High-performance Liquid Chromatography Analysis of Four *Leuzea carthamoides* Flavonoids

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Abstract

A simple and sensitive high-performance liquid chromatography method for the determination of four *Leuzea carthamoides* flavonoids, namely eriodictyol, patuletin, eriodictyol-7-β-glucopyranoside, and 6-hydroxykaempferol-7-*O*-(6"-*O*-acetyl-β-D-glucopyranoside), is presented. Using this method, quantitative composition of flavonoids ranged from 0.011% to 0.574% in dried plant material was determined. This method could be used in the future for the quantitative evaluation of major phenolic compounds in *L. carthamoides*.

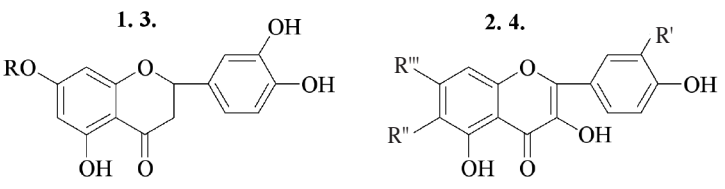
Introduction

Flavonoids form an important part of plant bioactive phenolic compounds and are an integral part of the human diet. They have been reported to exhibit a wide range of biological effects (1–6). According to their structural features, flavonoids are divided into various subclasses, such as flavones, flavonols, flavanones, or flavan-3-ols (catechins). All subtypes can also occur as flavonoid glycosides (7).

Leuzea carthamoides [*Rhaponticum carthamoides* (Asteraceae)] is an endemic plant of southern Siberia. Now, it is widely grown in Central and Eastern Europe. The principal bioactive constituents of the whole plant are ecdysteroids, flavonoids, and phenolic acids. *L. carthamoides* is an old folk medicine for fatigue, as a stimulant, for overcoming impotence, and to assist convalescence from long illness. It was found that *L. carthamoides* has a beneficial effect on memory and learning and can help break addictive behaviours. It increases working capacity in tired skeletal muscles, relieves neurosis, benefits anabolic and adaptogenic processes and improves metabolism of lipids and carbohydrates (8–10). This plant is contained in several preparations such as those for reducing body weight activity (Sternax) or improving erection function activity (Hard wood).

Generally, the percentages of bioactive components in a plant vary depending on the environmental conditions, like sunlight, water, or mineral nutrients, which are responsible for maintaining optimal growth (11). The quantitative determination of bioactive compounds could help to explain the required biological activity of the whole plant extract, which is considered to be one of the criteria of quality. For this reason, it is necessary to analyze the content of bioactive compounds in the consumer-available herbal drugs.

In this study, the procedure of preparation of whole *L. carthamoides* extract and simple and sensitive high-performance liquid chromatography (HPLC) method for quantitative determination of four flavonoids, namely eriodictyol, patuletin, eriodictyol-7-β-glucopyranoside, and 6-hydroxykaempferol-7-*O*-(6"-*O*-acetyl-β-D-glucopyranoside) (Figure 1) is presented. These flavonoids were previously isolated in our laboratory from *L. carthamoides* and evaluated as high antioxidant active (12).



Flav.	R	R'	R''	R'''
1.	H	–	–	–
2.	–	OH	OCH_3	OH
3.	glucopyranoside	–	–	–
4.	–	H	OH	(6"-acetyl)glucopyranoside

Figure 1. Flavonoids isolated from *L. carthamoides*.

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Experimental

Chemicals

Eriodictyol, patuletin, eriodictyol-7-β-glucopyranoside, and 6-hydroxykaempferol-7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside) were previously isolated and identified at the Department of Pharmaceutical Botany and Ecology, Faculty of Pharmacy, Charles University (12). Acetonitrile-gradient grade LiChrosolv, sodium acetate (99%) and glacial acetic acid (100%) were purchased from Merck (Darmstadt, Germany). Ethanol (95%) was purchased from Sigma-Aldrich (Prague, Czech Republic). The dried powdered leaves of *L. carthamoides* (Willd.) DC (Asteraceae), were obtained from Radka Simakova (Medicinal plants cultivation, Ohnisov, Czech Republic). Water was reverse osmosis pure.

Sample preparation

Dried leaves of *L. carthamoides* (0.5 g) were boiled for 30 min in 20 mL of 95% EtOH. Thereafter, the mixture was filtered on Büchner funnel. The residue was washed with 2 × 3 mL 95% EtOH, filtered, and put to the main plant extract. The residue was boiled again for 30 min in 20 mL of 95% EtOH, and the extraction procedure was repeated again. Both plant extracts were put together and evaporated. This raw extract (144.5 mg) was dissolved in 12 mL of water–acetonitrile 50:50 (v/v), treated 30 min in ultrasonic bath, and centrifuged at 10,000 rpm for 5 min. The clear supernatant was separated, filtered through a Target HPLC Syringe Filter (pore size 0.45 µm), and injected into the HPLC system. This sample was measured in 12 mg/mL, 6 mg/ml and 3 mg/mL concentrations three times each and statistically evaluated. Tested flavonoids were dissolved in water acetonitrile 50:50 (v/v) in 240, 120, and 30 µg/mL concentrations and injected into the HPLC system. Each concentration was measured three times.

Apparatus

The HPLC system consisted of a P200 gradient pump (Spectra-Physics Analytical, Fremont, CA), a 7125 injection valve (10 µL loop) (Rheodyne, Cotati, CA), an UV1000 detector (Spectra-Physics Analytical, Fremont, CA) and a CSW Chromatography Station 1.5 software (DataApex, Praha, Czech Republic).

Chromatographic conditions

For analyses, a 125 × 4 mm i.d. Nucleosil 100 – 5C18 AB column (Macherey Nagel, Düren, Germany) was used. The mobile phase was 10% acetonitrile and 90% acetate buffer. The acetate buffer was prepared with 0.1M sodium acetate solution. Finally the pH was adjusted to 4.8 with glacial acetic acid. The mobile phase was delivered isocratically at a flow-rate of 1 mL/min. The absorbance was measured at 260 nm. All chromatograms were obtained at room temperature (25°C).

Results and Discussion

In this article, quantitative determination of four flavonoids in *L. carthamoides* by HPLC is presented. For obtaining of the sufficient amount of natural compounds from the leaves of *L. carthamoides*, the sonication or hot ethanol extraction were performed. The sonication was performed for 30 min in 95% ethanol. The next procedure of sample preparation was analogous to each other. The more efficient hot ethanol extraction (10% more yield, compared to sonication) was selected for the next experiment. From the tested absorbance (250, 260, 270, 280, 290, 300, and 310 nm) of the standard flavonoid mixture, 260 nm was selected as the most efficacious wavelength of the UV detector.

During the development of the appropriate HPLC method, two different columns were used. Except the chosen method described in experimental section, another column was examined [250 × 4 mm i.d. Lichrospher 60 RP-select B (5 µm) column; Merck, Darmstadt, Germany]. Using this column, several mobile phases were used: A, 5% acetonitrile and 95% water; B, 25% acetonitrile and 75% water; C, 10% acetonitrile and 90% water (acetate buffer, pH 4); D, 10% acetonitrile and 90% water (acetate buffer pH 4) with a gradient of 10% to 50% ACN (30 min) were examined. None of these was able to detect the constituents of the extract.

On the contrary, the best results were achieved with (125 × 4 mm i.d. Nucleosil 100 – 5C18 AB column; Macherey-Nagel, Düren, Germany), which seems to be ideal for this kind of separation. The best separation result was achieved with the 10% acetonitrile and 90% acetate buffer at a flow rate of 1 mL/min.

Chromatograms of the flavonoid standards are shown in

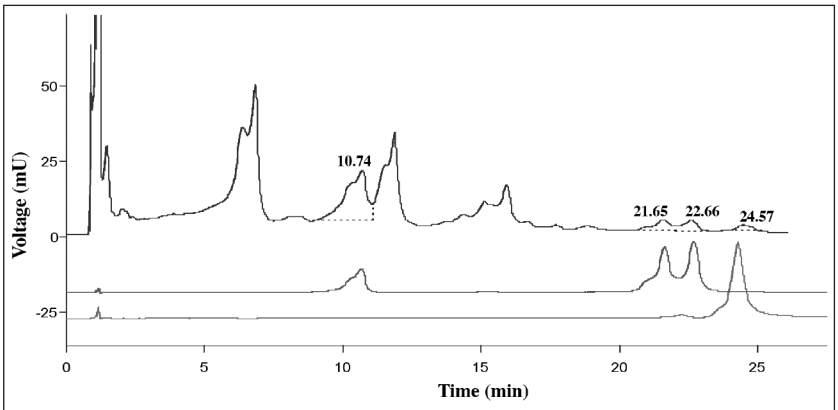

Figure 2. Chromatograms of flavonoid standards (lower and middle line) and *L. carthamoides* extract (upper line).

Table I. HPLC Characteristics of Flavonoids*

Compound	Equation of the calibration curve	Correlation coefficient	Retention time (min)
3.	$y = 9{,}58459x$	$r = 0{,}999965$	10.74
4.	$y = 21{,}4742x$	$r = 0{,}999636$	21.65
1.	$y = 9{,}63225x$	$r = 0{,}999917$	22.66
2.	$y = 34{,}7840x$	$r = 0{,}999937$	24.57

* Eriodictyol = 1; patuletin = 2; eriodictyol-7-β-glucopyranoside = 3; and 6-hydroxykaempferol-7-*O*-(6''-*O*-acetyl-β-D-glucopyranoside) = 4.

Figure 2 as the middle and lower line, while the chromatogram of *L. carthamoides* extract is situated at the top of the Figure 2. The results of correlation coefficients in Table I show good reproducibility of the analytical method. Calculated LOD are for the compounds were are follows: eriodictyol, 100 ng/mL; patuletin, 50 ng/mL; eriodictyol-7-β-glucopyranoside, 100 ng/mL; and 6-hydroxykaempferol-7-*O*-(6"-*O*-acetyl-β-D-glucopyranoside), 50 ng/mL. The values of retention times of flavonoids are shown in Table I and Figure 2.

In this article, quantitative determination of four flavonoids in *Leuzea carthamoides* by HPLC is presented. The optimal extraction procedure and isocratic HPLC program were developed to separate all four flavonoids. The assay is simple, rapid, sensitive, and reproducible. With the minor variations (e.g. prolongation of the analysis time, or change to the gradient elution) this assay could be applied for not only quantification of four flavonoids, but also for the quality evaluation of other constituents of *L. carthamoides*.

Acknowledgments

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