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CHEMICAL COMPOSITION, ANTI-INFLAMMATORY AND ANALGESIC ACTIVITIES OF *INULA VISCOSA* (L.) AITON AQUEOUS EXTRACT

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ABSTRACT. In the present study, the phenolic content of *Inula viscosa*, a medicinal plant traditionally used to treat inflammation in Algeria, was determined using ultra-high-performance liquid chromatography coupled with a diode array detector and an electrospray mass spectrometer (UHPLC-DAD-ESI-MS^2). The anti-inflammatory activity was evaluated *in vitro* using a nitric oxide scavenging assay and *in vivo* using an experimental model of carrageenan-induced acute inflammation. A tail pressure test was used to assess the analgesic activity. Additionally, an *in-silico* study was conducted to investigate further the interaction mechanisms between 1,5-*O*-dicaffeoylquinic acid (1,5-*O*-CQA), the main compound identified, and cyclooxygenases 1 and 2, two enzymes linked to inflammation. The results showed that the aqueous extract was rich in caffeoyl acid derivatives, mainly caffeoyl hexaric acids, described for the first time in this species. The biological activity assays demonstrated that *I. viscosa* exhibited strong anti-inflammatory and analgesic activities compared to ascorbic acid, a powerful antioxidant that can reduce inflammation by neutralizing free radicals. The main compound identified in the *I. viscosa* extract showed high affinity for the binding sites of cyclooxygenases 1 and 2 in the molecular docking study. Overall, the findings of this study further support the traditional use of *I. viscosa* as a remedy for inflammatory diseases.

KEY WORDS: Analgesic activity, Anti-inflammatory activity, *Inula viscosa*, Molecular docking

INTRODUCTION

Inflammation is a beneficial mechanism employed by the body to fight against pathogens. During an inflammatory reaction, immune cells are mobilized and play an essential role in neutralizing

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the pathogen, and numerous pro-inflammatory mediators such as cytokines, prostaglandin E2 (PGE2), and nitric oxide (NO) are also released [1]. This inflammatory system can sometimes become harmful when it malfunctions, leading to pathological conditions [2].

Inflammation therapy comprises key mechanisms that can be employed as therapeutic targets. Glucocorticoids and nonsteroidal anti-inflammatory drugs (NSAIDs) are considered to be the main classes of drugs used to control inflammation. Glucocorticoids act by inhibiting prostaglandin production through interaction with specific steroid receptors. The mode of action of NSAIDs is related to the inhibition of cyclooxygenase (COX) enzymes, which are responsible for converting arachidonic acid into prostaglandins. In addition to their action on inflammation, NSAIDs are also used to treat pain [3]. These drugs have long been used to control inflammation. Their use, particularly in chronic inflammatory pathologies requiring prolonged treatment, is, however, associated with numerous deleterious effects on human health [4]. Thus, more efforts should be made to discover new substances with anti-inflammatory activity. Medicinal plants, particularly those known for their traditional use in managing inflammation and pain, remain the only therapeutic resource for many populations. However, they must be regarded as a source of new active drugs or as inspiration for their synthesis.

Inula viscosa (L.) Aiton is a plant from the Asteraceae family, predominantly found in the Mediterranean, Africa, Europe, and Asia [5]. It is recognized in traditional medicine for its anti-inflammatory [6], antipyretic [7], antiseptic, and antiemetic [8] properties. It is also used to treat gastrointestinal disorders [9] and skin diseases [10, 11]. Studies have also shown that *I. viscosa* extracts possess antioxidant, antifungal [12], anti-inflammatory [13], antibacterial, hypoglycemic, hypolipidemic, anticancer, antiparasitic, and phytotoxic effects [14, 15].

The chemical composition of some *I. viscosa* extracts, namely the ethanol, methanol, and ethyl acetate fractions, has previously been reported [12, 16, 17]. Although these earlier studies described the phytochemical composition of *I. viscosa*, none included quantification. Only more recent research has provided both the profile and quantification of its specialized metabolites [15]. As far as we know, information concerning the chemical composition of the aqueous extract of this species has yet to be reported. Therefore, this study was conducted to offer further information regarding the chemical composition and bioactivity of this species. In this context, the aqueous extract from Algerian *I. viscosa* was evaluated for its anti-inflammatory and analgesic activities, as well as its chemical composition, to provide scientific evidence supporting the plant's traditional uses.

EXPERIMENTAL

Chemicals and reagents

Ascorbic acid, β-nicotinamide adenine dinucleotide (β-NADH), phenazine methosulfate (PMS), and nitro blue tetrazolium chloride (NBT) were purchased from Sigma (St. Louis, MO, USA). Standards used for the identification of phenolic compounds and for calibration curve preparation were obtained from EXTRA SYNTHESE (Genay Cedex, France). HPLC-grade acetonitrile and formic acid were purchased from Panreac (Barcelona, Spain). Carrageenan was obtained from Fluka (Bucharest, Romania). All other chemicals were of analytical grade.

Instruments and apparatuses

For the UHPLC-DAD-ESI-MS² and biological analyses, various instruments and apparatuses were used. The UHPLC system (Model: Ultimate 3000, Dionex Co., San Jose, CA, USA) was used for the separation of chemical constituents. A mass spectrometer (Model: LTQ XL, Thermo Scientific, San Jose, CA, USA) was coupled to the UHPLC system for compound identification and characterization. A Hypersil Gold C18 chromatographic column (Thermo Scientific, USA) was employed as the stationary phase for efficient separation. Data acquisition and processing

were performed using the Xcalibur Qual Browser software (Thermo Scientific, USA). For the evaluation of paw edema in the in vivo anti-inflammatory assay, a plethysmometer (Model: BX-plethy, Bioseb, Pinellas County, FL, USA) was used to measure paw volume changes accurately. Additionally, an analgesimeter (Model: BIO-RP-M, Bioseb, France) was employed to assess nociceptive thresholds in the analgesic activity tests.

Sample preparation

Aerial parts of *Inula viscosa* (L.) Aiton were collected in Jijel (Northeast of Algeria; 36° 46′ 17″ N, 5° 46′ 34″ E) and identified by Prof. Sarri Djamel (University of M'sila, Algeria). A specimen (AIV 11/18) was deposited in the Herbarium of the VARENBIOMOL research unit (Frères Mentouri University 1, Constantine, Algeria). The leaves were shade dried at ambient temperature (25 °C), and then ground into a fine powder. The extraction was carried out using water. To prepare the aqueous extract, 10 g of the plant powder was added to 200 mL of distilled water, boiled for 15 min and then filtered. The resulting solution was frozen, freeze-dried and then stored at 4 °C until future use.

Standard solution preparation

The extract was dissolved in distilled water to prepare a stock solution at a concentration of 10 mg/mL for quantitative analysis by UHPLC-DAD-ESI-MSn. The solution was subsequently filtered to remove any undissolved particles.

Chemical composition of I. viscosa extract

UHPLC-DAD-ESI-MSn profiling of the *I. viscosa* aqueous extract was carried out using a Ultimate 3000 (Dionex Co., San Jose, CA, USA) apparatus equipped with a binary pump, an automatic sampler and a diode array detector (Dionex Co., San Jose, CA, USA). Mass spectrometry (MS) analysis was conducted using a Thermo LTQ XL mass spectrometer (Thermo Scientific, San Jose, CA, USA) outfitted with an electrospray ionization interface (ESI). Separation was performed at room temperature (25°C) using a Hypersil Gold (Thermo Scientific, USA) C18 column (100 mm length; 2.1 mm i.d.; 1.9 µm particle diameter, end-capped). Formic acid in water (solvent A) and acetonitrile (solvent B) were used as analyte solvent of the extract (1 mg/mL), with an injection volume of 10 µL and flow rate of 2 mL/min. Gradient elution was 5% (solvent A) for 14 min, 40% (solvent A) over 2 min, 100% (solvent A) over 7 min. The column was re-equilibrated with 5% of solvent A for 10 min. The scan covered the mass range from m/z 100 to 2000. UV-Vis spectral data were collected between 200 and 500 nm. Spectra were recorded in negative mode. MS and MS/MS data were processed using the Thermo XcaliburQual Browser data system (Thermo Scientific, USA), and compounds were tentatively identified by comparing their mass spectra and retention times with those previously reported in the literature.

The following external standards were used for the quantification of phenolic compounds: quercitrin, chlorogenic acid, hydroxybenzoic acid, p-coumaric acid, and caffeic acid. These standards were selected based on their structural similarity to the compounds detected in the *I. viscosa* extract and were used to construct calibration curves for external quantification.

In vitro anti-inflammatory activity

The anti-inflammatory activity was determined *in vitro,* as described by Afonso *et al.* [18], by the ability of the plant extract to act as a scavenger of the nitric oxide radical ($NO^{\bullet}$). In a 96-well plate, 100 µL of sodium nitroprusside (3.33 mM) solution prepared in PBS 100 mM (pH 7.4) was added to 75 µL of sample/standard. After incubation for 10 min at room temperature under light irradiation, 100 µL of Griess reagent (equal volume of 1% of sulfanilamide and 0.1% of naphthyl ethylenediamine dihydrochloride in 2.5% of phosphoric acid) was added to the mixture. The absorbance was recorded at 560 nm. Ascorbic acid was used as a reference standard.

The scavenging ability of $NO^{\bullet}$ was calculated using the following equation:

$$NO^{\bullet} \text{ Scavenging effect (\%)} = (A_C - A_S)/A_C \times 100$$

Where: *A*c refers to the absorbance of the control; *A*s refers to the absorbance of the sample. $NO^{\bullet}$ scavenging ability was expressed as the $IC_{50,}$ which is the concentration of sample/standard required to scavenge 50% of $O_2^{\bullet-}$.

In vivo anti-inflammatory activity

The *in vivo* anti-inflammatory activity of the aqueous extract of *I. viscosa* was demonstrated in an experimental model of carrageenan-induced acute inflammation of the mouse paw as described by Ndiaye *et al*. [19]. Acetylsalicylic acid was used as a positive control.

Swiss female mice weighing between 25 and 27 g were used to carry out this activity. All *in vivo* tests were approved by the Laboratory of Applied Animal Biology (Badji Mokhtar University, 23000 Annaba, Algeria), in accordance with the Guide for the Care and Use of Laboratory Animals (2011). The mice, fasted 12 h before the experiment, were divided into three groups. The plant extract and the positive control were administered by gavage according to the following protocol:

The plant extract and the positive control were administered by gavage following a standardized protocol in which the animals were divided into three groups. Group 1 received 1 mL of distilled water, Group 2 received 1 mL of the aqueous extract of *I. viscosa* administered at a dose of 200 mg/kg selected on the basis of prior optimization, and Group 3 received 1 mL of acetylsalicylic acid administered at a dose of 300 mg/kg.

Thirty minutes after the oral administration of the plant extract and acetylsalicylic acid, 0.1 mL of a 1% carrageenan solution was administered into the subplanter region of the right hind paw of the mouse in the three groups.

Four hours after the administration of carrageenan, volumes of the right and left hind paws of each mouse were measured using a plethysmometer (BX-plethy, Bioseb, Pinellas County, FL, USA). The extent of edema was measured by the following formula:

$$\%\text{Paw edema inhibition} = (V_c - V_t)/V_c \times 100$$

V_c, Volume of paw edema in the control group; Vt, Volume of paw edema in the test-treated group.

In silico anti-inflammatory activity

Preparation of COX-1, COX-2, and ligand for molecular docking: All molecular docking calculations were performed using AutoDock Vina and Chimera software. The crystal structures of COX-1 and COX-2 were downloaded from the protein data bank (PDB: 3KK6 for COX-1 and 3LN1 for COX-2). The Chimera program was used for the preparation of the target protein. All non-standard residues except the co-crystallized ligand, celecoxib, were removed. Hydrogen atoms were added, and charges were assigned to the structures. The celecoxib molecule was separated from the native protein structure for the docking validation protocol. The molecule considered for docking was obtained from Pubchem (http://pubchem.ncbi.nlm.nih.gov/); 1,5-*O*-diCQA ID: 5280343. Molecular mechanics with Avogadro was applied for ligand minimization. The ligand was prepared in Chimera by adding charges necessary for docking simulations.

Docking validation

To validate the docking simulation with AutoDock Vina**,** redocking was performed, which consists of re-docking the co-crystallized ligand (celecoxib) into COX-1 and COX-2. The root mean square deviation (RMSD) quantifies the deviation between the atomic coordinates of the co-crystallized ligand and the best docking pose generated by AutoDock Vina**.** The protocol is

considered valid when the RMSD value is less than **2 Å**. The pose with the lowest Vina score was selected as the most suitable binding model between the ligand and the enzymes.

The protein data bank IDs of COX-1 and COX-2 are 3KK6 and 3LN1, respectively. The ligand was docked into the protein pdb file. The actives sites were defined with Chimera software as a box centered at the following coordinates: center_x = -12.4, center_y = -16.4 and center_z = -15.2 for COX-1 and center_x = -18, center_y = -16 and center_z = -17 for COX-2. The box dimensions were set to:size_x = 31.9Å, size_y = 43.04Å, and size_z = 7.06Å for COX-1 and size_x = 29.8Å, size_y = 22.35Å, and size_z = 15.16Å for COX-2.

In vivo analgesic activity

The analgesic activity of the *I. viscosa* aqueous extract was evaluated using the tail pressure test method [20]. A digital rodent pincher analgesimeter (BIO-RP-M, Bioseb, France) was used to induce pain. Mice were divided into two groups. The first group served as control and received distilled water by gavage, and the second group received a single oral dose of 200 mg/kg of *I. viscosa* aqueous extract. The pain sensitivity threshold was measured 30 minutes later.

The method consists of exposing the middle part of the tail of a restrained mouse to a progressively increasing force. The animal withdraws its tail in response to the applied pressure. The pain threshold, measured in newtons, is automatically displayed on the electronic device. When the animal withdrew its tail, the test was interrupted to record the pain threshold.

Statistical analysis

Data were analyzed using MINITAB software (version 16, State College, PA, USA) with one-way ANOVA, followed by Tukey's honestly significant difference (HSD) post hoc test at $p < 0.05$. Results are presented as the mean ± standard deviation (m ± SD) from three independent assays.

RESULTS AND DISCUSSION

LC-MS characterization of I. viscosa extract

Inula viscosa aqueous extract was analyzed by UHPLC-UV-MS/MS. The identification of the chemicals was accomplished by comparing the retention time and MS data from samples with those of reference standards injected under the same chromatographic conditions or by comparing their UV-Vis, MS, and MS/MS spectra data with those reported in the literature. Table 1 presents the identified and quantified compounds in the aqueous extract of *I. viscosa*, listed in the order of their elution, and Figure 1 shows the corresponding chromatogram.

Caffeoyl acid derivatives are prevalent in the *I. viscosa* aqueous extract. Several chlorogenic acids were identified, but the predominant ones are the hexaric acid derivatives (Table 1). Compounds **4** and **6**, having a similar ion $[M-H]^-$ at m/z 371, showed cleavage of one caffeoyl moiety, resulting in m/z 209 fragments, and were assigned to monocaffeoylhexaric acid isomers. Compounds **10**, **12**, **13**, and **14** presented a pseudomolecular ion $[M-H]^-$ at m/z 533, and two fragments were observed at m/z 353 and m/z 209, resulting from the cleavage of one or two caffeoyl moieties. These compounds were tentatively identified as dicaffeoylhexaric acid isomers I, II, III, and IV. Compounds **20** and **21**, with a pseudomolecular ion $[M-H]^-$ at m/z 695, released fragments at m/z 533, 353, and 209, resulting from the successive loss of caffeoyl moieties, and were assigned to tricaffeoylhexaric acid isomers I and II, respectively. Compound **22** was assigned to tetracaffeoylhexaric acid based on its MS pattern, which showed a molecular ion $[M-H]^-$ at m/z 857 and MS fragments at m/z 695, 533, 353, and 209 [26].

In addition, eight caffeoylquinic acid isomers and four other compounds were also present in the aqueous extract of *I. viscosa*. Compounds 3, 5, and 7 were assigned to 1-*O*-caffeoylquinic acid**,** 3-*O*-caffeoylquinic acid**,** and 1-*O*-caffeoylquinic acid**,** respectively, based on their

pseudomolecular ions $[M-H]^-$ at m/z 353 and their typical base peak at m/z 191, resulting from the loss of a caffeoyl residue (162 Da) [21].

Table 1. Phenolic composition of *I. viscosa* aqueous extract by UHPLC-DAD-ESI/MS.

Peak	Rt (min)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Identified compound	Quantification ($\mu g.mg^{-1}$extract)
1	1.63	191	110	Citric Acid	NQ
2	3.40	315	225, 153, 165, 109	Hydroxytyrosol hexoside	NQ
3	3.91	353	191, 179	3-*O*-CQA	4.22±0.35
4	4.22	371	353, 209, 191	Caffeoyl hexaric acid I	4.88±0.30
5	5.26	353	191, 179, 135	4-*O*-CQA	4.91±0.28
6	5.64	371	353, 209, 191	Caffeoyl hexaric acid II	4.57±0.32
7	8.02	353	191, 179	1-*O*-CQA	15.14±0.40
8	8.47	189	171, 142, 127, 99, 87	Unknown	1.85±0.05
9	8.82	179	135	Caffeic acid	5.18±0.64
10	9.35	533	371, 353, 209	Dicaffeoyl hexaric acid I	7.99±0.13
11	10.04	515	353, 335, 179	1,3-*O*-diCQA	4.63±0.44
12	10.15	533	371, 209, 191	Dicaffeoyl hexaric acid II	5.38±0.32
13	10.85	533	371, 353, 209	Dicaffeoyl hexaric acid III	5.86±0.30
14	11.42	533	371, 353, 209	Dicaffeoyl hexaric acid IV	8.41±0.11
15	11.89	477	301	Quercetin glucuronide	16.70±1.69
16	12.52	515	353, 299, 203	1,4-*O*-diCQA	6.14±0.98
17	12.70	515	353, 335, 299, 203, 179	3,4-*O*-diCQA	10.13±0.41
18	12.91	515	353, 335, 191	1,5-*O*-diCQA	106.26±1.60
19	13.59	515	353, 299, 255, 203	4,5-*O*-diCQA	11.92±1.00
20	13.71	695	533, 371	Tricaffeoyl hexaric acid I	36.20±3.54
21	13.97	695	533, 371	Tricaffeoyl hexaric acid II	6.36±0.11
22	15.58	857	695, 533, 371	Tetracaffeoyl hexaric acid	75.81±1.11

Retention time (RT), molecular ion ($[M-H]^-$), not quantified (NQ), caffeoylquinic acid (CQA). All peaks were identified by comparing their retention periods and MS and UV spectra to those of references used under the same chromatographic conditions, or with MS and UV-Vis data already published in the literature. The quantification of chemicals was carried out using the approach of external standards and calibration curves constructed using pure compounds that are structurally closest to them. However, peaks 1 and 2 were not quantified.

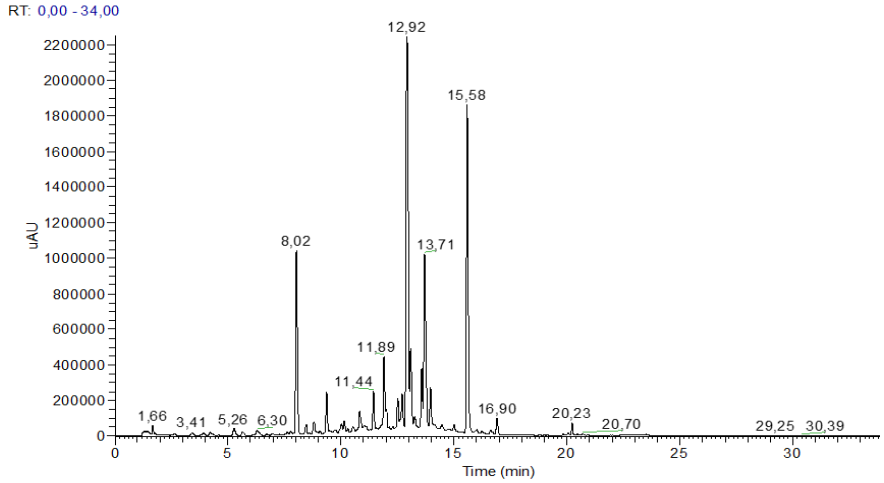

Figure 1. UHPLC-DAD-ESI-MS² chromatogram of *Inula viscosa* aqueous extract recorded at 280 nm, highlighting major detected peaks. Key analytical parameters: C18 column, gradient elution, flow rate of 2 mL/min, injection volume of 10 µL.

Compounds **11**, **16**, **17**, **18**, and **19**, with similar molecular ions at m/z 515 and fragments at m/z 353, 191, and 179**,** corresponding to monocaffeoylquinic acid, quinic acid, and caffeic acid moieties, respectively, were assigned to dicaffeoylquinic acids. Based on their retention times and spectral data, these compounds were identified as 1,3-*O*-diCQA, 1,4-*O*-diCQA, 3,4-*O*-diCQA, 1,5-*O*-diCQA, and 4,5-*O*-diCQA, dicaffeoylquinic acid isomers [21-23].

Compound **1**, which shows a molecular ion $[M-H]^-$ at m/z 191, had a fragment ion at m/z 111**,** suggesting the loss of neutral molecules CO, CO_2, and H_2O [28]. Compound **2** displayed a molecular ion $[M-H]^-$ at m/z 315 and released an MS^2 fragment at m/z 153, attributed to hydroxytyrosol, which is yielded from the elimination of a hexose moiety. Therefore, this compound is assigned as hydroxytyrosol-hexoside. Compound **9** was tentatively identified as caffeic acid by comparison with a pure standard injected under the same conditions. Its UV spectrum and MS data further support this identification. Compound **15**, having a molecular ion at m/z 477 and an intense peak at m/z 301 resulting from the loss of a glucuronic acid moiety, was assigned to quercetin-O-glucuronide.

It should be noted that the aqueous extract is particularly rich in caffeoyl hexaric acids. These compounds, which are associated with various biological activities, were previously identified in the hydroalcoholic extracts of Xerolekia speciosissima and Galinsoga parviflora aerial parts, two other species from the Asteraceae family [25, 26].

Our results support previous research reporting that hydroxycinnamic acids are the most abundant phenolic compounds identified in the Asteraceae family [27, 28]. These findings also revealed that the phenolic compounds identified in this study differ from those found in a previous investigation by Brahmi-Chendouh *et al.* [17], which analyzed the ethyl acetate extract of the same species.

In vitro and in vivo anti-inflammatory activity

In the present study, we also describe the anti-inflammatory activity of *I. viscosa* aqueous extract. Specifically, this activity was evaluated based on the extract's ability to scavenge $NO^{\bullet}$ radicals using an *in vitro* assay. The results, expressed as IC_{50} values, the concentration of the sample or standard required to scavenge 50% of $NO^{\bullet}$, are presented in Table 2. Additionally, the anti-inflammatory effect was assessed using a carrageenan-induced acute paw edema model in mice.

Table 3 shows the percentage inhibition of paw edema caused by carrageenan following oral administration of 200 mg/kg of *I. viscosa* aqueous extract and 300 mg/kg of acetylsalicylic acid.

Table 2. *In vitro* anti-inflammatory activity of *I. viscosa* aqueous extract by $NO^{\bullet}$ scavenging assay.

Sample	$NO^{\bullet}$ (IC_{50} µg.ml^{-1})
Aqueous extract	37.02±2.57^{a}
Ascorbic acid	144.58±7.03^{b}

IC_{50} values correspond to the concentration that yields a 50% inhibition. Different small letters in each column are statistically different at $p < 0.05$ (HSD Tukey test).

The *I. viscosa* aqueous extract showed a strong anti-inflammatory effect (IC_{50} = 37.02 ± 2.57 µg/mL), which was more than three times greater than that of ascorbic acid used as the reference (IC_{50} = 144.38 ± 7.03 µg/mL) (Table 2). The use of ascorbic acid as a reference compound in this study is justified by its well-documented modulatory effects on inflammatory responses [29, 30].

As shown in Table 3, oral administration of 200 mg/kg of *I. viscosa* aqueous extract reduced paw edema by 54.60% after 4 hours. In comparison, acetylsalicylic acid, administered orally at a dose of 300 mg/kg, inhibited carrageenan-induced paw edema by 59.57%, highlighting the strong anti-inflammatory effect of this species.

Table 3. In vivo anti-inflammatory activity of *I. viscosa* aqueous extract.

Sample	Volume of paw edema (mL) and % inhibition of paw edema (%)
Control	0.0587±0.018
Aqueous extract	0.0266±0.0081(54.60%)
Acetylsalicylic acid	0.0238±0.0074(59.57%)

Aqueous extract was orally administered at 200 mg/kg. Acetylsalicylic acid was orally administered at 300 mg/kg.

Inflammation is a natural defense mechanism involving immune cells such as neutrophils, macrophages, and lymphocytes, but it can become harmful when dysregulated [31]. The carrageenan-induced mouse paw edema model, commonly used to evaluate anti-inflammatory activity, reflects inflammation linked to the activation of cyclooxygenase enzymes (COX-1 and COX-2), which are key targets of nonsteroidal anti-inflammatory drugs (NSAIDs) [32].

Our study shows that the aqueous extract of *Inula viscosa* effectively prevents carrageenan-induced inflammatory edema in vivo. Additionally, the extract demonstrated significant nitric oxide ($NO^{\bullet}$) radical scavenging activity in vitro, indicating its anti-inflammatory potential. These findings suggest that the identified compounds, particularly caffeoylquinic and hexaric acid derivatives, may act as natural inhibitors of inflammatory mediators, offering a promising alternative to conventional NSAIDs. This highlights the pharmacological novelty of these molecules in the context of inflammation.

Since antiquity, different parts of traditional medicinal plants have been used to treat a range of inflammation-related conditions [33]. Data from earlier studies support our findings and demonstrate that extracts from *I. viscosa* act as anti-inflammatory agents. This species has previously been reported to decrease nitric oxide ($NO^{\bullet}$) production and inhibit the expression of inducible nitric oxide synthase (iNOS) in a dose-dependent manner. Furthermore, pretreatment of Swiss mouse with an oral dose of 200 mg/kg of *I. viscosa* aqueous extract reduced paw edema at 3 and 6 hours following carrageenan stimulation [13]. Among fourteen Mediterranean plant species evaluated for their ability to suppress pro-inflammatory cytokines using an ELISA assay, *I. viscosa* extracts were reported to be the most effective [34]. In another study, Ouahchia *et al.*

[34], found that methanolic extracts and decoctions of *I. viscosa* leaves and flowers, administered orally at doses of 400, 600, and 800 mg/kg, significantly reduced paw edema in mouse after 4 hours. Additionally, 7-O-methylaromadendrin, a flavanone previously isolated from *I. viscosa*, exhibited both *in vitro* and *in vivo* anti-inflammatory activities [10].

In silico activity

Molecular docking, a key technique in structure-based drug design, plays a crucial role in streamlining and accelerating the drug discovery process [35, 36]. In the present study, the major compound identified in *I. viscosa* aqueous extract was docked to the binding sites of the key regulatory enzymes COX-1 and COX-2, which are linked to inflammation.

COX-1 and COX-2 are internal membrane-bound enzyme isoforms. These enzymes catalyze the conversion of arachidonic acid to prostaglandins and other prostanoids involved in various physiological processes [37]. COX-1 is constitutively expressed under normal physiological conditions and plays a role in protecting the stomach lining and maintaining renal function. In contrast, COX-2 is an inducible enzyme expressed at sites of inflammation and in certain cancers. Inhibition of COX-1 and COX-2 is a key target in the treatment of inflammation, and many nonsteroidal anti-inflammatory drugs (NSAIDs) exert their effects by inhibiting these enzymes. Traditional NSAIDs inhibit both isoforms, whereas the newer class of COX-2-selective drugs preserves the normal physiological functions mediated by COX-1 [37, 38].

The re-docking of the native ligand, celecoxib, into COX-1 and COX-2 was performed to validate the docking protocol. The root mean square deviation (RMSD) between the X-ray structures of COX-1 and COX-2 and the best docked ligand poses was calculated using Dock RMSD, yielding values of 0.740 Å and 0.478 Å, respectively. These results clearly indicate that the docking protocol is reliable and suitable for determining the optimal conformations and binding energies of the molecules under study within the COX-1 and COX-2 active sites. Figure 2 shows the superposition of the co-crystallized celecoxib and the best docked pose.

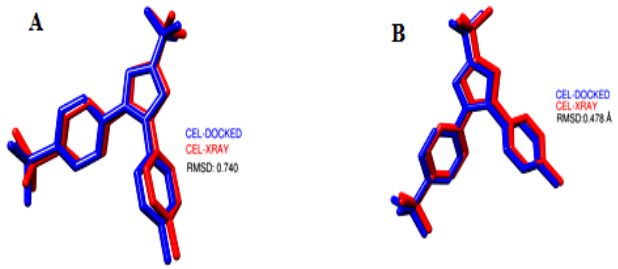

Figure 2. Superposition of X-ray celecoxib and the best docking pose in the active sites of COX-1 (A) and COX-2 (B). Hydrogen atoms are omitted for clarity.

The docking energies of the molecule under study are reported in Table 4. Figure 3 shows 2D maps of different interactions of 1,5-*O*-diCQA with COX-1 and COX-2.

Table 4. Molecular docking energies of 1,5-*O*-diCQA.

	Celecoxib (ΔG kcal/mol)	1,5-*O*-diCQA (ΔG kcal/mol)
COX-1	-11.0	-6.8
COX-2	-12.5	-7.8

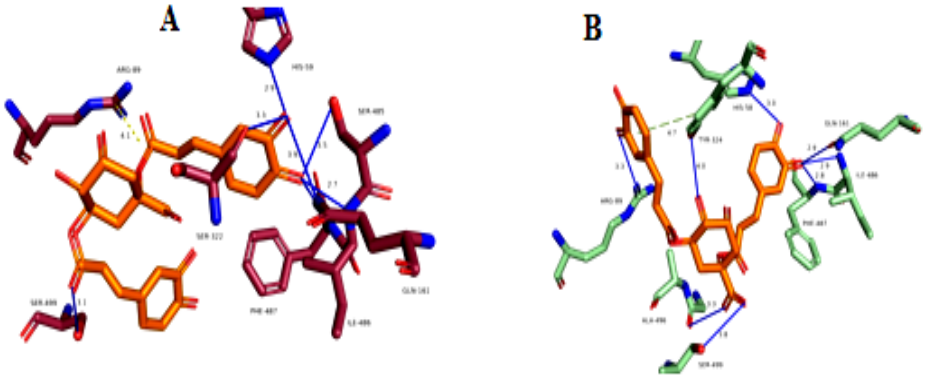

Figure 3. 2D representation of interactions of 1,5-*O*-diCQA with COX-1 (A) and COX-2 (B).

Based on the docking interaction data, it is clear that 1,5-*O*-diCQA has the best binding affinity (lower binding energy of –7.8 kcal/mol) for the binding site of the COX-2 enzyme. In contrast, a higher binding energy was recorded with COX-1 (–6.8 kcal/mol). Moreover, 1,5-*O*-diCQA interacts with COX-2 by forming hydrogen bonds with His58, Arg89, Gln161, Tyr324, Ile486, Phe487, Gly495, and Ser499, as well as hydrophobic interactions with Leu61, Val85, Tyr324, Ile486, Phe487, Val492, Ala496, and Leu500 (Figure 3B). On the other hand, 1,5-*O*-diCQA interacts with COX-1 by engaging different amino acids (Figure 3A), forming hydrogen bonds with His59, Gln161, Ser322, Ser485, Ile486, Phe487, and Ser499, as well as hydrophobic interactions with Val318, Leu321, Tyr354, Trp356, Ile486, Phe487, and Ile492. For both COX-1 and COX-2, π-stacking interactions were observed at the Arg89 and Tyr324 residues (Figure 3). Celecoxib, used as a reference, displayed stronger binding energies of -11.0 kcal/mol (COX-1) and -12.5 kcal/mol (COX-2). Although 1,5-*O*-diCQA showed a moderate binding affinity compared to the standard NSAID celecoxib, its docking scores of -6.8 kcal/mol (COX-1) and -7.8 kcal/mol (COX-2) still suggest a favorable interaction with both enzymes, highlighting its potential to inhibit these targets, which are known to be involved in the inflammatory process

Considering that 1,5-*O*-diCQA is the most abundant compound in the *I. viscosa* aqueous extract, the docking results support the promising *in vitro* and *in vivo* anti-inflammatory activities observed in the present study. Although 1,5-*O*-diCQA was used in the molecular docking study, it is evident that the main interactions involve the caffeoyl moiety, specifically through its hydroxyl and carbonyl groups, suggesting that other caffeoyl derivatives present in the *I. viscosa* aqueous extract may also contribute to its anti-inflammatory activity.

Overall, our results suggest that compounds from the *I. viscosa* species may act as anti-inflammatory agents. This effect may be attributed to 4,5-dicaffeoylquinic acid, which has previously been reported to exhibit protective effects against sepsis induced by cecal ligation and puncture (CLP) in mice *in vivo* [38].

Analgesic activity

Table 5 shows the results of the *in vivo* analgesic activity of *I. viscosa* aqueous extract in the tail pressure pain model in mice. The threshold sensitivity to pain, measured using a digital pressure-based analgesimeter, was expressed in newtons. Compared to the control group (10.05 ± 2.49

newtons), pretreatment of mice with an oral dose of 200 mg/kg of *I. viscosa* aqueous extract significantly increased the pain threshold 30 minutes after administration (16.06 ± 3.29 newtons).

Table 5. *In vivo* analgesic activity of *I. viscosa* aqueous in the tail flick model of pain in the mouse.

Sample	Pain threshold (newton)
Control (distilled water, po)	10.05±2.49[a]
Aqueous extract (200 mg/kg, po)	16.06±3.29[b]

Aqueous extract was orally administered at 200 mg/kg. po: per oral. Different small letters in each column are statistically different at $p < 0.05$ (HSD Tukey test).

In the present study, pretreatment of mouse with an oral dose of 200 mg/kg of *I. viscosa* aqueous extract caused a significant increase in pain threshold sensitivity 30 minutes after administration. This activity may be attributed to the inhibitory effect of *I. viscosa* on the release of pain mediators involved in the nociceptive response. During inflammation, these mediators are released at the site of inflammation. Among them are prostaglandins, which are produced from arachidonic acid via the action of cyclooxygenases (COX) [43]. Chemical compounds present in the aqueous extract of *Inula viscosa* may exert their effects by blocking COX activity.

Nitric oxide ($NO^{\bullet}$), produced by macrophages during inflammatory processes, is another important chemical mediator of pain. Therefore, the significant $NO^{\bullet}$ scavenging activity of *I. viscosa* aqueous extract observed in the present study may contribute to its analgesic effect.

Phytochemical analysis of the *I. viscosa* aqueous extract revealed an abundance of caffeoylquinic acid derivatives, which have been previously reported to possess analgesic properties. One study showed that 3,5- and 4,5-di-O-caffeoylquinic acids contribute to the analgesic activity of a polar extract from *Lychnophora ericoides* roots in the acetic acid-induced mouse writhing test [44]. Additionally, chlorogenic acid has demonstrated analgesic effects in the formalin-induced pain test [45].

Finally, it is important to highlight that *I. viscosa* has been previously evaluated for its potential toxicity, and studies suggest that this medicinal plant is safe and suitable for use in traditional medicine [46, 47].

CONCLUSIONS

The present study characterized the chemical composition of *I. viscosa* from Algeria. The extract's biological activity was evaluated for its anti-inflammatory and analgesic effects. An *in silico* study was conducted to further investigate the interaction between the main identified compound and cyclooxygenases 1 and 2, two enzymes associated with inflammation. UHPLC-DAD-ESI/MS analysis revealed that the *I. viscosa* extract is rich in caffeoylhexaric acids, which are described here for the first time in this species**.** Moreover, the biological assays showed that the aqueous extract of *I. viscosa* possesses strong anti-inflammatory and analgesic activities, and the *in silico* study supports these findings. Previous studies indicating that this medicinal plant is non-toxic, along with the results obtained in the present study, further support the use of *I. viscosa* in folk medicine as a remedy for inflammatory diseases. They also highlight it as a promising candidate for the discovery of new compounds with anti-inflammatory activity.

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