The Analysis of Flavonoids from Inula helenium L. Flowers and Leaves

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Objective: In this study we investigated the flavonoids in the leaves and flowers of Inula helenium L..
Material and method: Flowers and leaves were harvested from the Medicinal Plant Garden of the University of Medicine and Pharmacy of Tîrgu Mureş. Dried vegetable product was extracted with methanol and in order to investigate aglycones we prepared hydrolised extracts. We screened the vegetable product for flavonoids using a TLC method, then we determined the total flavonoid content by a spectrophotometric method. HPLC-MS and HPLC-UV methods were used to determine polyphenols in methanolic and hydrolised extracts.
Results: Flavonoid content varies from 1.83% in leaves to 1.43% in flowers, equivalent in hyperoside. We identified the flavonols quercetin and kaempferol and the glycosides isoquercitrin, quercitrin and rutoside. Isoquercitrin is the major compound, in a concentration of 0.898 mg/g dry vegetable product.
Conclusions: Our study shows that the leaves and flowers of Inula helenium L. contain a high percentage of flavonoids with pharmacological activities.

Keywords: Inula helenium L., flavonoids, TLC, spectrophotometry, HPLC

Introduction

Inula helenium L. (elecampane) is known for the medicinal uses of its roots. Inulae radix represents a rich source of inulin and contains up to 3% volatile oil rich in sesquiterpene lactones [1–4]. There are few reports about the composition of the aerial part of the plant. Small quantities of lactones are present in the aerial part of the plant, mainly germacranolides (11,(13)-dehydroeryolin; 4α,5α,-epoxy-10α-14-H-inuviscolide; carabrone) and eudesmanolides (2α-hydroxyalantolactone) [5]. The flavonoids quercetin, its glucoside quercetin-7-triglucosid and 3-methyl quercetin are present in the flowers [1].

Polyphenols are a large and diverse class of compounds, which naturally occur in a wide range of plants. The flavonoids are the largest and best-studied group among polyphenols due to the considerable interest in their pharmacological effects. They have antioxidative, antiinflammatory activities [6,7], antihypertensive effect [8] and in vitro studies showed that they inhibit enzymatic activities involved in several types of tumor cells [1,9].

Considering the use of the aerial part of the plant in phytotherapy we aimed to conduct a comprehensive analysis of flavonoids present in flowers and leaves. Firstly, we investigated flavonoids in flowers and leaves extracts using thin layer chromatography, then we quantified flavonoids by a spectrophotometric method. HPLC-MS and HPLC-UV methods [11–17] were used to determine major flavonoids in methanolic extracts.

Material and methods

Flowers (F) and leaves (L) of Inula helenium L. were harvested at the beginning of July 2010 from the Medicinal Plant Garden of The University of Medicine and Pharmacy of Tîrgu Mureş. The vegetable product was dried at room temperature.

1). TLC identification of flavonoids

Extraction

1 g plant material was extracted with 99% MeOH (L1, F1) and with 60% MeOH (L2, F2) at 60˚C, for 5 minutes, filtered and 10 µl solution volumes were applied on the chromatographic plates.

Hydrolysis

Acid hydrolysis was carried out with 25% HCl, over a period of 50 minutes using 2 g plant material (L3 and F3), according to the specifications of "Crataegi folium cum flos" monography (FR X) [18] 10 µl solution volumes were applied on the chromatographic plates.

The separation of flavonoids was performed on silicagel plates (Alugram Sil G, MN, GERMANY) using 3 mobile phases [19,20]:

I. anhydrous formic acid – glacial acetic acid – water – ethyl acetate (11:11:26:100);
II. toluol – ethyl acetate – formic acid (50:40:10);
III. n-hexane – ethyl acetate – acetic acid (62:28:10).

After development the plates were heated at 100–105°C, then evenly sprayed with NEU/PEG-4000 and analysed in visible light and UV_{325} light.

Standard substances

Rutoside, quercetol, caffeic acid, chlorogenic acid (Merck).

Table I. The 18 standard polyphenols used for the determination of flavonoids and equations

No.	Compound	Retention time (min)	Calibration curve equation	No.	Compound	Retention time (min)	Calibration curve equation
1	caftaric acid	2.10	–	10	rutin	20.2	A = 0.226 + 13.47 x
2	gentisic acid	2.15	–	11	myricetin	20.7	A = -0.544 + 26.45 x
3	caffeic acid	5.6	–	12	fisetin	22.6	A = 0.241 + 19.19 x
4	chlorogenic acid	5.6	–	13	quercitrin	23.0	A = 0.047 + 10.69 x
5	p-cumaric acid	8.7	A = - 0.325 + 33.23 x	14	quercetin	26.8	A = -1.152 + 36.32 x
6	ferulic acid	12.2	A = - 1.016 + 39.55 x	15	patuletin	28.7	A= - 0.429 + 31.44 x
7	sinapic acid	14.3	A = - 0.236 + 37.10 x	16	luteolin	29.1	A = - 0.760 + 28.97 x
8	hyperoside	18.6	A = 0.107 + 19.29 x	17	kaempferol	31.6	A = - 1.270 + 30.15 x
9	isoquercitrin	19.6	A = - 0.273 + 12.97 x	18	apigenin	33.1	A = - 0.908 + 20.40 x

* A = peak area in mAU×s, x = concentration in μg mL^{-1}

2). Quantitative spectrophotometric determination

Flavonoids were determined using a spectrophotometric method (FR X) using $AlCl_3$ reagent, according to the specifications of "Crataegi folium cum flos" monography [18].

Absorbances of sample solutions were registred at λ = 425 nm. Results were calculated using specific absorbance of hyperoside A 1% 1 cm = 500.

3). Identification and quantification of flavonoids by HPLC-MS and HPLC-UV analysis

Extraction

1 g leaves (L) and 1 g flowers (F) respectively were extracted with 99 % MeOH.

Hydrolysis

Methanolic extracts of leaves (Lh) and flowers (Fh) were evenly diluted with hydrochloric acid 2N and heated at 80°C on a water bath for 60 min. The evaporated volume was replaced with the same quantity of distilled water. Two samples of each extract were analysed.

HPLC-MS system

HP 1100 Series HPLC equiped with a binary pump (HP 1100 Series), auto-sampler (HP 1100 Series), thermostat (HP 1100 Series), UV detector (HP 1100 Series) and Agilent Ion Trap 1100 VL mass spectrometer.

Chromatographic conditions

The HPLC separation was performed on a Zorbax SB-C18 column (100 mm × 3.0 mm i.d., 3.5 μm) and a Zorbax SB-C18 precolumn. The mobile phase consisting of methanol and 0.1% (V/V) acetic acid was used in the following gradient: start with 5% methanol; from 35 to 38 min increased to 42% methanol; then to 45 min 5% methanol. The flow rate was 1 ml/min and the sample injection volume was 5 μl. Column temperature was set at 48°C. UV absorbtion was monitored at 330 nm to 17 minutes and at 370 nm to 38 minutes.

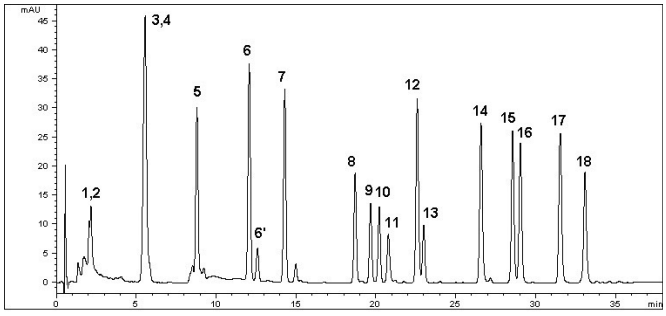

Fig. 1. HPLC chromatogram of the 18 polyphenols standards mixture, UV detection at 330 and 370 nm

MS conditions

Analysis was carried out using ESI (electrospay) ion source, in a negative ionozation mode, a nitrogen sprayer (70 psi pressure); nitrogen drying gas; flow rate 12 L/min; at 360°C; capilary voltage 3000 V. Analysis mode: monitoring of specific ions (polyphenolcarboxylic acids) or AUTO MS (flavonoids and their aglycones).

HPLC-UV detection of polyphenolic compounds

Polyphenolcarboxylic acids were detected at 330 nm while flavonoids and their aglycones at 370 nm.

The quantitative analysis was performed using calibration curves. The calibration curve for each standard was prepared in the concentration range of 0.5–5 μg/ml. Due to incomplete separation of the pairs caftaric acid/gentisic acid and caffeic acid/chlorogenic acid, for these compounds we retrieved only qualitative data.

Results

1). TLC analysis

Mobile phase I separated efficiently extracts L2 and F2 (MeOH 60%), showing more blue zones in the lower part than extracts L1 and F1 (MeOH 99%). This solvent mixture was not suitable for L3 and F3 extracts (hydrolised). System II, consisting of more lipophylic solvents, was used for extracts L2, F2 (MeOH 60%) and L3, F3 (hydrolised). All extracts show a light blue zone in the range of caffeic acid (Rf = 0.56) and chlorogenic acid (Rf = 0.1). The major orange zone in F2 and F3 extracts is due to quercetin (Rf = 0.62) and probably its glycosides situated immediately under quercetin.

To separate the zone corresponding to quercetin and its glycosides we prepared bidimensional chromatograms.

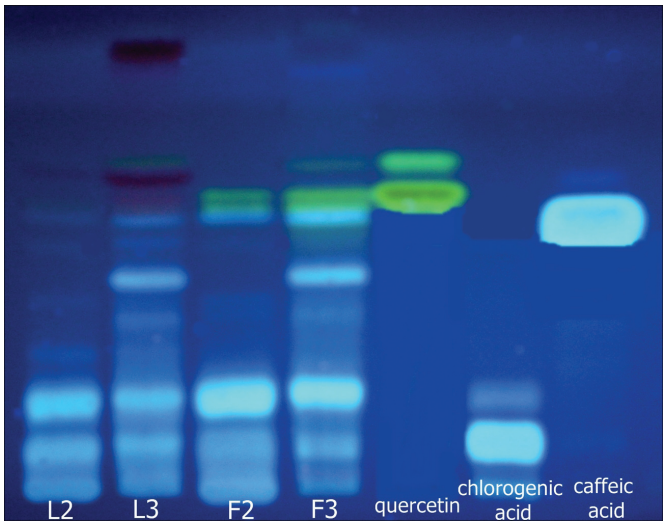

Fig. 2. TLC chromatogram of the methanolic (60%) extracts (L2, F2) and of hydrolised extracts (L3,F3), in UV_{325} light

Table II. Flavonoid content in flowers and leaves, spectrophotometric assay

Concentration of flavonoids hyperoside %g/g vegetable product	
Leaves	Flowers
1.776	1.278
1.757	1.432
1.962	1.583
1.831	1.431

System II was used as first developer and system III as the second one. Leaves and flowers of Inula helenium L. show a similar TLC pattern of flavonoids in the hydrolised extracts, two (L3), respectively three (F3) orange zones, a yellow zone (L3), respectively two yellow zones (F3) (Figure 3).

2). Quantitative analysis

The total flavonoid content of the hydrolised extracts of samples was determined by an UV-VIS spectrophotometric method. Aglycones were extracted from the hydrolised extracts with ethyl acetate and chelated with AlCl3. Our results show that the flavonoid content varies between 1.43% and 1.83% and that leaves contain higher amounts

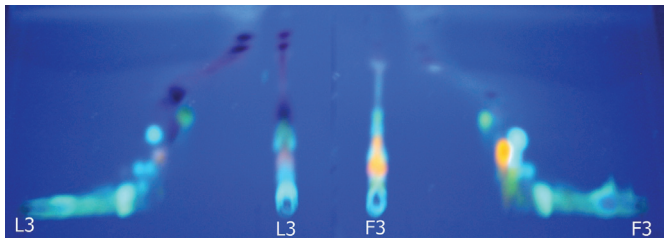

Fig. 3. TLC bidimensional chromatograms of hydrolised extracts of leaves (L3) and flowers (F3), in UV_{325} light

Table III. Identification of polyphenolic standards in samples

No	Compound	Retention time (min)	Leaves				Flowers			
			L		Lh		F		Fh	
			UV	MS	UV	MS	UV	MS	UV	MS
1	gentisic acid	2.15		+		+				+
2	caffeic acid	5.6		+		+		+		+
3	chlorogenic acid	5.6		+		+		+		+
4	p-cumaric acid	8.7		+	+	+				+
5	ferulic acid	12.2								+
6	isoquercitrin	19.6					+	+		
7	rutin	20.2	+	+						
8	quercitrin	23.0	+	+			+	+		
9	quercetol	26.8			+	+	+	+	+	+
10	patuletin	28.7			+	+				
11	kaempferol	31.6			+	+			+	+

of flavonoids. The flavonoid concentrations in hyperoside % in dry plant material are presented in Table II.

3). HPLC assay

Results of quantitative determinations are shown in figure 8.

Discussions

Our study determines qualitatively and quantitatively the flavonoids in the flowers and leaves of Inula helenium, data not found in literature. To our knowledge there is only one reference regarding flavonoid content in this plant [1].

TLC analysis showed that quercetin and its derivatives are present in both leaves and flowers. The investigated extracts also contain polyphenolic acids, corresponding to chlorogenic and caffeic acids.

The quantitative assay revealed high concentrations of flavonoids, a content of flavonoids over 1.5% g/g is com-

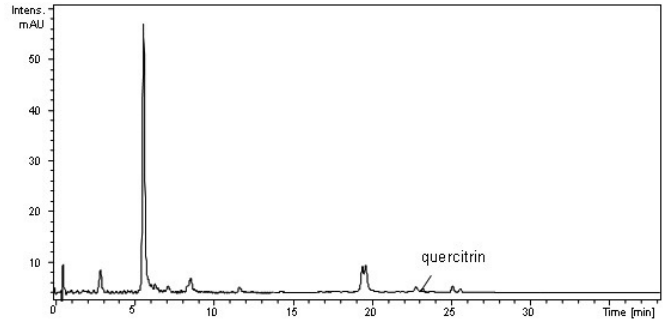

Fig. 4. TLC chromatogram of the methanolic (60%) extracts (L2, F2) and of hydrolised extracts (L3,F3), in UV_{325} light

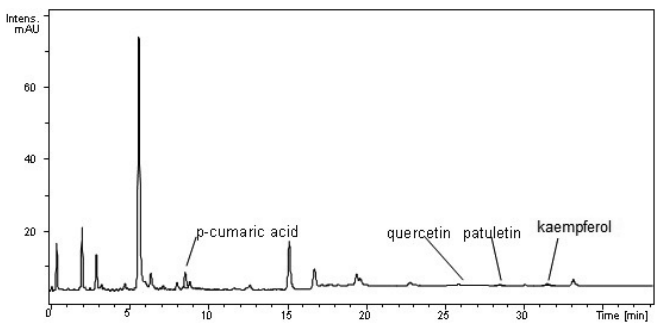

Fig. 5. TLC bidimensional chromatograms of hydrolised extracts of leaves (L3) and flowers (F3), in UV_{325} light

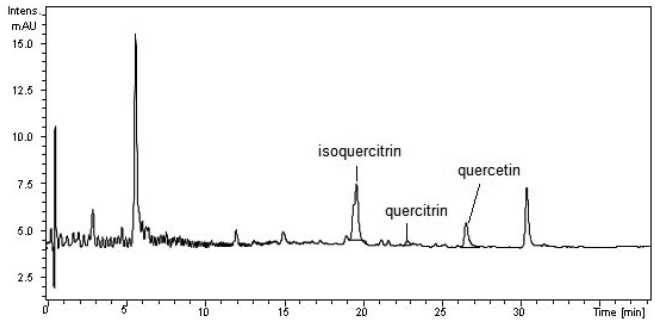

Fig. 6. TLC chromatogram of the methanolic (60%) extracts (L2, F2) and of hydrolised extracts (L3,F3), in UV_{325} light

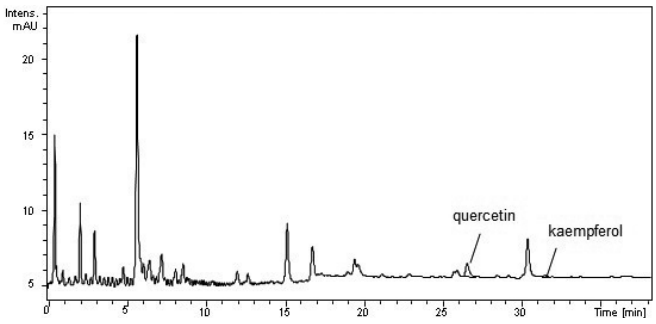

Fig. 7. TLC chromatogram of the methanolic (60%) extracts (L2, F2) and of hydrolised extracts (L3,F3), in UV_{325} light

parable to other official plant drugs included in European Pharmacopoeia Ed. 6. [21].

Eleven polyphenolic compounds were identified by HPLC-MS and the major components were determined by HPLC-UV. The HPLC analysis confirmed TLC results, quercetin and the polyphenolic acids: gentisic, caffeic, chlorogenic, p-cumaric were identified in both leaves and flowers extracts.

In the methanolic extracts of flowers we identified the aglycone quercetin and its glycosides isoquercitrin, quercitrin and in leaves we identified the glycosides rutoside and quercitrin.

The hydrolised extracts of both flowers and leaves contain the flavonol aglycones quercetin and kaempferol, patuletin is present only in leaves.

We could not identify using this method quercetin-7-triglucosid and 3-methyl quercetin, flavonoids mentioned in the literature [1].

The flavonol isoquercitrin (quercetin 3-O-β-D-glucopyranoside) is the main component of flowers methanolic extract 0.898 mg/g. This compound was isolated in some plant species like Crataegus sp. [22], Argemone platyceras [23], and in the genus Hyptis [7]. Studies showed strong antioxidant activity of isoquercitrin comparable to that of the extract of Gingko biloba used as standard [7,24].

Conclusions

The relatively high percentage of flavonoids with pharmacological activities in leaves and flowers of Inula helenium L. makes it a valuble vegetable product. To consider the use of this product in phytotherapy we need to further determine the composition of the aerial part of the plant.

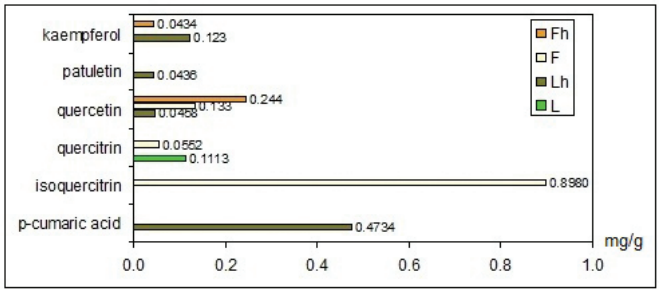

Fig. 8. TLC chromatogram of the methanolic (60%) extracts (L2, F2) and of hydrolised extracts (L3,F3), in UV_{325} light

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