Exploring *Habenaria intermedia* D. Don Tubers for Phytochemical and Anti-depressant Properties

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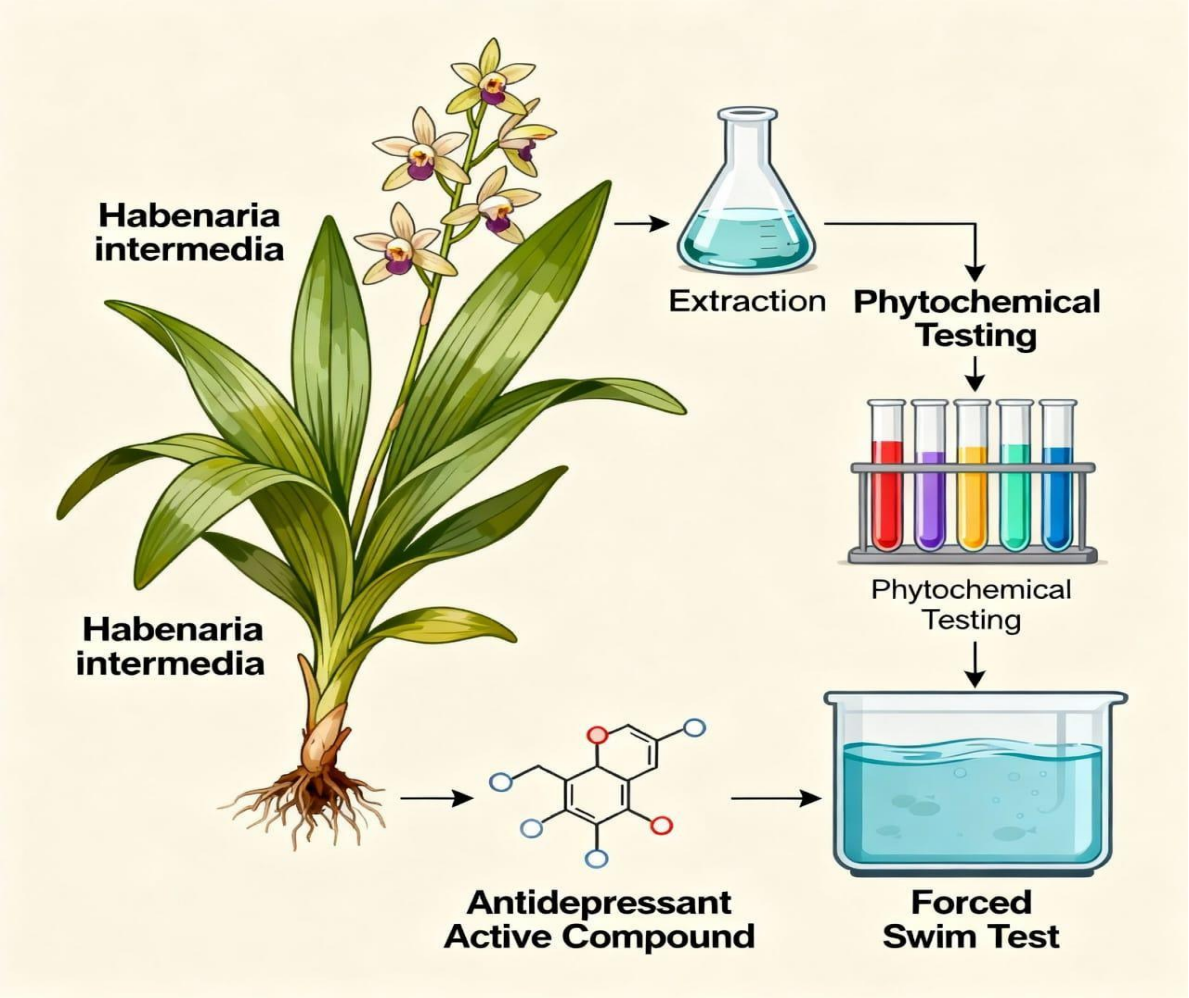
Abstract

The research aimed to assess the antidepressant properties of various extracts and fractions from correctly identified tubers of *Habenaria intermedia* D. Don (Vriddhi; Orchidaceae), with a focus on validating traditional claims through systematic phytochemical and pharmacological investigation using the FST (forced swim test) in mice. The tubers were successively extracted with solvents of ascending polarity, namely n-hexane, chloroform, methanol, and water. Only crude extracts containing abundant phyto-constituents were selected for antidepressant assays in mice, administered orally at doses of 100, 200, and 400 mg/kg. The test groups were statistically compared with imipramine (15 mg/kg, i.p.), which served as the reference antidepressant drug. The pharmacologically active methanolic extract was subsequently fractionated using n-hexane, ethyl acetate, and 1-butanol. Each obtained fraction was administered orally to mice at doses of 25 and 50 mg/kg to assess antidepressant activity. Among all extracts, the methanol extract at 400 mg/kg exhibited maximal antidepressant efficacy, comparable to imipramine and superior to other extracts at tested doses. During fractionation, only the ethyl acetate fraction (EAF) at 50 mg/kg demonstrated significant antidepressant activity, equivalent to the standard medication. The activity of other fractions was markedly lower. The research confirms traditional claims about the antidepressant potential of *Habenaria intermedia* tubers. Phytochemical analysis revealed that phenolic and flavonoid compounds in the tubers may contribute to these effects, indicating promise for the development of natural product-based therapeutics targeting depression disorders.

Major Findings: Significant antidepressant activity was observed in mice administered with the methanol extract and ethyl acetate fraction of *Habenaria intermedia* tubers, which supports their use in traditional medicine.

Keywords: Antidepressant, Forced swim test, *Habenaria intermedia*, Orchidaceae, Vriddhi, *etc.*



Graphical Abstract

1. Introduction: The world's number one factor contributing to mental health-related disability is depression, a serious global mental health issue. When depression first appears, it often happens in mid- to late-adolescence (between the ages of 14 and 25), with a median 12-month prevalence of 4-5%.[1] Major depressive disorder (MDD) hurts relationships, career, and education, and is potentially linked to premature death, including suicide, obesity, and cardiac illness. When physical health issues are comorbid with depression in persons over the age of 18, the functional impacts of depression can be more severe, complicating treatment options. Commonly prescribed medications for treating depression include selective serotonin reuptake inhibitors (SSRIs), tricyclic and tetracyclic antidepressants, as well as monoamine oxidase inhibitors (MAOIs). The adverse effects of these antidepressant medications include headaches, mydriasis, constipation, dry mouth, transient exhaustion, and restlessness. Many allopathic medicines have been made to reduce depression. But these medicines have many side effects. Plants or herbs are a natural way to relieve depression. Traditional Ayurvedic medicine has long valued *Habenaria intermedia* D. Don, commonly known as Vriddhi, for its diverse therapeutic benefits, including use as a rejuvenator, brain tonic, and adaptogen. Its tubers have been employed to enhance vitality, support the immune system, and treat nervous disorders, reflecting centuries of empirical use in boosting mental and physical well-being. Our findings suggest that the plant *Habenaria intermedia* D. Don, or Vriddhi, may provide an effective natural treatment for depression with minimal or no adverse effects.[3, 4] This is an orchid species native to the Himalayan region, found at elevations ranging from 2000 to 3300 meters across Pakistan, Bhutan, Nepal, and India, particularly in temperate areas like Kashmir, Himachal Pradesh, Uttarakhand, and Sikkim. Among the over 600 Habenaria species worldwide, around 100 species occur in India. Phytochemical analyses of *H. intermedia* have revealed the

presence of flavonoids, tannins, steroids, and coumarin glycosides. Notably, this species contains substantial amounts of phenolic acids, including hydroxyl benzoic acid and gallic acid, which contribute to its significant antioxidant properties and ability to scavenge free radicals[6, 7]. Contemporary research indicates that flavonoids, aside from their antioxidant effects, may also affect brain amine metabolism in neuronal and neuroendocrine cells, potentially playing a beneficial role in certain neurological conditions, including depression. Despite these findings, scientific investigations specifically addressing the antidepressant potential of *H. intermedia* remain insufficient[8]. The edible tubers are known to be palatable, soothing, and traditionally used as aphrodisiacs, depuratives, anthelmintics, and tonics. They have also been utilized in treating conditions such as leprosy, dermatological issues, and asthma, and are included in the formulation of Chyavanprasha, a widely used polyherbal tonic. The plant's rejuvenating effects in traditional medicine suggest that it could also offer benefits for depression[9, 10]. Based on the reported presence of flavonoids, phenolic acids, and coumarins in H. intermedia, and given the well-established antidepressant mechanisms of these phytochemical classes in other medicinal plants, it was hypothesized that the tuber extracts of H. intermedia would produce significant antidepressant effects in the Forced Swim Test, potentially mediated by these bioactive constituents.

2. Materials and Methods

2.0 Study Location

All procedures, including extraction, phytochemical screening, and pharmacological assays, were conducted at the Department of Pharmacognosy and Pharmacology, ASBASJSM College of Pharmacy, Bela (Ropar), Punjab, India (July–October 2024).

2.1 Plant Material, Solvents, Chemicals, Reagents, and Instruments

The tubers of *Habenaria intermedia* were procured in July 2024 from Hans Raj and Sons, situated at 6549-A, Khari Baoli, New Delhi, India. Botanical authentication of the plant material was conducted by Dr. Alok Goyal, Scientist In-Charge at the Natural Products Field Laboratory, National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab, India, dated August 5, 2024. A voucher specimen was prepared and deposited in the NIPER herbarium, and the accession number NIP-H-3012 has been assigned to ensure permanent and verifiable botanical identification. This voucher specimen serves as a critical reference for the reproducibility and validation of the phytopharmacological investigations carried out using this plant material. To prepare the plant extracts and fractions, LR-grade solvents (E Merck, Delhi, India) such as n-hexane, chloroform, methanol, ethyl acetate, and 1-butanol were utilized. The current research effort was conducted using the following instruments: a water bath (Perfit, Ambala, Haryana, India), a digital weighing balance, a hot air oven, a rotary vacuum evaporator, and a Soxhlet apparatus.

2.2 Preparation of Various Extracts and Fractions of *Habenaria intermedia* Tubers:

In a grinder, the *Habenaria intermedia* tubers were ground into a powder. Ten kilograms of dried powdered plant material were added to a thimble composed of fine filter paper. Next, the plant was thoroughly extracted using n-hexane in a Soxhlet apparatus until a small amount of droplets that were collected from the apparatus, and a few milliliters of the n-hexane layer evaporated without leaving any residue, confirming complete removal of non-polar constituents. To obtain a chloroform extract, the marc was dried, packed in a thimble, and thoroughly extracted using a Soxhlet device. To obtain the methanol extract, the same process was used once the chloroform extraction was finished. The plant material was boiled for two hours on a hot plate with distilled water to prepare the water extract. Using a rotating vacuum evaporator, the solvents from the crude extracts were recovered at lowered pressure.

A round-bottom flask containing 250 g of methanol extract was filled with 500 ml of distilled water. The material was triturated with a glass rod for thirty minutes to create the extract suspension in distilled water. It was then divided using 50 milliliters of n-hexane by boiling it to 50 degrees Celsius for 30 minutes while stirring constantly. After cooling the contents, the upper layer, known as the n-hexane layer, was separated. New 500 milliliters of n-hexane were added to the extract. The process of partitioning using n-hexane was repeated until a few milliliters of the n-hexane layer evaporated without leaving appreciable residue on the watch glass. To ultimately produce the n-hexane fraction (HF), all of the separated n-hexane layers were combined and condensed under low pressure. To obtain the ethyl acetate fraction (EAF), the remaining bioactive extract underwent a similar process. A similar process was used to obtain 1-butanol fraction (BF) from the residual bioactive extract. The residual bioactive extract (RBE) was also concentrated via sequential partitioning with n-hexane, ethyl acetate, and 1-butanol. The existence of distinct groups of

phytoconstituents was checked in all plant extracts and fractions of the bioactive extract. [12, 13, 14]

2.2.1 Quantitative Phytochemical Analysis

Total phenolic content (TPC) and total flavonoid content (TFC) of the crude extracts (CE, ME) and solvent fractions (EAF, BF) were determined using the Folin–Ciocalteu and aluminum chloride colorimetric methods. Results were expressed as mg gallic acid equivalents (GAE)/g extract and mg quercetin equivalents (QE)/g extract.

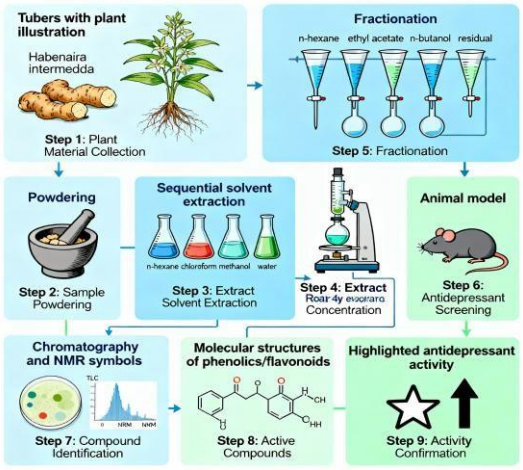


Figure1. Extraction and Solvent Fractionation Scheme of *Habenaria intermedia* Tubers

2.2.1 (A) Estimation of total phenols content using Folin Ciocalteu's assay

The conventional method was used to evaluate the total phenol content of *H. intermedia* tubers (Madaan *et al*., 2011).

Preparation of standard curve

100 ml of 50% methanol was used to dissolve 10 mg of gallic acid (100 µg/ml), which was further diluted to 20, 40, 60, 80, 100, or 120 µg/ml. In a test tube, one millilitre of each dilution was taken and diluted with ten milliliters of distilled water. After adding 1.5 ml of Folin Ciocalteu's reagent, the mixture was given five minutes to incubate at room temperature. Each test tube received 4 ml of 20% (w/w) Na2CO3, which was then agitated, adjusted with distilled water to reach 25 ml, and allowed to stand at room temperature for 30 minutes. Using a UV/VIS spectrophotometer, the absorbance of the standard was measured at 765 nm in comparison to distilled water as the blank.

Preparation of test samples

H. intermedia tubers (200 g) were extracted in a Soxhlet device using petroleum ether at a temperature between 60 and 80ºC (500 ml). The plant's marc was then extracted using 500 milliliters of methanol in a Soxhlet system to get methanol extract (ME). The ME (15 g) of plant material was separately suspended uniformly in water (100 ml), placed in a round bottom flask, and partitioned with ethyl acetate (50 ml) by heating at 50ºC for 30 min along with constant stirring. Eight more times, this partitioning process was carried out using each solvent. All the separated layers of ethyl acetate were pooled and condensed under reduced pressure to yield ethyl acetate fraction (EAF).

150 mg of ME was mixed with 15 ml of 50% methanol and extracted three times using maceration for two hours. The mixture was then filtered and the remaining volume was filled up to 25 ml in a volumetric flask with 50% methanol. One ml aliquot of the material was taken in a test tube and diluted with 10 ml of distilled water. After adding 1.5 ml of Folin Ciocalteu's reagent, the mixture was given five minutes to incubate at room temperature. After adding 4 ml of 20% (w/w) Na2CO3, adjusting with distilled water to reach 25 ml, stirring, and letting it stand at room temperature for 30 minutes, the process was completed. The sample's absorbance was measured at 765 nm in comparison to distilled water, or the blank. A similar process was used to prepare the EAF test sample. The sample's absorbance was measured at 765 nm using a UV/VIS spectrophotometer in comparison to distilled water as the blank.

2.2.1 (B) Quantification of total phenols content

An absorbance standard curve was created and plotted against the concentration of gallic acid. Gallic acid equivalents were calculated using the standard curve's regression equation. The proportion w/w (mean ± S.D.) was used to express the results. The following formula was used to determine the sample's percentage of total phenolic content:

$$\text{Total phenolic content (\% w/w)} = GAE \times V \times D \times 10^{-6} \times 100 / W.$$

Where V is the sample's total volume (ml), D is the dilution factor, W is the sample weight (g), and GAE is the gallic acid equivalents (µg/ml).

3.9.Estimation of total flavonoids content using aluminium chloride assay

The conventional approach was used to assess the total flavonoid content of H. intermedia tubers (Madaan et al., 2011).

Preparation of standard curve

100 milliliters of methanol (200 µg/ml) containing 20 mg of quercetin was then diluted to 30, 60, 90, 120, 150, or 180 µg/ml. Separately, 1.5 ml of 95% methanol, 0.1 ml of aluminum chloride (10%), 0.1 ml of 1M potassium acetate, and 2.8 ml of distilled

water were combined with the standard solutions (0.5 ml). Using a UV/VIS spectrophotometer, the absorbance of the reaction mixture was measured at 415 nm following a 30-minute incubation period at room temperature. The same volume of distilled water was used in place of 10% of aluminum chloride in the blank.

Preparation of test samples

Two hundred fifty milligrams of ME and EAF were dissolved separately in twenty-five milliliters of methanol (made as described in the section preparation of test samples in total phenols content estimations). Similar to how the section "preparation of standard curve in total flavonoids content estimations" describes, 0.5 ml of ME and EAF were reacted with aluminum chloride to determine the flavonoid content. The same volume of distilled water was used in place of 10% of aluminum chloride in the blank.

Quantification of total flavonoids content

An absorbance standard curve was created and plotted against the concentration of quercetin. Quercetin equivalents were found using the standard curve regression equation. The proportion w/w (mean ±S.D.) was used to express the results. The following formula was used to determine the sample's percentage of total flavonoid content:

$$\text{Flavonoids content (\% w/w)} = QE \times V \times D \times 10^{-6} \times 100 / W$$

Where V is the sample's total volume (ml), D is the dilution factor, W is the sample weight (g), and QE is the quercetin equivalents (µg/ml).

Total phenols and flavonoids content of *H. intermedia* tubers

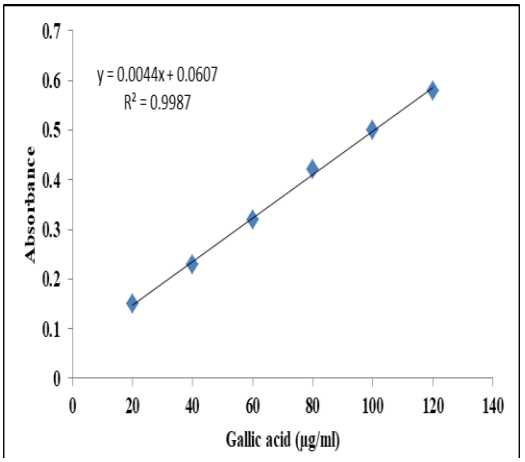
Total phenols and total flavonoids were quantitatively determined using standard curves for quercetin (linearity: 30 to 180 mg/ml; r2 = 0.9991; figure 3B) and gallic acid (linearity: 20 to 120 mg/ml; r2 = 0.9987; figure 3A), respectively. Table 7 makes clear that the plant's EAF had a greater concentration of flavonoids and total phenols than ME. This finding implies that ethyl acetate might be used to extract the majority of the phenols and flavonoids in the ME.

H. intermedia tubers were extracted using n-hexnae in a Soxhlet device to defatten them. Methanol extract (ME) was then obtained by extracting the plant's marc with methanol in a Soxhlet equipment. It was intended to isolate the phenol and flavonoid-rich fraction from plant methanol extracts using a standard process because phenols and flavonoids are the main class of phytoconstituents found in plants. It was discovered that the ME yield of *H. intermedia* tubers was 4.11 percent w/w. In comparison to ME, the EAF yield of *H. intermedia* tubers was reported to be 21.90% w/w.

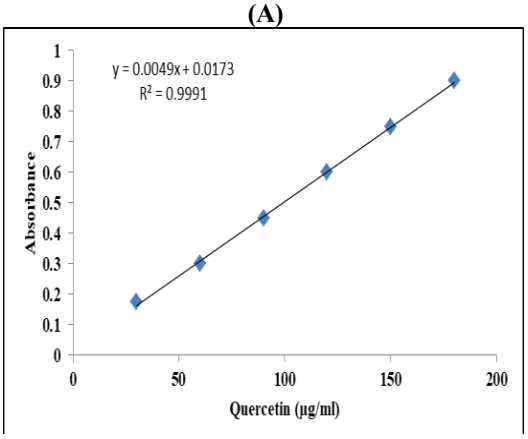
Table 7: Total phenols and flavonoids content in ME and its EAF *H. intermedia* tubers.

Test sample	Total phenols content (% w/w) Mean^n ± S.D.	Total flavonoids content (% w/w) Mean^n ± S.D.
ME	9.05 ± 0.56	3.25 ± 0.10
EAF	12.56 ± 0.79	6.40 ± 0.13

n=3



(A)



(B)

Figure 3: Gallic acid vs absorbance (A) and quercetin versus absorbance (B) are standard plots.

2.3 Anti-depressant Activity

2.3.1 Animals

Swiss Albino mice, both male and female, weighing from 20 to 30 grams, were utilized to test the antidepressant effects. These animals were obtained from the AIIMS (All India Institute of Medical Sciences), New Delhi. Ethical approval for the study was granted by the Institutional Animal Ethics Committee (IAEC) of ASBASJSM College of Pharmacy, Bela (Ropar), Punjab, with approval

number ASCB/IAEC/19/24/19. Initially, the mice were kept under quarantine for health observation before being transferred to the main housing area. They were acclimated to laboratory conditions in the Central Animal House Facility of ASBASJSM College of Pharmacy for one week. The mice were housed in polypropylene cages with dust-free rice husk bedding, maintained at a temperature of 23±2ºC and relative humidity of 40±10%. They were given a standard pellet diet with free access to water. Experiments were carried out daily from 9:00 AM to 5:00 PM. All animal care and handling complied strictly with the guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Forests & Environment, Government of India, ensuring ethical treatment and welfare standards.

2.3.2 Vehicle and Standard Drugs

The test doses of the crude extracts, fractions, and reference drug were prepared using distilled water with 2% Tween 80 as the solvent. Imipramine, given orally at a dose of 15 mg/kg, was utilized as the standard antidepressant for comparison in the study.

2.3.3 Acute Toxicity Studies

The acute oral toxicity of the crude extract (CE) and methanol extract (ME) from *Habenaria intermedia* tubers was assessed based on the OECD guideline 423. In each test batch, six mice were given a single oral dose of 2000 mg/kg to evaluate any possible toxic effects. This standardized testing method ensures the safety of the extracts before conducting further pharmacological studies [15, 16].

a. Experimental Design

Two experimental designs were developed, each comprising 14 groups of mice for the study. The doses chosen for the ethyl acetate (EAF) and butanol (BF) fractions were lower than those of the crude extract due to their greater concentration of phytochemicals. Preliminary pilot tests showed that doses higher than 50 mg/kg did not improve behavioral outcomes, whereas doses less than 25 mg/kg resulted in minimal antidepressant effects, establishing 25 and 50 mg/kg as the most suitable and scientifically sound doses for testing these fractions. Dose selection was also supported by preliminary pilot studies in which doses above 50 mg/kg showed no additional improvement, while doses below 25 mg/kg produced negligible effects.

2.4.1 Experimental Protocol I - This was created to evaluate the antidepressant potential of several crude extracts of *H. intermedia* tubers, encompassing groups 1 through 8. Group 1 was the control group and was given vehicle (0.25 ml, p.o.); Group 2 was the standard group and was given imipramine (15 mg/kg, p.o.); Groups 3,4 and 5 received doses of CE of 100, 200, and 400 mg/kg, respectively; Groups 6, 7 and 8 received doses of ME of 100, 200, and 400 mg/kg.

2.4.2 Experimental Protocol II- The purpose of groups 9 through 14 was to evaluate the antidepressant potential of different *H. intermedia* tuber fractions. Group 9: This group was given a vehicle (0.25 ml, p.o.); Group 10: This group was given imipramine (15 mg/kg, p.o.); Groups 11 and 12: This group was given a dose of 25 and 50 mg/kg of EAF; Groups 13 and 14: This group was given a dose of 25 and 50 mg/kg of BF.[17, 18, 19]

For Protocol II, a fresh, concurrent control group was employed to ensure independent comparison and eliminate behavioural variability. Although a washout period was available for animals from Protocol I, new animals were assigned for the control and treatment groups in Protocol II to meet standard behavioural research guidelines.

Experimental Protocol	Group	Treatment	Dose	Route of Administration
I	1	Vehicle (Control)	0.25 ml	p.o
I	2	Imipramine (Standard)	15 mg/kg	p.o.
I	3	Crude Extract (CE)	100 mg/kg	p.o.
I	4	Crude Extract (CE)	200 mg/kg	p.o.
I	5	Crude Extract (CE)	400 mg/kg	p.o.
I	6	Methanolic Extract (ME)	100 mg/kg	p.o.
I	7	Methanolic Extract (ME)	200 mg/kg	p.o.

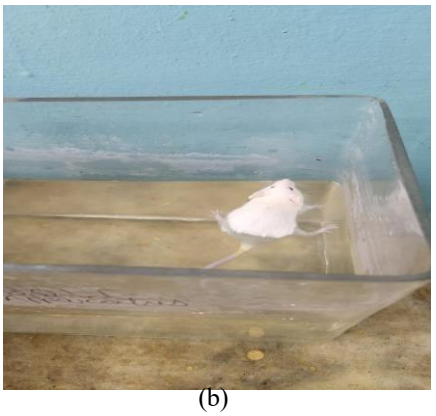
I	8	Methanolic Extract (ME)	400 mg/kg	p.o.
II	9	Vehicle (Control)	0.25 ml	p.o.
II	10	Imipramine (Standard)	15 mg/kg	p.o.
II	11	Ethyl Acetate Fraction (EAF)	25 mg/kg	p.o.
II	12	Ethyl Acetate Fraction (EAF)	50 mg/kg	p.o.
II	13	Butanolic Fraction (BF)	25 mg/kg	p.o.
II	14	Butanolic Fraction (BF)	50 mg/kg	p.o.

Table1. Outlines the Animal groups, Treatments, respective doses, and the Route of Administration used in the Study

2.5 Forced Swim Test

Water was filled to a 15 cm depth in a plexiglass cylinder measuring 40 cm in height and 18 cm in diameter, with the water temperature maintained at 25 ± 2ºC throughout the test. After administration of the vehicle, test compound, or standard drug, mice were placed in the cylinder to swim for six minutes. During this period, the total duration of immobility when the animal floated with its nose above the water surface was recorded [as 4].

(a)



(b)

Figure 2. Animal exposed to Force swim test (a) immobile phase (b) mobile phase

2.6 Statistical Analyses

The results were expressed as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was performed to compare the test treatments with the control and standard drug, followed by the application of the Student Newman-Keuls post hoc test for multiple comparisons [20].

3. Results and Discussion

The Soxhlet extraction of *Habenaria intermedia* tubers yielded percentages of 0.60% for the n-hexane extract (HE), 0.51% for the chloroform extract (CE), 3.52% for the methanol extract (ME), and 2.68% for the water extract (WE). Medicinal plants are extensively utilized to treat a wide range of ailments due to their diverse pharmacological properties, such as anticancer, antimicrobial, antioxidant, anti-inflammatory, analgesic, antidiabetic, antihypertensive, and antidiarrheal effects. The therapeutic efficacy of such plants is predominantly attributed to their phytochemical constituents, either alone or synergistically. Key phytoconstituents exhibiting diverse bioactivities include alkaloids, flavonoids, phenolics, tannins, saponins, steroids, glycosides, and terpenes. Preliminary phytochemical screening, conducted using classical qualitative assays, identified varying chemical constituents across the crude extracts of *H. intermedia* tubers: HE contained fixed oils; CE showed presence of alkaloids and coumarins; ME exhibited flavonoids, tannins, and coumarins; and WE contained carbohydrates and proteins[18]. Based on these initial screenings, only CE and ME contained significant bioactive phytomolecules, whereas HE and WE lacked such constituents. Consequently, acute toxicity and antidepressant activity studies were performed exclusively on CE and ME extracts. In accordance with OECD-423 guidelines, an acute oral toxicity study was conducted on CE and ME at a dose of 2000 mg/kg

body weight administered orally. No mortality or adverse effects were observed in mice during the 24-hour observation period. Autonomic, neurological, and behavioral parameters remained within normal limits, and thus the extracts were classified as "unclassified" in terms of toxicity. Subsequently, no further testing at lower doses was deemed necessary.

Phytochemical screening of HE and WE confirmed the absence of major bioactive phytoconstituent classes. Therefore, only CE and ME were evaluated for antidepressant activity using the forced swim test (FST) model. Immobility time during the test was used as an indicator of antidepressant effect, with statistical analyses comparing test groups to controls and standard drug treatments. Results (refer to Table 1 and Figure 3) revealed that among the crude extracts tested, only ME demonstrated significant antidepressant efficacy relative to the control. While ME at doses of 100 and 200 mg/kg notably reduced immobility duration compared to control, these reductions did not reach levels comparable to the standard antidepressant. However, at 400 mg/kg, ME produced antidepressant effects statistically equivalent to the conventional drug (Table 2). By contrast, CE exerted only a modest antidepressant effect at all tested doses. Although CE significantly decreased immobility time compared to controls, its efficacy was. Rather than reiterating the findings, the discussion should contextualize how the antidepressant effect of the methanol extract aligns with known flavonoid- and phenolic-mediated mechanisms reported in related species. Critical interpretation should highlight how this study advances understanding of *Habenaria intermedia*'s neuropharmacological potential by linking the observed behavioural effects with established phytochemical actions. Overall, these findings identify ME as the most pharmacologically active antidepressant fraction of *Habenaria intermedia* tubers.

Table 2: Anti-depressant Activity of CE and ME of *H. intermedia* Tubers using FST

Treatment	Dose (mg/kg)	Mean^{n} immobility time (sec)
Control	Vehicle	285.80 ± 8.10^{a}
Imipramine	15	61.80 ± 5.80*
CE	100 200 400	191.20 ± 11.81^{*a} 165.60 ± 6.26^{*a} 145.00 ± 7.90^{*a}
ME	100 200 400	103.20 ± 5.80^{*a} 88.20 ± 6.01^{*a} 65.40 ± 9.78*

Data are expressed as mean ± S.D. (n = 5 animals per group). Statistical significance was determined using one-way ANOVA followed by Student–Newman–Keul's test. *P<0.05 vs. control group; aP<0.05 vs. standard medication (imipramine)

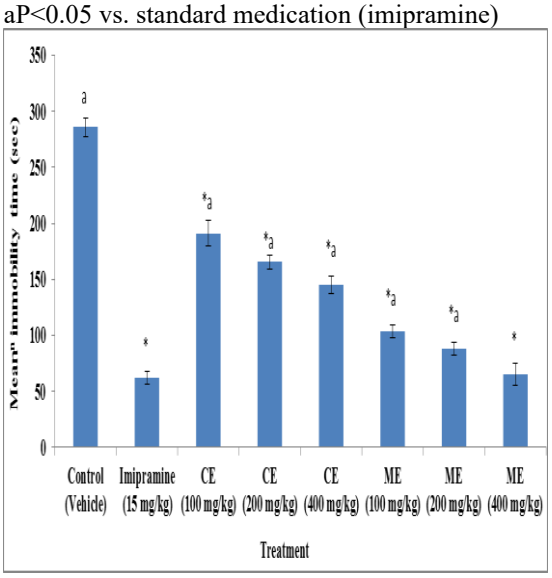


Figure 3. Duration of immobility following treatment with vehicle, imipramine, and different *H. intermedia* tuber extracts. ME = methanol extract; ChE = chloroform extract. Statistical significance is indicated as *P<0.05 vs. control; aP<0.05 vs. imipramine.

The methanol extract demonstrated the highest antidepressant activity among the fractions. This bioactive methanol extract was further separated into fractions based on increasing polarity, yielding the following: n-hexane fraction (HF) at 1.70%, ethyl acetate fraction (EAF) at 21.60%, 1-butanol fraction (BF) at 18.23%, and the remaining bioactive extract (RBE) at 54.89%, all expressed as % w/w relative to the original methanol extract. The results of chemical testing suggested that HF and RBE did not show any sign of presence of phytomolecules. Only EAF contained all three types of natural compounds such as phenols, flavonoids, coumarins whereas BF contained coumarins as natural compounds.

Only the ethyl acetate fraction (EAF) and the 1-butanol fraction (BF) were evaluated for antidepressant effects in mice using the forced swim test (FST). The average immobility times after administration of EAF (25 or 50 mg/kg, orally), BF (25 or 50 mg/kg, orally), imipramine (15 mg/kg, orally), and the control (vehicle, orally) are shown in Table 3 and Figure 4. At 50 mg/kg, EAF significantly reduced immobility time compared to the control, with effects comparable to the standard drug. In contrast, BF did not

significantly reduce immobility time and thus did not exhibit therapeutic efficacy similar to the standard treatment.

Table 3. Antidepressant Activity of EAF and BF of *H. intermedia* Tubers using FST

Treatment	Dose (mg/kg)	Mean[n] immobility time (sec)
Control	Vehicle	271.80 ± 5.80[a]
Imipramine	15	65.60 ± 7.30[*]
EAF	25 50	81.80 ± 5.80[*a] 68.40 ± 5.94[*]
BF	25 50	180.60 ± 7.47[*a] 155.00 ± 5.24[*a]

Data are expressed as mean ± S.D. (n = 5 animals per group). Statistical significance was determined using one-way ANOVA followed by Student–Newman–Keul's test. *P<0.05 vs. control group; aP<0.05 vs. standard medication (imipramine)

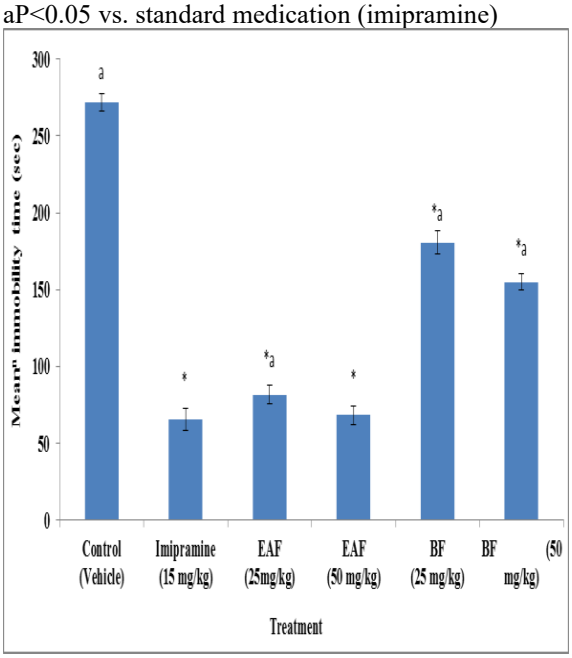


Figure 4. Duration of immobility following treatment with vehicle, imipramine, and different fractions of the bioactive methanol extract of *H. intermedia*. EAF = ethyl acetate fraction; BF = 1-butanol fraction. Statistical significance: *P<0.05 vs. control; aP<0.05 vs. imipramine.

The forced swim test, a well-researched experimental technique, was used to evaluate the antidepressant properties of *Habenaria intermedia* tubers. Mice are made to swim in a restricted space from which they are unable to escape during this test. Mice initially attempt to get out of the confined area while staying mobile. Mice adjust to their distinctive immobility after a few seconds. As a measure of the test drugs' antidepressant effectiveness, the length of immobility is noted[21].

The model was selected because it works well, is inexpensive, easy to use, takes less time, doesn't require any prior training for the mice, and doesn't give the animals much distress when handled. The concept is mostly based on the findings that mice that are made to swim in a confined area from which they are unable to escape exhibit immobility as a typical trait. This conduct is indicative of a state of despair that can be lessened by a number of therapeutically effective medications for depression in humans. The reduction in motor activity, which is determined by the amount of time the animal spends immobile, is the final sign of depression in animals[22, 23].

The presence of flavonoids, phenols, and coumarins in the bioactive extract and/or fraction of aerial parts of *H. intermedia* tubers was revealed by preliminary phytochemical tests. Our findings are consistent with the literature that has been reported to show antidepressant activity for flavonoids such as naringenin[24] icariin[25], hesperidin[26], chrysin[27], phenols such as gallic acid, protocatechuic acid, gentisic acid, salicylic acid and syringic acid, ferulic acid, ellagic acid[28, 29] and coumarins such as scopoletin[30], psoralen [31], lacinartin[32], auraptene, and umbelliferon. Based on an exhaustive scrutiny of the literature survey suggested that the antidepressant activity of *H. intermedia* tubers may be due to the flavonoids, phenols, and/or coumarins present therein[33,34].

The observed antidepressant activity is consistent with the known neuroprotective, antioxidant, and monoaminergic modulatory effects of flavonoids, phenolic acids, and coumarins. While these phytochemical classes have documented roles in influencing neurotransmission and stress-related pathways in other plant species, the specific molecular mechanisms underlying the antidepressant effect of *H. intermedia* remain unconfirmed. Future mechanistic studies are required to determine the precise pathways responsible for its neuropharmacological activity[35, 36].

4. Conclusion

The study identified the ethyl acetate fraction as the most active and demonstrated that phenolic and flavonoid classes are likely contributors to the antidepressant effect based on qualitative phytochemical screening. While these findings support the traditional use of *H. intermedia*, comprehensive isolation and structural elucidation of its active constituents are required to achieve complete phytochemical characterization in future research.

5. Acknowledgment

The authors duly acknowledge the Department of Pharmacognosy and the Head of the Department of Pharmacology, Dr. Ajay Singh Kushwah, and Associate Professor Dr. Rahul Kumar Sharma of ASBASJSM College of Pharmacy, Bela (Ropar), Punjab, India, for providing necessary instrumentation facilities for the completion of research work and valuable assistance in animal handling during the course of this research.

6. Author Contribution

Navjit Kaur conceptualized the study and was responsible for its overall validation. The methodology was developed jointly by Shipra Thapar and Navjit Kaur. Both Navjit Kaur and Shipra Thapar conducted formal analysis, while the investigation and data curation were carried out by Shipra Thapar. Shipra Thapar prepared the original draft of the manuscript, with both authors involved in the review and editing process. Supervision of the research was provided by Navjit Kaur and Shipra Thapar. All authors have read and agreed to the published version of the manuscript.

7. Conflict of Interest

No Conflict of Interest

8. References

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