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PHYTOCHEMICAL PROFILING AND PHARMACOLOGICAL EVALUATION OF *HELIANTHEMUM PILOSUM* L.

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ABSTRACT. *Helianthemum pilosum* L. *(Cistaceae),* traditionally used in the Algerian Sahara to treat gastrointestinal disorders, remains insufficiently characterized regarding its phytochemical composition and pharmacological properties. This study investigated the phytochemical profile, LC–MS/MS metabolite composition, and in vitro and in vivo biological activities of sequential solvent extracts from its aerial parts. Sequential fractionation of the hydromethanolic extract using dichloromethane, ethyl acetate, and n-butanol yielded fractions with distinct phytochemical profiles. The hydromethanolic extract (HPM) exhibited the highest total phenolic and flavonoid contents (165.88 mg GAE/g and 149.6 mg QE/g, respectively), and the n-butanolic extract (HPB) showed the strongest DPPH radical-scavenging activity (IC_{50} = 0.024 µg/mL). The antibacterial activity was moderate, particularly against *E. coli*, *K. oxytoca, and K. enterobacter.* Acute toxicity testing showed that *H. pilosum* extracts were non-toxic at 2000 mg/kg body weight. In vivo assays demonstrated significant analgesic, antipyretic, anti-inflammatory, antidiarrheal, and wound-healing activities. LC-MS/MS analysis identified 16 bioactive metabolites, with Shikimic acid, gallic acid, protocatechuic acid, quercetin-3-D-xyloside, quercetin, kaempferol-3-glucoside, trans-ferulic acid, vanillin, and p-coumaric acid as the major constituents. These findings validate the traditional use of *H. pilosum* and highlight its potential as a source of bioactive phenolic compounds for future pharmacological investigations.

KEY WORDS: *Helianthemum pilosum* L., Phytochemical study, Medicinal plant, Flavonoids, Biological activities

INTRODUCTION

Human populations have possessed empirical knowledge of the medicinal properties of herbs since their inception, as demonstrated by prehistoric records and subsequent cultural traditions [1]. Medicinal herbs are abundant in bioactive constituents, including alkaloids, phenolics, terpenoids, and essential oils, and many plant-derived substances have been standardized or semi-synthesized for use in approved pharmaceuticals. Recent analyses of drug approvals demonstrate that natural products and their derivatives remain a substantial source of pharmacologically relevant scaffolds, especially in the development of anti-infective and anticancer agents [2-4].

Simultaneously, international health organizations have established regulatory frameworks to guarantee the quality and safety of plant materials, encompassing GACP (Good Agricultural and Collection Practices) and quality standards for herbal medicinal products, thereby improving reproducibility and facilitating safer clinical application [5-7]. These advancements reinforce the relationship between traditional medicinal knowledge and modern pharmacological science, underscoring the necessity for thorough pharmacological and phytochemical investigations of local therapeutic herbs, especially in North Africa [8]. In Algeria, steppe and pre-Saharan

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ecosystems, exemplified by *Stipa tenacissima* formations, are molded by significant edaphic limitations that affect vegetation structure and local plant utilization patterns [9]. *Helianthemum pilosum* L. (*Cistaceae*), commonly known as *Eldjerache*, is acknowledged in reputable floristic literature and online taxonomic databases but is inadequately represented in ethnopharmacological studies [10-12]. In traditional medicine, the species is conventionally employed for the management of acute diarrhea and various intestinal ailments in humans and livestock, with decoctions occasionally combined with olive oil to mitigate adverse effects. We therefore hypothesized that *H. pilosum* extracts are rich in phenolics and flavonoids that confer significant biological activities. To test this hypothesis, it was selected for comprehensive phytochemical analysis of its extracts and isolated secondary metabolites containing primary active constituents responsible for antioxidant, antimicrobial, anti-inflammatory, antidiarrheal, and wound-healing activities.

Accordingly, the scientific objective of this study was to evaluate the biological, therapeutic, and pharmacological potential of the crude extracts and isolated compounds. This work is, to our knowledge, the inaugural thorough report on the phytochemical composition, biological activities, and LC–MS/MS profile of *H. pilosum*, providing novel chemical and pharmacological insights into an overlooked species of the Algerian flora, thus adding a significant study to ethnopharmacology. Taxonomic authentication and voucher specimen details are provided in the Materials and Methods section.

MATERIALS AND METHODS

Chemical products

Most reagents were purchased from Prochima-Sigma (Industrial Service Zone No. 2, Lot No. 6, Khemisti El Mostakbal, Hennaya, Tlemcen, Algeria; ISO 9001:2015 certified). Solvent selection and preparation were performed in accordance with the procedures described in Plant Drug Analysis [13]. For each extraction, 200 g of *H. pilosum* aerial parts were subjected to extraction using methanol (99.9%), LiChrosolv, Germany; n-hexane (95%), BIOCHIM Chemopharma, France; dichloromethane (99.9%), Honeywell, France/Germany; ethyl acetate (99.9%), Carlo Erba, Milan, Italy; n-butanol (99.9%), Sigma-Aldrich, Germany; chloroform (99.4%), Honeywell, Germany; acetone (99.9%), glacial acetic acid (99%), and formic acid (99%), supplied by Eden-Labo, Algeria. Antimicrobial assays were conducted using Mueller-Hinton agar (Biokar Diagnostics, France) in accordance with EUCAST guidelines [14]. Sodium diclofenac injectable solution (75 mg/3 mL) and paracetamol tablets (500 mg) were obtained from SAIDAL Group. Loperamide capsules (4 mg) were supplied by Pharmalliance. Acetic acid (99%) was purchased from Eden-Labo, whereas brewer's yeast was obtained from Biomax. *Carrageenan* was sourced from Wako Chemical Industries Ltd., and castor oil was supplied by CAP Pharm. Sulfadiazine 1% cream and pure vaseline were provided by Novapharm and Labioco, respectively.

Preparation of plant extracts

The aerial parts of *Helianthemum pilosum* collected from the Bir El-Ater region, eastern Algeria, were milled into a powder after being air-dried. The powder was macerated in hydromethanolic solvent ($MeOH/H_2O$) (80:20, v/v) for 48 h with intermittent stirring. Following through Whatman filter paper, the crude extract was successively divided into more polar solvents (n-hexane, dichloromethane, ethyl acetate, and n-butanol), each for a minimum of six hours. Three times, this partitioning process was repeated using fresh solvent at each step. The mixed organic extracts were concentrated under decreased pressure at 45 ± 2 °C using a BÜCHI Rotavapor R-100 (Flawil, Switzerland) and subsequently dried to constant weight in an incubator. The obtained

extracts comprised the hydromethanolic extract (HPM), *n-hexane* extract (HPH), dichloromethane extract (HPD), ethyl acetate extract (HPA), n-butanol extract (HPB), and a residual aqueous phase. All samples were weighed and then maintained at ambient temperature in the absence of light until further analysis [14].

Preliminary screening for phytochemicals

The extracts were subjected to phytochemical screening at the VPRS Laboratory using the standard procedures described by Wagner and Bladt [13]. The following qualitative and quantitative assays were performed.

Total phenolic content (TPC)

The Folin-Ciocalteu reagent (FCR), already diluted 1:10 with distilled water, was combined with 0.5 mL of each methanol extract solution to assess the total phenolic content. 2.0 mL of 7.5% (w/v) Na_2CO_3 was administered after a 5-minute incubation period. A UV-Vis spectrophotometer measured the absorbance at 760 nm after the mixture was maintained at ambient temperature in the absence of light for 30 to 40 minutes. A calibration curve for gallic acid (100-500 µg/mL) was employed to assess total phenolic content (TPC), thereafter expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g). Each assay was conducted in triplicate. [15].

Total flavonoid content (TFC)

The total flavonoid content (TFC) was evaluated using the aluminum chloride ($AlCl_3$) colorimetric method [16]. Each extract was diluted in methanol and filtered using Whatman filter paper; then, 1.0 mL of the filtrate was combined with 1.0 mL of 2% (w/v) $AlCl_3$ dissolved in methanol. The reaction mixture was incubated at ambient temperature in the absence of light, after which the absorbance was measured at 430 nm utilizing a UV-Vis spectrophotometer. Quantification was conducted utilizing a quercetin calibration curve spanning from 50 to 400 µg/mL, yielding a correlation coefficient of $R^2 \geq 0.99$. Results were reported as milligrams of quercetin equivalents per gram of dry extract (mg QE/g), with all studies conducted in triplicate. The assay relies on the establishment of a stable combination between flavonoids and aluminum ions, producing detectable absorbance at 430 nm under specified assay conditions.

$$\mathrm{TFC} = \frac{\mathrm{R}\times\mathrm{F.D}\times\mathrm{V}\times 100}{\mathrm{W}}$$

Where R is the value obtained from the standard calibration curve, F.D is the factor of dilution, V is the volume of the stock solution, 100 corresponds to the conversion factor for 100 g of plant material, and W is the weight of the plant material used.

In vitro tests

Determination of total antioxidant activity via DPPH assay

The radical-scavenging assay of DPPH was performed by preparing concentrated solutions of each extract in methanol and then diluting them to reach final concentrations of 0.1, 0.2, 0.4, 0.6, and 0.8 µg/mL [17]. For each dilution, 20 µL of DPPH solution (100 µg/mL in methanol) was added. The reaction mixtures were carefully blended and incubated for one hour at 37 °C in darkness. Absorbance was later measured at 517 nm using a UV-Vis spectrophotometer [18-20].

Antibacterial activity assay (Disk diffusion method)

MHA *(Mueller-Hinton agar)* was prepared from a commercially available dehydrated medium in compliance with the manufacturer's directives and standardized microbiological procedures based on the original method of *Mueller and Hinton* (1941) and current CLSI recommendations [21, 22]. Briefly, the medium was suspended in distilled water with continuous agitation, heated to boiling until completely dissolved, sterilized, and allowed to cool to 45-50 °C before use. The final pH was calibrated to 7.2 - 7.4, and the medium was dispensed onto sterile 9-cm Petri plates to achieve a consistent agar depth of 4 mm. Bacterial inoculum was prepared by suspending freshly isolated colonies in 5 mL of sterile 0.9% saline solution. The resulting suspensions were uniformly spread over the agar surface and allowed to dry for up to 30 min at 30-37 °C. Sterile paper disks impregnated with plant extract solutions at concentrations of 600, 300, 150, and 75 mg/L were then applied to the inoculated plates using sterile forceps. Dilutions were produced in sterile physiological saline, and a solvent control was incorporated in each test. Plates were incubated at 37 °C for 16-18 hours in an inverted position, after which antibacterial activity was evaluated by measuring the diameters of the inhibitory zones, including the disk diameter, with a ruler accurate to 0.5 mm, as specified in the *Kirby-Baue*r disk diffusion method [23].

In vivo evaluation

Preparation of experimental rats and extract

Wistar rats were housed under standard laboratory conditions and acclimatized for at least 5 days prior to the experiments. Animals were randomly allocated and individually identified. Environmental conditions, including temperature, relative humidity, and light/dark, were regularly controlled, and food, along with water, was supplied ad libitum, in accordance with OECD guidelines [24].

The cooked aqueous extract (HPC) was prepared as a decoction by boiling the plant material for 30 minutes, then filtering through Whatman filter paper. The filtrate was subsequently dried in an oven at 60 °C to obtain a powdered extract. This extract, together with the hydromethanolic extract (HPM), was used in animal experiments [25].

Acute oral toxicity test

The rat is the preferred species in this test, but other species can be used. The rats were aged 8 to 12 weeks and weighed between 150 and 250 grams. The rats were subjected to a fasting period of 4-5 hours prior to administration, with unrestricted access to water but no food. Preliminary acute oral toxicity was assessed following a single administration of the test extracts by gastric gavage. Three animals were utilized in accordance with OECD Guideline No. 423 for Acute Oral Toxicity, employing the Acute Toxic Class Method, which was authorized on 17 December 2001 for the experimental phase, with a limit dose of 2000 mg/kg body weight established. Two treatment groups received a single oral dose of 2000 mg/kg body weight of *H. pilosum* extract, namely the cooked aqueous extract (HPC) and the hydromethanolic extract (HPM), whereas the control group was supplied with 2 mL of physiological saline. Animals were continuously observed for the first 24 hours following treatment and subsequently examined daily for 14 days to detect signs of toxicity, behavioral alterations, and mortality. The LD50 value was established based on the observed toxicological findings [24, 26].

Analgesic activity (acetic acid–induced writhing test)

The analgesic efficacy was evaluated using the acetic acid-induced writhing method with some changes [25]. The assessment involved quantifying the abdominal contractions, or "writhings," elicited by an intraperitoneal injection of 0.1% acetic acid, given 30 min after oral administration.

Six groups of Wistar rats (n = 3 per group) were used, with the sample size selected according to the 3Rs principle (reduction) to minimize animal use while enabling a preliminary pharmacological evaluation. Group 1: Negative control (physiological saline); Group 2: Positive control (diclofenac, 10 mg/kg BW, intraperitoneal); Group 3: *H. pilosum* cooked aqueous extract (HPC) at 100 mg/kg BW; Group 4: *H. pilosum* cooked aqueous extract (HPC) at 300 mg/kg BW; Group 5: *H. pilosum* hydromethanolic extract (HPM) at 100 mg/kg BW; Group 6: *H. pilosum* hydromethanolic extract (HPM) at 300 mg/kg BW.

The % inhibition of writhing was determined using the following equation:

$$\%\ \text{analgesic activity} = \frac{\text{No. of control writhes} - \text{No.of treated writhes}}{\text{No. of control writhes}} \times 100$$

Antipyretic activity

Prior to the trial, rats were acclimatized, subjected to laboratory conditions, and shown to be healthy. Six groups were established, and all animals underwent a 24-hour fast while having unrestricted access to water. Baseline rectal temperatures were measured with a digital thermometer immediately before the onset of pyrexia. Fever was induced through subcutaneous injection of a 20% aqueous suspension of brewer's yeast (*Saccharomyces cerevisiae*) 10 mL/kg BW [25]. After 18 hours, rectal temperatures were measured again. Pyrexia was confirmed by an increase of > 0.3 °C relative to baseline. Animals were then treated orally according to the following grouping: Control group: Distilled water (10 mL/kg); Reference group: *Paracetamol* (150 mg/kg); Test groups: *H. pilosum* extracts administered at 100 and 300 mg/kg BW.

The percentage reduction of fever was computed using the following formula [27]:

$$R\ (\%) = \frac{(\text{Tp}-\text{Tn})}{\text{Tp}-\text{Ti}} \times 100$$

Where R% is the percentage reduction of fever, Tp is the temperature after induction of pyrexia, Tn is the temperature recorded after a given treatment time, and Ti is the initial (baseline) temperature.

Anti-inflammatory activity

The extracts' anti-inflammatory effectiveness was evaluated using the carrageenan-induced paw edema model [28]. The assay evaluates the efficacy of *H. pilosum* extracts in suppressing inflammation triggered in rat hind paws by a 1% carrageenan solution. Rats underwent a 16-hour fasting period before the experiment, with unrestricted access to water. Paw volume was measured with a digital caliper one hour before extract administration [29]. Extracts were administered orally via a gastric tube according to the following design: Negative control group: 2 mL of distilled water (oral); Positive control group: Diclofenac 10 mg/kg BW (intraperitoneal); Test groups: *H. pilosum* cooked aqueous extract (HPC) and hydromethanolic extract (HPM) at doses of 100 and 300 mg/kg BW.

One hour post-treatment, inflammation was elicited via subplantar injection of 0.1 mL of 1% carrageenan into the right hind paw pad (U/FPP). Paw volume was recorded hourly for 5 hours [30].

The percentage augmentation in paw volume (PA) and the % inhibition of oedema (PI) was calculated using the following formulas [31].

$$PA = \frac{t_t - t_0}{t_0} \times 100$$

$$PI = \frac{PATe - PATr}{PATe} \times 100$$

Where t_0 is the initial paw thickness before oedema induction; t_t is the paw thickness measured after treatment; PA is the average percentage augmentation in paw volume; PI is the % inhibition of oedema; PA_{te} represents the percentage increase in thickness of the paw in the control group; and PA_{tr} represents the percentage increase in thickness of the paw in the treated group [32].

Antidiarrheal activity (Castor oil–induced diarrhea model)

Antidiarrheal activity was evaluated as follows: Rats fasted for 24 hours before the experiment and had unrestricted access to water. Each rat was housed in a separate cage lined with clean blotting paper [33].
Six experimental groups were established: Control group: Received 2 mL of distilled water (oral); Reference group: Treated with *Loperamide* (4 mg/kg BW); Test groups (4): Rats received H. pilosum cooked aqueous extract (HPC) or hydromethanolic extract (HPM) at doses of 100 and 300 mg/kg BW.

Thirty minutes after administration of the treatments, each animal was administered 1 mL of castor oil orally. Rats were monitored for a duration of 5 hours, and the severity of diarrhea was assessed by counting the number of diarrheal droppings in each cage and comparing them with the control group.
The % inhibition of diarrhea was determined using this formula [34]:

$$\%\ \text{Inhibition diarrhea} = [(A - B)/A]) \times 100$$

A represents the average number of defecations in the control group, while B denotes the total number of defecations recorded after treatment.

Wound-healing activity

This study sought to assess the wound-healing potential of the aerial parts of *H. pilosum* using an experimental model of second-degree burns in rats.

Preparation of ointments

Pure Vaseline was gradually incorporated into the dried aqueous extract of *H. pilosum* with gentle stirring until a homogeneous mixture was obtained. *Sodium benzoate* was added as a preservative at a concentration of 150 mg per 100 g of ointment. Two formulations containing 3% and 10% extract were prepared. The ointments were transferred to hermetically sealed containers and stored at ambient temperature, protected from direct light.

Induction of second-degree burns

The wound-healing model was performed according to the following experience: Rats were divided into four groups (n = 3 per group): Group 1: Untreated rats (negative control); Group 2: Rats treated with sulfadiazine cream (positive control); Group 3: Rats administrated with *H. pilosum* ointment (3%); Group 4: Rats treated with *H. pilosum* ointment (10%) [35].

Animals were anesthetized with a 3% chloral hydrate solution administered intraperitoneally (1 mL/100 g BW) [36]. Second-degree burns were induced by applying a metal cylinder (3 cm in diameter), previously heated for 5 minutes, onto the shaved dorsal skin of the rats with gentle pressure for 20 seconds [37].

Wound progression and healing were monitored daily for 14 days using a scoring system that evaluates changes in burn appearance and tissue regeneration [38].

Characterization of natural substances

Separation and purification of compounds by TLC–CC

Column chromatography (CC) is a widely used technique for the separation and isolation of bioactive phytochemicals from plant extracts. Fractionation is achieved by sequential elution with solvents of increasing polarity to obtain chemically distinct fractions. Thin-layer chromatography (TLC) analyses were performed using three to four solvent systems to optimize resolution of individual constituents. Solvent systems were selected to establish a polarity gradient, ranging from non-polar (e.g., hexane) to polar mixtures (e.g., methanol- or water-based systems), enabling efficient separation and visualization of compounds. Prior to isolation, each extract was screened by analytical TLC using small aliquots of the selected mobile phases to determine appropriate elution conditions. Fractions displaying similar TLC profiles were pooled, while compounds presenting single, well-defined spots were considered purified. Purified isolates were subsequently collected in clean, labeled tubes for further characterization.

Liquid chromatography-Mass spectrometry

The chemical components of the HPD, HPA, and HPB fractions of *H. pilosum* were examined utilizing a UPLC-ESI-MS/MS system (6460 Triple Quadrupole with Agilent Jet Stream Technology). For analysis, 50 mg of each sample was placed into an Eppendorf tube and extracted using 2 mL of methanol. The mixture was extensively vortexed and underwent liquid-liquid partitioning using hexane. Following centrifugation at 9000 rpm for 10 minutes, 100 µL of the methanolic phase was extracted and diluted to a final volume of 900 µL with a water/methanol mixture (1:1, v/v) containing 0.1% formic acid. The solution was subsequently filtered before injection into the LC-MS/MS equipment. Chromatographic analysis was performed under the following conditions: column temperature at 40 °C, flow rate at 0.4 mL/min, and injection at 5.12 mL volume [39].

UV-Visible spectroscopy

UV-visible spectroscopy is a qualitative analytical technique used to record absorbance spectra, identify characteristic chromophores, and support the structural validation of isolated compounds. It is widely employed in natural products chemistry for preliminary identification of functional groups and conjugated systems. In the present study, UV spectra of the 17 purified fractions of *H. pilosum*, previously isolated by column chromatography, were recorded using a UV-Vis spectrophotometer with 1 cm quartz cuvettes. Measurements were performed in methanolic solution.

All in vitro assays (antioxidant and antibacterial) were performed in triplicate (n = 3) and expressed as mean ± SD. Antibacterial activity was evaluated by measuring the inhibition zone diameter (mm) in 90 mm Petri dishes. Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparison test for inter-dose comparison.

All in vivo assays (acute toxicity, analgesic, antipyretic, anti-inflammatory**,** antidiarrheal and wound healing) were performed with 3 animals per group (n = 3) and expressed as mean ± SEM. Wound-healing data were analyzed by two-way ANOVA (time × treatment) followed by Bonferroni post-hoc test.

All graphs and calculations were performed using OriginPro 9.0 (OriginLab Corporation, Northampton, MA, USA) and GraphPad Prism 10.4.1 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

RESULTS AND DISCUSSION

Phytochemical characterization and extraction yield

The extraction yields of the different solvent fractions of *H. pilosum*. Among all fractions, the ethyl acetate extract (HPA) showed the highest yield, suggesting a preferential solubilization of medium-polarity constituents in this solvent system.

Preliminary phytochemical screening validated the existence of multiple categories of secondary metabolites characteristic of the species, indicating a rich and diverse phytochemical profile revealing a very high abundance (+++) of flavones, free flavonoids (genins), glycoside flavonoids, cyanidins, anthracene derivatives, O-heterosides with reduced genins, coumarins, saponins, mucilage, reducing compounds and tannins; a moderate abundance (++) of leuco-anthocyanins, C-heterosides, combined anthracene derivatives, cardiac glycosides, catechuic tannins and total polyphenols; a low abundance (+) of alkaloids (≥1% and <1%), sterols, triterpenes and essential oils; whereas, absent (-) of flavanones, anthocyanins, catechols, free anthracene derivatives, O-heterosides, carotenoids, gallic acid and tannins.

Quantitative analysis of the hydromethanolic extract (HPM) revealed high levels of total phenolic content (TPC: 165.88 mg GAE/g) and total flavonoid content (TFC: 149.6 mg QE/g), highlighting the abundance of antioxidant secondary metabolites that may influence the documented biological activity.

In vitro tests

Antioxidant activity (DPPH assay)

The antioxidant efficacy of *H. pilosum* extracts was assessed via the DPPH free-radical scavenging assay, which measures the capacity of a sample to convert the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH·) into its non-radical form (DPPH-H). The IC_{50} value, which denotes the concentration necessary to scavenge 50% of DPPH radicals, is inversely correlated with antioxidant capacity; diminished IC_{50} values signify enhanced activity. The IC_{50} values for the various extracts are presented in Table 1.

Among the tested extracts, the n-butanolic fraction (HPB) exhibited the strongest DPPH radical-scavenging activity with an IC_{50} of 0.024 µg/mL, followed by the dichloromethane extract (HPD, IC_{50} = 0.077 µg/mL), the hydromethanolic extract (HPM, IC_{50} = 0.118 µg/mL), the ethyl acetate extract (HPA, IC_{50} = 0.161 µg/mL) and the n-hexane extract (HPH, IC_{50} = 0.284 µg/mL). All extracts showed lower activity than the reference standard ascorbic acid (IC_{50} = 0.0013 µg/mL).

These results listed in Table 1 confirm that *H. pilosum*, particularly the HPB and HPD extracts, possesses strong antioxidant potential, consistent with its high polyphenol and flavonoid content.

Table 1. Antioxidant activity (IC_{50}) of *H. pilosum* plant extracts.

Extract	IC_{50} (µg/mL)
HPH	0.284
HPD	0.077
HPA	0.161
HPB	0.024
HPM	0.118

Ascorbic Acid	0.0013

(n=3). $p < 0.001$.

Antibacterial activity

The antibacterial activity of *H. pilosum* extracts is summarized in Table 2. The extracts HPA, HPB, and HPM showed a resistant inhibitory activity. A slightly intermediate activity was also observed for the dichloromethane extract (HPD) against *Escherichia coli* at 600 mg/L, ethyl acetate extract (HPA) against *K. oxytoca* at 300 mg/L, and n-butanolic extract (HPB) against *K. enterobacter* at 300 mg/L.

Table 2. Antibacterial activity of *H. pilosum* extracts.

	Inhibition zone diameter (mm)					
Extract	HPD		HPA		HPB	
C (µg/mL)	300	600	300	600	300	600
Escherichia coli	7***	8***	3	3	4	3
K. pneumoniae	3	4	4***	7***	6.5**	7**
R. ornithinolytica	3	3	3	3.5*	3	3
K. oxytoca	5**	3***	8***	7**	3	3
Proteus mirabilis	3.5	3	3	2.5	3.5	3
K. enterobacter	3	4	4	6.5**	8***	7**
C. leishmaniosis	2.5	2.5	2.5	2.5	2.5	2.5

(n=3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

In vivo tests

Acute oral toxicity

Oral administration of cooked extract (HPC) and hydromethanolic extract (HPM) at doses up to 2000 mg/kg body weight did not induce mortality in any treated animals. Acute toxicity testing was conducted according to standard acute toxicity guidelines, indicating that the extracts can be classified as non-toxic under the tested conditions. No deaths or severe toxicological signs were observed during the initial 24 h or throughout the 14-day observation period. Only mild and transient behavioral changes, such as reduced locomotor activity, transient lethargy, tremors, or scratching, were occasionally recorded, without associated morbidity or mortality [40]. Consequently, no mortality was observed during 14 days up to 2000 mg/kg (BW) of *H. pilosum* extracts; thus, LD50 is considered >2000 mg/kg, with values of 2071 ± 12.25 for HPC and 2000 ± 11.43 for HPM at a dose of 350 mg orally (n=3).

Analgesic activity

Table 3 shows the analgesic activity in rats treated with *H. pilosum* plant extracts and in the presence of diclofenac as a reference. The protective effect of diclofenac produced 92.69% inhibition. whereas HPC and HPM at 300 mg/kg produced 77.08% and 84.63% inhibition, respectively.

Table 3. Analgesic activity of *H. pilosum* extracts in rats.

Oral dose (mg/kg)	N°. Writhes	% Inhibition
Physiologic water	132.33 ± 8.73	-
Diclofenac inj (10)	9.66 ± 0.58	92.69
HP Cooked (100)	69 ± 4	47.86
HP Cooked (300)	30.33 ± 1.15	77.08
HP Methanol (100)	33.33 ± 1.53	74.81
HP Methanol (300)	20.33 ± 0.58	84.63

(n=3). P < 0.001

Antipyretic activity

Figure 1 (A) shows the percentages of pyrexia inhibition produced by the tested treatments. Subcutaneous injection of brewer's yeast caused a marked elevation in rectal temperature 18 h after administration, confirming successful induction of experimental pyrexia in rats. Paracetamol produced the most pronounced antipyretic effect, reducing fever by 90.26% relative to the distilled-water control group. Table 4 shows that *H. pilosum* extracts also exhibited appreciable antipyretic activity, with the hydromethanolic extract (HPM, 100 mg/kg BW) and the cooked aqueous extract (HPC, 100 mg/kg BW) reducing pyrexia by 53.32% and 52.24%, respectively, after 4 h of treatment.

Table 4 Antipyretic activity by *H. pilosum* extracts in rats.

Oral dose (mg/kg bw)	Percentage reduction of fever (R%)			
	R1% (1h)	R2% (2h)	R3% (3h)	R4% (4h)
Distilled water	3.03 ± 4.1	7.89 ± 6.0	12.29 ± 6.9	11.23 ± 8.1
Paracetamol 150	30.69 ± 20.3*	57.59 ± 26.0*	76.89 ± 15.4***	90.26 ± 11.4***
HPC 100	10.56 ± 15.4	31.98 ± 15.7*	44.13 ± 10.4**	52.24 ± 6.2***
HPC 300	13.24 ± 4.3*	22.72 ± 6.6*	30.03 ± 12.1	37.15 ± 13.4*
HPM 100	12.15 ± 21.1	20.25 ± 28.7	32.09 ± 26.3	53.32 ± 20.6*
HPM 300	17.87 ± 5.7*	36.82 ± 15.0*	33.57 ± 6.6*	38.26 ± 9.7*

(n=3). *p < 0.05, **p < 0.01, ***p < 0.001.

Anti-inflammatory activity

In this model, carrageenan-induced acute inflammation was evaluated in rats. Two *H. pilosum* extracts, cooked-aqueous (HPC) and hydromethanolic (HPM), at 100 and 300 mg/kg BW doses, in the presence of diclofenac (10 mg) as a reference, were studied. The results are presented in Table 5, illustrated in Figure 1 (B) and Figure 2 (A). Five hours after induction of oedema, the volume of oedema decreased significantly to 96.35% for the diclofenac, and 84.24% for the HPM at the 300 mg/kg dose, then 82.06% for the HPC at the same dose. These results indicate a marked but incomplete inhibition of carrageenan-induced oedema.

Table 5. Anti-inflammatory activity (1% carrageenan paw oedema) in rats.

Oral dose	Paw oedema inhibition (PI %)				
(mg/kg BW)	PI1% (1h)	PI2% (2h)	PI3% (3h)	PI4% (4h)	PI5% (5h)
Distil water	-	-	-	-	-
Diclofenac	41.95 ± 1.79	54.09 ± 1.23	69.12 ± 0.02	84.74 ± 1.38	96.35 ± 0.61
HPC (100)	17.27 ± 2.72	33.54 ± 3.19	49.68 ± 1.40	63.61 ± 2.18	70.87 ± 2.21
HPC (300)	20.40 ± 4.33	36.44 ± 3.71	61.57 ± 1.70	71.09 ± 2.11	82.05 ± 0.81
HPM (100)	16.98 ± 0.32	34.36 ± 4.28	52.48 ± 2.01	66.19 ± 1.73	74.14 ± 1.27

HPM (300)	22.14 ± 4.00	38.06 ± 3.93	59.83 ± 2.39	72.42 ± 2.08	84.23 ± 1.27

(n=3). $p < 0.001$.

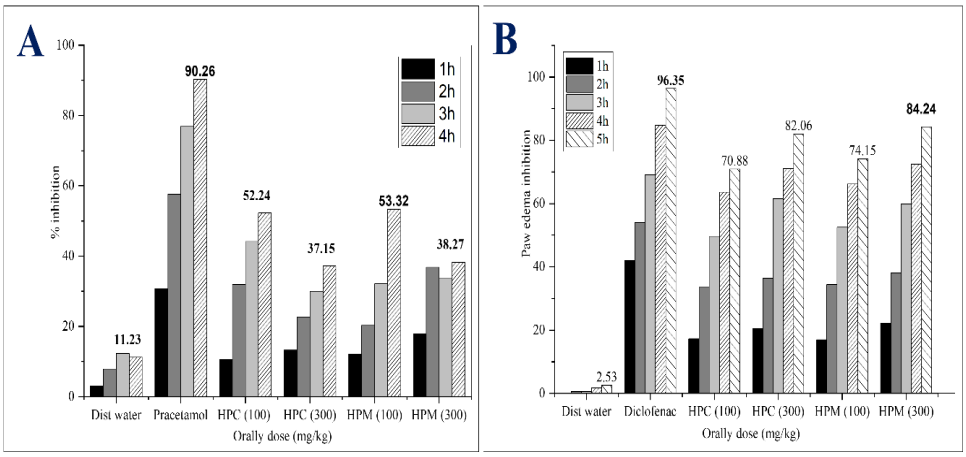

Figure 1. (A) Antipyretic activity by *H. pilosum*. (B) anti-inflammatory activity by *H. pilosum*.

Antidiarrheal activity

As illustrated in Table 6, *H. pilosum* extracts showed marked protective effects against castor oil-induced diarrhea. At 300 mg/kg, the cooked aqueous extract (HPC) and the hydromethanolic extract (HPM) reduced the number of diarrheal droppings from 104 in the negative control group to 23 and 14, respectively. Both extracts also diminished the frequency and severity of defecation and delayed the onset of diarrhea. The strongest inhibition was recorded for HPC 300 and HPM 300, with values of 77.95% and 86.28%, respectively, exceeding those observed at the lower doses and approaching or surpassing the effect of loperamide (82.82%).

Table 6. Antidiarrheal activity of *H. pilosum* extracts in rats.

Treatment (mg/kg)	Mean N° of defecations (5h)	% Inhibition
Dist. water 2 mL	104 ± 0.35	-
Loperamide	17.87 ± 0.70	82.82
HPC (100)	43.67 ± 2.93	58.01
HPC (300)	22.93 ± 1.01	77.95
HPM (100)	42.07 ± 0.64	59.55
HPM (300)	14.27 ± 0.90	86.28

(n=3). $p < 0.001$

Wound-healing activity

The wound-healing activity of *H. pilosum* ointments was assessed in a second-degree burn model in rats using a standardized scoring system. The untreated group showed delayed and incomplete healing, with persistent necrosis at day 4 (score 5) and only partial recovery at day 14 (score 4.33). The *sulfadiazine*-treated group exhibited marked improvement over time, reaching near-complete re-epithelialization by day 14 (score 0.66), confirming its strong healing effect. Both *H. pilosum* formulations significantly accelerated wound repair after 14 days. The 3% ointment showed the

highest efficacy (score 1.66), while the 10% ointment also improved healing (score 2.66), though to a lesser extent. Overall, both treatments enhanced burn wound recovery, with the 3% formulation demonstrating the most pronounced effect (Table 7 and Figure 2 (B)).

Table 7. Wound-healing activity of *H. pilosum* extracts in rats.

	Scores (n)			
Time (days)	8d	10d	12d	14d
Untreated	4.67 ± 0.58	4.33 ± 0.58	4.00 ± 0.00	4.33 ± 0.58
Sulfadiazine	3.00 ± 0.00**	1.67 ± 0.58**	1.00 ± 0.00***	0.67 ± 0.58**
HP 3%	3.67 ± 0.58	2.33 ± 0.58*	2.00 ± 0.00***	1.67 ± 0.58**
HP 10%	4.00 ± 0.00	3.33 ± 0.58	3.00 ± 0.00***	2.67 ± 0.58*

(n=3). *p < 0.05, **p < 0.01, ***p < 0.001

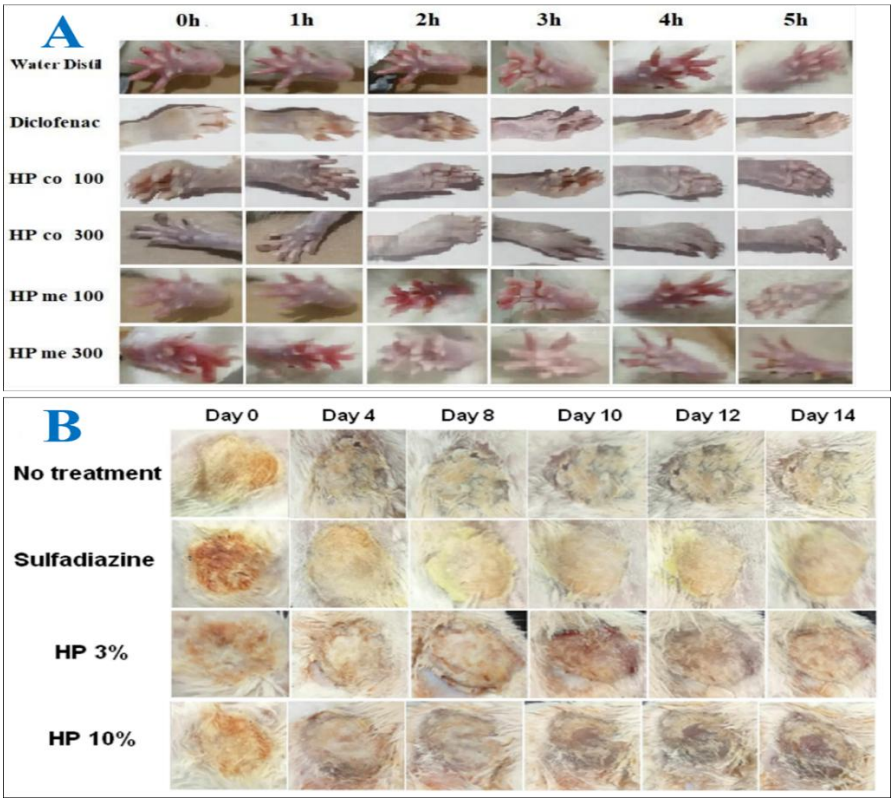

Figure 2. (A) Images of anti-inflammatory activity, (B) Images of the wound-healing activity.

Characterization of natural substances

Column chromatography

Chromatographic fractionation of *H. pilosum* extracts yielded 17 purified fractions obtained by successive elution with solvents of increasing polarity. These included 5 fractions from the dichloromethane (HPD), 7 from the ethyl acetate extract (HPA), and 5 from the n-butanol extract

(HPB). This distribution highlights the chemical diversity of the plant, with compounds ranging from low to high polarity: non-polar to moderately non-polar constituents in HPD and polar flavonoid- and glycoside-rich compounds in HPB. Overall, the results confirm the efficiency of the chromatographic method in separating the complex phytochemical profile of *H. pilosum* [41, 42].

LC-MS/MS analysis

UPLC-ESI-MS/MS analysis of the HPD, HPA, and HPB extracts enabled the tentative detection of 18 compounds belonging to different phytochemical classes, including: flavonoids (isoquercitrin, quercetin-3-D-xyloside, kaempferol-3-glucoside, fisetin, quercetin, naringenin, and kaempferol); organic acids (shikimic acid); phenolic acids (gallic acid, protocatechuic acid, chlorogenic acid, p-coumaric acid, salicylic acid, and trans-ferulic acid); and phenolic aldehydes (hydroxybenzaldehyde and vanillin); alkaloid (caffeine); saponin (diosgenin) [43]; the results are presented in (Figure 3 and Table 8).

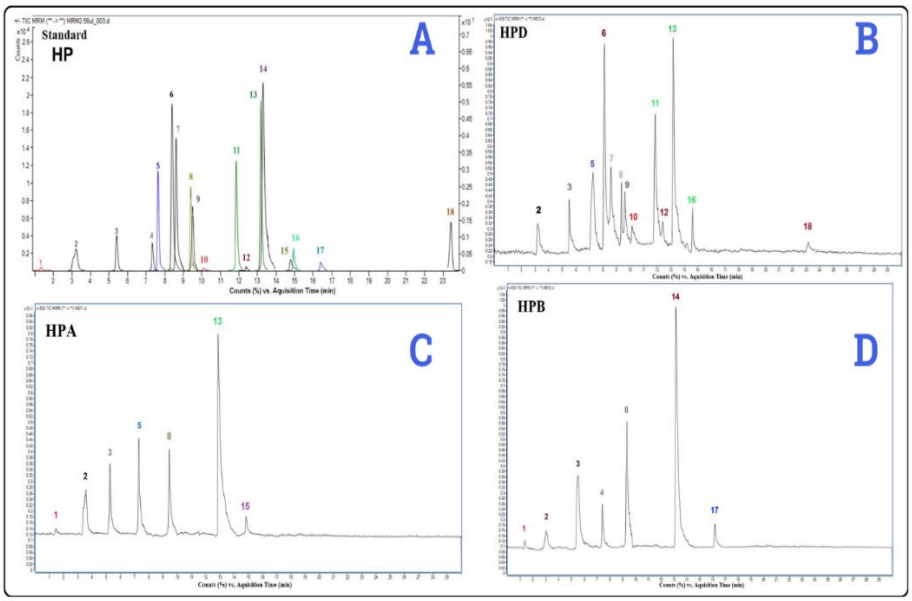

Figure 3. (A, B, C, D) LC/ MS spectra of detected compounds from *H. pilosum* extracts.

These fractions showed a chromatographic profile strongly dominated by shikimic acid (74.42%), gallic acid (10.01%), protocatechuic acid (3.04%), quercetin-3-D-xyloside (1.81%), quercetin (1.70%), kaempferol-3-glucoside (1.53%), *trans*-ferulic acid (1.46%), vanillin (1.36%), and p-coumaric acid (1.35%) as the major constituents. This pattern indicates several abundant phenolics and flavonoids constituents [44].

The HPB fraction showed the greatest distribution, representing 55.03% of the total quantified compounds, followed by HPA (34.05%) and finally HPD (10.92%). The wider distribution of flavonoids and phenols in HPB and HPA indicates greater biological activity [45].

Table 8. UPLC-ESI-MS/MS analysis of *H. pilosum* fractions.

N°	Extract	(mg/g) of extract	tR (min)	Ionization mode (m/z)	m/z	Tentatively identified compound	Molecular formula
1	HPD	ND	1.304	$[M-H]^-$	173	Shikimic acid	$C_7H_{10}O_5$
	HPA	0.694					
	HPB	1.242					
2	HPD	0.068	3.761	$[M-H]^-$	169	Gallic acid	$C_7H_6O_5$
	HPA	0.065					
	HPB	0.127					
3	HPD	0.024	5.373	$[M-H]^-$	153	Protocatechuic acid	$C_7H_6O_4$
	HPA	0.025					
	HPB	0.030					
4	HPD	ND	7.326	$[M-H]^-$	353	Chlorogenic acid	$C_{16}H_{18}O_9$
	HPA	ND					
	HPB	0.002					
5	HPD	0.012	7.712	$[M+H]^+$	123	Hydroxybenzaldehyde	$C_7H_6O_2$
	HPA	0.003					
	HPB	ND					
6	HPD	0.001	8.396	$[M+H]^+$	195	Caffeine	$C_8H_{10}N_4O_2$
	HPA	ND					
	HPB	ND					
7	HPD	0.035	8.600	$[M-H]^-$	151	Vanillin	$C_8H_8O_3$
	HPA	ND					
	HPB	ND					
8	HPD	0.009	9.307	$[M+H]^+$	165	*p*-coumaric acid	$C_9H_8O_3$
	HPA	0.019					
	HPB	0.007					
9	HPD	0.015	9.722	$[M+H]^+$	139	Salicylic acid	$C_7H_6O_3$
	HPA	ND					
	HPB	ND					
10	HPD	0.038	9.939	$[M-H]^-$	193	*trans*-ferulic acid	$C_{10}H_{10}O_4$
	HPA	ND					
	HPB	ND					
11	HPD	0.014	11.93	$[M-H]^-$	463	Isoquercitrin	$C_{21}H_{20}O_{12}$
	HPA	ND					
	HPB	ND					
12	HPD	0.047	12.347	$[M-H]^-$	433	Quercetin-3-D-xyloside	$C_{20}H_{18}O_{11}$
	HPA	ND					
	HPB	ND					
13	HPD	0.017	13.139	$[M+H]^+$	449	Kaempferol-3-glucoside	$C_{21}H_{20}O_{11}$
	HPA	0,023					
	HPB	ND					
14	HPD	ND	13.302	$[M+H]^+$	287	Fisetin	$C_{15}H_{10}O_6$
	HPA	ND					
	HPB	0.003					
15	HPD	ND	14.841	$[M+H]^+$	303	Quercetin	$C_{15}H_{10}O_7$
	HPA	0.044					
	HPB	ND					
16	HPD	0.021	14.471	$[M+H]^+$	273	Naringenin	$C_{15}H_{12}O_5$
	HPA	ND					
	HPB	ND					
17	HPD	ND	16.124	$[M+H]^+$	287	Kaempferol	$C_{15}H_{10}O_6$
	HPA	ND					

	HPB	0.010					
18	HPD	0.008	23.398	$[M+H]^+$	415	Diosgenin	$C_{27}H_{42}O_3$
	HPA	ND					
	HPB	ND					

ND: not detected

These polarity-dependent differences may partly explain the biological divergence observed between the three fractions. HPB displayed the strongest antioxidant activity in the DPPH assay, and the three fractions showed moderate antibacterial activity, particularly against *Escherichia coli, K. oxytoca,* and *K. enterobacter* [46, 47].

Link between identified compounds and biological activities

The LC-MS profiling revealed that shikimic acid, although a non-phenolic cyclitol, is the biosynthetic precursor of phenylpropanoids and exerts significant anti-inflammatory and analgesic effects via inhibition of NF-κB and MAPK pathways and suppression of TNF-α and IL-6 [48]. Its high percentage explains the potent anti-inflammatory, analgesic, and wound-healing effects of HPB/HPA fractions in vivo. Gallic acid, with three hydroxyl groups, is one of the strongest ROS scavengers and disrupts bacterial cell walls and membrane integrity, supporting the strong DPPH activity and the antibacterial zones against *E. coli, K. Enterobacter* and *K. oxytoca* (8.0 mm). Protocatechuic acid enhances antioxidant capacity due to the catechol moiety. Flavonols (quercetin-3-D-xyloside, quercetin, and kaempferol-3-glucoside) with a 3',4'-dihydroxy B-ring and 2,3-double bond-4-oxo conjugation show antioxidant activity 4-fold higher than Trolox and inhibit *S. aureus* α-haemolysin and increase membrane resilience [49]. Finally, trans-ferulic and p-coumaric acids are reported as the main antibacterial hydroxycinnamates against enterobacteria [50]. The synergism between shikimic acid as a major scaffold and these phenolics/flavonoids justifies the superior activity of the hydromethanolic extract (HPM). Trace compounds (caffeine 0.001 and diosgenin 0.008 µg/mg) were below LOQ (< 0.01 µg/mg) and excluded.

CONCLUSION

This study presents the inaugural comprehensive and pharmacological characterization of *Helianthemum pilosum* L., a traditionally used but poorly documented medicinal species from southern Algeria. The hydromethanolic extract and its derived extracts displayed marked differences in both chemical composition and biological performance, with HPM showing the highest total polyphenol and flavonoid contents and HPB exhibiting the strongest antioxidant activity. Selective antibacterial effects were observed mainly against *Escherichia coli* and *Klebsiella* spp. In vivo experiments demonstrated that the extracts were well tolerated at 2000 mg/kg body weight and possessed substantial analgesic, antipyretic, anti-inflammatory, antidiarrheal, and wound-healing effects. UPLC-ESI-MS/MS profiling enabled the tentative identification of 18 compounds belonging to multiple phytochemical classes, revealing distinct extract-dependent signatures. In particular, HPD was strongly dominated by gallic acid, quercetin-3-D-xyloside, trans-ferulic acid, and vanillin, whereas HPA displayed higher relative levels of shikimic acid, gallic acid, and naringenin. Finally, HPB was strongly dominated by shikimic acid, gallic acid, and protocatechuic acid. Overall, these findings highlight marked chemical differences between the three fractions that may contribute to their distinct biological activities. Overall, the current findings provide scientific validation for the traditional use of *H. pilosum* and identify this species as a viable source of bioactive compounds with potential pharmacological relevance. The observed chemical divergence among fractions further underlines the importance of extraction and fractionation procedures in the valorization of medicinal plants. Future investigations should

aim to isolate the main active compounds, validate their biological effects in mechanistic models, and establish robust analytical markers for extract standardization.

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Conflict of interest

The authors confirm that this manuscript is original and has not been published before. There are no conflicts of interest related to this work.

Declaration of generative AI in scientific writing

The authors declare that no generative AI or AI-assisted technologies were used in the writing, editing, analysis, or preparation of this manuscript.

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