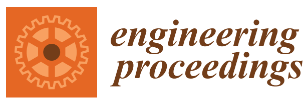

Proceeding Paper

Characterization of Phenolic Compounds of *Arnica montana* Conventional Extracts †

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Abstract: *Arnica montana* L. (Asteraceae family) is a plant commonly used in traditional medicine, and several reports have characterized this plant's bioactivities, especially its phenolic compounds. These compounds are well known for their numerous beneficial biological properties. Consequently, industry stakeholders from the feed, food, cosmetic, and pharmaceutical sectors are seeking extracts rich in phenolic compounds, which could be interesting for the development of bio-based applications. The objective of the present study was to characterize the phenolic profile of this species to lay the groundwork for further optimization studies to obtain the highest amount of phenolic compounds. Therefore, *A. montana* was extracted with an ethanol/water ratio of 80:20 (*v*/*v*) at room temperature for 1 h, and phenolic compounds were identified and quantified through UPLC (HPLC Dionex Ultimate 3000) with a mass detector (TSQ Quantis). In the extract, phenolics belonging to different groups were identified, namely eriodictyol-*O*-glucuronide (flavanone), hispidulin and luteolin (flavones), kaempferol and 6-methoxykaempferol, (flavonols), p-coumaric, feruloylquinic, caffeoylquinic, and dicaffeoylquinic isomers (hydroxycinnamic acids). However, only four of them could be quantified: kaempferol and the three hydroxycinnamic acids. The total phenolic content (mg/g of dry sample) was estimated to be 27.34 mg/g, with the most prevalent compounds being the dicaffeoylquinic acids (accounting for 79.5% of the total phenolics). It has been demonstrated that dicaffeoylquinic acids present anti-inflammatory and antioxidant activities, which have been linked to several beneficial effects. Thus, obtaining phenolic-rich extracts of *A. montana* may allow us to exploit this plant's significant biological properties, and it could be a new ingredient for developing new applications in the nutraceutical, cosmetic, and/or pharmaceutical industries.

Keywords: *Arnica montana*; phenolic compounds; extraction; characterization; caffeoylquinic acid

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1. Introduction

Arnica montana (AM) L., belonging to the Asteraceae family, is a plant species widely used in traditional medicine globally. It has been employed to treat various disorders (mainly inflammatory problems), such as skin inflammation, bruises, sprains, or rheumatic pain. In fact, this plant has been reported to exhibit antioxidant, anti-inflammatory, and

antimicrobial effects [1]. Recently, there has been a growing interest in this species and its possible industrial applications. Different bioactive compounds have been identified in AM, including sesquiterpene lactones, which are associated with anti-inflammatory properties, and phenolic compounds (mainly phenolic acids and flavonoids), known for their antioxidant and antimicrobial activities [2]. Nowadays, phenolic compounds are one of the most studied groups of bioactive compounds in plants, algae, and foods. Numerous scientific studies have supported their beneficial properties in enhancing human health (including antioxidant, anti-inflammatory, antimicrobial, and anticancer properties, as well as many others) at in vitro, in vivo, and clinical levels [3,4]. Consequently, industry stakeholders in the feed, food, cosmetic, and pharmaceutical sectors are actively searching for extracts rich in phenolic compounds, which could be valuable for the development of bio-based applications. Therefore, the objective of the present study was to characterize the phenolic profile of this species to lay the groundwork for further optimization studies to obtain phenolic-rich extracts that could serve as new ingredients for bio-based applications.

2. Materials and Methods

2.1. Sample Preparation and Extraction

Dried flowers of *Arnica montana* L. (AM) were purchased from Pinisan (Madrid, Spain) on November 2022. The samples were crushed and sieved to obtain a homogeneous matrix. Conventional extraction was performed as follows: two grams of sample were mixed with 40 ethanol/water 80:20 (*v*/*v*), obtaining a solid/liquid ratio of 50 g/L, and the mixture was constantly stirred for one hour at room temperature. The extracts were centrifuged at 3500 rpm, and the supernatant obtained was vacuum-filtered. Then, aqueous ethanol extracts were filtered via syringe filters of 0.2 µm pore size and transferred to sample vials prior to injection for further analysis.

The extraction efficiency, calculated based on the dry weight of the extract after evaporation of the solvent, was also evaluated, and in this study, it is expressed as mg of extract per g of sample (mg E/g S). For this, 5 mL of extracts were transferred into previously prepared crucibles. The crucibles were then subjected to a 24 h drying period at 60 °C in the dark, followed by an additional 24 h drying period at 104 °C until complete dryness. Then, the crucibles were weighed. The mass of dry residue in relation to the initial mass was calculated to determine the extraction yield.

2.2. Identification and Quantification of Phenolic Compounds

HPLC-MS/MS analysis of phenolic profile was carried out in a Dionex Ultimate 3000 UPLC+ (Thermo Scientific, Waltham, MA, USA) system coupled with a triple quadrupole mass spectrometer TSQ Quantis (Thermo Scientific, USA). Compound separation was carried out with a Waters Spherisorb S3 ODS-2C18 (3 µm, 4.6 mm × 150 mm, Waters, Milford, MA, USA) column thermostatted at 35 °C. The solvents employed were (A) 0.1% formic acid in water and (B) acetonitrile. The elution gradients employed were 15% B (5 min), 15–20% B (10 min), 20–25% B (10 min), 25–35% B (10 min), 35–50% B (10 min), and the column was subjected to re-equilibration using a flow rate of 0.5 mL/min. The sample injection volume was 10 µL, and after chromatographic separation, the eluate was introduced into the triple quadrupole mass spectrometer.

Mass detection was performed using a TSQ Quantis (ThermoFinnigan, San Jose, CA, USA) equipped with an electrospray ion (ESI) source working in negative mode. Mass analysis was performed via selected reaction monitoring (SRM). The following parameters were used as universal conditions: sheath gas—30 Arb; auxiliar gas—10 Arb; ion transfer tube temperature—325 °C; and vaporizer temperature—350 °C. In order to determine the optimal conditions for identification and quantification, the SRM parameters of each compound were optimized (precursor/product ion combination, retention time, collision energy, and RF lens voltage). Whenever feasible, standards were utilized in this process, and prior research findings were taken into account for guidance. Quantification was performed using the calibration curves of commercially available phenolic standards.

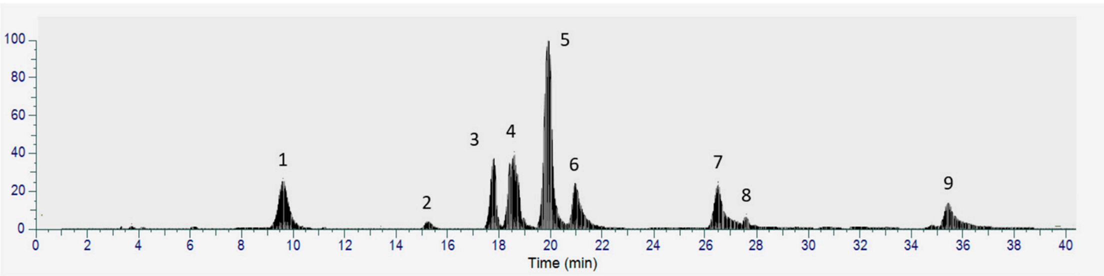
Total phenolic compound contents were calculated as the sum of the quantifiable compounds. The results are expressed in mg per g of dry sample (mg/g S). The purity of the extract in phenolic compounds was also calculated, and in this study, these values are expressed as mg of total phenolics per g of dry extract (mg/g E).

3. Results and Discussion

3.1. Characterization of AM Conventional Extracts

The SRM scan mode allows for the simultaneous identification and quantification of the compounds present in the sample, enabling the monitoring of the fragmentation of the selected precursor ions into product ions. For this work, the AM phenolic compounds were first investigated using a compilation of phenolics and insights from previous studies [5,6]. The objective was to initially characterize the compounds present in the conventional extracts. In total, up to 50 different phenolic compounds were investigated in the samples. After characterization, only the compounds positively identified were chosen for the further optimization of the most favorable conditions for identification and quantification.

Figure 1 displays the SRM chromatogram of the conventional AM extracts, highlighting the identified phenolic compounds. Most compounds belong to the hydroxycinnamic acids class, including caffeoylquinic acid (*m*/*z* 353), feruloylquinic acid (*m*/*z* 367), p-coumaric acid (*m*/*z* 163), and three di-caffeoylquinic acid isomers (*m*/*z* 515), corresponding to peaks 1, 2, 3, 4, 5, and 6. The remaining peaks correspond to one flavanone, two flavones, and two flavonols. In peak 4, eriodictyol-*O*-glucuronide (*m*/*z* 463), the only flavanone, co-eluted with a di-caffeoylquinic acid isomer. In peak 7, luteolin and kaempferol (both *m*/*z* 285) also co-eluted, but their distinct fragmentation pattern allowed us to identify them separately. Finally, peaks 8 and 9 correspond to 6-methoxykaempferol (*m*/*z* 315) [7] and hispidulin (*m*/*z* 299).



Peak	RT	[M-H]- (*m*/*z*)	Transitions (*m*/*z*)	Identification
1	9.60	353	178, 191	Caffeoylquinic acid
2	15.25	367	134, 191	Feruloylquinic acid
3	17.78	163	93, 119	*p*-coumaric acid
4	18.60	463	271, 300	Eriodictyol-*O*-glucuronide
		515	191, 353	Dicaffeoylquinic acid isomer
5	19.91	515	191, 353	Dicaffeoylquinic acid isomer
6	20.95	515	191, 353	Dicaffeoylquinic acid isomer
7	26.50	285	239, 187	Kaempferol
		285	151, 133	Luteolin
8	27.58	315	271, 300	6-Methoxykaempferol
9	35.41	299	198, 271	Hispidulin

Figure 1. SRM chromatogram of conventional AM extracts and retention time and mass spectral data of identified phenolic compounds.

Considering the good results achieved when identifying the phenolic compounds, a more in-depth study to validate the present HPLC-MS/MS method in terms of linearity, precision, repeatability, stability, and recovery has been planned [8].

3.2. Quantification of Phenolic Compounds

For the quantification of the identified phenolic compounds, standards were only available for kaempferol. Thus, quantification was carried out using compounds from similar

groups; ferulic acid was employed to quantify the hydroxycinnamic acids, kaempferol was employed to quantify the flavonols, and luteolin was employed to quantify the flavones as well as the flavanone.

Although up to nine compounds were identified, only kaempferol, p-coumaric, caffeoylquinic, and dicaffeolyquinic acids could be quantified. The total phenolic content was estimated to be 27.34 mg/g S, with the most prevalent compounds being the the dicaffeoylquinic acid isomers (accounting for 79.5% of the total phenolics), followed by kaempferol (18.4%) (Figure 2). Considering that the extraction yield was 159.4 mg E/g S, the purity of the conventional extract reached 171.6 mg/g E.

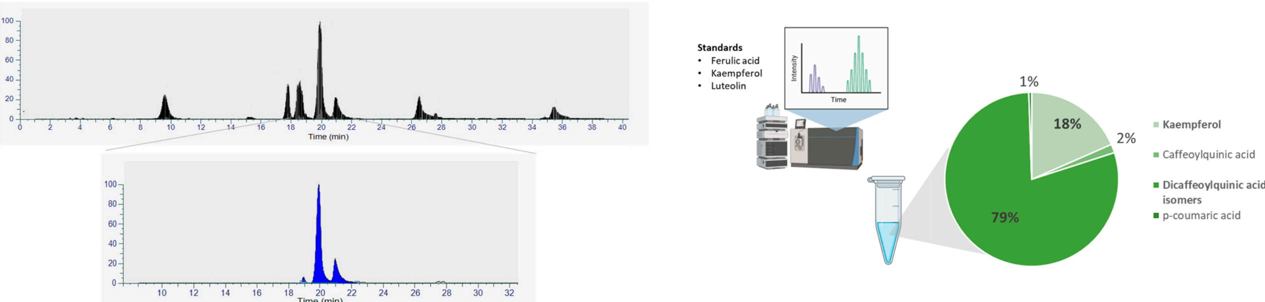


Figure 2. (**Left**) SRM chromatogram of all phenolic compounds (black) and the SRM of dicaffeoylquinic acid isomers (blue). (**Right**) percentage of quantified phenolic compounds in conventional extracts of AM.

Numerous studies have reported the beneficial effects of dicaffeoylquinic acid isomers, including their antioxidant, anti-inflammatory, and antimicrobial activities. For example, various dicaffeoylquinic acid isomers have exerted protective effects against Aβ-induced neurotoxicity in neuroblastoma SH-SY5Y cells in vitro, mainly through antioxidant mechanisms [9]. Another study showed that these compounds successfully prevented cancer proliferation and metastasis on breast cancer cell lines, inducing cell cycle arrest and apoptosis [10]. The biological potential of dicaffeoylquinic acids has been also assessed in animal models. For instance, in a study by Chen and colleagues, it was reported that 3,5-dicaffeoylquinic acid was the main bioactive compound in *Ilex kaushue* extracts, displaying significant anti-inflammatory effects in a lipopolysaccharide-induced acute lung injury model [11]. Similarly, kaempferol has been widely described as a potent bioactive compound with diverse properties, including antioxidant, anti-inflammatory, anticancer, antimicrobial neuroprotective, and hepatoprotective effects, as supported by in vitro, in vivo, and clinical studies [12–14]. Considering the previous studies that support the beneficial properties of these compounds, AM could be considered as a promising source of bioactive compounds. Thus, further studies are expected to increase the extraction yields and attainment of phenolic compounds. These extracts could have relevant applications in diverse fields, including the nutraceutical, cosmeceutical, pharmaceutical, and technological sectors.

4. Conclusions

For the present study, the phenolic compounds present in conventional *Arnica montana* extracts were characterized and quantified. In total, nine compounds belonging to four different phenolic classes were identified. The total phenolic content was estimated to be 27.34 mg/g of the sample, with the major compounds being dicaffeoylquinic acid isomers and kaempferol, accounting for 79.5 and 18.4% of the total content, respectively. Given the significance of these compounds as bioactive agents, this species could be considered as a matrix for further optimization studies for the attainment of phenolic- rich extracts for bio-based industrial applications.

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Conflicts of Interest: The authors declare no conflict of interest.

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