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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 their bioactives, especially phenolic compounds. These compounds are well known for their numerous beneficial biological properties. In consequence, industry from the feed, food, cosmetic and pharmaceutical sectors are seeking extracts with high content in phenolic compounds that 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, as a first step for further optimization studies to obtain the highest amount of phenolic compounds. Therefore, *A. montana* was extracted with ethanol:water 80:20 (*v*/*v*) at room temperature during 1 hour and phenolic compounds were identified and quantified through UPLC (HPLC Dionex Ultimate 3000) coupled to a mass detector (TSQ Quantis). In the extract, up to nice phenolics were identified, belonging to different groups, 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 in 27.34 mg/g, being the major compound dicaffeoylquinic acids (79.5 % of the total phenolics). It has been demonstrated that dicaffeoylquinic acids present anti-inflammatory and antioxidant potential, which have been linked to several beneficial effects. Thus, obtaining rich phenolics extracts of *A. montana* may display significant biological properties and could be a new ingredient for developing new applications in nutraceutical, cosmetic or pharmaceutical industries.

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

Citation: P. Garcia-Oliveira; F. Chamorro; P. Donn; P. Garcia-Perez; S. Seyyedi-Mansour; A. Silva; J. Echave; J. Simal-Gandara; L. Cassani Characterization of Phenolic Compounds of *Arnica Montana* Conventional Extracts. **2023**, *4*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s):

Received: date
Accepted: date
Published: date

1. Introduction

Arnica montana (AM) L., belonging to the Asteraceae family, is a 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, anti-cancer and many others) at in vitro, in vivo and clinical levels [3,4]. Consequently, industry 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, as a first step for further optimization studies to obtain phenolic-rich extracts, which could serve as new ingredients for bio-based applications.

2. Materials & Methods

2.1. Sample Preparation and Extraction

Dried flowers of *Arnica montana* L. (AM) were purchased from Pinisan (Madrid, Spain; www.pinisan.com). 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 remained for one hour at room temperature with constant stirring. The extracts were centrifuged at 3500 rpm and the supernatant obtained was vacuum filtered. Then, aqueous ethanol extracts were filtered by syringe filters of 0.2 μm pore size and transferred to sample vials prior to injection for further analysis.

The extraction efficiency was also evaluated, calculated based on the dry weight of the extract after evaporation of the solvent, expressed as mg of extract per g of sample (mg E/ g S). To do this, 5 mL of extracts were transferred into previously prepared crucibles. The crucibles were then subjected to a 24-hour drying period at 60°C in the dark, followed by an additional 24-hour 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

The HPLC-MS/MS analysis of phenolic profile was carried out in a Dionex Ultimate 3000 UPLC+ (Thermo Scientific, USA) system coupled to 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, USA) column thermostatted at 35 °C. The solvents employed were (A) 0.1% formic acid in water and (B) acetonitrile. The elution gradient employed was 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 re-equilibration of the column, with a flow rate of 0.5 mL/min. The injection volume of sample was 10 μL, and the eluate after chromatographic separation 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 in 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 curve of phenolic standards commercially available.

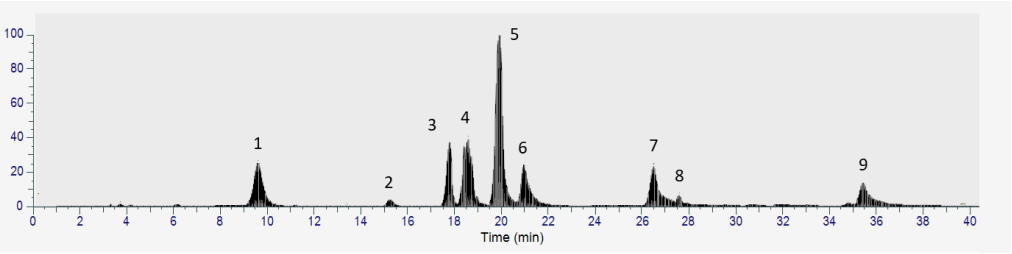
Total phenolic compounds were calculated as the sum of the quantifiable compounds. The results were expressed in mg per g of dry sample (mg/g S). The purity of the extract in phenolic compounds was also calculated, which was expressed as mg of total phenolics per g of dry extract (mg/g E).

3. Results and discussion

3.1. Characterization of AM Conventional Extracts

The SRM scan mode allows simultaneous identification and quantification of the compounds present in the sample, monitoring the fragmentation of the selected precursor ions into product ions. In this work, a first exploration of AM phenolic compounds was conducted 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 this 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 AM conventional extracts, highlighting the identified phenolic compounds. Most compounds belong to the hydroxycinnamic acids class: 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 with similar *m*/*z* 285, were also co-eluting, but their distinct fragmentation pattern allowed 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 AM conventional extracts and retention time and mass spectral data of identified phenolic compounds.

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

3.2. Quantification of Phenolic Compounds

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

group: ferulic acid was employed for quantifying hydroxycinnamic acids, kaempferol for flavonols and luteolin for 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. Total phenolic content was estimated in 27.34 mg/g S, being the major compounds the dicaffeoylquinic acid isomers (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.

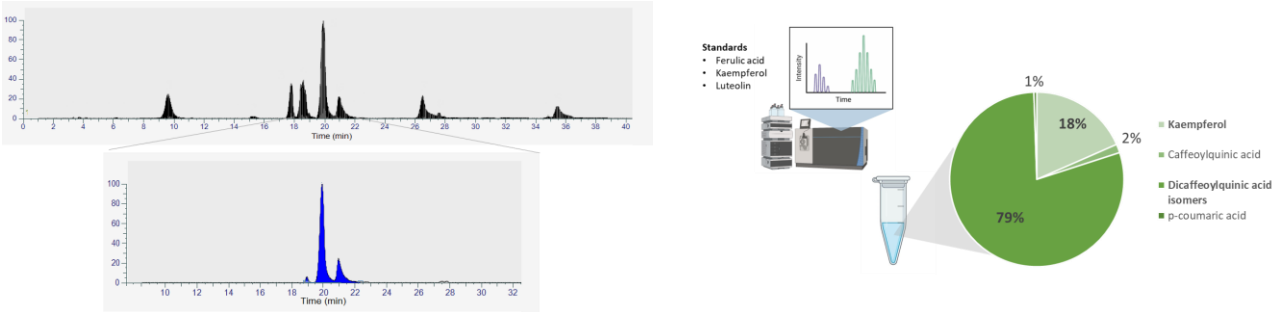


Figure 2. Left) SRM chromatogram of all phenolic compounds and the SRM of dicaffeoylquinic acid isomers. Right) percentage of quantified phenolic compounds in conventional extracts of AM.

Numerous studies have reported the beneficial effects of dicaffeoylquinic acid isomers, especially 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, the study of Chen and colleagues reported that 3,5-dicaffeoylquinic acid was the main bioactive compound in *Ilex kaushue* extract, 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 could be expected to increase the extraction yields and obtaining of phenolic compounds. These extracts could have relevant applications in diverse fields, including nutraceutical, cosmeceutical, pharmaceutical and technological sectors.

4. Conclusions

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

Author Contributions: Conceptualization, P.G.O., L.C. and M.A.P.; methodology, P.G.O and F.C.; validation, P.G.O., F.C. and L.C.; formal analysis, P.G.O. and P.G.P.; investigation, P.G.O., P.D and S.S.M.; resources, J.S.G. and M.A.P.; data curation, P.G.P.; writing—original draft preparation,

P.G.O., P.D and S.S.M.; writing—review and editing, L.C. and M.A.P.; visualization, P.G.O.; supervision, L.C. and M.A.P.; project administration, L.C. and M.A.P.; funding acquisition, J.S.G. and M.A.P. All authors have read and agreed to the published version of the manuscript.

Funding: The research leading to these results was supported by MICINN supporting the Ramón y Cajal grant for M.A. Prieto (RYC-2017-22891) and the contract for P. Garcia-Oliveira, by the European Union through the "NextGenerationEU" program supporting the "Margarita Salas" grant awarded to P. Garcia-Perez. The authors would like to thank the EU and FCT for funding through the programs UIDB/50006/2020; UIDP/50006/2020; LA/P/0008/2020 and also to Ibero-American Program on Science and Technology (CYTED— GENOPSYSEN, P222RT0117).

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