-H]- at m/z 179 and dicaffeoyl quinic acid, with [M-H]- at m/z 515. Quercetin-3-O-beta-glucopyranoside (with [M+H]+at m/z 495) alantolactone (with [M+H]+at m/z 232) have been also identified in significant amounts.

The major compound was caffeic acid; followed by dicaffeoyl quinic acid, quercetin-3-*O*-beta-glucopyranoside, alantolactone while the minor identified component was kaempherol-7-*O*-dipentoside.

Caffeic acid and its derivatives have been well-represented components in F2 fraction. Thus, dicaffeoyl quinic acid having [M-H]- at m/z 515, caffeic acid showing [M-H]- at m/z 179, 3-feruloyl-4-caffeoyl-quinic acid with [M-H] - at m/z 515, and 3,4,5-tricaffeoyl quinic acid with [M-H] - at m/z 677 were identified. Dicaffeoyl quinic acid, caffeic acid, and quercetin rhamnosyl glucoside were the most abundant compounds in the F2 fraction the less represented being 3-feruloyl-4-caffeoyl-quinic acid. These differences are reflected in the antioxidant capacity. It is well known that antioxidant capacity of a substrate can be measured through the ability of the substrate to intercept free radicals by scavenging [29]. It was stated that single electron transfer assays measure the colour changes of an oxidant due to reduction by an antioxidant, the extent of the colour changes being correlated with the extract's antioxidant concentrations.

Free radical-scavenging effect of our extracts was determined by DPPH method, based on the measurement of the reducing ability of antioxidants toward DPPH stable radical [29]. The chemical reaction mechanism of the DPPH assay is primarily based on single electron transfer (SET) reactions, while hydrogen-atom transfer (HAT) is a marginal reaction [30]. The method is stable and quantifies samples with hydrophilic or lipophilic antioxidants. It was found that a long-lived nitrogen radical unlike radicals present in living organisms and has no similarity to the highly reactive and transient peroxyl radicals that are involved in lipid peroxidation [29]. Our results are in agreement with those reported by other authors [31-33] which evidenced that antioxidant activity is correlated with phenolic constituents. Also, a pharmaceutical product comprising water-soluble chitosan and an ethanolic extract of *I. helenium* with anti-inflammatory properties was patented [34]. Measurements of antioxidant activity of *I. helenium* root extracts showed a variation between them (Table 3), which can be correlated with the flavonoid and total phenolic contents [35]. It seems that high antioxidant activity corresponds to high total phenolic content as well.

The antioxidant activity is probably based on the ability of the phenolics and also other biomolecules (tannins, terpenes) present in elecampane to act as donors of hydrogen atom.

The pharmacological activity of medicinal plants is usually due to their secondary metabolites. In the case of elecampane, this activity is related to the presence of mainly sesquiterpene lactones such as alantolactone, isoalantolactone, and dihydroalantolactone [36]. Numerous studies have been attributed the anti-inflammatory activity of phenolic constituents to their antioxidant activity [37-39] while some authors consider that the hydrogen-donating antioxidant activity of these inhibitors is unlikely to be the sole explanation for their effects at the cellular level [40,41].

The anti-inflammatory effect of *Inula helenium* root extracts was examined using the fresh egg albumin-induced edema model. Thus, the acute inflammatory responses induced by egg albumin (very similar with carrageenan injection) involve three phases of chemical mediator release in an orderly sequence [42]. For the first 1.5 h, an initial phase takes place with the release of histamine and serotonin and for the subsequent 1.5–2.5 h, a second phase is mediated by bradykinin. The third and final phase occurs between 2.5 and 5 h and is presumably mediated by prostaglandins (PGs). In the present study the initial and second anti-inflammatory phases were evaluated.

Table 2. HPLC-MS data for constituents present in fractions 1 and 2 of *Inula helenium* L. root extract.

HPLC		LC-MS data (m/z)		Tentative identification	Fraction
Rt (min)	Data λmax (nm)	[M+H]⁺ /[M-H]⁻	Ion fragments		
4.37	218, 326	/343		unknown	F2
10.11	278	/289	289, 245, 203 187,161	epicatechin	F2
10.28	214, 254	447	447, 315, 153,151	3,5-dihydroxybenzoic acid-*O*-glucosyl-xyloside	F1
14.4		/441	441, 359, 263, 201,137,108	catechin gallate	F1
14.6	277	/137	137,112	p-hydroxybenzoic acid	F2
16.9	218, 326	/353	191,179, 161,131	chlorogenic acid	F1, F2
18.4	205	232	232,171	alantolactone	F1 (9.9%) F2
22.93	240, 322	/179	179,135	caffeic acid	F1 (12.4%) F2 (12.1%)
23.7	276	212	181, 149, 121 , 105	9,10-dihydroxy-8-methoxythymol	F2
26.7	286	/355	134,178,193	ferulic acid-4-*O*-glucoside	F2
27.7	284	320/	320, 235, 233, 232, 194, 163, 145, 133, 105	dehydro-9,10-isobutyryloxythymol	F2
29.8	246	/341	341,251,179, 161	caffeic acid hexose	F2
30.1	265, 360	/595	300,271,179	unknown	F1
36.1	252, 367	495	325,163,252	galloyl-caffeoylhexose	F1
40.13	262, 312, 360	551	551,363,287	kaempherol-7-O-dipentoside	F1 (1.6%)
41.5	254, 360	465	465,303	quercetin-7-*O*-galactoside	F2
44.83	246	/515	353, 191, 179,135	dicaffeoyl quinic acid	F1 (10.6%) F2 (21.7%)
48.5	236, 274, 326	/609	300, 301, 271, 255, 179, 151	quercetin-rhamnosyl-glucoside	F2 (15.8%)
49.57	216, 244, 328	495	495,433,303	quercetin-3-O-beta-glucopyranoside	F1 (10.5%)
49.574	320	745	745, 615, 539, 384,305	dihydroquercetin pentosyl rutinoside	F2
52.06		/529	529,283,253, 235,173	5-O-feruloylquinic acid	F1
52.3	236, 284,	/529	485, 447, 387, 367, 353, 349, 335, 193, 173	3-feruloyl-4-caffeoyl-quinic acid	F2 (1.9%)
55.8	242, 326	/677	515, 514, 497, 353, 335, 299, 255, 179	3,4,5-tricaffeoyl quinic acid	F2
56.4	276	/327	327,351	9-O-beta-D-glucopyranosyl-9-hydroxythymol	F1
60.42	298	597	597, 515, 433, 269,187	dihydrocaffeic acid derivative	F1
62.38	254, 334	519	519, 271	6"-O-malonyl genistein	F1

The paws were monitored and an increase in the linear diameter of the point where the phlogistic agent was administrated served as indication of inflammation. Assessment showed that sub-plantar injection of the fresh egg albumin led to an increase in the hind paw diameters of the control and untreated rats. Data are presented in Table 4.

Table 4 shows the results of the effect of root extracts of *I. helenium* on egg albumin-induced rat paw edema.

Both extracts did not exert any considerable anti-inflammatory effect by blocking the release of mediators from egg albumin (e.g. histamine). A significant (P<0.01) anti-inflammatory effect was demonstrated by the standard drug, ASP 100 (100 mg kg⁻¹), which reduced egg albumin, induced edema. The analysis of variance test (ANOVA) was performed and the results of ANOVA followed up with Tukey's range test are presented in Table 5.

Table 3. Total antioxidant capacity, total phenolic and total flavonoid contents of the *Inula helenium* root extracts under study.

Extract sample	TAC (mg ascorbic acid g⁻¹)[a]	Total phenolic content (mg gallic acid g⁻¹)[b]	Total flavonoid content (mg rutin g⁻¹)[c]
F1	9.4 ± 0.1	5.06 ± 0.06	15.6 ± 0.2
F2	23.13 ± 0.04	42.2 ± 0.07	50.0 ± 0.1

[a, b, c] *Average ± standard deviation*

Table 4. Mean values of volumes (mL) for rat hind paw induced at different times after administration of phlogistic agent.

Time (min)	Hind paw volume after 30 min		Hind paw volume after 60 min		Hind paw volume after 120 min		Hind paw volume after 180 min	
Groups	mean±SD (mL)	Variance (%)	mean±SD (mL)	Variance (%)	mean±SD (mL)	Variance (%)	mean±SD (mL)	Variance (%)
F1	1.063 ± 0.433	0.188	1.226 ± 0.500	0.250	1.168 ± 0.476	0.227	0.941 ± 0.383	0.147
F2	0.977 ± 0.398	0.159	1.053 ± 0.430	0.185	0.85 ± 0.35	0.120	0.773 ± 0.315	0.099
Control	1.356 ± 0.553	0.306	1.005 ± 0.409	0.168	0.767 ± 0.313	0.098	0.645 ± 0.263	0.069
ASP 100	0.745 ± 0.303	0.092	0.574 ± 0.235	0.055	0.472 ± 0.192	0.037	0.467 ± 0.189	0.036

Table 5. ANOVA summary and Tukey test analysis for anti-inflammatory test (independent samples).

Source	Sum of squares (SS)	Degree of freedom (df)	F	P
Treatment (between groups)	0.610868	3	5.56	0.012592
Tukey HSD test HSD[0.05]=0.4 HSD[0.01]=0.53	F1 vs. F2 F1 vs. control F1 vs. ASP 100 F2 vs. control F2 vs. ASP 100 control vs. ASP100	non-significant non-significant $P<0.01$ non-significant non-significant non-significant		

HSD= the absolute difference between any two sample means required for significance at the designated level; HSD[0.05] for the 0.05 level; HSD[0.01] for the 0.01 level

F1 extract exhibits a weak anti-inflammatory activity in the first 30 minutes, but until 180 min this fraction seems to act as a pro-inflammatory agent. This could be explained by its rapid degradation or by low amounts of flavonoids as anti-inflammatory compounds. After 120 min, *Inula helenium* extracts fail to sustain an efficient anti-inflammatory action. The difference between control and F1/F2 is non-significant, as evidenced by Tukey test analysis. The results showed that both extracts have a non-significant influence on the inflammatory mediators such as histamine and serotonin. More studies involving all the inflammation phases need to be carried out.

4. Conclusions

Use of chromatographic systems coupled with mass spectrometry contributed to rapid identification of the main compounds present in the two extracts of *I. helenium* roots. It is extremely important to point out that there is a positive correlation between antioxidant activity potential and the amount of phenolic compounds in the extracts.

Both *I. helenium* root extracts contain phenolic acids (caffeic, chlorogenic, dicaffeoyl quinic, hydroxibenzoic), terpenes (alantolactone), and different flavonoids (epicatechin, catechin gallate, ferulic acid-4-O-glucoside, dihydroquercetin pentosyl rutinoside, kaempherol-7-O-dipentoside, quercetin-3-O-β-glucopyranoside).

The anti-inflammatory effect of *Inula helenium* root extracts was examined using the fresh egg albumin-induced edema model. These did not exert any significant anti-inflammatory effect by blocking the mediators that are released by egg albumin (*e.g.* histamine). A significant (P<0.01) anti-inflammatory effect was demonstrated by the standard drug, ASP 100 (100 mg kg⁻¹), which reduced egg albumin, induced edema. More investigation involving all the inflammation phases needs to be carried out.

Pharmacology and pharmacokinetics experiments accompanied with rational and accurate clinical practice of different *Inula helenium* extracts are greatly needed for this important herbal plant.

Acknowledgements

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