

Cirsium vulgare leaves: isolation and identification of phenolic compounds

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There are a few new researches on the pharmaceutical and nutraceutical application of *Cirsium vulgare*, but they do not reflect the full potential use of this plant. For the best of our knowledge, no study has been conducted so far to determine the impact of extraction methods and conditions on the extraction yields of phenolic compounds from *Cirsium vulgare*. We aimed to investigate a simple and sensitive HPLC-PDA method to determine phenolic compounds in *Cirsium vulgare* extracts. Three extraction techniques as maceration (ME), ultrasound-assisted extraction (UAE) and heat-reflux extraction (HRE) were applied and compared for sample preparation to obtain a high recovery of the phenolic compounds. The developed HPLC-PDA method can be used for the preparations of extracts with a high content of bioactive compounds for both pharmaceutical and nutraceutical applications. Moreover, it was validated and used for the qualitative and quantitative evaluation of phenolic compounds. A simple extraction procedure for phenolic compounds from *Cirsium vulgare* leaves was optimized in this work through the use of the heat-reflux extraction method with 50% ethanol and extraction time of 1.5 h.

Keywords: *Cirsium vulgare*, bull thistle, phenolic compounds, apigenin-7-O-glucoside, rutin, isoquercitrin, hyperoside, chlorogenic acid, HPLC

INTRODUCTION

Cirsium vulgare (Savi) Ten. is also known as spear thistle, bull thistle or common thistle. It is a species of the Asteraceae, genus *Cirsium*. This plant is known as a biennial native in most of Europe, Western Asia and Northwestern Africa. Bull thistle is an intrusive weed that grows in meadows, orchards, roadsides and among cereals. This plant bears leaves that end in extended, very sharp thorns and are beige in colour. *Cirsium vulgare* produces a lot of seeds that have small feathers and are fixed to the base by means of a ring until they mature. The roots of this plant are taproots. They are thin and run deep, and can develop several smaller lateral roots [1]. A few decades

before, bull thistle was only used as a folk medicine herb. History shows that bull thistle was known for its therapeutic properties in the treatment of sore jaws, rheumatic joints and bleeding piles. A hot infusion of the whole plant has been used as a herbal steam for treating rheumatic joints. Bleeding piles have been treated by a decoction of the whole plant, used both internally and externally [2]. In the past twenty years, *Cirsium vulgare* has received a few studies about its hepatoprotective [3], anti-oxidant [4], anti-bacterial [5], anti-rheumatic [6–7] effects and neurological disease treatment [8]. Other plants from the Asteraceae family, *Cirsium* genus, also showed anti-cancer [9–11], anti-diabetic [12] effects, Alzheimer's disease [13] and osteoarthritic [14] treatments, but no studies have been performed with our main studied plant

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Cirsium vulgare with respect to these effects. Knowing that bull thistle has such a variety of effects on the living organism, it has been further explored that these effects can be caused by its active ingredients.

Studies show that the main active compounds in *Cirsium* genus plant material are flavonoids, sterols and triterpenes, alkaloids, polyacetylenes, acetylenes and hydrocarbons, sesquiterpene lactones, phenolic acids, lignans, and a few other compounds [15]. According to data provided in the literature, *Cirsium vulgare* accumulates apigenin-7-O-glucoside, which is biologically active in anti-inflammatory, anti-spasmodic and antibacterial effects. Genkwanin-4'-O-glucoside, which is cytotoxic, arabinosyl-galactose, kaempferol-3-O-glucoside, quercetin-3-galactoside and quercetin-3-O-glucoside have an anti-microbial effect [15–17]; phenolic acids in *Cirsium vulgare* plant material are p-coumaric, caffeic, ferulic, p-hydroxybenzoic, protocatechuic and vanillic acids [17–18]. The reported secondary metabolites in *Cirsium vulgare* are Δ -5-avenasterol, brassica-sterol, lupeol, β -sitosterol and stigmasterol.

Phenolic compounds are the main active compounds responsible for various effects. However, there is just a few studies about the determination of phenolic compounds in *Cirsium vulgare*. The aim of this study was to establish an optimal extraction method of phenolic compounds from *Cirsium vulgare* plant material. The extraction method of phenolic compounds from plant material should be simple, safe, inexpensive and suitable for industrial applications.

That is why we focused on finding the most conventional extraction methods which would fit the optimal extraction description in our research. These conventional extraction methods, including maceration and reflux extraction, usually use organic solvents and require a large volume of solvents and long extraction time. Maceration is a simple extraction method with the disadvantage of a low extraction efficiency and long extraction time, but its positive side is that it may be used for the extraction of thermolabile components. Reflux extraction is more efficient than maceration and requires less extraction time and solvent. It cannot be used for the extraction of thermolabile products. Ultrasound-assisted extraction (UAE), also called sonication, uses ultrasonic wave energy in the extraction. Ultrasound-assisted analysis accelerates the dissolution and diffusion of the solute as well as the heat transfer, which

improves the extraction efficiency. Other advantage of the ultrasound-assisted analysis is that this method includes a low solvent and energy consumption and reduces needed extraction temperature and time. This method is suitable for the extraction of a thermolabile and unstable compound and is commonly used for the extraction of many types of natural products [19]. As a consequence, we decided to use these three different extraction methods in our studies. Phenolic compounds obtained by applying three extraction techniques (ME, UAE and HRE) were analysed using the HPLC-PDA method. Results were compared in order to select the best yield of the main phenolic compounds from the plant material. This study represents a powerful tool for the extraction and analysis of phenolic compounds from *Cirsium vulgare* plant leaves and it can be used for the preparation of extracts with a high content of phenolic compounds for both pharmaceutical and nutraceutical applications.

EXPERIMENTAL

Plant materials

Bull thistle samples were collected at the Šiauliai Academy Botanical Garden. The object of the research was bull thistle leaves which were collected during May 2020. Plant leaves were dried at 40°C in a drying chamber. Bull thistle leaves were grounded to a fine powder using an Ultra Centrifugal Mill ZM 200 (Retsh, Hann, Germany). Grinding was performed using a 0.5 mm trapezoid hole sieve. The final moisture content of the herb was $7.37 \pm 0.3\%$.

Solvents and reagents

HPLC-grade and analytical-grade reagents were used: trifluoroacetic acid, acetonitrile (Sigma Aldrich, Steinheim, Germany); standards of rutin, apigenin, chlorogenic acid, hyperoside and isoquercitrin (Sigma Aldrich, Steinheim, Germany); ethanol (96%) (Vilniaus Degtinė, Vilnius, Lithuania). The water used for sample preparation was produced using a Super Purity Water System (Millipore, USA).

Extraction of plant material

Ultrasound-assisted extraction (UAE)

0.1 ± 0.001 g of dried and milled leaves was mixed with 50% ethanol in a 30 mL round bottom flask. Extraction was performed using an ultrasound bath

(frequency 38 kHz) (Cambridge, UK, Grant Instruments TM XUB12 Digital) for 10, 15, 30, 45 and 60 min. The obtained extract was filtered through 0.22 µm PVDF syringe filters.

Heat-reflux extraction (HRE)

0.1 ± 0.001 g of dried and milled leaves was mixed with 50% ethanol in a 250 mL round bottom flask and was placed under reflux for 30 min, 1, 1.5, 2 and 3 h at 90°C temperature. The obtained extract was filtered through 0.22 µm PVDF syringe filters.

Maceration extraction (ME)

0.1 ± 0.001 g of dried and milled leaves was mixed with 50% ethanol in a 250 mL round bottom flask and extraction was performed for 6, 12, 24 and 48 h. The obtained extract was filtered through 0.22 µm PVDF syringe filters.

HPLC-PDA conditions

Qualitative and quantitative analysis was carried out on a Waters Alliance 2695 liquid chromatograph equipped with a Waters 996 photodiode array detector (PDA) and an ACE C18 (250 × 4.6 mm × 5 µm) column (Advanced Chromatography Technologies, Aberdeen, Scotland). The mobile phase consisted of solvents A (trifluoroacetic acid (0.1%)) and B (acetonitrile). The linear gradient elution profile was as follows: 95% A/5% B at 0 min, 85% A/15% B at 8 min, 80% A/20% B at 30 min, 60% A/40% B at 48 min, 50% A/50% B at 58–65 min, 5% A/95% B at 66–70 min and 95% A/5% B at 71 min. The flow rate was 1 mL/min, and the injection volume was 10 µL. Absorption was measured in the range from 330 to 360 nm. Detection of phenolic compounds was performed using standards of apigenin-7-O-glucoside, rutin, isoquercitrin, hyperoside and chlorogenic acid.

The extraction conditions were evaluated on the basis of the amount of five main identified compounds: apigenin-7-O-glucoside, chlorogenic acid, rutin, hyperoside and isoquercitrin.

Statistical analysis

Data is presented as the mean ± standard deviation (SD). All experiments were performed in triplicate. Statistical analysis of the results was performed with SPSS 25.0 (IBM Corporation, Armonk, NY, USA). One-way ANOVA was used to investigate the differences between extractions. Post hoc com-

parisons of the means were performed according to the Tukey's HSD test. The level of significance was taken as value of $p < 0.05$.

RESULTS AND DISCUSSION

In many studies, methanol is used as a solvent for the quantitative and qualitative evaluation of phenolic compounds. Various phenolic compounds were identified in *Cirsium vulgare* or other *Cirsium* species. According to Sieliwoniuka et al., the identified polyphenols in methanolic extracts from *C. vulgare* inflorescences were the following: chlorogenic acid, caffeic acid, luteolin 7-O-glucoside, quercetin 3-O-glucoside, kaempferol 3-O-galactoside, apigenin 7-O-glucoside, apigenin 7-O-glucuronide, kaempferol 3-O-rhamnoside, apigenin 7-O-methylglucuronide and apigenin. The qualitative composition of other extracts was analogous to the composition of the methanolic extract. An exception was the aqueous extract in which they did not detect apigenin 7-O-methylglucuronide and apigenin – compounds with a low polarity [20]. Nazaruk et al. investigated the aqueous extracts of various *Cirsium* species. The results showed that chlorogenic acid was detected in all cases, gallic acid dominated in *Cirsium palustre*, and in *Cirsium vulgare* only traces of phenolic acids (protocatechuic, chlorogenic, vanillic, caffeic and p-coumaric acid) were observed [21]. The same author, Nazaruk et al. (2017), performed other experiment with petrol and chloroform-made extracts. Fatty acids (0.1 mg/g), phytosterols (total 76.9 mg/g) and triterpenes (155.9 mg/g) were determined using extracts obtained with petrol and fatty acids (58.0 mg/g), phytosterols (total 45.5 mg/g) and triterpenes (48.8 mg/g) were determined using extracts obtained with chloroform from *Cirsium vulgare* flower heads [22]. Using RP-HPLC, Kozyra et al. determined the presence of free phenolic acids, which were gallic, protocatechuic, gentisic, hydroxybenzoic, vanillic and caffeic acids found, and additionally chlorogenic and syringic acids were identified. After acid hydrolysis, p-coumaric and ferulic acids were detected, while after alkaline hydrolysis – caffeic, ferulic and p-coumaric acids were identified [23]. Based on the results of other research, the determination of phenolic compounds such as rutin, apigenin and chlorogenic acid in *Cirsium arvense* and *Cirsium rivulare* species were performed [24]. Based on the studies performed by other scientists,

HPLC methodology using eight standards was used. In this study, scientists used naryangin, valic acid, chlorogenic acid, caffeic acid, rutin, luteolin, apigenin and p-coumaric acid as standards. The results of these studies showed that *Cirsium vulgare* extracts contained chlorogenic acid, rutin and p-coumaric acid [25]. No other studies identified isoquercitrin as an active compound in methanolic or ethanolic extract, except us. There are some studies with other *Cirsium* species – *Cirsium setosum* – whose results showed the identification of flavonoid quercetrin as an active compound using liquid chromatography-mass spectrometry method [26]. Some studies with methanolic extracts identified apigenin-7-O-glucoside in other *Cirsium* species, such as *C. ligulare*

(27.75 mg/g), *C. decussatum* (very small amount) and *C. eriophorum* (3.06 mg/g) [27]. Kozyra et al. (2015) performed RP-HPLC analysis, which enabled identification of the following bioactive substances: chlorogenic, caffeic, p-coumaric, protocatechuic, p-hydroxybenzoic, vanillic, syringic and trans-cinnamic acids, luteolin-7-glucoside, apigenin-7-glucoside, kaempferol-3-glucoside, linarin, apigenin, rutoside, luteolin and kaempferol in *Cirsium canum* inflorescences [28].

The HPLC-PDA method was validated and used for the qualitative and quantitative evaluation of phenolic compounds. The validation data are provided in the Table. Five main phenolic compounds were identified in the extracts (Fig. 1).

Table. Validation data

Active compound	Limit of detection	Limit of quantification	Range	R ²	Repeatability	Accuracy	Formula
Chlorogenic acid	0.03 µg/ml	0.11 µg/ml	14.5–0.113 µg/ml	0.9999	0.21%	0.32%	3.14e+004X+5.89e+001
Apigenin-7-O-glucoside	0.320 µg/ml	0.078 µg/ml	20.0–0.625 µg/ml	0.9999	0.41%	0.63%	3.53e+004X-1.41e+002
Rutin	0.099 µg/ml	0.198 µg/ml	12.65–0.790 µg/ml	0.9999	0.43%	0.98%	1.51e+004X-1.53e+003
Hyperoside	0.132 µg/ml	0.264 µg/ml	16.9–0.528 µg/ml	0.9997	0.47%	1.02%	2.13e+004X+5.81e+002
Isoquercitrin	0.109 µg/ml	0.217 µg/ml	27.85–0.870 µg/ml	0.9999	0.45%	0.86%	2.14e+004X+2.09e+003

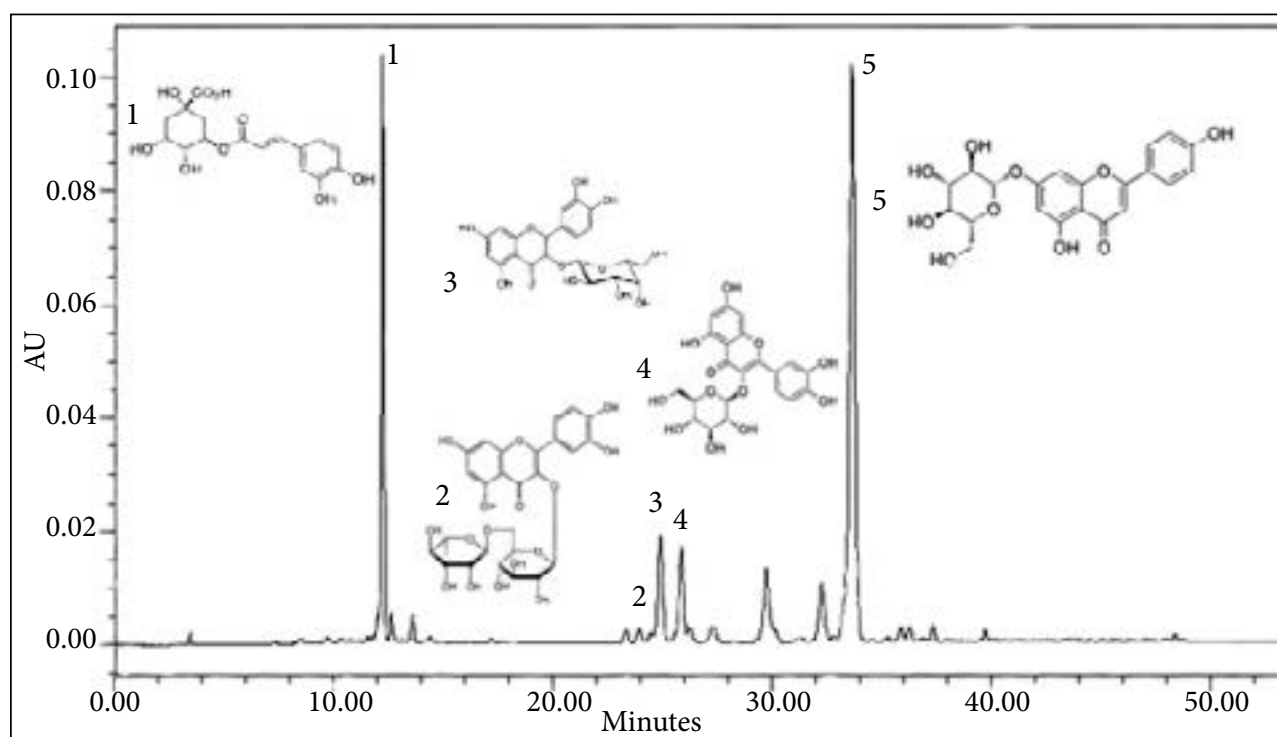


Fig. 1. Phenolic compounds identified in *Cirsium vulgare* extracts: 1, chlorogenic acid; 2, rutin; 3, hyperoside; 4, isoquercitrin; 5, apigenin-7-O-glucoside

Solvent selection is one of the most important steps of the extraction. The extraction of natural products progresses through the following stages: the solvent penetrates into the solid matrix, the solute dissolves in the solvents, the solute is diffused out of the solid matrix and the extracted solutes are collected. Any factor enhancing the diffusivity and solubility in the above steps will facilitate the extraction. The properties of the extraction solvent, the particle size of the raw materials, the solvent-to-solid ratio, the extraction temperature and the extraction duration will affect the extraction efficiency. The selection of the solvent is crucial for the solvent extraction. Alcohols (ethanol and methanol) are most common and universal solvents in the solvent extraction for phytochemical investigation. The greater the solvent-to-solid ratio is, the higher the extraction yield is [19].

Depending on the polarity of the phenolic compounds, alcohols such as methanol and ethanol were considered to be most suitable, but the literature data showed that the best solvent for the extraction of bioactive substances from *Cirsium vulgare* should be methanol or diethyl ether [4]. Our study showed significantly better results with ethanol as a solvent than with water used as an extractant. Figure 2 shows significantly different yields of the phenolic compounds used in our research in applying different solvents for the extraction: water and ethanol 50%. As it follows, using water as a solvent, no isoquercitrin, hyperoside and rutin were detected in our extracts made with water as a solvent compared to extracts made with ethanol.

Having evaluated the influence of the concentration of ethanol used for the extraction (from 50 to 96%) on the content of the main phenolic compounds, the obtained results showed a significant difference. The highest yield of active compounds such as apigenin-7-O-glucoside (6.958 ± 0.35 mg/g) and chlorogenic acid (9.038 ± 0.45 mg/g) was obtained by the extraction with 50% ethanol. This concentration was selected. Since ethanol possesses the most appropriate polarity of the above-mentioned compounds, 50% ethanol was selected as an extraction solvent for further studies.

The experiments with extracts showed the correlation between the extraction time and the amount of phenolic compounds. For example, during the maceration, the best time to extract the main phenolic compounds was 6 h, except for apigenin-7-O-glucoside, which was best to detect after 48 h. The highest amounts of extracted phenolic compounds, using maceration, were the following: rutin 0.1523 ± 0.008 mg/g, hyperoside 0.8199 ± 0.041 mg/g, isoquercitrin 0.6968 ± 0.035 mg/g, chlorogenic acid 3.7418 ± 0.187 mg/g and apigenin-7-O-glucoside 9.0057 ± 0.45 mg/g (Fig. 3).

The experiment also showed the impact of time on the ultrasound-assisted extraction. The main phenolic compounds found in extracts, except chlorogenic acid, demonstrated the highest amounts of compounds after 30 min of the ultrasound-assisted extraction. The highest amounts of extracted phenolic compounds, using the ultrasound-assisted extraction, were those: rutin 0.1695 ± 0.008 mg/g,

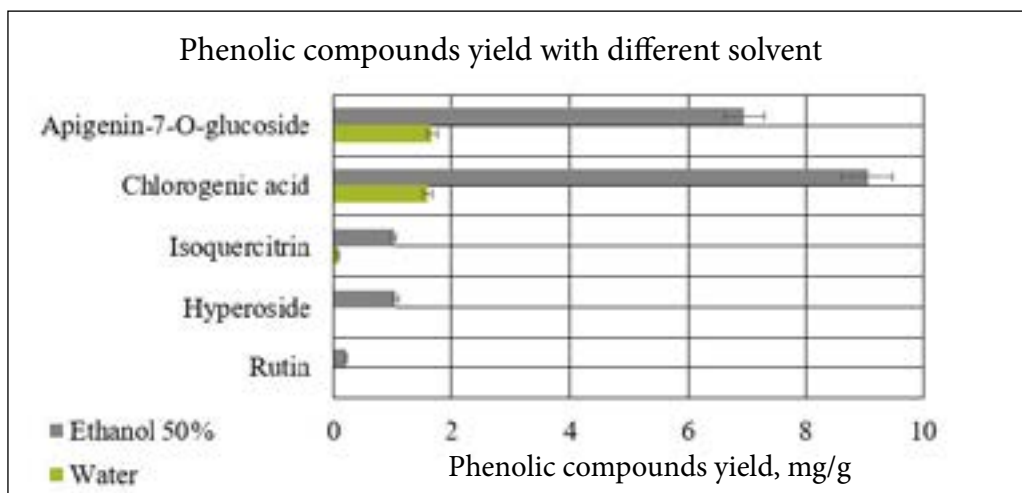


Fig. 2. The yield of phenolic compounds with different solvent

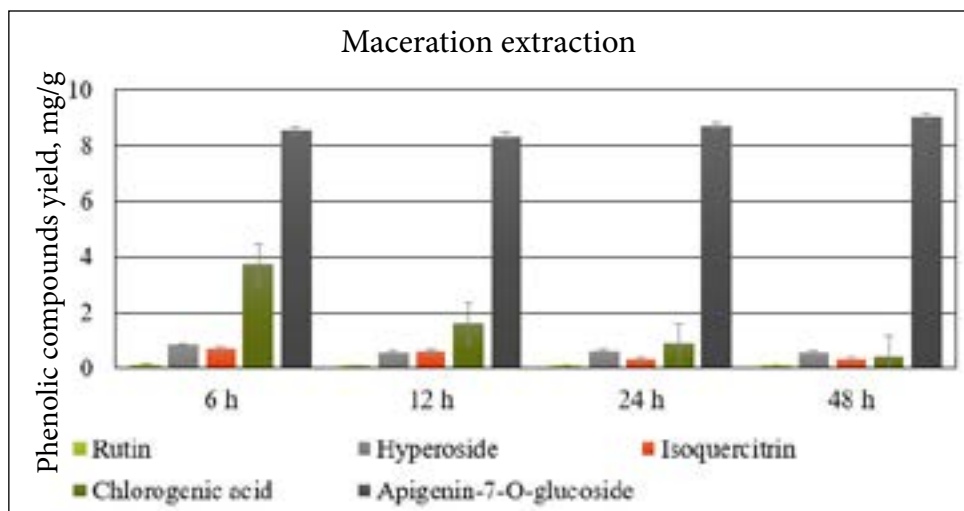


Fig. 3. Maceration extraction (ME) time and the yield of phenolic compounds

hyperoside 0.8638 ± 0.043 mg/g, isoquercitrin 0.873 ± 0.044 mg/g and apigenin-7-O-glucoside 7.604 ± 0.38 mg/g. The highest amount of chlorogenic acid was determined using the ultrasound-assisted extraction for 15 min (chlorogenic acid 7.314 ± 0.366 mg/g) (Fig. 4).

The highest amounts of flavonoids were obtained throughout heating with reflux: rutin 0.2372 ± 0.012 mg/g, hyperoside 1.331 ± 0.067 mg/g, isoquercitrin 1.2624 ± 0.063 mg/g, chlorogenic acid 13.098 ± 0.655 mg/g and apigenin-7-O-glucoside 9.915 ± 0.496 mg/g. No dependence on the extraction time was determined in this case. The best results were obtained after 90 min by the heat-reflux extraction (Fig. 5).

In summary, the best extraction conditions for phenolic compounds were heat-reflux extraction for 90 min. Apigenin-7-O-glucoside and chlorogenic acid were mainly detected by this method.

In order to obtain a high yield of phenolic compounds, several extraction techniques, including maceration, ultrasound-assisted extraction and heat-reflux extraction, were used in this study. Each extraction technique was optimized through the appropriate adjustment of the extraction time. The extraction time and method validation have not been used before for the determination of phenolic compounds in *Cirsium vulgare* plant leaves. The extraction time was only studied in some research performed with Asteraceae family herbs, for

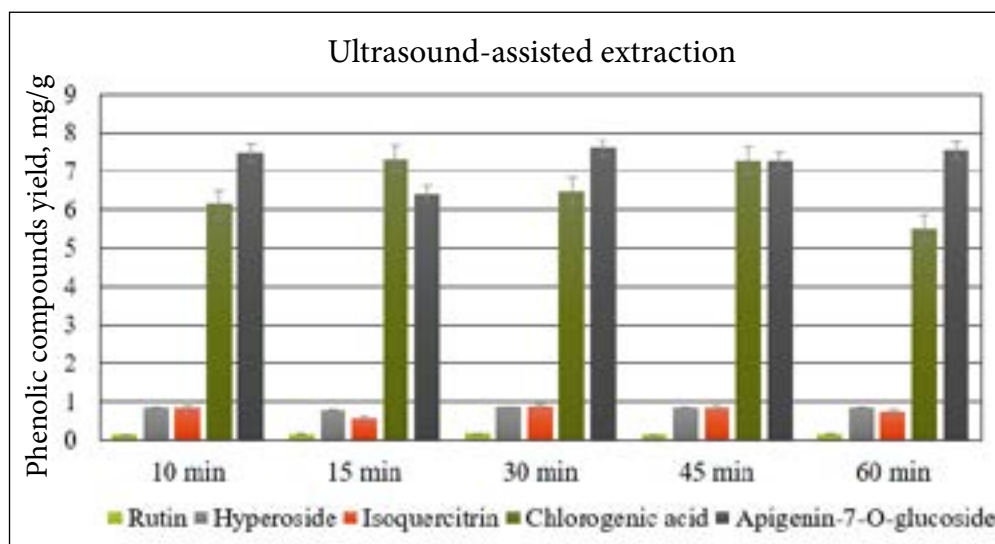


Fig. 4. Ultrasound-assisted extraction (UAE) and the yield of phenolic compounds

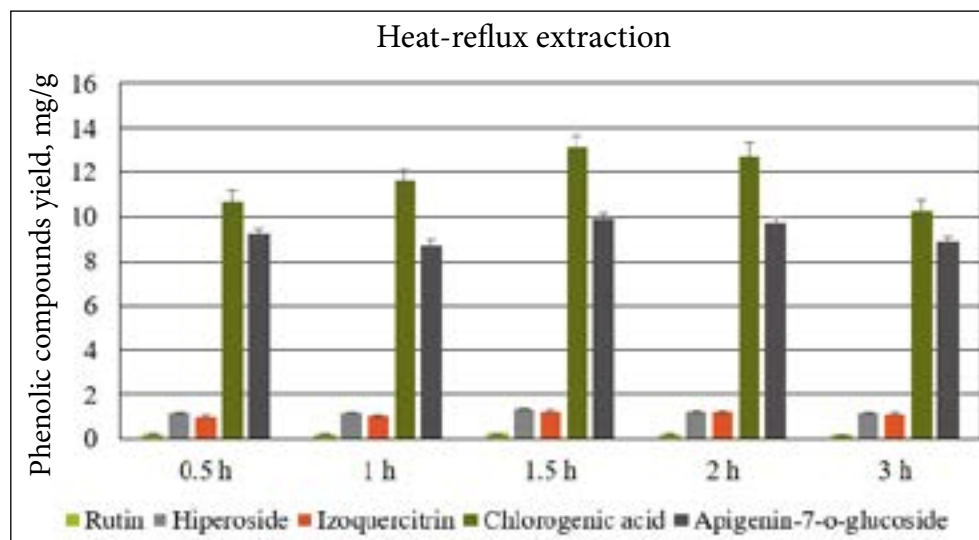


Fig. 5. Heat-reflux extraction time and the yield of phenolic compounds

example, *Calendula officinalis*. This study showed that the extraction time for the effect of the extraction method on the recovery of rutin, made a big impact in this research. Martins et al. pointed out that the ultrasound-assisted extraction and maceration extraction results of rutin yields were better with the time of extraction less than 35 min [29]. The impact of extraction time performing heating with reflux for the determination of phenolic compounds has not been described with Asteraceae family plants before.

As there are just a few studies performed on the *Cirsium vulgare* plant, we tried to compare and examine the extract method validations of other *Cirsium* species, for example, *Cirsium yildizianum*. According to Martinez et al., the best extraction method for *Cirsium* species, *C. yildizianum*, was homogenizer-assisted extraction. HAE methanol extract presented the highest phenolic (37.10 mg gallic acid equivalent/g) and flavonoid (46.78 mg rutin equivalent/g) contents [30].

Comparing the same extraction methods in the Asteraceae family, there were some researches which investigated extraction methods such as maceration and ultrasound-assisted extraction. The results showed that using extrahent ethanol and ultrasound-assisted extraction rutin yield was larger than using maceration extraction [29]. Other study showed that using different methods of extract obtainment (90°C hot-air, 70°C hot-air, shade-, and freeze-drying) the high-performance liquid chromatography-electrospray ionization-

mass spectrometry (HPLC-ESI-MS) aimed at a significant decrease of the total phenolic contents of *Cirsium japonicum* under the hot-air-drying condition, especially the chlorogenic acid content. Chlorogenic acid has been reduced for 85 and 60% for 90 and 70°C hot-air-drying, respectively [31].

With reference to the study results, it can be concluded that by heat-reflux extraction for 90 min using 50% ethanol as a solvent can cause the highest yield of phenolic compounds.

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CIRSIIUM VULGARE LAPAI: FENOLINIŲ JUNGINIŲ IŠSKYRIMAS IR IDENTIFIKAVIMAS

Santrauka

Nors yra keletas naujų tyrimų apie dygiosios usnies (*Cirsium vulgare*) panaudojimą farmacijoje ir mityboje, jie neatspindi visų šio augalo teikiamų galimybių. Kiek mums žinoma, iki šiol nebuvo atlikta tyrimų, kaip ekstrahavimo metodas ir sąlygos paveikia fenolinių junginių iš *Cirsium vulgare* išėigą. Mūsų tikslas: pritaikyti paprastą ir jautrų HPLC-PDA metodą fenoliniams junginiams *C. vulgare* ekstraktuose nustatyti. Siekiant išgauti kuo didesnę fenolinių junginių išėigą, mėginio ruošimui pritaikyti trys ekstrahavimo būdai: maceracija (ME), ekstrakcija ultragarsu (JAE) ir *heat-reflux* ekstrakcija (HRE). HPLC-PDA metodu galima paruošti ekstraktus, kuriuose yra daug biologiškai aktyvių junginių, farmacijos ir / ar mitybos reikmėms. Dar jis tinkamas kokybiniam ir kiekybiniam fenolinių junginių nustatymui. Studijos metu pritaikyta paprasta fenolinių junginių ekstrakcijos iš *Cirsium vulgare* lapų metodika naudojant 1,5 valandos trukmės *heat-reflux* ekstrakciją su 50 % etanoliumi.