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Original Research

Study of Antidepressant, Anxiolytic, Sedative-Hypnotic, and Anticonvulsant Activities of *Haplophyllum acutifolium* (DC.) G.Don Aqueous Extract in Mice

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Abstract

In traditional Persian medicine, *Haplophyllum acutifolium* (DC.) G.Don has been utilized as a treatment for central nervous system (CNS) disorders with sedative-hypnotic effects, as recommended by Avicenna. This plant is recognized for its pharmacological characteristics stemming from the existence of various bioactive compounds including alkaloids, coumarins, flavonoids, and lignans. However, a comprehensive investigation of the sedative-hypnotic, anxiolytic, antidepressant, and anticonvulsant activities of *H. acutifolium* extracts has yet to be conducted. The present investigation aimed to assess the sedative-hypnotic, anxiolytic, antidepressant, and anticonvulsant potential of various extracts obtained from *H. acutifolium* in NMRI and Swiss mice. Total of 250 mice randomly distributed into specific groups based on the test type. The sedative-hypnotic, anxiolytic, antidepressant, and anticonvulsant properties of extracts of *H. acutifolium* were assessed using various tests, including open field, the pentobarbital-induced sleep, elevated plus maze, forced swimming, maximal electroshock, and pentylenetetrazol tests. Aqueous, methanol and dichloromethane extracts were administrated intraperitoneally at doses of 25, 50, 100, 200 and 400 mg/kg. Additionally, the concentration of quercetin in *H. acutifolium* aqueous extract was determined using reversed-phase high-performance liquid chromatography (RP-HPLC). Total phenolic (TPC) and flavonoid contents (TFC) were assessed by spectroscopic methods as well. Upon behavioral tests, it was found that the aqueous extract has the biggest sedative-hypnotic, anxiolytic, antidepressant, and anticonvulsant effects. TPC and TFC of *H. acutifolium* hydrolyzed aqueous extract were measured as 61.48 ± 2.34 mg GAE/g and 21.13 ± 3.15 RE/g respectively on the basis of colorimetric findings. The RP-HPLC analysis revealed the average quercetin content of 49.87 ± 0.24 mg/100 g dry matter in the aqueous extract. The findings provide a reliable approach for measuring the quantity of quercetin existing in hydrolyzed *H. acutifolium* aqueous extract, as well as its neuropharmacological effects.

Keywords: Anticonvulsant; Anxiolytic; *Haplophyllum acutifolium*; High-performance liquid chromatography; Quercetin; Sedative-hypnotic

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Introduction

Several epidemiological studies have reported that insomnia affects approximately 12-20% of the worldwide population [1]. Insomnia is characterized by difficulties in initiating/maintaining sleep, as well as premature awakening [2]. Subsequent studies have demonstrated that insomnia is a commonly occurring comorbid condition among individuals who suffer from depression and anxiety. Hence, various treatment strategies including pharmacotherapy and behavioral therapies are utilized to tackle these disorders [3]. The United States Food and Drug Administration (FDA) has approved benzodiazepines, non-benzodiazepines, melatonin agonists, H_1-receptor antagonists, and orexin antagonists as promising therapeutic agents for the management of insomnia. Furthermore, certain antidepressants, such as amitriptyline and trazodone, as well as anticonvulsants like gabapentin and pregabalin, have been utilized as treatments for insomnia [4]. Research findings indicate that prescription drugs administered for a singular neurological disorder may elicit either a constructive or adverse outcome on other neurological disorders. Specifically, medications utilized to treat depression have a significant effect on the convulsive thresholds, which may vary depending on the duration of administration. Recent investigations propose that the extended usage of certain antidepressants may either reduce or augment the anticonvulsant properties of particular antiepileptic drugs [5,6].

In spite of appropriate therapeutic responses to existing medications for insomnia, patients suffer from some adverse effects [7]. To address such problems, natural products can be considered as an alternative therapy. For example, *Artemisia dracunculus* L. and *Stachys lavandulifolia* Vahl showed potential for addressing depression [8]. Iran is well-known for employing herbal remedies for tackling neurological disorders, such as insomnia, depression, and anxiety. *Rosa damascena* Mill., *Crocus sativus* L., *Melissa officinalis* L., *Valeriana officinalis* L., *Passiflora incarnata* L., *Sesamum indicum* L., and *Matricaria chamomilla* L. are examples of such medicinal plants, used to treat sleep disorders. Besides, *Citrus aurantium* L. demonstrates anxiolytic, and *Hypericum perforatum* L. shows antidepressant-like effects [9-11].

The *Haplophyllum* genus (Rutaceae) is represented in the Iranian flora with a total of 18 species, of which nine are endemic to the region. From a botanical perspective, the species are predominantly classified as perennial herbs, although some low shrub forms can also be observed. They possess inflorescences arranged in cymes and accompanied by bracts. Petal colors range from pale white to vibrant yellow. These species exhibit ten stamens with filaments that are free and notably expanded at the base; while the inner surface of the filaments is covered in fine pubescent [12,13]. The distribution range of this genus is extensive, as it includes a wide variety of regions including Morocco, Spain, Romania, Somalia, China, Turkey, Iran, and Central Asia [12]. Certain taxa belonging to the genus *Haplophyllum* have undergone great investigations regarding their noteworthy antimicrobial, antileishmanial, anti-inflammatory, gastroprotective, hepatoprotective, neuroprotective, cytotoxic, and anticancer properties [14]. Different phytochemical compounds have been isolated from *Haplophyllum* species, the most important classes of secondary metabolites in this genus are alkaloids, lignans, phenolic compounds, and flavonoids [15,16]. The flavonol, quercetin, has been detected in several species within the *Haplophyllum* genus [17-19].

Morphologically, *Haplophyllum acutifolium* (DC.) G.Don has five sepals and yellow flowers. The ovary of the specimen is composed of five locules. The stamens, totaling ten in number, possess filaments that are separate and enlarged below and on the inner surface. The capsules, on the other hand, remain indehiscent [20]. Ethnobotanical investigations show that the citizens of eastern Turkmen Sahra, located in Iran use aerial parts of *H. acutifolium* decoction to promote wound healing and treat skin inflammations [13]. *H. acutifolium* (Syn. *H. perforatum, Ruta acutifolia, R. flexuosa, R. perforata,* and *R. sieversii*) has been used as a medicinal plant with neuroprotective properties including sedative-hypnotic effects, as documented in Avicenna's Canon of Medicine [21,22].

In this study, sedative-hypnotic, antidepressant, anxiolytic, and anticonvulsant effects of the extracts of *H. acutifolium* were assessed using behavioral tests in mice. Besides, the quercetin content in *H. acutifolium* hydrolyzed aqueous extract was investigated by reversed-phase high-performance liquid chromatography (RP-HPLC) equipped with a diode array detector (DAD). The total phenolic content (TPC) and total flavonoid content (TFC) of the aqueous extract were measured, as well.

Materials and Methods

Plant Collection and Identification

The aerial parts of *H. acutifolium* were collected from Golestan province, Iran in June 2022. Subsequently, it was identified by a qualified botanist at the Traditional Medicine and Materia Medical Research Center (TMRC), Shahid Beheshti University of Medical Science, Tehran, Iran (voucher specimen# THRC498).

Plant Extraction

The air-dried plant material (300 g) underwent initial grinding to obtain a fine powder before being sieved (Pharmacopoeia no. 3 sieve, 50 mesh). Then, about 300 g aliquots of powdered plant material were accurately weighed and extracted three times (macerated for 24 h) using 1000 mL of distilled dichloromethane, methanol, and water solvents, respectively. The extracts were filtered using Whatman filter paper and transferred to a Heidolph rotary evaporator (Schwabach, Germany) to obtain

a concentrated extract.

Chemicals

The solvents used for plant extraction and HPLC analysis were of suitable purity and were purchased from Merck Co. (Darmstadt, Germany). The following compounds, namely, Aluminum chloride, Folin-Ciocalteu, gallic acid, rutin, hydrochloric acid, quercetin, midazolam, naloxone, diazepam, Tween 80 (polyoxyethylene sorbitan monooleate), sodium pentobarbital, pentylenetetrazol (PTZ), fluoxetine, imipramine, and flumazenil, were purchased from Sigma-Aldrich Co. (Steinheim, Germany). The Millipore Milli-Q purification system (Bedford, MA, USA) was employed to prepare purified water.

Animals and Study Design

Male Swiss mice (for the forced swimming test) and NMRI albino mice (for the rest of the behavioral tests) weighing between 20-25 g were utilized in the experiments. The animals were bred at the School of Pharmacy, Shahid Beheshti University of Medical Sciences, and kept in a controlled environment, maintaining a temperature of 25 ± 2 ºC and a light/dark cycle of 12 hours. The light period lasted from 07:00 to 19:00. The mice were given free access to food and water, except during experimental trials. Behavioral observations were conducted on randomly assigned groups (n=8) between 10:00 AM and 3:00 PM. In each experiment, the animals were categorized into different groups. One group served as the control group and received a normal saline solution (0.9% W/V) plus 1% Tween 80; while the other groups were considered experimental groups. In the experimental groups, the animals were administered intraperitoneally with various doses (25, 50, 100, 200, and 400 mg/kg) of the extracts in order to evaluate their pharmacological effects. The animals were acclimated to the laboratory environment for an hour before testing began. The research adhered to the laboratory animal use and care principles outlined in the European Community guidelines (EEC Directive of 1986; 86/609/EEC). The study protocol received approval from the Ethical Committee of Shahid Beheshti University of Medical Sciences in Tehran, Iran (R.SBMU. ACE.1402.059).

Behavioral Assessments

Open Field Test (OFT)

This comprised a locomotor behavior trial, followed by an experiment in an open-field arena (40 × 40 × 40 cm; Plexiglas; transparent). Mice were let into the arena 30 min after i.p. injections of the plant extracts and recorded on how long it will take an animal to move 30 meters. The total distance moved, based on Jones et al. (2009), was measured for each mouse using the video-tracking system EthoVision® XT version 8 (Noldus Information Technology, Wageningen, The Netherlands) [23].

Pentobarbital-induced Sleeping Test

The study investigated the hypnotic properties of *H. acutifolium* using the pentobarbital-induced loss of righting reflex test according to the method performed by Almasirad et al. in 2021 [24]. The extract was administered at doses of 50, 100, and 200 mg/kg, with normal saline plus 1% Tween 80 as a vehicle and diazepam (2 mg/kg) as a positive control. The sleep duration was measured based on the interval between the loss and return of the righting reflex after sodium pentobarbital (40 mg/kg) injection. To determine the potential mechanism of action, flumazenil (10 mg/kg), an antagonist of benzodiazepine receptors, and naloxone (1 mg/kg), an antagonist of opioid receptors, were given 15 minutes before treatment.

As we will show in the results section, in both open-field and pentobarbital-induced sleep tests, the aqueous extract was more potent than the prepared extracts. Consequently, aqueous extract was selected for further behavioral studies and the determination of TPC and TFC.

Elevated Plus Maze (EPM)

The EPM apparatus used in this study was comprised of four arms, each measuring 40 cm in length. Two of these arms were enclosed by walls that were 20 cm in height; while the other two were open. The arms were arranged such that the two closed arms faced each other, as did the two open arms. The maze was elevated 50 cm above the ground. Anxious mice instinctively tend to avoid open spaces and prefer closed ones; therefore, the possible anxiolytic effects of *H. acutifolium* were evaluated using a method previously used by Mariah Mesquita et al. [25]. The data were analyzed using Ethovision® XT software.

Forced Swimming Test (FST)

We also investigated the antidepressant-like properties of *H. acutifolium* using the forced swimming test based on the Porsolt method. The forced swimming test (FST) is an experimental procedure developed for the assessment of the efficacy of antidepressants in rodents. This assessment is grounded in the animal's reaction to a situation that induces stress, specifically its willingness to escape. In this study, male Swiss albino mice were put in a cylindrical plexiglass container filled with water at a fixed temperature (22-25 °C), 30 minutes after being administered with different doses of the extract or vehicle (control) *via* intraperitoneal injection. The diameter and depth of the container were 14 and 30 cm, respectively. During an eight-minute observation period, the duration of immobility was measured specifically within the final six minutes. The "immobility time" is used to describe the duration during which animals cease struggling to escape and instead try to float on the surface of the water. Following their removal from the water, the animals were placed in a warm environment to undergo drying (26).

Pentylenetetrazol (PTZ) and Maximal Electroshock (MES) Tests

The anticonvulsant activities of the aqueous extract were evaluated using two seizure models: MES and PTZ. These experiments aimed to assess the anticonvulsant properties of the aqueous extract using established seizure models, providing insights into its efficacy in preventing seizures induced by maximal electroshock or pentylenetetrazol. The MES test followed the parameters of 60 Hz frequency, 50 mA current intensity, and 0.2 seconds duration as employed by Toolabi et al. (2020). The PTZ test was carried out following the methodology described by Khoramjouy et al. (2021). Both tests were performed 30 minutes after administering the aqueous extract or vehicle. The MES test involved counting the number of animals that showed hind limb tonic extensions (HLTE), representing the presence of seizures. In the PTZ test, the mortality rate of animals following seizures during 24 hours after PTZ injection was considered as indicator of the effectiveness of the aqueous extract in preventing PTZ-induced seizures. [27-29].

Determination of TPC and TFC

In order to determine the TPC in the aqueous extract of *H. acutifolium*, the Folin-Ciocalteu method was utilized. To conduct the experiment, a volume of 25 µL of the sample solution was mixed with 125 µL of a 10% Folin-Ciocalteu reagent. Following 5 minutes of incubation, a solution of sodium carbonate (Na_2CO_3) with a concentration of 7.5% (V/V) was added to the mixture in 96-well plates. The plates were covered with aluminum foil and shaken at 80 rpm for two hours before measuring absorbance at a wavelength of 760 nm using a Bio-Tek Powerwave XS microplate spectrophotometer (Winooski, Vermont, USA). A calibration curve was established using five concentrations of gallic acid (20, 40, 80, 160, and 200 µg/mL). The calibration curve produced an equation ($y = 0.0045x + 0.0905$, R_2=0.99) that was utilized to determine the quantity of TPC in milligrams of gallic acid equivalent per gram of dry extract (mg GAE/g dry matter). The experiment was replicated three times and reported as mean ± SD values [30].

The quantification of TFC was conducted using a solution consisting of 4% sodium hydroxide (NaOH), 5% sodium nitrite ($NaNO_2$), and 10% Aluminum chloride ($AlCl_3$) in distilled water. To conduct the experiment, a 96-well plate was used. Each well initially contained 100 µL of distilled water and 7.5 µL of $NaNO_2$ solution. Then, 25 µL of the sample solution was added to each well. After a six-minute interval, a mixture comprising 7.5 µL $AlCl_3$, 100 µL NaOH, and 10 µL distilled water was introduced into every well. The spectrophotometer was used to determine the absorption at a wavelength of 510 nm after waiting for fifteen minutes. Calibration curves were plotted using five concentrations of rutin (20, 40, 60, 80, and 100 µg/mL) as standards with an equation derived from the standard curve ($y=0.0208x+0.1919$ with R^2=0.99). To determine the TFC, the standard curve was used to calculate the amount of rutin equivalent in milligrams per gram of dry extract.

Both experiments were conducted with three replications and the reported values included both the mean and standard deviation. [30].

RP-HPLC-DAD Analysis of Quercetin

Sample Preparation

Firstly, thin-layer chromatography fingerprinting of the aqueous extract, quercetin, and the hydrolyzed extract was performed. The spots represented the presence of quercetin in the hydrolyzed aqueous extract. Due to the presence of a high content of glycosylated flavonoids, we used the hydrolyzed extract for the HPLC analysis. Hence, HCl (1.1 M) was added to the aqueous extract before HPLC analysis. A specific weight of 30 mg of the hydrolyzed aqueous extract was measured and subsequently dissolved in a solution consisting of 1 ml of H_2O:ACN (1:1), which was then diluted to a concentration of 10 mg/ml. Before the HPLC analysis, the solution underwent filtration using a 0.22 µm syringe filter.

Preparation of Standard Solutions

The stock solution was transferred to an Eppendorf tube and subsequently diluted with solvents, thereby producing the requisite working solutions. The standard solution of quercetin was freshly prepared in H_2O:ACN (1:1), at varying concentrations (1, 5, 10, 25, 50, and 100 µg/mL).

Chromatographic Instrumentation and Conditions

HPLC analysis of *H. acutifolium* hydrolyzed aqueous extract was performed with a Shimadzu SCL-10AVP HPLC system (Kyoto, Kyoto Prefecture, Japan). It consists of LC-10ADVP micro piston pump with seal wash, a column oven, four-channel membrane degassers (DGU-14A), and a diode array detector (SPD-10AVP, 190-600 nm). In this study, a Machery-Nagel C_{18} column (250 mm × 4.6 mm, 5 µm; Düren, Nordrhein-Westfalen, Germany) was utilized. The mobile phase consisted of 0.1% formic acid in H_2O (A) and ACN (B). The flow rate used during the experiment was 0.8 mL/min and the column temperature was set to 35 °C. The gradient program for quercetin quantification and detection in *H. acutifolium* was as follows: 0-5 min, 90% (B); 5-20 min, 90-60% (B); 20-30 min, 60-50% (B). The injection volume was 20 µL, the total run time was 33 minutes, and a detection wavelength of 370 nm was employed. [31].

Method Validation

The assay was also validated according to linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ) as presented by ICH guidelines

(ICH, 2015) [32]. The linearity of the method was tested using the correlation coefficient (R^2) obtained from the equation for the calibration curve. Recovery rates were calculated at two concentration levels to guarantee the precision of the method. Consequently, we analyzed the concentration of quercetin before and after being spiked with the standard quercetin. Recoveries were calculated according to the equation recovery (%) = (practical contents − theoretical contents)/amount spiked × 100%. The intra-day precision variations were checked by determining the standard samples of low, medium, and high concentrations in six replicates daily. The inter-day precision variations were determined by analyzing the standard samples at three concentrations for three consecutive days. The stability of the sample solutions was examined at various time points (0, 2, 4, 8, 12, 24, and 48 hours) over a period of two days under room temperature condition. Additionally, the LOD and LOQ were established based on signal-to-noise ratios of approximately 3 and 10, respectively.

Statistical Analysis

Statistical analysis was conducted using the GraphPad Prism software version 9 (San Diego, CA, USA). The normality of the data was assessed using the D'Agostino and Pearson normality test. The experimental results were reported as the mean ± standard error of the mean (SEM). One-way analyses of variance (ANOVA) were performed, followed by Tukey's post hoc multiple comparison tests. Statistical significance was attributed to differences with a p-value < 0.05. To analyze the variations among different groups, significance levels of *p< 0.05, **p< 0.01, ***p< 0.001, and ****p< 0.0001 were compared to the control group (n=8 in all groups).

Results

Evaluation of locomotor activity in open field test

The parameter under investigation pertains to the cumulative displacement and the impact of plant extracts on animal locomotion. The study records the total distance moved in centimeters over 10 minutes, under the influence of extracts obtained from the aerial parts of *H. acutifolium* at different doses of 25, 50, 100, and 200 mg/kg. The results illustrated a significant reduction in motor activity among animals treated with the extracts in comparison with the animals in the control group which may indicate the sedative effects of the extracts. The aqueous extract provided the most significant sedative effect, as evidenced by its ED_{50} value of 36.7 mg/kg (Table 1, Figure 1).

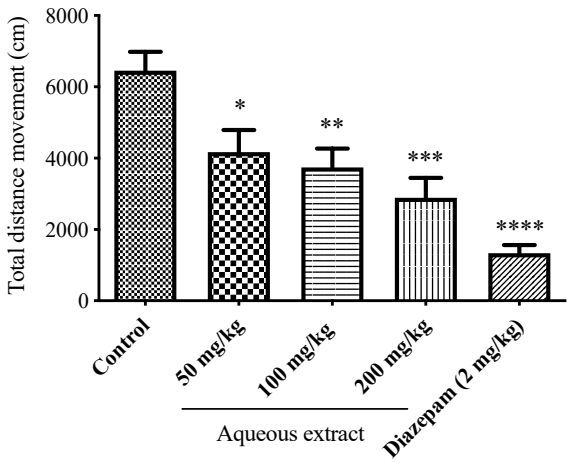

Figure 1. Effects of *H. acutifolium* aqueous extract on locomotor activity in open field test. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 compared with the control group. The results were analyzed by one-way ANOVA followed by Tukey's test. Data are described as mean + SEM (n = 8).

Hypnotic effects in pentobarbital-induced sleep test

As depicted in the result, the extracts were administered at varying doses of 25, 50, 100, and 200 mg/kg. The results showed a significant increase in sleep time treated with the extracts, as compared to those in the control group. Among them, the aqueous extract had the highest hypnotic effect with an ED_{50} value of 12.8 mg/kg (Table 1). As shown in table 1, in both open-field and pentobarbital-induced sleep tests, the aqueous extract showed better effects (lower ED_{50}s) compared to the other extracts. Therefore, we used the aqueous extract for the rest of the behavioral tests.

To investigate the potential mechanism of action of aqueous extract, naloxone (1 mg/kg), an antagonist of opioid receptors, and flumazenil (10 mg/kg), an antagonist of benzodiazepine receptors, were administered intraperitoneally in the pentobarbital-induced sleep test. Figure 2

Table 1. Sedative and hypnotic effects of extracts obtained from the aerial parts of *H. acutifolium* and diazepam (n=8)

Extracts and samples	ED_{50} (95% CI) mg/kg Open field test	ED_{50} (95% CI) mg/kg Pentobarbital-induced sleep test
CH_2Cl_2 extract	63.7 (42.0 to 96.5)	54.78 (38.3 to 78.0)
Aqueous extract	36.7 (28.1 to 47.9)	12.8 (8.2 to 19.8)
MeOH extract	122.3 (99.0 to 150.9)	57.10 (41.7 to 80.8)
Diazepam	2.02 (1.7 to 2.4)	1.93 (1.7 to 2.1)

shows that naloxone and flumazenil were unable to inhibit the sedative effect of an aqueous extract in a pentobarbital-induced sleep test.

Elevated plus maze

In the EPM model, the anxiolytic effect of a substance is evaluated based on the cumulative duration of the mouse's presence in the open arms. Figure 3 illustrates the negative control group, a positive control group treated with midazolam diluted with normal saline (2 mg/kg), and mice treated with varying doses (50, 100, and 200 mg/kg) of the aqueous extract. Results indicated a significant increase in time spent in the open arms by mice treated with the *H. acutifolium* aqueous extract at a dose of 200 mg/kg compared to the control group ($p < 0.01$). Thus, this extract at doses of 200 mg/kg demonstrated anti-anxiety effects in the EPM model (Figure3).

Immobility time in the forced swimming test

The forced swimming test is conducted to assess depression-like behavior. It involves measuring the amount of time the animals spend immobile and providing information about the potential antidepressant effects of a substance. Figure 4 illustrates a notable difference in the duration of immobility among the experimental groups. The animals were treated with the aqueous extract at doses of 50, 100, and 200 mg/kg. The extract administered at doses of 100 and 200 mg/kg exhibited a significant reduction in the immobility time of animals in water compared to the control group ($p < 0.01$ and $p < 0.001$ respectively). Fluoxetine (32mg/kg) and Imipramine (32mg/kg) were administered as positive controls.

Anticonvulsant activity in the PTZ and MES Tests

In this study, a total of eight animals were given varying doses (100, 200, and 400 mg/kg) of the aqueous extract. The results indicate that the percentage of animal protection differed between the groups. The ED_{50} values of the aqueous extract were determined through PTZ and MES tests. The calculated values can be found in tables 2 and 3.

TPC and TFC

TPC and TFC of *H. acutifolium* hydrolyzed aqueous extract was calculated as 61.48 ± 2.34 mg GAE/g and 21.13 ± 3.15 RE/g dry matter, respectively.

Quercetin content

The quercetin calibration curve was obtained by injecting the working solutions into the column and conducting HPLC analysis. The calibration curves were plotted using quercetin working standard solutions at six different concentration points to assess linearity, accuracy, precision, LOD, and LOQ. These evaluations were performed to verify the validity of the method. The curve areas were utilized to create calibration curves, which were subse-

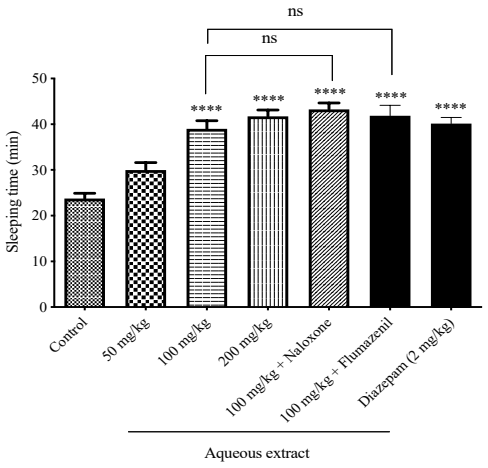

Figure 2. Effects of *H. acutifolium* aqueous extract with naloxone (1 mg/kg) and flumazenil (10 mg/kg) on the sleeping time in a pentobarbital-induced sleep test. ****p < 0.0001 compared with the control group. The results were analyzed by one-way ANOVA followed by Tukey's test. Data are described as mean + SEM (n = 8).

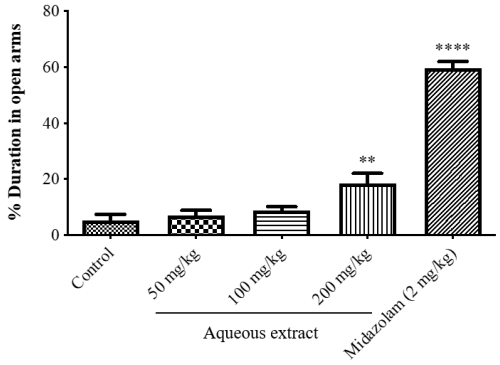

Figure 3. Effects of *H. acutifolium* aqueous extract on the percent of duration in open arms in the EPM test. Midazolam (2 mg/kg) was used as a positive control. **p < 0.01, ****p < 0.0001 compared with the control group. The results were analyzed by one-way ANOVA followed by Tukey's test. Data are described as mean + SEM (n = 8).

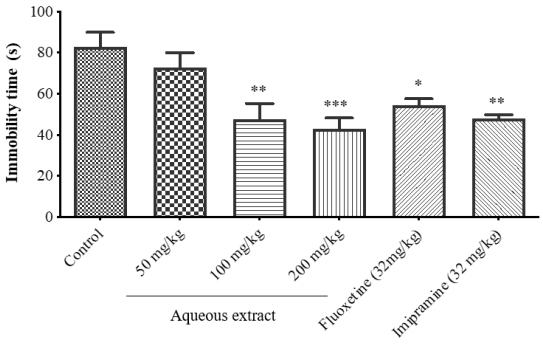

Figure 4. Effects of *H. acutifolium* aqueous extract on the immobility time in the forced swimming test. Fluoxetine (32 mg/kg) and Imipramine (mg/kg 32) were used as positive controls. **p < 0.01, ***p < 0.001 compared with the control group. The results were analyzed by one-way ANOVA followed by Tukey's test. Data are described as mean + SEM (n = 8).

Table 2. Anticonvulsant effect of *H. acutifolium* aqueous extract in the PTZ test

Dose (mg/kg)	% Protection	Findings	% Mortality	ED_{50} (95% CI) mg/kg PTZ test
100	0	ns	100	342.6 (297.7 to 403.0)
200	20	ns	80	
400	60	* /*p* value=0.0108	40	

Table 3. Anticonvulsant effect of *H. acutifolium* aqueous extract in the MES test

Dose (mg/kg)	% Protection	Findings	% Mortality	ED_{50} (95% CI) mg/kg MES test
100	0	ns	100	322.6 (225.4 to 664.1)
200	30	ns	70	
400	60	* /*p* value=0.0108	40	

quently used for quantitative analysis. The average quercetin content was calculated as 49.87 ± 0.24 mg/100 g dry matter in the *H. acutifolium* hydrolyzed aqueous extract. Two sample chromatograms are shown in figure 5.

Analytical performance characteristics

Validation of the method used to quantify the content of quercetin in hydrolyzed aqueous extract of *H. acutifolium* was conducted to identify the linearity, precision, accuracy, LOD, and LOQ of the chromatographic analysis. The performance characteristics of the method are demonstrated in table 4. To establish a standard curve for quercetin, the response (peak area) was plotted against the nominal concentration. Table 4 displays the achievement of good linearity (R^2 = 0.998), which confirms the capability of the method to accurately quantify quercetin content within the expressed concentration range. Accuracy was assessed by computing recovery rates in the matrix at two different concentration levels. The study demonstrated recovery rates within the range of 96.32% to 101.69%, suggesting that the method's recovery was deemed acceptable. As all acquired values for RSD% of intra-day and inter-day analysis were below 5%, it could be inferred that the method demonstrates a high level of precision and reliability for the upcoming quantification of quercetin. The results achieved for LOD and LOQ were 0.81 ng/mL and 2.67 ng/mL, respectively. It is worth mentioning that LOD and LOQ pertain to the solutions of standards rather than the corresponding samples.

Discussion

Due to the high prevalence of mental and neurological disorders such as depression, anxiety, seizures, and insomnia, exploring the putative treatments for the management of these conditions is of interest. The growing attention to the therapeutic properties of natural products in neurological disorders has led to a concerted effort to assess the effectiveness of medicinal plants [33].

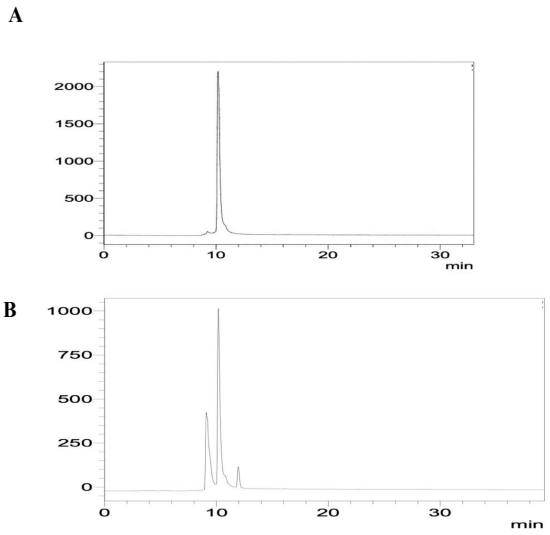

Figure 5. RP-HPLC-DAD chromatograms of a standard solution (50 µg/mL) of quercetin (A: above) and *H. acutifolium* hydrolyzed aqueous extract (10 mg/mL) (B) at 370 nm.

As mentioned before, according to Avicenna's Canon of Medicine, *H. acutifolium* was used for the treatment of neurological disorders [22]. The main characteristics of mental disorders are a disturbance in the equilibrium of CNS receptors and neurotransmitters, an overactive state of immune-inflammatory responses, and a perturbation in customary synaptic plasticity [34-36]. Moreover, there are a number of preclinical studies confirming the positive effects of phenolic compounds on psychiatric disorders [40,41]. Previous studies on *Haplophyllum* species have detected a variety of phytochemical compounds in this genus, including alkaloids, coumarins, and lignans [37,38]. Thus, this study revealed the potential impact of the phenolic and flavonoid components (such as quercetin) of *H. acutifolium* in animal models. Additionally, one of the bioactive flavonoids in the *H. acutifolium* aqueous

Table 4. Method performance characteristics for quercetin determination in *H. acutifolium* hydrolyzed aqueous extract.

Parameter	Result
Retention time	10.1 min
Concentration linear range	1-100 µg/mL
R^2	0.998
Recovery	96.32-101.69%
Intra-day RSD (RSD%)	1.28-2.72%
Inter-day RSD (RSD%)	2.51-4.43%
LOQ	2.67 ng/mL
LOD	0.81 ng/mL

extract, quercetin, was specifically analyzed and determined using RP-HPLC-DAD.

The OFT was executed after i.p. administration of the extracts at various doses, to reject the possibility that the decrease in immobility time was due to the psychostimulant properties of the extracts, which could produce a false positive outcome in the FST. Additionally, the OFT was utilized to quantify the sedative impact of the extract. The findings from the OFT, specifically the average distance traveled by the test animal, indicate that the extracts at various dosages exhibited a significant decrease in the animal's motor activity.

Additionally, the extracts displayed significant effects in the pentobarbital-induced sleep test, demonstrating a highly noticeable hypnotic effect comparable to the 2 mg/kg dose of diazepam, which served as the positive control. Thus, among the aqueous, methanolic, and dichloromethane extracts, and aqueous and methanolic extract, the aqueous extract had the highest sedative and hypnotic effects. Aqueous extract holds promise as a potential candidate for assessment as a hypnotic medication. To determine the potential mechanism of action, flumazenil (10 mg/kg), an antagonist of benzodiazepine receptors, and naloxone (1 mg/kg), an antagonist of opioid receptors, were given 15 minutes before treatment. However, none of them could prevent the hypnotic effect of the extract (Figure 2). According to these findings, it can be concluded that opioid or benzodiazepine receptors are probably not involved in the hypnotic effects of the plant extract.

In the EPM test, which is a tool for evaluating anti-anxiety effects, the aqueous extract showed a dose-dependent effect. Nonetheless, the effect was significantly less pronounced than that produced by midazolam (the positive control). This shows that the anxiolytic effects of the aqueous extract may be weaker in comparison to benzodiazepines.

The results obtained from the FST indicated that the i.p. administration of 200 mg/kg of the aqueous extract derived from the aerial parts of the *H. acutifolium* plant causes a significant reduction in the immobility rate of mice compared to the vehicle group. This suggests that the aqueous extract of *H. acutifolium* has dose-dependent antidepressant-like effects. The dose of 200 mg/kg showed a significant effect compared to the positive control groups (fluoxetine and imipramine). Thus, it can be concluded that the aqueous extract of the plant has the potential to become an antidepressant. An animal study on mice found that oral administration of quercetin at the dose range of 20-40 mg/kg can counteract the symptoms of anxiety and depression caused by corticotropin-releasing factors [39]. This flavonoid protects the body against stress by enhancing neurogenesis and neural plasticity and increasing signaling activity for brain-derived neurotrophic factor, tropomyosin kinase B receptor. Additionally, quercetin also has the potential to improve depressive-like behaviors and exerts this effect by reducing inflammatory responses, enhancing the activity of antioxidant enzymes, reducing oxidative stress markers, and improving changes in the hypothalamus-pituitary-adrenal axis [40]. In addition, the antidepressant effect of rutin is related to its capacity to increase serotonin and noradrenaline in the synaptic cleft [41].

The aqueous extract exhibited a significant dose-dependent anticonvulsant effect, with an effective dose of 400 mg/kg in both the PTZ and MES seizure tests. These findings underscore the need for dosage optimization and further investigation into the pharmacokinetic and safety profiles of the extract for potential clinical use in epilepsy. Furthermore, previous research has demonstrated the potential of several plant extracts, which contain quercetin and its derivatives, to have antiseizure properties [42]. Quercetin (100 mg/kg, i.p.) exhibits protective properties against seizures induced by PTZ and kainic acid. It effectively reduces both the seizure score and duration; thus, demonstrating its anticonvulsant effects. Nonetheless, certain studies suggest that the anticonvulsant effects of quercetin may be limited [43]. The underlying mechanisms responsible for the anticonvulsant effects of quercetin can be attributed to its anti-inflammatory impacts as well as antioxidant properties [44,45]. Additionally, quercetin applies its anticonvulsant effects through the modulation of neurotransmitter expression [46].

Conclusion

Based on the obtained results, it can be concluded that the aqueous extract of the aerial parts of *H. acutifolium* has a considerable impact on promoting sleep, diminishing anxiety, controlling seizures, and alleviating depression. Additionally, it is evident that phenolic compounds and flavonoids, as part of the main compounds of *H. acutifolium*, have a critical role in the reported pharmacological effects of the extract. However, additional explorations are needed to identify the phytochemical components of the plant and discover the mechanisms related to the observed effects.

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Conflict of Interests
The authors declare that they have no Conflict of Interest.

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