Research Article

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Mineral composition, principal polyphenolic components, and evaluation of the anti-inflammatory, analgesic, and antioxidant properties of *Cytisus villosus* Pourr leaf extracts

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Abstract: *Cytisus villosus* Pourr. (*C. villosus*) is a medicinal plant belonging to the Fabaceae family, which grows in the Mediterranean area. It is used in traditional medicine against diseases related to inflammation. The objective of the present study was to identify the mineral and polyphenolic composition as well as to evaluate some biological properties including antioxidant, anti-inflammatory, and analgesic activities of *C. villosus* leaf aqueous extract. The chemical constituents were identified and quantified using ultra performance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) methods. The antioxidant properties of *C. villosus* leaves were tested using reducing power (RP), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) assays. The anti-inflammatory potency was evaluated *in vitro* and *in vivo* using the albumin denaturation test and the carrageenan test, respectively. Furthermore, the analgesic effect was performed *in vivo* using tail flick, acetic acid-induced contortion, and plantar tests. Mineralogical analysis revealed that potassium and calcium were the most abundant minerals. The analysis and quantification of the phytochemical composition using UPLC-ESI-MS/MS showed that quinic acid (57.478 ± 1.72 mg/kg) was the major compound of the aqueous extract, followed by salicylic acid (17.38 ± 0.2 mg/kg), isoquercetin (16.895 ± 1.01 mg/kg), and gallic acid (15.914 ± 1.51 mg/kg). The extracts showed potent antioxidant activity for all tests used. The highest antioxidant activity was recorded for the DPPH, ABTS and RP methods, with an IC_{50} of 3.94 ± 0.09, 2.88 ± 0.07, and 1.94 ± 0.10 µg/mL, respectively. Additionally, using the most frequent analgesic assays, the aqueous extract at a dose of 500 mg/kg exhibited a potent analgesic activity. Notably, an interesting inhibition of albumin denaturation was recorded with an IC_{50} of 383.94 µg/mL, corroborating the *in vivo* test. Overall, the results presented here may represent a scientific basis for the traditional use of *C. villosus* in the treatment of inflammation-related diseases.

Keywords: antioxidant activity, anti-inflammatory, analgesic activity, *Cytisus villosus*, UPLC-ESI-MS/MS

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1 Introduction

The inflammatory response is a process marked by the stimulation of immune and non-immune cells that defend

the body against infections and diseases by eliminating harmful chemicals and promoting tissue repair and regeneration [1]. However, if defensive systems are overwhelmed, inflammation may cause significant alterations in all tissues and organs, which could lead to an increased risk of various chronic pathologies such as cancer, neurodegeneration, diabetes, and hypertension [2].

Inflammation treatments are systemically associated with non-steroidal anti-inflammatory drugs (NSAIDs), which act through the inhibition of cyclooxygenases. However, the use of NSAIDs has been linked with a risk of adverse events [3,4], which have a significant impact on morbidity and represent a substantial increase in healthcare costs [5]. The development of new drugs is consequently an ongoing challenge. Among the options for drug development are plants that constitute a source of bioactive molecules [6].

The plant known as *Cytisus villosus* Pourr. belongs to the Cytisus genus within the Leguminosae (Fabaceae) family. Typically, this shrub can grow to a height of 1–2 m and features erect stems that give rise to numerous branches. The young branches are angular and covered with long white hairs. The flowers are large, streaked yellow with a papilionaceous corolla. The flowering takes place in April–May [7]. This genus is present in abundance in the Mediterranean basin and used in ancient therapeutic practices as a cure for specific ailments, entailing diabetes and liver diseases, as well as has antioxidant, anxiolytic, diuretic, cardiotonic, and antifungal properties [7–11]. Furthermore, *Cytisus villosus* Pourr. is rich in phenolic and flavonoid compounds [12,13] Moreover, the plant is linked with numerous biological effects, such as anti-oxidant, anti-inflammatory, and anti-microbial activities [14].

The current study's objectives are to assess the antioxidant, analgesic, and anti-inflammatory properties of *Cytisus villosus* Pourr. leaf as well as its chemical and mineral composition.

2 Materials and methods

2.1 Plant material

The *Cytisus villosus* Pourr. specimen was gathered from the Ketema locality in February 2022 (N: 34°47′56, W: 4°37′44, altitude: 642.1 m) and then identified and cataloged with a reference sample (ANC0039/2022). The plant has been stored at the herbarium of NAMAP (National Agency for Medicinal and Aromatic Plants), Morocco.

2.2 Preparation of plant extracts

The maceration method was used to extract the leaf powder of *C. villosus*. To obtain the extracts, 100 g of the raw material was macerated at room temperature with either 1 L of distilled water or with ethanol/water (70:30, v/v). The solution was then filtered through Whatman paper no.1 and evaporated using a rotary evaporator at 45°C under reduced pressure. The resulting aqueous and hydroalcoholic extracts had a yield of 19.23% and 15.21% (w/w), respectively, and were stored at 4°C for further use.

2.3 Chemical reagents and standard compounds

Standard compounds, quinic acid, isoquercetin, gallic acid, *p*-coumaric acid, ferulic acid, salicylic acid, vanillic acid, gentisic acid, 4-OH-phenylacetic acid, caffeic acid, quercetin, 3,4,5-trimethoxycinnamic acid (sinapic acid methyl ether), and avicularin, were purchased from Merck and Carl Roth GmbH (Darmstadt, Germany). Protocatechuic acid, cynaroside, and aromadendrin were purchased from PhytoLab (Dettendorfer, Germany). Astragalin and quercetin were acquired from Extrasynthese (Lyon, France). Gallic acid, Folin–Ciocalteu reagent, catechin, butylated hydroxytoluene (BHT), 2,2′-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Indomethacin, diclofenac sodium, and aspirin were purchased from Pharma5 (Bouskoura, Morocco). The other reagents utilized in the study were of analytical grade and utilized as received.

2.4 Analysis of mineral elements

The mineral composition of *C. villosus* leaves (silver, aluminum, copper, boron, cobalt, chromium, titanium, iron, calcium, potassium, magnesium, manganese, sodium, nickel, phosphorus, palladium, silicon, tin, zinc, and vanadium) was determined using the calcination method (ICP-AES), as performed previously by Silva et al. [15].

2.5 Determination of the total flavonoid content (TFC), total phenolic content (TPC), and proanthocyanidin content (PC)

The quantification of the TPC was carried out through the Folin–Ciocalteu assay, and the results were reported as milligrams of gallic acid equivalent per gram of dry weight extract (mg GAE/g extract) [16].

The TFC of all extracts was estimated using the protocol detailed by Amezouar et al. [17]. The results were expressed as milligram quercetin equivalent per gram dry weight (mg QE/g extract). The PC content of all extracts was measured following the procedure of Sayah et al. [18]. The results are presented as milligrams of catechin equivalent per gram of dry weight of extract (mg EC/g extract).

2.6 Identification and quantification of phenolic compounds by UPLC-ESI-MS/MS

The acquity liquid chromatography (UPLC) system coupled to a Waters Xevo TQ-S triple quadrupole system (Waters, United States) was used to analyze the aqueous extract of *C. villosus* according to the methodology described by Zhu et al. [19]. The molecules were identified by checking the typical fragment with those in an in-house developed library of molecules spectra (see supplementary file). The polyphenols were detected in the negative mode of ESI with the deprotonated [M–H]-ion selected as the precursor ion, and optimized mass spectrometry was used to determine the content of the 32 phenolic compounds among the 33 phenolic compounds detected. Table 1 summarizes the precursor-to-product ion transitions.

2.7 Antioxidant activity

The ability of our examined extracts to scavenge the DPPH (DPPH$^{\bullet}$) (2,2-diphenyl-1-picrylhydrazyl) radical was measured as described by El Omari et al. [20]. For the preparation of the DPPH solution, 0.005 g of DPPH was dissolved in 200 mL of ethanol (96%) to obtain an absorbance of 0.700 ± 0.01 at 515 nm. Then, 25 µL of the extract at different dilutions (0–1,044 µg/mL) was mixed with 825 µL of the DPPH reagent. The reaction preparation was carefully vortexed and then left in the dark at the laboratory temperature for 30 min. The coloration produced was determined at 517 nm using a spectrophotometer (UV-1700APC, China). The results obtained are compared with the BHT standard. The inhibitory percentage was determined using the following formula:

$$\% \text{ Inhibition} = \frac{(\text{Control} - \text{Sample})}{\text{Control}} \times 100. \quad (1)$$

The ability of *C. villosus* extracts to inhibit the cation-based ABTS (ABTS$^{\bullet+}$) radical (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) was determined by the method described by Miguel et al. [21]. An aqueous solution of ABTS (7 mM) was combined with 2.5 mM potassium persulfate to regenerate ABTS$^{\bullet+}$. For 16 h, the combination was left at room temperature and in the dark. Using a spectrophotometer, the mixture was adjusted with ethanol (98%) to give an absorbance of 0.70 at 734 nm. Then, 25 µL of the extract at different concentrations was mixed with 825 µL of ABTS$^{\bullet+}$ reagent. An absorbance measurement was performed at 734 nm after 6 min. The antioxidant standard was ascorbic acid. According to equation (1), the ABTS radical scavenging activity was calculated: The IC_{50} values were expressed in µg/mL.

The method recommended by Bougandoura and Bendimerad [22] was used to evaluate the reducing potential of the studied extracts, and ascorbic acid was used as a standard reference. The IC_{50} (µg/mL) of the different assays was determined using different concentrations, in which the % inhibition was between 20 and 80%.

2.7.1 Total antioxidant capacity (TAC)

The phosphomolybdate (PPM) method described in previous study [17] was used to determine TAC. Ascorbic acid was used to establish the calibration range, and the results were expressed as milligrams of ascorbic acid equivalent per gram of crude plant (mg AAE/g E).

2.8 Experimental animal

In this study, adult Wistar rats were used in the experiments. The rats were bred in the NAMAP animal facility (Morocco). All experimental rats were housed in standard environmental conditions (5% humidity, 22 ± 3°C, 55 ±, and 12 h light/dark cycles) and had free access to tap water. Normal (distilled water) and experimental rats (extracts or drugs) were subjected to a fasting period of 16 h before the experiment. All experimental procedures were conducted according to the rules established in the "Guide for the Care and Use of Laboratory Animals," established by the National Academy of Sciences [23].

Table 1: Multiple reaction monitoring transitions for the analyzed phenolic compounds

Compound no.	Analyte	Retention time (min)	Precursor ion (*m/z*)	Product ions (*m/z*)	Ionization mode (+/−)
I	Pyrocatechol	5.44	109.2	80.93	ES−
			109.2		
II	Gallic acid	4.33	169	125	ES−
			169	79	
III	Protocatechuic acid	6.25	153	109	ES−
			153		
IV	Vanillic acid	8.92	167	152	ES−
			167		
V	Gentisic acid	8.06	153	109	ES−
			153		
VI	Salicylic acid	12.44	137	93	ES−
			137		
VII	4-OH-Phenylacetic acid	7.3	151	107	ES−
			137		
VIII	Quinic acid	2.16	191	85	ES−
			191	93	
IX	*p*-Coumaric acid	11.5	163	119	ES−
			163		
X	Caffeic acid	9.58	179	135	ES−
			179	107	
XI	Sinapinic acid	12.2	223	208	ES−
			223	164	
XII	Sinapic acid methyl ether	14.71	237	102	ES−
			237	132	
XIII	Ferulic acid	11.94	193	134	ES−
			193	178	
XIV	Hydroferulic acid	10.12	195	121	ES−
			195	93	
XV	Isoquercetin	13.18	463	300	ES−
			463	271	
XVI	Cynaroside	12.93	447	285	ES−
			447	151	
XVII	Astragalin	14.03	447	284	ES−
			447	255	
XVIII	Quercetin	14.24	447	300	ES−
			447	271	
XIX	Aromadendrin	14.15	287	259	ES−
			287	125	
XX	Avicularin	13.91	433	300	ES−
			433	271	
XXI	Luteolin	16.49	285	133	ES−
			285	107	
XXII	Rutin	12.78	609	300	ES−
			609	271	
XXIII	Kaempferol	17.99	285	93	ES−
			285	146	
XXIV	Naringenin	16.73	271	151	ES−
			271	119	
XXV	Isorhamnetin	17.92	315	300	ES−
			315	151	
XXVI	Taxifolin	12.45	303	285	ES−
			303	125	
XXVII	Quercetin-3-*O*-glucuronide	13.48	477	301	ES−
			477	151	
XXVIII	Apigetrin	14	431	268	ES−

(*Continued*)

Table 1: *Continued*

Compound no.	Analyte	Retention time (min)	Precursor ion (*m/z*)	Product ions (*m/z*)	Ionization mode (+/−)
			431	107	
XXIX	Apigenin	17.74	269	117	ES−
			269	149	
XXX	Phloridzin	13.77	435	273	ES−
			435	167	
XXXI	Quercetin	16.53	301	151	ES−
			301	179	
XXXII	Daidzin	15.49	253	224	ES−
XXXIII	Resveratrol	15.39	227	158	ES−

ES−: negative electrospray.

2.9 Evaluation of anti-inflammatory activity

To assess the anti-inflammatory potency, the carrageenan-induced rat paw edema test, as per Winter et al. [24], was employed. Each treatment was administered to a group of six rats. The aqueous extract of *C. villosus* (500 mg/kg bw) was orally administered 30 min before carrageenan injection. Positive and negative controls, indomethacin (10 mg/kg bw) and distilled water (5 mL/kg), were used, respectively. The paw volume was measured before carrageenan injection, and at 1, 3, and 6 h after carrageenan injection using a LE 7500 plethysmometer.

Using equation (2), the anti-inflammatory potency was calculated as a percent inhibition:

$$\text{Inhibition (\%)} = \frac{(V_c - V_t)}{V_c} \times 100, \tag{2}$$

where V_c is the average increase in the paw volume of the control group and V_t is the average increase in the paw volume of the treatment group.

2.10 Inhibition of albumin denaturation

To study the *in vitro* anti-inflammatory efficacy of the aqueous extract of *C. villosus* leaves, we used the albumin denaturation assay according to Lekouaghet et al. [25]. The percentage of protein denaturation was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{(\text{Control} - (\text{Sample} - \text{White}))}{\text{Control}} \times 100. \tag{3}$$

2.11 Analgesic activity study

2.11.1 Writhing test

Contortion provoked by acetic acid was performed as previously reported by Sayah et al. [26]. The weighed rats (180–200 g) were randomized into three groups of six rats each. Group 1 (control) was treated with 0.9% saline, group 2 was pre-treated with aspirin (150 mg/kg bw), group 3 received the *C. villosus* extract (500 mg/kg bw) 30 min after the treatments, 3.75 mL/kg bw of intraperitoneal acetic acid solution (3%) was injected to cause contortions.

The contortion numbers were counted during a 10 min observation period after the injection of acetic acid. The inhibition (%) of abdominal constrictions was determined as follows :

$$\text{Inhibition (\%)} = \left(1 - \frac{W_t}{W_c}\right) \times 100, \tag{4}$$

where W_t and W_c represent the contortion numbers in the treated and control group, respectively.

2.11.2 Tail flick test

This test was conducted as previously detailed by Sood et al. [27]. Wistar albino rats (180–200 g) were separated into four groups. Each group consisted of six rats. The first group was considered as a control, the second group was used as a reference (aspirin 150 mg/kg), and the third and fourth groups received aqueous extracts of *C. villosus* at two doses: 250 and 500 mg/kg p.o.

The tail response was recorded (ANALGESY, METER LE 7106) as the radiant heat sensation from the spine, and a cut-off time of 20 s was maintained. The tail test was performed in each batch before treatment and 30, 60, 90, and 120 min after drug administration.

2.11.3 Plantar test

To determine the nociceptive response to thermal stimuli, we performed a plantar test (UGO BASILE model 37370) following the method previously reported by El Youbi et al. [28] using four groups of six rats each. Group I (normal group): the animals received distilled water. Group II (standard reference group): the animals received aspirin at a dose of 150 mg/kg by gavage. Groups III and IV (experimental group): the animals received *C. villosus* extract at two different doses (250 and 500 mg/kg p.o.). The latency of the paw withdrawal response was measured automatically at 30, 60, 90, and 120 min.

2.12 Statistical analysis

The findings are reported as mean ± standard error. Graph Pad Prism 8.02 was used to conduct all statistical analyses. Comparisons of *C. villosus* leaf extracts were performed by analysis of variance (ANOVA) and multiple comparisons were performed by Tukey's test. Differences were considered significant when $P \leq 0.05$.

3 Results and discussion

3.1 Mineral composition

The mineral content of the *C. villosus* leaf is summarized in Table 2. Importantly, potassium (1130 mg/kg) was the most abundant element, followed by calcium (234 mg/kg), magnesium (90 mg/kg), phosphorus (48.9 mg/kg), and silicon (12.9 mg/kg), while the concentrations of aluminum, sodium, iron, and manganese were 6.07, 2.26, 1.75, and 1.44 mg/kg, respectively. The concentrations of copper, zinc, boron, nickel, chromium, cobalt, silver, tin, vanadium, lead, and titanium were the least abundant. Notably, the potassium, calcium, magnesium, copper, phosphorus, and iron involved in the organism's defense system against oxidative stress, which may help protect it from ailments [29–31].

3.2 TFC, TPC, and PC contents

The results, presented in Figure 1, indicate that the aqueous extracts of *C. villosus* have greater concentrations of TFC and PC (337.12 ± 1.2 mg GAE/g and 183.88 ± 2.97 mg EC/g E, respectively) when compared to hydroethanolic extracts. Furthermore, the hydroethanolic extract has a greater flavonoid content (46.33 ± 0.45 mg EQ/g E) than the aqueous extract. Our findings are in accordance with the preceding investigations that showed the abundance of phenols in *C. villosus* [13,32]. Larit et al. [14] recorded the aerial part of *C. villosus*, which contained 363.0 ± 8.32 mg GAE/g E phenols and 21.16 ± 1.022 mg QE/g E flavonoids from the n-butanol extract.

3.3 Phenolic compound quantification in the aqueous extract of *C. villosus*

The aqueous extract of *C. villosus* contained 32 polyphenolic compounds (Table 3). The identified chemicals contained polyphenolics including phenolic acids, cinnamic acids, and flavonoids. The major phenolic components identified (Table 3) are (mg/kg) quinic acid (57.478 ± 1.72), salicylic acid (17.38 ± 0.21), isoquercetin (16.895 ± 1.01), gallic acid (15.914 ± 1.51), protocatechuic acid (12.935 ± 0.40), ferulic acid (11.544 ± 0.25), *p*-coumaric acid (8.873 ± 0.81), vanillic acid (5.377 ± 0.56), gentisic (4.021 ± 0.31), cynaroside (3.145 ± 0.18), caffeic acid (2.711 ± 0.04), quercetin (2.197 ± 0.01), astragalin (1.811 ± 0.61), 4-OH-phenylacetic

Table 2: Mineral composition of the leaves of *C. villosus*

Minerals (mg/kg)									
B	Ag	Ca	P	Al	Pb	Cu	V	Zn	Si
0.552	0.0780	234	48.9	6.07	0.008	0.710	0.060	0.654	12.9
Co	K	Ti	Fe	Cr	Mg	Na	Mn	Ni	Sn
0.085	1130	0.002	1.75	0.194	90.0	2.26	1.44	0.239	0.065

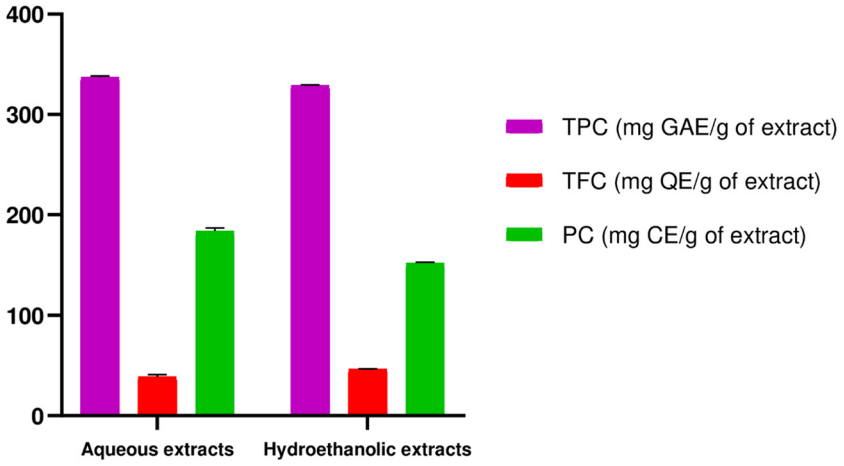

Figure 1: PC, TFC, and TPC contents of aqueous and hydroethanolic extracts of *C. villosus* leaves (mean ± SE).

Table 3: Phenolic components identified in the aqueous extract of *C. villosus* (n = 3)

Class	Compounds	Molecular formula	PubChem number	Mass (mg/kg)
Simple phenols	Pyrocatechol	$C_6H_6O_2$	289	0.128 ± 0.074
Phenolic acids	Gallic acid	$C_7H_6O_5$	370	15.914 ± 1.51
	Protocatechuic acid	$C_7H_6O_4$	72	12.935 ± 0.40
	Vanillic acid	$C_8H_8O_4$	8468	5.377 ± 0.56
	Gentisic acid	$C_7H_6O_4$	3469	4.021 ± 0.31
	Salicylic acid	$C_7H_6O_3$	338	17.38 ± 0.21
Phenylacetic acids	4-OH-Phenylacetic acid	$C_8H_8O_3$	127	1.635 ± 0.31
Quinate precursors	Quinic acid	$C_7H_{12}O_6$	6508	57.478 ± 1.72
Hydroxycinnamic acid derivatives	*p*-Coumaric acid	$C_9H_8O_3$	637542	8.873 ± 0.81
	Caffeic acid	$C_9H_8O_4$	689043	2.711 ± 0.04
	Sinapinic acid	$C_{11}H_{12}O_5$	637775	0.73 ± 0.41
	Sinapic acid methyl ether	$C_{12}H_{14}O_5$	735755	0.978 ± 0.06
	Ferulic acid	$C_{10}H_{10}O_4$	445858	11.544 ± 0.25
	Hydroferulic acid	$C_{10}H_{12}O_4$	17865499	0.247 ± 0.049
Flavonoids	Isoquercetin	$C_{21}H_{20}O_{12}$	5280804	16.895 ± 1.01
	Cynaroside	$C_{21}H_{20}O_{11}$	5280637	3.145 ± 0.18
	Astragalin	$C_{21}H_{20}O_{11}$	5282102	1.811 ± 0.61
	Quercetin	$C_{21}H_{20}O_{11}$	5280459	1.545 ± 0.55
	Aromadendrin	$C_{15}H_{12}O_6$	122850	1.334 ± 0.09
	Avicularin	$C_{20}H_{18}O_{11}$	5490064	0.893 ± 0.17
	Luteolin	$C_{15}H_{10}O_6$	5280445	0.765 ± 0.09
	Rutin	$C_{27}H_{30}O_{16}$	5280805	0.764 ± 0.01
	Kaempferol	$C_{15}H_{10}O_6$	5280863	0.598 ± 0.11
	Naringenin	$C_{15}H_{12}O_5$	439246	0.512 ± 0.05
	Isorhamnetin	$C_{16}H_{12}O_7$	5281654	0.479 ± 0.140
	Taxifolin	$C_{15}H_{12}O_7$	471	0.423 ± 0.78
	Quercetin-3-*O*glucuronide	$C_{21}H_{18}O_{13}$	5274585	0.32 ± 0.012
	Apigetrin	$C_{21}H_{20}O_{10}$	5280704	0.642 ± 0,07
	Apigenin	$C_{15}H_{10}O_5$	5280443	0.27 ± 0.098
	Phloridzin	$C_{21}H_{24}O_{10}$	6072	0.052 ± 0.001
	Quercetin	$C_{15}H_{10}O_7$	5280343	2.197 ± 0.01
Isoflavonoids	Daidzin	$C_{21}H_{20}O_9$	107971	nd
Stilbenes	Resveratrol	$C_{14}H_{12}O_3$	445154	0.028 ± 0.001

nd: not determined.

acid (1.635 ± 0.31), quercetin (1.545 ± 0.55), aromadendrin (1.334 ± 0.09), 3,4,5-trimethoxycinnamic acid (0.978 ± 0.06), and avicularin (0.893 ± 0.17). A few other phenolic compounds were also identified at lower concentrations and some of them were hardly detectable, such as luteolin, rutin, sinapinic acid, kaempferol, naringenin, isorhamnetin, taxifolin, quercetin-3-*O*-glucuronide, apigenin, hydroferulic acid, pyrocatechol, phloridzin, and resveratrol (Figure 2).

In the aqueous extract of *C. villosus* from Algeria, 21 phenolic compounds were identified by Bouziane et al. [12], including apigenin-C-hexoside, quercetin-3-*O*-glucoside, myricetin-3-*O*-glucoside myricetin-3-*O*-rutinoside, myricetin-*O*-rhamnoside, kaempferol-3-*O*-rutinoside, myricetin-*O*-coumaroylrutinoside, and quercetin-*O*-rhamnoside. The richness and diversity of the polyphenol content of *C. villosus* offer it many therapeutic possibilities. Notably, ferulic acid has anti-inflammatory and antioxidant capacities [33], and it seems to protect against cardiovascular [34] and renal diseases [35]. Quercetin has been known as an antiviral, antiulcer, anti-inflammatory, and antihypertensive agent [36,37].

3.4 Antioxidant activity

In the current investigation, the PPM assay was used to evaluate the TAC, whereas ABTS, DPPH and reducing power (RP) assays were used to assess the antioxidant activity (Table 4). The molybdate test indicated that the hydroethanolic extract had a high TAC value of 101.14 ± 3.02 mg EAA/g extract, while the aqueous extract showed an antioxidant capacity of 94.18 ± 2.31 mg EAA/g extract. For the DPPH method, the aqueous extract had the most important antioxidant power, with an IC_{50} (3.94 ± 0.09 µg/mL) compared with the hydroethanolic extract and BHT (IC_{50} = 4.81 ± 0.061 and 4.15 ± 0.19 µg/mL, respectively). Importantly, the ABTS test indicated that the aqueous extract had a higher level of free radical scavenging activity (IC_{50} = 2.88 ± 0.07 µg/mL), while the hydroethanolic extract had a lower level, with an IC_{50} value of 3.32 ± 0.12 µg/mL. These results were equivalent to the activities of ascorbic acid (2.14 ± 0.07 µg/mL).

The RP method confirmed that the effect of the aqueous extract is dose-dependent and higher than that of the hydroethanolic extract and ascorbic acid, with IC_{50} values of 1.94 ± 0.10 and 2.23 ± 0.06 µg/mL, respectively.

The abundance of phenolic molecules with strong antioxidant activity in *C. villosus* extracts justifies these results [38]. According to the literature, our results are superior to those reported by Bouziane et al. [12] where it was reported that the aqueous extract of *C. villosus* from Algeria exhibited IC_{50} values of 59 ± 2 and 468 ± 34 µg/mL for the DPPH and ABTS tests, respectively. Another study by Aourahoun et al. [39] performed on *C. villosus* leaves recorded an IC_{50} of 19.17 µg/mL for the hydroalcoholic extract in the DPPH assay.

The antioxidant activity of the plant studied in this research may be attributed to the correlation between the antioxidant tests and potassium activation in the enzymes that promote the biosynthesis of flavonoids and phenolic compounds [40], which is the most abundant mineral in the leaves of *C. villosus*. Additionally, common antispasmodic, antimicrobial, anti-inflammatory, and antioxidant properties have been associated with plant metabolites such as proanthocyanidins and flavonoids [41]. Isoquercetin was the most potent DPPH radical scavenger of the quercetin derivatives [42]. Similarly, Yasuda and co-authors [43] proved the DPPH radical scavenging abilities of gallic acid, the key structure of hydrolyzable tannins.

3.5 Anti-inflammatory effect

Our findings (Figure 3) indicated that the inhibition percentages of the aqueous extract of *C. villosus* (for 500 mg/kg bw) were 42.85 ± 1.27, 57. 95 ± 1.28, and 77.49 ± 0.59% after 1, 3, and 6 h of carrageenan injection, respectively. This effect is remarkable to that of indomethacin (10 mg/kg bw), which is 47.72 ± 0.58, 58.89 ± 0.42, and 75.29 ± 1.07%. Our results show better inhibition percentages relative to results reported by Aourahoun et al. [39] for the hydroethanolic extract of *Cytisus triflorus* at doses of 200 and 400 mg/kg bw, which exhibited inhibition percentages of 44.19 and 60.50%, respectively. However, our findings are lower than those previously published by Madoui [44], where the inhibition percentages were 80.05 and 88.56% at 4 and 6 h, respectively, for the 400 mg/kg dose of the crude extract of *Cytisus triflorus*, as the first phase of inflammation is mediated by bradykinin, serotonin, and histamine, whereas the second phase is mediated by prostaglandins and other cytokines [45,46]. The paw edema, which was caused by the accumulation of leukocytes near the inflammatory site, was significantly suppressed by the aqueous extract of *C. villosus*, a very rich extract in polyphenols. This is exemplified by luteolin derivatives, which protect against GalN/LPS-induced hepatotoxicity through the regulation of inflammatory mediators and phase II enzymes [47]. Moreover, the essential phenolic molecules that we discovered in the aqueous extract of *C. villosus* such as quercetin, sinapic acid, ferulic acid, caffeic acid, and gallic acid possess effective anti-inflammatory effects [48–53]. A study on

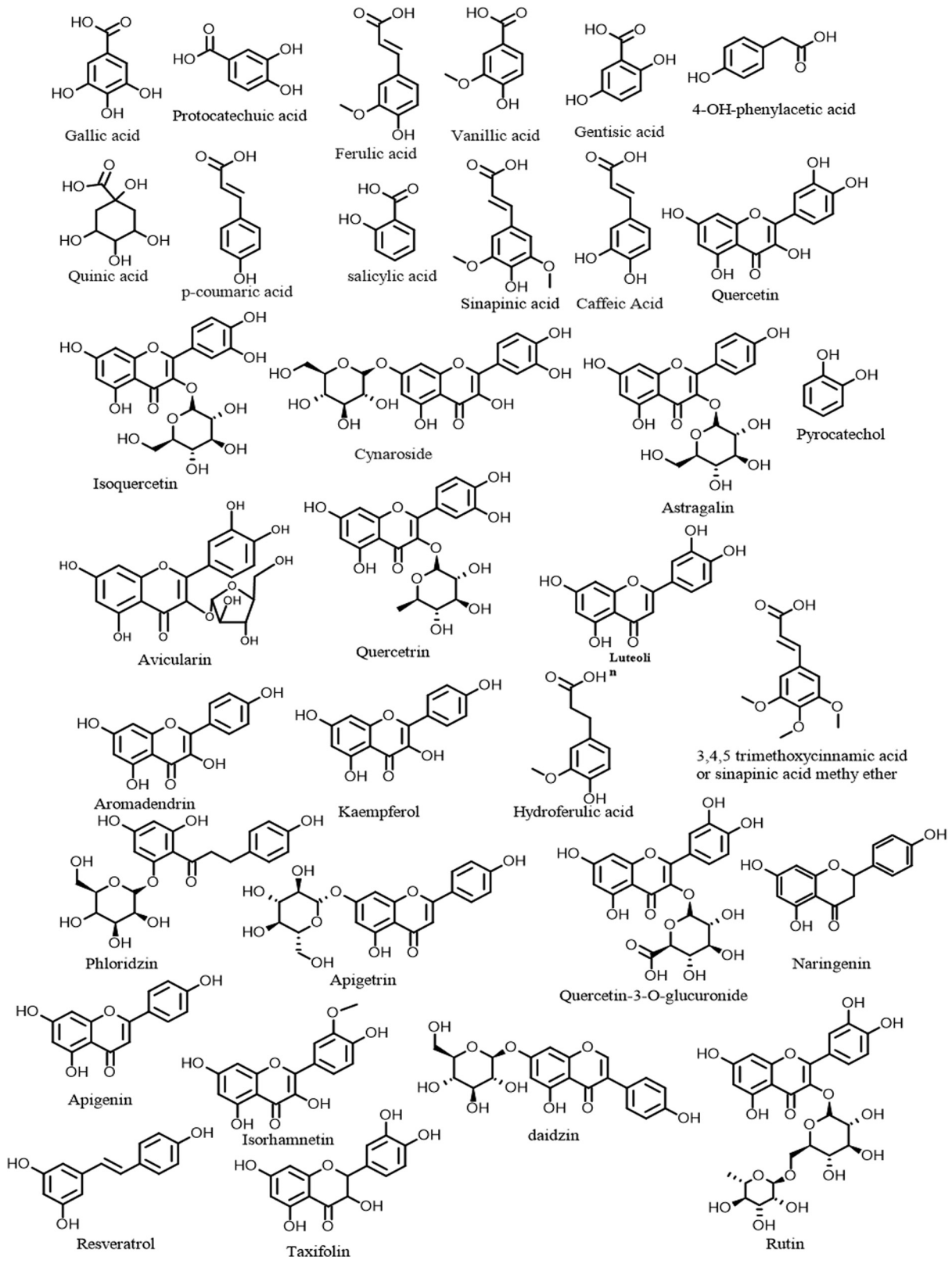

Figure 2: Structures of the main polyphenols detected in the aqueous extract of *C. villosus*.

Table 4: Antioxidant activities (ABTS, DPPH, RP, and molybdate) of *C. villosus* extracts (mean ± SEM)

	IC_{50} (µg/mL)			
	Aqueous extracts	**Hydroethanolic extracts**	**BHT**	**Ascorbic acid**
DPPH	3.94 ± 0.09	4.81 ± 0.061*	4.15 ± 0.19	—
ABTS	2.88 ± 0.07*	3.32 ± 0.12**	—	2.14 ± 0.07
RP	1.94 ± 0.10*	2.69 ± 0.06**	—	2.23 ± 0.06
Molybdate (mg AAE/g E)	94.18 ± 2.31	101.14 ± 3.02	—	—

*$P < 0.05$; **$P < 0.01$

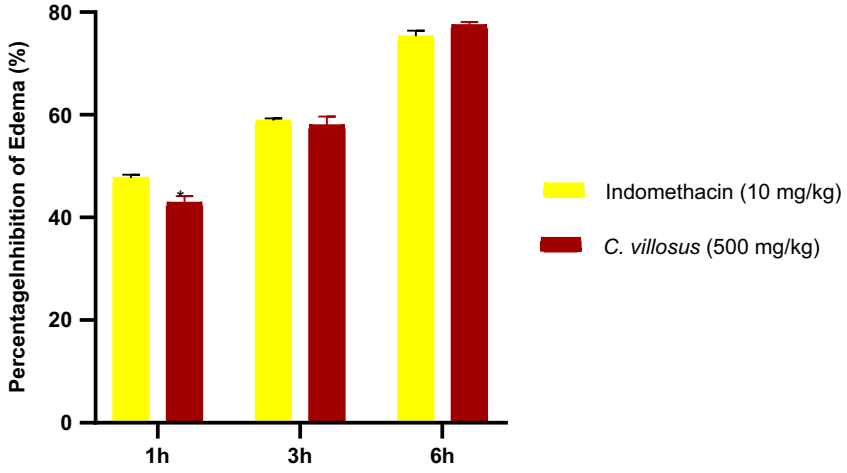

Figure 3: Anti-inflammatory effects of aqueous extracts of *C. villosus*. Mean ($n = 6$) ± SE; **$P < 0.001$.

testicular tissue of young rats by Saygin et al. [54] indicated a strong anti-inflammatory effect of gallic acid by reducing the formation of PGE 2 and calcitonin, and also decreases the expression of IL-6, TNF-α, and TGF-β in rats [48]. In the *in vivo* model, caffeic acid decreases PGE_2 and NO formation and also decreases COX-2, iNOS, and TNF-α expressions through reverse regulation of NF-κB transcription [55]. Using another inflammatory model, Doss et al. revealed that ferulic acid exhibited very interesting anti-inflammatory properties [56]. Lee reported that sinapic acid reduces TNF-α in TNBS-induced colonic inflammation in mice [57]. Importantly, Lesjak et al. [58] found that quercetin reduced the effects of 12-LOX and COX-1, the enzymes involved in the inflammatory response, in a measure-dependent manner.

3.6 Inhibition of albumin denaturation

The results of anti-inflammatory activity tests, which were conducted using albumin denaturation, are displayed in Figure 4. According to the results, the aqueous

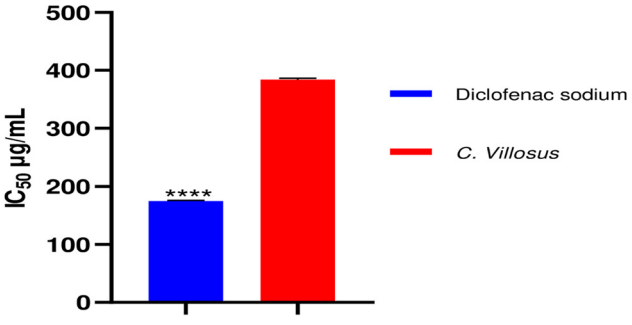

Figure 4: IC_{50} values of the inhibition of albumin denaturation of *C. villosus* and diclofenac sodium. Mean ($n = 3$) ± SE. The results are considered significantly different for ****$P < 0.0001$.

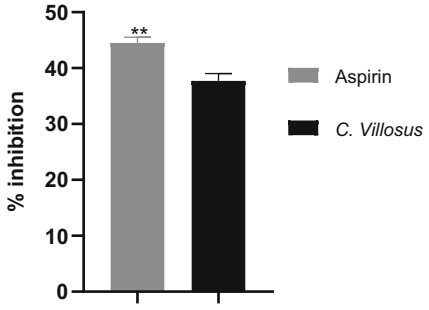

Figure 5: Efficacy of the *C. villosus* aqueous extract on acetic acid-induced writhing in rats. Mean ($n = 6$) ± SE; **$P < 0.01$.

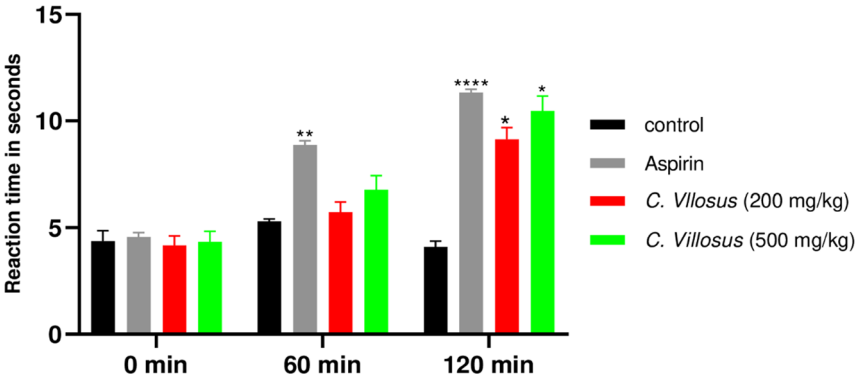

Figure 6: Latency of the tail flick of *C. villosus*. The results are mean of six experiments ± SEM. $^{*}P < 0.05$.

extract has a lower inhibition than the positive control diclofenac sodium ($P < 0.0001$) with an IC_{50} = 383.94 and 174.96 µg/mL, respectively.

The phytochemical analyses performed indicate that the aqueous extract shows significant amounts of condensed tannins and flavonoids, which may be responsible for this effect. Numerous research studies have demonstrated the well-known and interesting anti-inflammatory properties of flavonoids and condensed tannins [59,60]. The study by Sadique et al. [61] proved that flavonoids trigger STAT-1 and NF-kβ factor by blocking the signal transducer, which would inhibit inflammation.

3.7 Analgesic effects

The peripheral anti-nociceptive performance in rats was determined using the acetic acid-induced torsion method. This approach is widely recognized as a model of visceral inflammatory pain that allows a rapid assessment of this type of analgesic activity. In this method, the release of a number of inflammatory mediators, including cytokines, serotonin, and histamine, is induced by administering acetic acid [62]. Oral dosing of *C. villosus* (500 mg/kg bw) and aspirin (150 mg/kg bw) exhibited both analgesic activities, with 44.54 and 37.73% contortion inhibition, respectively (Figure 5).

The central analgesic actions of the aqueous extract and aspirin were assessed using tail-flick and plantar methods. The results are presented in Figures 6 and 7, respectively, and compared with aspirin. In the tail flick method, the aqueous extract (200 and 500 mg/kg) induced significant ($P < 0.05$) analgesic activity, after 120 min. The highest reaction times for the aqueous extract (200 and 500 mg/kg)-treated groups were 9.13 and 10.46 s, respectively, at 120 min, whereas it was 4.1 and 11.33 s for the saline- and aspirin-treated groups. Similarly, in the plantar method, oral application of an aqueous extract of *C. villosus* (250, 500 mg/kg) induced significant analgesic activity in a dose-related manner. A peak analgesic activity was reached within 1 h. The pain tolerance times were 4.25 s ($P < 0.05$) and 5.92 s ($P < 0.01$) for the aqueous extract at 200 and 500 mg/kg, respectively, whereas it was 7.55 s ($P < 0.0001$) in the case of aspirin.

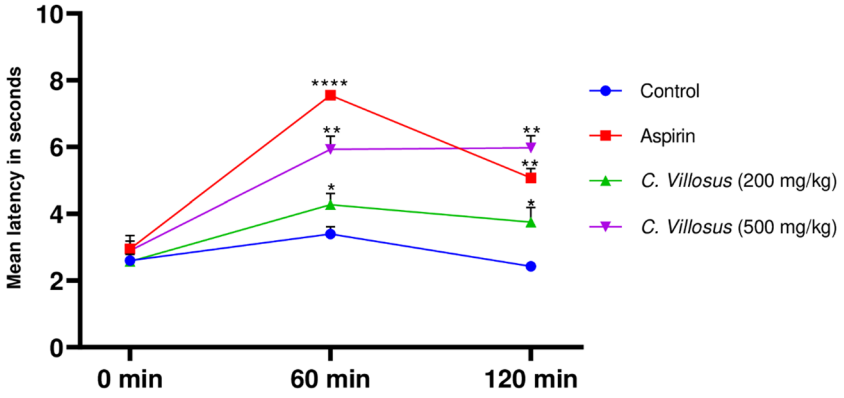

Figure 7: Effect of *C. villosus* aqueous extract in the plantar test. Mean ($n = 6$) ± SEM. $^{*}P < 0.05$.

The aqueous extract of *C. villosus* may contain phytochemicals that can block the cyclooxygenase enzyme to diminish pain at the peripheral level or that can intervene on opioid receptors at the central level to reduce this pain. The UPLC analysis of the aqueous extract of our plant shows the presence of analgesic molecules such as quercetin, ferulic acid, quinic acid, and gallic acid that have shown strong analgesic properties *in vivo* [63,65–67]. In the acetic acid-induced pain assay, quercetin (100–500 mg/kg, po) reduced the nociceptive defense response in a drug-related manner [67]. Additionally, quercetin reduced the pain caused by formalin in both phases and reduced the pain caused by capsaicin and glutamate by 75.5 and 68.2%, respectively [67]. Notably, Zhao et al. [63] showed that quercetin reduces the sensation of pain in the feet of mice and improves pain sensitivity, thus showing its analgesic properties. Moreover, ferulic acid increases the nociceptive threshold at doses of 40 and 80 mg/kg in the thermal hyperalgesia test [64]. Di-caffeoyl quinic acids from *Lychnophora ericoides* showed considerable analgesic action in the acetic acid-induced contortion assay [65]. In addition, da Silva et al. [66] revealed that gallic acid isolated from the leaves of *Calophyllum brasiliense* had a notable analgesic effect in the contortion test in mice. The results obtained above proved that the aqueous extract of *Cytisus villosus* has anti-inflammatory and analgesic properties, which confirms the fact that the traditional application of this plant could treat various diseases associated with inflammatory pain.

4 Conclusion

This study highlights the anti-inflammatory, analgesic, and antioxidant abilities of the aqueous extract of the phytodrug *Cytisus villosus* realized by *in vivo* and by *in vitro* tests. Furthermore, UPLC analysis revealed the content of several phenolic molecules with known anti-inflammatory and analgesic effects. These findings indicate that the aqueous extract has the potential to be used in developing a novel analgesic and anti-inflammatory functional food ingredient derived from natural products. Additional investigation is required, however, to confirm these activities by determining the action and toxicities on non-target organisms.

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Conflict of interest: The authors declare that there is no conflict of interest.

Ethical approval: The conducted research is not related to either human or animal use.

Data availability statement: All data generated or analyzed during this study are included in this published article.

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