

Evaluation of phytoconstituents of *hygrophila auriculata* as sedatives and hypnotics: *in-vivo* and *in silico* studies

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The study explores the potential of *Hygrophila auriculata*, also known as “Ikshugandha” in Ayurvedic literature, to alleviate insomnia by extracting phytoconstituents and investigating its *in-vivo* sedative-hypnotic activity. GC-MS analysis of seed and leaf extracts revealed the presence of phytoconstituents like β -stigmasterol, lupeol, and gamma-sitosterol. The receptor $\alpha 1$ - $\beta 2$ - $\gamma 2$ subtype in combination with GABA and diazepam was selected for the docking investigation. The highest docking scores were -9.46 Kcal/mol for stigmasterol and -9.40 Kcal/mol for lupeol. The *in-vivo* sedative-hypnotic activity was assessed using the Hole cross test, Hole board test, open field test, and the thiopental-induced sleeping duration test. The oral administration of ethyl acetate subextract and unsaponifiable fraction resulted in a significant reduction in the number of holes traversed and dips and a similar reaction for the open field test. In the thiopental-induced sleeping test, the hydroalcoholic extract, ethyl acetate subextract, and unsaponifiable fraction were observed to improve sleep time compared to the control group. The substance was considered safe up to 400 mg/kg for leaf extract and 200 mg for seed extract. These findings suggest the plant is a promising candidate for future pharmacological studies.

Keywords: *Hygrophila auriculata*. Sedative-hypnotic. *In-vivo* study. Molecular docking. Diazepam. Gas chromatography-mass spectrometry.

INTRODUCTION

Sleep problems are a prevalent health concern among the general population. These disorders are linked to decreased physical and psychological activity, and they can have significant societal, economic, and personal implications (Roth, 2005, Neubauer, Flaherty, 2009). Benzodiazepines are currently the most often given medications for sleep disorders. But the therapeutic use of benzodiazepines is associated with adverse effects including drug dependence, tolerance, rebound insomnia, amnesia, and psychomotor impairment. Thus, during the last 10 years, research has been conducted to identify innovative, less harmful hypnotic and sedative medications from both traditional and alternative medicine. Traditionally, safe treatments for a wide range of diseases impacting human health have been offered

by medicinal plants (Panara, Nishteswar, Nariya, 2021). Within this model, ethnomedical knowledge of therapeutic plants with varying pharmacological effects is available. Utilizing the same knowledge, *Hygrophila auriculata* (Schumacher) Heine was chosen. *Hygrophila auriculata* and *Hygrophila spinosa* belong to the family Acanthaceae. Other names of this plant are *Asteracantha longifolia* (Tejashri, Hasabe, Dhole, 2018), *Kolshinda* and *Talimkhana* in Marathi, *Kokilaksha* in Sanskrit, *Kuliyakhara* in Bengali, *Ekharo* in Gujarati, *Talmakhana* in Hindi and Urdu, *Kolavali* in Kannada, and *Golmidi* in Tamil. The plant was also named *ikshura*, and *ikshagandha* based on its morphology, which refers to “plant looking like eyes of the kokila (Indian cuckoo).” It is erect and spreads across the plains of India, particularly in swampy areas. Annual grow to a height of 1-1.5 meters (Patra, Jha, Murthy 2009). In Indian traditional medicine, this plant has been used as a diuretic, tonic, aphrodisiac, antibacterial, analgesic, and demulcent (Patra, *et.al.* 2009; Saha, Paul, 2017).

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The literature survey indicates that plants contain a variety of phytoconstituent including terpenoids, saponins, steroids, flavonoids, alkaloids, fatty acids as described in Figure 1 (Mohanty, Rautaray, 2020; Katariya *et al.*, 2021; Sarvananda, Premarathna, 2018).

The chemical structures of some phytoconstituents of *Hygrophila auriculata* are represented in Figure 2.

H. auriculata's seeds, roots, stems, leaves, aerial parts, complete plant, and even ash have been used to treat numerous diseases, according to traditional and ethnomedical literature (Ahmed *et al.*, 2001). According to the Ayurvedic system, it is categorized as "Seethaveeryam" and "Mathuravipaka". The seeds are used to treat blood problems, biliousness, gonorrhoea, spermatorrhoea and fever, in addition to being included in a variety of aphrodisiacs and tonic confections (Chauhan, Dixit, 2010). The leaves have aphrodisiac, tonic, diuretic, sweet and hypnotic properties. They can be used to treat inflammations, cough, diarrhoea, thirst, urinary calculi and urine discharges (Kuru *et al.*, 2014). The pharmacological actions of *Hygrophila auriculata* was depicted as shown in Figure 3.

The majority of natural product-based drug development initiatives employ crude plant extracts. These kinds of initiatives are helpful in forecasting how small molecules will interact with potential therapeutic targets. The molecular docking studies predicts effectiveness of the molecule for identified target (Sahu *et al.*, 2024; Tiwari, Kartikay, 2024; Ganeshpurkar *et al.*, 2019). An important analytical method that has been modified to assess the phytoconstituents found in plant extracts together with their structures is Gas chromatography - Mass spectrometry (GC-MS). This method produces chemical fingerprints with exceptional accuracy and precision due to its enhanced separation potency. Furthermore, GC-MS can provide quantitative data in addition to the linked mass spectral database, which is extremely valuable for establishing a correlation between bioactive substances and the pharmacological uses (Thamer, Thamer 2023; Ralte *et al.*, 2022; Satpathy, Patra, Ahirwar 2018; Salve, Bhuktar, 2017; Patra *et al.*, 2010).

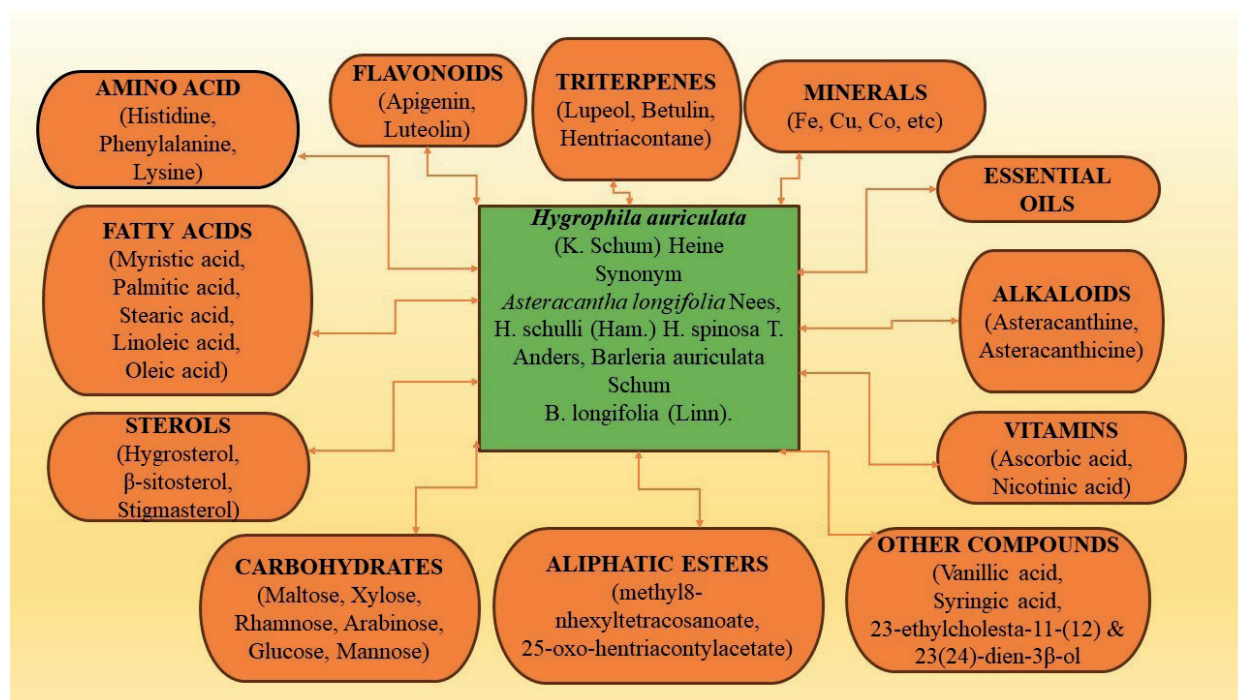


FIGURE 1 - Phytochemical perspective of *Hygrophila auriculata*.

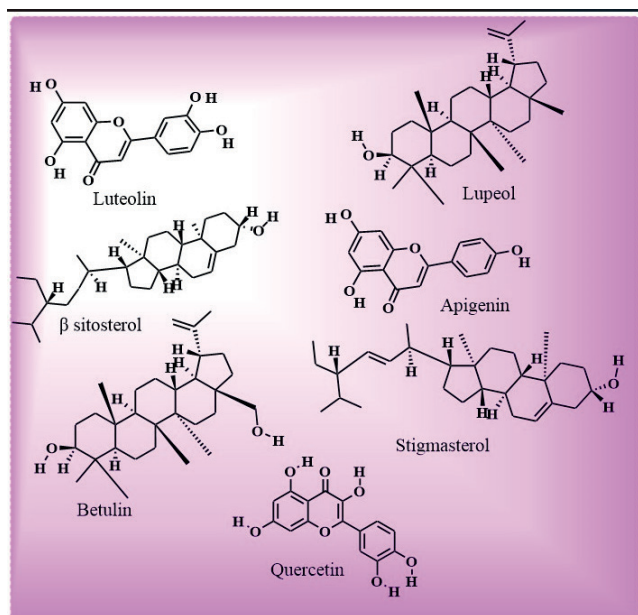


FIGURE 2 - 2D Chemical structure of phytoconstituents of *Hygrophila auriculata*.

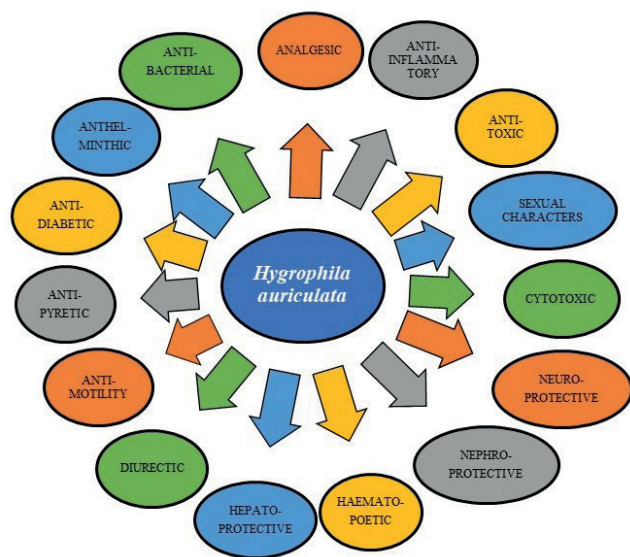


FIGURE 3 - Pharmacological actions of *Hygrophila auriculata*.

MATERIAL AND METHODS

Plant Collection

In October of 2022, the fresh plants of *Hygrophila auriculata* were gathered from the field region of Markol

chokadi, which is located on Narsarovar road in Sanand, India. Dr. Hitesh Solanki, a professor in the Department of Botany at the School of Science at Gujarat University, verified the authenticity of the plant specimen. The authentication number is GU/BOT/AH1 (certificate attached in supplementary Figure S1).

Chemicals

Folin-Ciocalteu Reagent (FCR) was procured from LOBA Chemie Pvt. Ltd, Mumbai, India. Gallic acid monohydrate (Standard), Sodium carbonate, Methanol, Rutin, Aluminium chloride, Sodium hydroxide, Sodium nitrite, Hexane, n-Butanol, and Ethyl acetate were of analytical grade and procured from Acros Organics, Powai, Maharashtra, India. (Lists of chemicals and instruments attached in supplementary Table T1 and T2)

Extraction of Plant Material

Extraction method for leaves

100g of dried leaves was extracted in a Soxhlet extractor at 80°C using a hydro-alcoholic solvent 50% Water-Methanol solvent. The extract was fractionated in order of increasing polarity to obtain ethyl acetate, hexane, and n-butanol fractions (Satpathy *et.al.*, 2018).

Extraction method for seeds

The process was begun with extracting seeds using petroleum ether. This was done continuously for 48 hours at 60°C. The extract was then reduced to one-fourth of its original volume. After that, it was mixed with a 10% w/v aq. potassium hydroxide solution and saponified at 60°C for 8 hours. Once the saponification process was completed, the solution was cooled and partitioned with diethyl ether three times. The organic layer was collected and concentrated afterwards.

Phytochemical study (Qualitative and Quantitative)

Qualitative phytochemical screening

The qualitative phytochemical screening was conducted to identify chemical compounds in *H. auriculata* leaves and seeds extracts. To identify alkaloids, few mg or 0.5 gm of extract was mixed with 5ml of dil. hydrochloric acid and then filtered. The sample was tested with Dragendroff's reagent and Picric acid test. To detect flavonoids, Shinoda's test and Ferric chloride test was performed. For phytosterols, Salkowski's test and Liebermann-Burchard's test was done. In order to test for terpenoids, 5ml of sample was dissolved in 2ml of chloroform evaporated on water bath and then 3ml conc. sulphuric acid was added (boiled on water bath). The tannins were identified by 10% w/v sodium hydroxide test and Bromine water test whereas phenolic compounds were identified by lead acetate test and Ferric chloride test. The presence of saponins was detected by Foam test. The detailed procedure (Silva, Abeyundara, Aponso, 2017) was given in supplementary(P1).

Quantitative Phytochemical Estimation

Total Phenolic content

To achieve a concentration of 1000 µg/ml, 10 milligrams of gallic acid was properly weighed and dissolved in 10 mL of methanol. To make a 100 µg/ml solution, transfer the 1000 µg/ml standard solution to a 100 ml volumetric flask and add methanol to the mark. The solution was further diluted to achieve a concentration of 10-70 µg/ml. Mix 0.5 ml of normal gallic acid solution (10-70 µg/ml) with 2 ml of Folin-Ciocalteu reagent (10%v/v) and 4 ml of sodium carbonate solution (7.5% w/v). The prepared solutions were incubated at room temperature for 30 minutes before measuring the absorbance at 765 nm with a UV-VIS spectrophotometer. To prepare the sample, 20 mg

of extract was properly weighed and diluted in 10 ml of methanol, yielding a concentration of 2000 µg/ml. 0.5 mL of the above solution was made as a reference solution, and the absorbance was measured.

Total Flavonoid Content

50 mg of Rutin was accurately weighed and dissolved in 50 ml of methanol. Then, the solution was further diluted to get various dilution of working standard solution to obtain concentration range of 100 – 900 µg/ml. 0.6 ml of the sample (standard rutin solution 100 – 1000 µg/ml) was mixed with 6.8 ml of the methanol and subsequently 0.3 ml sodium nitrite solution (10% w/v) and 0.3 ml of aluminium chloride solution (10% w/v) was added. Then, after 5 min 2 ml NaOH (1% w/v) was added and immediately the absorbance was measured at 506 nm using UV-VIS spectrophotometer. 20 mg of sample was taken and dissolved in 10 ml of methanol to attain a concentration of 2000 µg/ml. 0.6 ml of the sample was taken and prepared as standard solution. The absorbance was measured at 506 nm (Islam, Parvin, Islam, 2022; Saeed, Khan, Shabbir, 2012).

Gas Chromatography/Mass Spectrometry (GC-MS) analysis

10 mg extract was dissolved in 10 ml of methanol for hydroalcoholic extract, ethyl acetate extract and unsaponifiable fraction. Sample: solvent ratio of (1:1) was taken in methanol. The GC-MS reading of the unknown mass spectrum was completed by comparing the division patterns of the mass spectra with the known and standard compound provided in the database of NIST 17 (National Institute of Standard and Technology) for unsaponifiable extract of seed and hydroalcoholic extract of leaves, and NIST 14 for ethyl acetate extract of leaves of *H. auriculata*. The compound was identified by their GC retention time. The average peak area, total peak area, and relative percentage of each compound were compared, and the name, molecular formula, and molecular weight of each detected compound were

determined. The GC-MS Programming was given in following Tables I-III.

***In Vivo* study: Sedative-Hypnotic Activity**

Swiss albino mice weighing 15-25 g (male) were obtained from the Animal Resources Branch of Synbio Research Pvt. Ltd., Changodar, Ahmedabad. The animals were kept in conventional laboratory settings (relative humidity 55-60%, 12h light/dark cycle, room temperature $25 \pm 2^{\circ}\text{C}$) and supplied with standard pellets and clean water ad libitum during the acclimation period. The animals were acclimatized to the laboratory setting for seven days before the tests began. The animals were fasted overnight before the studies. All experimental protocols were approved by the Institutional Ethics Committee (Certificate no. LJIP/IAEC/2023-24/06, attached in supplementary Figure no. S7). For each experiment, the animals were placed into five groups, with five animals in each for the control, standard, and test samples. The test animals were given 200 mg/kg and 400 mg/kg body weight of hydroalcoholic extract and ethyl acetate fraction of the leaf (diluted in 1% Tween80) thirty minutes before the experiment. Unsaponifiable fraction of seed was administered at levels of 100 and 200 mg/kg body weight (oral route). The standard drug diazepam (1 mg/kg) was administered intraperitoneally 15 min before the experiments. In the sleeping time measurement test, thiopental sodium (40 mg/kg) was administered 15 min after diazepam or 30 min after vehicle (1% Tween 80) or extract administration. The control group received 0.1 ml/mouse of vehicle orally 30 minutes prior to the experiment.

Hole Cross test

A wooden box measuring 30cm×20cm×14cm was constructed with a partitioning wall and a 3cm diameter opening at a height of 7.5cm from the floor (Takagi, Watanabe, Saito, 1971). The animals were given either a vehicle, a medication, or extracts and then permitted to pass through the hole from one room to another.

Mice were observed for 3 minutes, with passage counts recorded at 30, 60, 90, and 120 minutes after treatment.

Open Field Test

This procedure was carried out with the open field equipment, which consisted of a half-square-meter wooden field with a sequence of squares divided by black strips. The wall was 25 cm high and put in a poorly lighted room. Mice were given vehicle, extract, or diazepam and placed in the center of an open field. The number of squares visited by the animals was then tallied for three minutes at 30-, 60-, 90-, and 120-minute intervals following the treatments (Gupta, Dandiya, Gupta, 1971).

Hole Board Test

The hole-board test was carried out using the previously reported approach by Ozturk *et al.* (1996), with minor adjustments. For this test, we used a wooden hole board measuring 40cm by 40cm with 16 uniformly placed holes (each 3cm diameter). The equipment was lifted to a height of 25 cm. After 30 minutes of vehicle or extract administration and 15 minutes of diazepam administration, each animal was allowed to move on the platform for 5 minutes. The number of head dips into the holes was tallied.

Thiopental Sodium-Induced Sleeping Time Determination

Thiopental sodium (TS) was given intraperitoneally to mice at a dose of 40 mg/kg thirty minutes after treatment with vehicle or extracts and 15 minutes after diazepam. The animals were then evaluated for the time it took to lose their righting reflex immediately after thiopental sodium injection (latent period), as well as the duration of sleep caused by TS (Turner, 1965).

Acute toxicity test

TABLE I - Details of Column used in Extraction

Extract	Column details
Ethyl acetate Leaf extract	Elite-5 MS capillary column (30 x 0.25mm ID x 1µMdf) was made of 95% polysiloxane and 5% biphenyl group
Hydroalcoholic Leaf extract & Unsaponifiable seed extract	SH-Rxi-5 Sil MS column (30.0m x 0.25mm ID x 0.25µm) was made of (1,4-bis(dimethylsiloxy)phenylene dimethyl polysiloxane)

TABLE II - Instrumental Programming for Gas Chromatography (*continues*)

Column Oven Temp.	50°C	
Injection Temp.	250°C	
Injection Mode	Split	
Flow Control Mode	Linear Velocity	
Pressure	103.5 kPa	
Total Flow	24.3 mL/min	
Column Flow	1.75 mL/min	
Linear Velocity	48.0 cm/sec	
Purge Flow	5.0 mL/min	
Split Ratio	Hydroalcoholic Leaf extract and Unsaponifiable seed extract	10:1
	Ethyl acetate Leaf extract	50:1
Total run time	50 min	
High Pressure Injection	ON	
High Press. Inj. Pressure	250 kPa	
High Press. Inj. Time	2.3 min	
Carrier Gas	Helium	
Carrier Gas Saver	OFF	
Splitter Hold	OFF	

TABLE II - Instrumental Programming for Gas Chromatography (*continues*)

Oven Temp. Program		
Rate (°C/ min)	Temperature (°C)	Hold Time (min)
-	50	2
10	200	10
10	300	10
GC- MS Programming		
Ion Source Temp	230°C	
Interface Temp.	250°C	
Solvent Cut Time	3 min	
Detector Gain Mode	Relative to the Tuning Result	
Detector Gain	0.97 kV +0.20 kV	
Threshold	0	

TABLE III - MS Programming for Leaf extract & Unsaponifiable seed extract

Extract	Hydro alcoholic extract and Unsaponifiable fraction	Ethyl acetate subextract
Start Time	3 min	5min
End Time	50 min	50 min
Acquisition Mode	Q3 Scan	EI Scan
Event Time	0.300 sec	0.5 sec
Scan Speed	3333	-
Start m/z	40	50
End m/z	800	600
Sample Inlet Unit	GC	

The acute toxicity test was performed to assess any potential toxicity. Swiss albino male mice (5) were given hydroalcoholic extract and the ethyl acetate sub extract at different doses (200 and 400 mg/kg, p.o. oral route), unsaponifiable fraction (100mg/kg and 200mg/kg, p.o. oral route), and a control group received 1% Tween80 (0.1ml/mouse). The groups were monitored for gross effects during the first 4 hours, and mortality was measured after 24 hours (Pattanayak, Sunita, 2008; Vyas, Gamit, Raval, 2020; Neharkar, Gautam, Pandhare, 2016).

In-Silico studies

Protein Preparation

The Protein Data Bank (<https://www.rcsb.org/>) provided information on the human GABAA receptor alpha1-beta2-gamma2 subtype in complex with GABA and diazepam (PDBID: 6X3X). The protein was prepared for molecular docking by removing water and ions, substituting them with polar hydrogen, and assigning Kollman charges with the MMFF94 force field (Kim *et al.*, 2020).

Ligand Preparation

Energy minimization was used to correct the geometry and eliminate steric conflicts among overlapping atoms in the compounds. Open Babel 3.1.1 to reduce energy of compounds (Ganeshpurkar *et al.*, 2019). The reduced ligands were then converted to an Auto Dock-compatible format called “pdbqt”. Following energy minimization, all compounds were subjected to molecular docking studies.

Grid generation and Molecular Docking

The residues surrounding the co-crystallized ligand were used to form a grid box around the active sites. Auto Dock employs auto grid 4.0 to build grid maps of

interaction energies with various atom types in ligands. Grid maps showing interaction energies with several atom types found in ligands (A, C, HD, N, NA, OA, Cl, and SA) were generated using Auto Dock’s auto grid 4.0. The grid box size was modified to ensure that all amino acid residues in the active pocket were covered. The grid points were set at 40, 40, and 40, with a spacing of 0.375 Å. The grid center’s x, y, and z coordinates were set to 145.474, 123.248, and 122.431 (respectively). Autodock Tools version 1.5.6 (Morris, 2009) and the Discovery Studio visualizer (Biovia *et al.*, 2015) were used to evaluate and illustrate the docking results.

RESULTS

Qualitative phytochemical screening

The leaves and seeds extract of *H. auriculata* were tested qualitatively for phytochemicals. Saponins, terpenoids, alkaloids, flavonoids, sterols, tannins, and steroids were found in the samples. Alkaloids were detected in all hydroalcoholic, ethyl acetate, and petroleum ether samples. Steroids and terpenoids were present in all extracts, although tannin and saponin were only present in the hydroalcoholic and ethyl acetate extracts. Flavonoids were found in the hydroalcoholic extract, ethyl acetate fraction, and n-butanol fraction (Table IV).

Quantitative phytochemical screening

The quantitative phytochemical screening of *H. auriculata* plant was performed for determining the total flavonoid and phenolic content present in plant (Table V).

GC-MS analysis of extract

The hydroalcoholic and ethyl acetate extracts of leaves and unsaponifiable extracts of seeds of *H. auriculata* were subjected to GC-MS analysis. A total of twenty-nine phytoconstituents were detected

TABLE IV - Qualitative phytochemical screening of leaves and seeds extract of *H. auriculata*

S. No.	Phyto-Constituents	Leaf				Seed	
		Hydro-alcoholic extract	Ethyl acetate fraction	n-butanol fraction	Hexane fraction	Petroleum-ether extract	Unsaponifiable fraction
1	Alkaloid	+	+	-	-	+	-
2	Flavonoid	+	+	+	-	-	-
3	Phy-tosterol	+	+	+	+	+	+
4	Terpenoid	+	+	+	+	+	+
5	Tannin	+	+	-	-	-	-
6	Saponin	+	+	-	-	-	-
7	Phenolic compounds	+	+	+	+	+	-

Note: “+” indicates Presence or “-” indicates absence of Phytochemical in plant.

TABLE V - Quantitative determinations of Flavonoid and Phenolic content (n=3) in *H. auriculata*

Part	Extract	Flavonoid (mg/gm)	Phenolic (mg/gm)
Leaf	Ethyl acetate	124.94±3.47	31.345±0.15
	n-butanol	44.95±2.55	33.33±1.68
	Hexane	183.27±1.92	24±0.15
	Hydro alcoholic	257.66±2.565	60.425±0.197
Seed	Unsaponifiable extract	40.49±165	17.14±0.15

in the hydroalcoholic (8), ethyl acetate leaf extracts (10) and unsaponifiable seed extract (11) as shown in Table VI and Figures 4-6. The GC-MS graph of all the extracts is attached in supplementary (Figure S2- S4).

***In Vivo* study: Sedative Hypnotic Activity**

Hole cross test

The sedative effects of extracts began by recording spontaneous locomotor activity in mice during hole

TABLE VI - Phytoconstituents detected in *Hygrophila auriculata* extracts (continues)

Peak No.	Phytoconstituent	RT	% Area	Molecular formula	MW (g/mol)
Unsaponifiable seed extract (Figure No. 4)					
1	Cyclotetrasiloxane, octamethyl	5.768	2.09	C ₈ H ₂₄ O ₄ Si ₄	296
2	Cycloheptasiloxane, tetradecamethyl	13.224	4.31	C ₁₄ H ₄₂ O ₇ Si ₇	518
3	Cyclooctasiloxane, hexadecamethyl	15.109	3.85	C ₁₆ H ₄₈ O ₈ Si ₈	593
4	Neophytadiene	17.558	1.62	C ₂₀ H ₃₈	278
5	Tetracontane	34.453	5.56	C ₄₀ H ₈₂	563
6	Dotriacontane	36.169	9.75	C ₃₂ H ₆₆	450
7	Tetrapentacontane	37.693	10.24	C ₅₄ H ₁₁₀	759
8	Campesterol	38.914	9.01	C ₂₈ H ₄₈ O	400
9	Stigmasterol	39.171	24.39	C ₂₉ H ₄₈ O	412
10	Gamma-sitosterol	39.837	4.83	C ₂₉ H ₅₀ O	414
11	Lupeol	40.927	3.88	C ₃₀ H ₅₀ O	426
Hydroalcoholic leaf extract (Figure No. 5)					
1	2-Heptanamine, 5-methyl	3.020	1.99	C ₈ H ₁₉ N	129
2	3,3,5-Triethoxy-1,1,1,7,7,7-hexamethyl-5-(trimethylsilyloxy)tetrasiloxane	9.719	22.38	C ₁₅ H ₄₂ O ₇ Si ₅	474
3	Cycloheptasiloxane, tetradecamethyl	13.147	3.31	C ₁₄ H ₄₂ O ₇ Si ₇	519
4	Bis(heptamethylcyclotetrasiloxo)siloxane	13.631	8.33	C ₁₄ H ₄₂ O ₉ Si ₈	578
5	Ammelide, N,N,O'-tetrakis(trimethylsilyl)	13.864	3.08	C ₁₅ H ₃₆ N ₄ O ₂ Si ₄	416
6	2-[(p-Trimethyl silyl oxy)phenyl]-2-[(p-trimethyl silyloxyethylen oxy) phenyl] propane	14.039	6.77	C ₂₃ H ₃₆ O ₃ Si ₂	416
7	Cyclooctasiloxane, hexadecamethyl	14.885	17.90	C ₁₆ H ₄₈ O ₈ Si ₈	592
8	Cyclononasiloxane, octadecamethyl	16.828	7.35	C ₁₈ H ₅₄ O ₉ Si ₉	667
Ethyl acetate leaf extract (Figure No. 6)					

TABLE VI - Phytoconstituents detected in *Hygrophila auriculata* extracts (continues)

Peak No.	Phytoconstituent	RT	% Area	Molecular formula	MW (g/mol)
1	2,3,5,8- Tetramethyl decan	10.927	3.728	C ₁₄ H ₃₀	198
2	1-Hexen-3-ol	12.888	3.180	C ₆ H ₁₂ O	100
3	2-Propanone,1,1-dibutoxy	13.968	2.024	C ₁₁ H ₂₂ O ₃	202
4	2-Di methyl (trimethylsilyl) silyloxytridecane	15.356	2.747	C ₁₈ H ₄₂ OSi ₂	330
5	Phthalic acid, octyl oct-3-yl ester	33.208	3.229	C ₂₄ H ₃₈ O ₄	390
6	Silane, dodecyldiethoxymethyl	37.396	34.442	C ₁₇ H ₃₈ O ₂ Si	276
7	Octasiloxane,1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexa decamethyl	38.211	3.053	C ₁₆ H ₃₀ O ₇ Si ₈	579
8	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandro-8-en-17-yl)	38.445	4.708	C ₂₇ H ₄₂ O ₄	430
9	1-(beta-d-Arabinofuranosyl)-4-difluoromethyl-5- bromouracil	39.087	2.141	C ₁₀ H ₁₁ BrF ₂ N ₂ O ₆	373
10	Silane, dodecyldiethoxymethyl	43.331	20.917	C ₁₇ H ₃₈ O ₂ Si	302

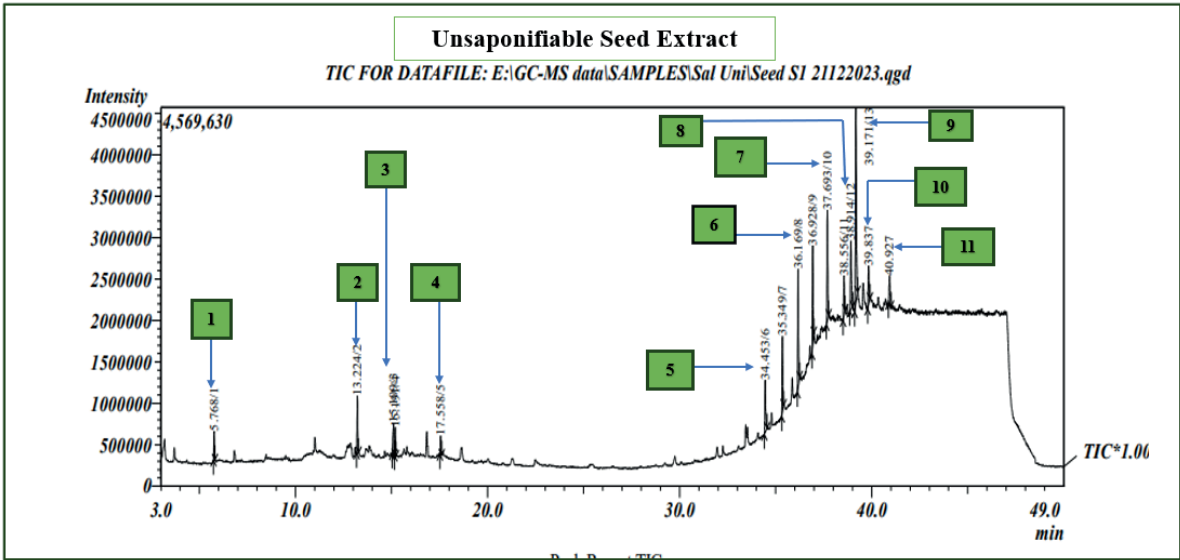
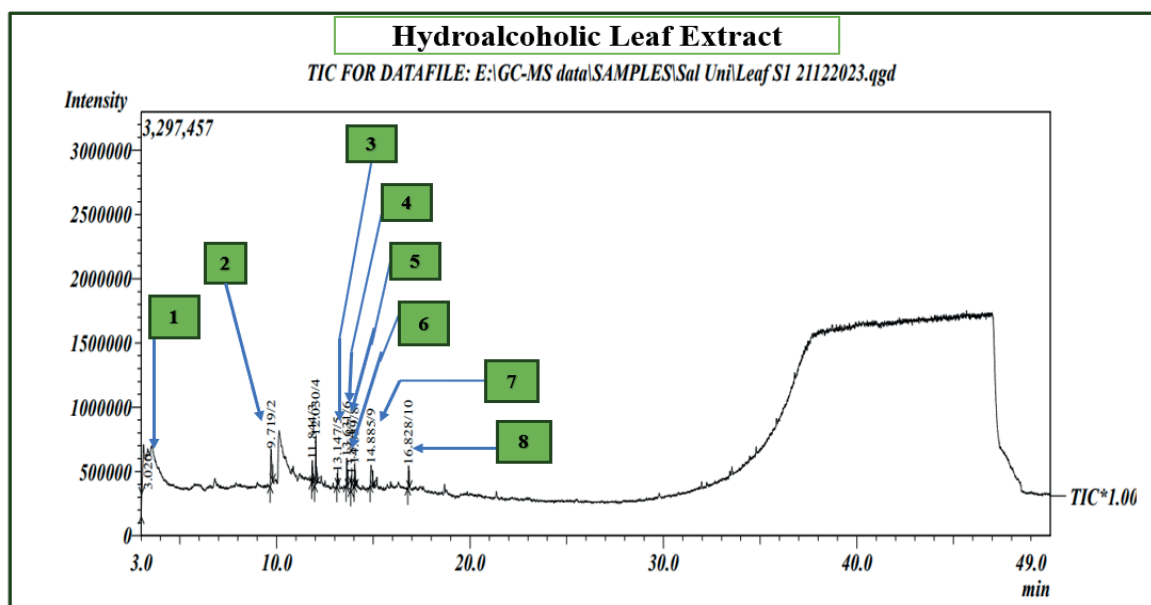
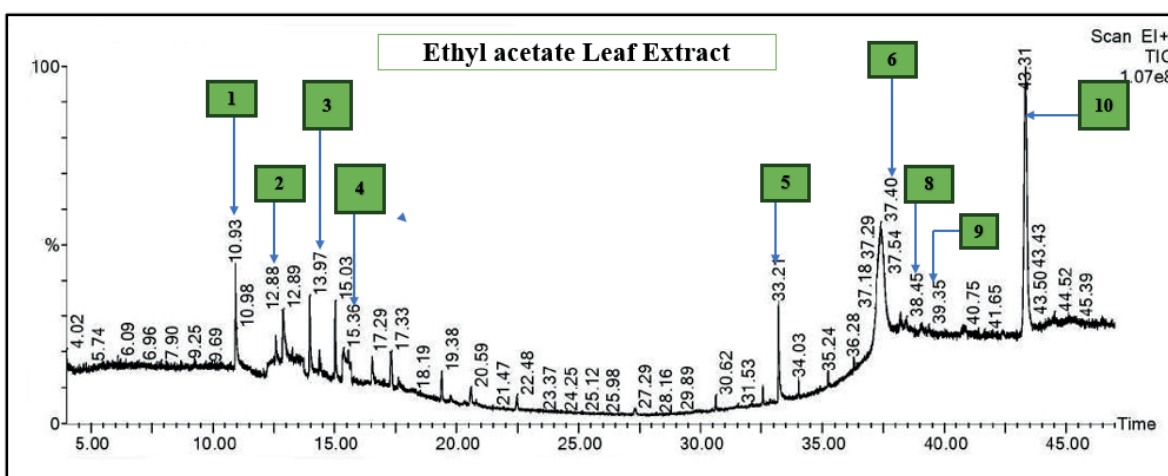


FIGURE 4 - GC-MS of unsaponifiable extract of seeds of *Hygrophila auriculata*.

FIGURE 5 - GC-MS of hydroalcoholic extract of leaves of *Hygrophila auriculata*.FIGURE 6 - GC-MS data of ethylacetate extract of leaves of *Hygrophila auriculata*.

cross. In these studies, any drugs with sedative effects caused a decrease in the number of movements, which was interpreted as a decrease in curiosity about the new surroundings (Takagi, Watanabe, Saito, 1971; Gupta *et al.*, 1971; Prut, Belzung, 2003). The results showed that oral administration of ethyl acetate subextract (400mg) and unsaponifiable fraction (200mg) resulted in a significant reduction in the number of holes crossed, as

illustrated in Figure S5. Figure 7 depicts chart diagram for comparing various extracts in different doses with the standard drug diazepam and a control group.

Note ∴ Statistical analysis for animal experiment was carried out using One-way ANOVA followed by Dunnet's multiple comparisons. The results obtained were compared with the vehicle control group, in which *P <

0.05, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ were considered to be statistically significant. All statistical analysis was performed using Graph Pad Prism 10.2.0 software.

Open Field Test

After administering ethyl acetate sub extract and unsaponifiable fraction, a suppressive effect was observed after 30 minutes and lasted up to 120 minutes. The ethyl acetate sub extract (400mg) and unsaponifiable fraction (200mg) significantly inhibited locomotion ($P < 0.05$ and $P < 0.01$) from 30 to 120 minutes of observation. The ability of the ethyl acetate sub extract and unsaponifiable fraction to inhibit locomotor activity indicates that the extract contains central nervous system depressant activity. Figure S5 depicts the *in vivo* effect of *H. auriculata* extracts on mice as well as a chart diagram (Figure 8) for comparing various extracts in different doses with the standard drug diazepam and the control group.

Hole-Board Test

The Hole-Board Test was an essential and favored method for determining the possible sedative and anxiolytic effects of any drug in mice. This test was useful since it was methodologically simple and easily exhibited varied behavioral reactions when the experimental animal was exposed to a foreign body or situation. Animals' head-dipping behavior was found to be directly tied to their emotions. Based on this observation, it was proposed that the expression of an anxiolytic state in animals could be reflected by an increase in head-dipping behavior (Takeda, Tsuji, Matsumiya, 1998), whereas a decrease in the number of head dips was found to be correlated with the depressant effect (Viola *et al.*, 1995). After administering the hydroalcoholic extract, ethylacetate subextract, unsaponifiable fraction, and diazepam to the hole-board test, All the extracts shows that, the number of head insertions decreases considerably compared to the control group ($P < 0.0001$). Ethylacetate

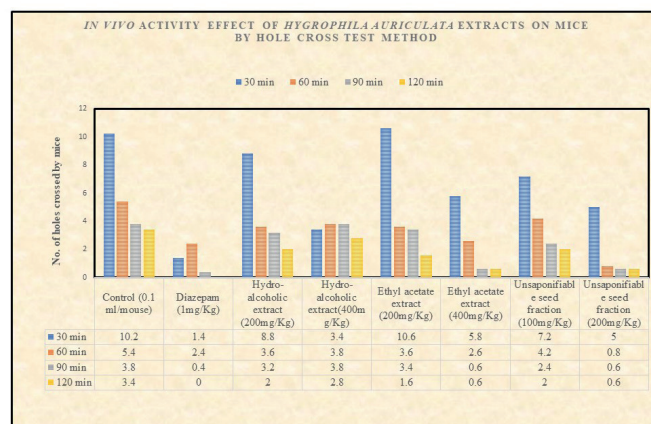


FIGURE 7 - Graph shows response obtained from Hole cross test.

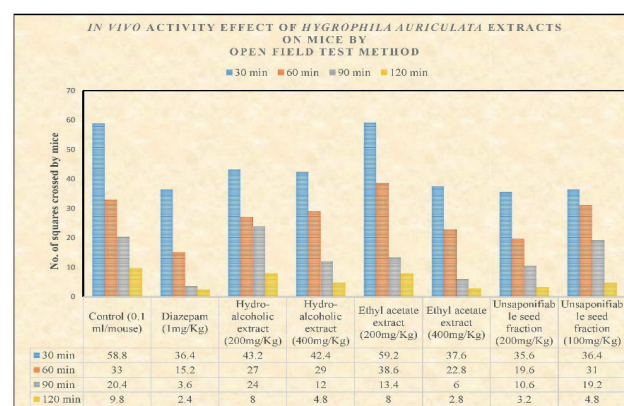


FIGURE 8 - Graph shows response obtained by Open field test method.

subextract(400mg) and unsaponifiable fraction(200mg) show the effect which is nearly equivalent to the reference drug diazepam. Figure S5 depicts the *in vivo* effect of *H. auriculata* extracts on mice using the hole board test method, in addition to chart diagram (Figure 9) for comparing various extracts in different doses with the standard drug diazepam and a control group.

Thiopental-induced sleeping time test

The Thiopental-induced sleeping time test was one of the most popular studies in the investigation of sedative-hypnotic medications. Barbiturates that depress the central nervous system, such as thiopental sodium,

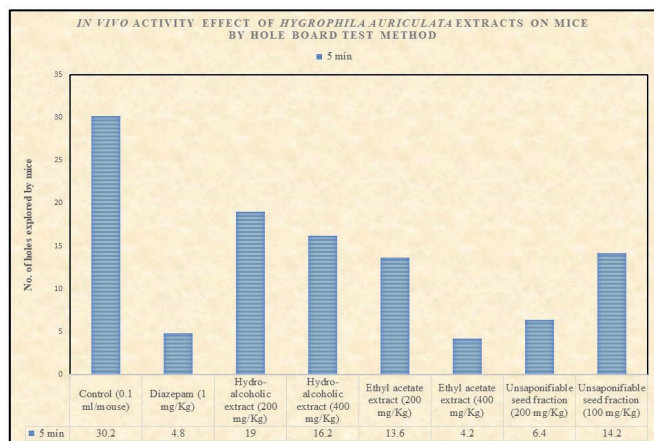


FIGURE 9 - Graph shows response obtained by Hole board test method.

bind to the barbiturate-binding site on the GABA receptor complex and cause GABA-mediated hyperpolarization of postsynaptic neurons (Moniruzzaman, Rahman, Ferdous, 2015). In the current investigation, the hydroalcoholic extract, ethylacetate subextract, and unsaponifiable fraction were observed to increase sleep time compared to the control group (Figure S5). The effect is nearly equivalent to the reference drug diazepam. Given that thiopental's inhibitory effect on the CNS was linked to GABAergic system activation (Sivam, Nabeshima, Ho, 1982; Steinbach, Akk, 2001), the current study found that the sedative-hypnotic effect of some *H. auriculata* components was mediated by GABAergic receptors. A chart diagram (Figure 10) for comparing various extracts in different doses with the standard drug diazepam and a control group.

Acute Toxicity Test

The hydroalcoholic extract and ethyl acetate sub extract did not cause any toxicological symptoms or mortality at the tested doses of 200 mg/kg and 400 mg/kg, respectively. Thus, the extracts (hydroalcoholic extract and ethyl acetate sub extract) were deemed safe up to a level of 400 mg/kg. In respect of, unsaponifiable fraction there were no toxicological symptoms or deaths

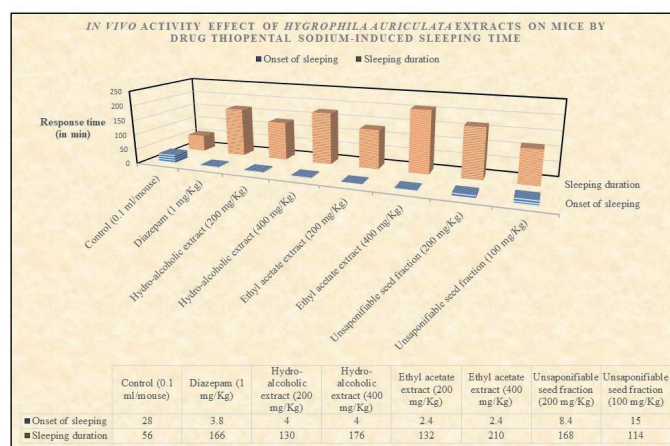


FIGURE 10 - Graph shows response obtained by Thiopental sodium-induced sleeping time.

detected at the tested doses (100 and 200 mg/kg). Thus, this was considered safe up to a dose of 200 mg/kg.

In-Silico Studies

Protein Preparation

A resolution 2.92Å crystal structure (PDB ID: 6x3x) to examine the human GABA-A receptor alpha1-beta2-gamma2 subtype when combined with GABA and diazepam (Biovia *et al.*, 2015). The protein structure was retrieved from the Protein Data Bank website. After missing residues and atoms were mended, water molecules were removed and hydrogen atoms were added using role-based criteria. Prior to using the conjugate gradient reduction technique for docking to avoid incompatibilities, the protein structure was reduced in 500 steps. Several validation procedures were utilized to evaluate the model's quality (Table VII and Figure 11). According to the Ramachandran analysis (Laskowski *et al.*, 1993), the residues were distributed as follows: 88.2 percent in the favored zone, 11.2% in the additional authorized zone, and 0% in the outlier zone. There were no amino-acid residues identified in the outlier area. Table XI shows the findings of the ERRAT (Colovos, Yeates, 1993)

TABLE VII - Assessment of Constructed Protein Model

Programs/ Parameters	Results
PROCHECK (Ramachandran Plot)	
Residues in most favoured region	88.5% (1353)
Residues in additional allowed regions	11.2% (171)
Residues in generously allowed regions	0.3% (4)
Residues in disallowed regions	0% (0)
ERRAT (Overall quality factor)	92.138%

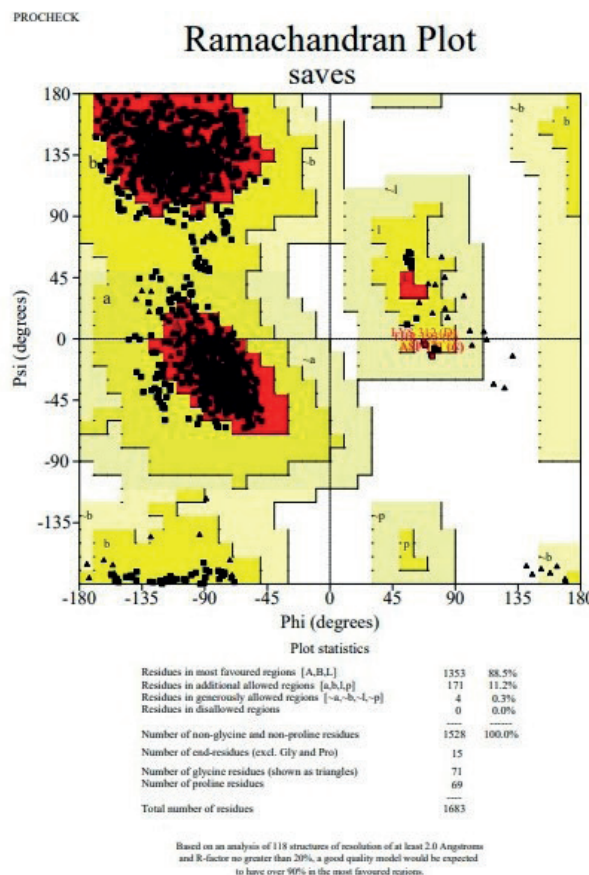
and Ramachandran plots. The ERRAT picture depicts an error function related to the location of a 9-residue sliding window that provides information on non-bonded interactions between certain atom types. This map resulted from the comparison of highly refined and less refined structures. In the ERRAT plot, the “overall quality factor” was defined as the fraction of proteins with projected error values smaller than the 95 percent statistical rejection limit. This figure no. 12 was created by comparing the actual and projected error values. The errat plots are attached in supplementary (Figure S6). The model accurately represented a protein, as indicated by its ability to satisfy the requirements reasonably.

Preparation of Ligand

After obtaining all molecules in Structured Data Format (SDF) from the “PubChem” database and converting them to Mol2 format, the energy was lowered using the Open Babel application, a simple interface offered by the Pyrx (Python Prescription Virtual Screening) module (Dallakyan, Olson, 2015). For docking, the Raccoon software was utilized.

Grid Generation and Molecular Docking

Molecular docking experiments were carried out on different phytoconstituents like Quercetin,

**FIGURE 11** - Ramachandran plot of Protein (PDB ID: 6x3x).

Lupeol, Beta-stigmasterol, Beta-sitosterol, Betulin, Apigenin, and Luteolin. In docking, Lupeol, Beta-stigmasterol, and Beta-sitosterol shown high binding affinity (Table VIII). The three-dimensional grid was constructed around interacting residues separated by 5 Å distances. Ileu228, Asn265, Leu285, Thr262, Met236, Thr262, and Leu232 are residues that occupy active sites. Diazepam’s docking score was -7.44 kcal/mol, indicating a conventional H-bond between NH and Leu253. The amino acid residues Ala289, Met236, Pro233, and Leu269 have pi-alkyl interactions. Furthermore, Thr251, Thr268, Thr256, Thr254, and Val252 demonstrated van der Waal interactions. As a result, the generated grid produced plausible outcomes that were compatible with the interactions of the co-crystallized ligand prior to docking (Figure 13).

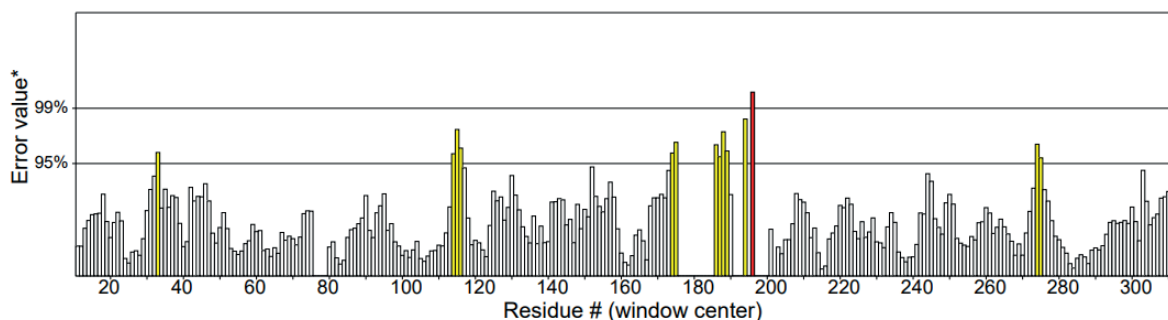


FIGURE 12 - Errat plot for protein (PDB ID: 6x3x).

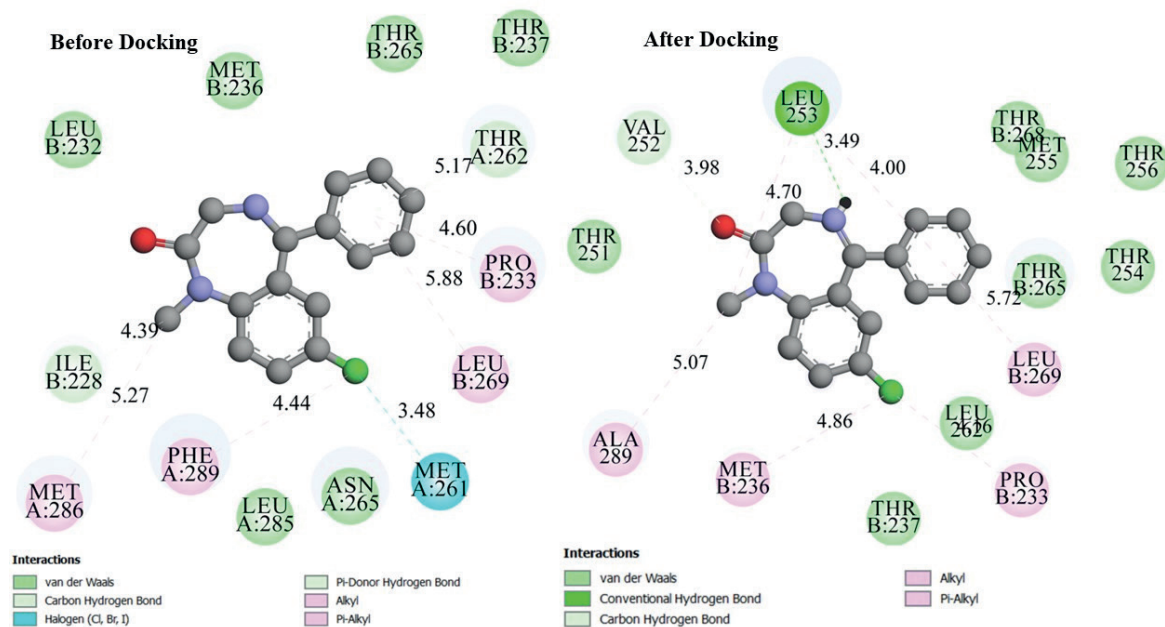


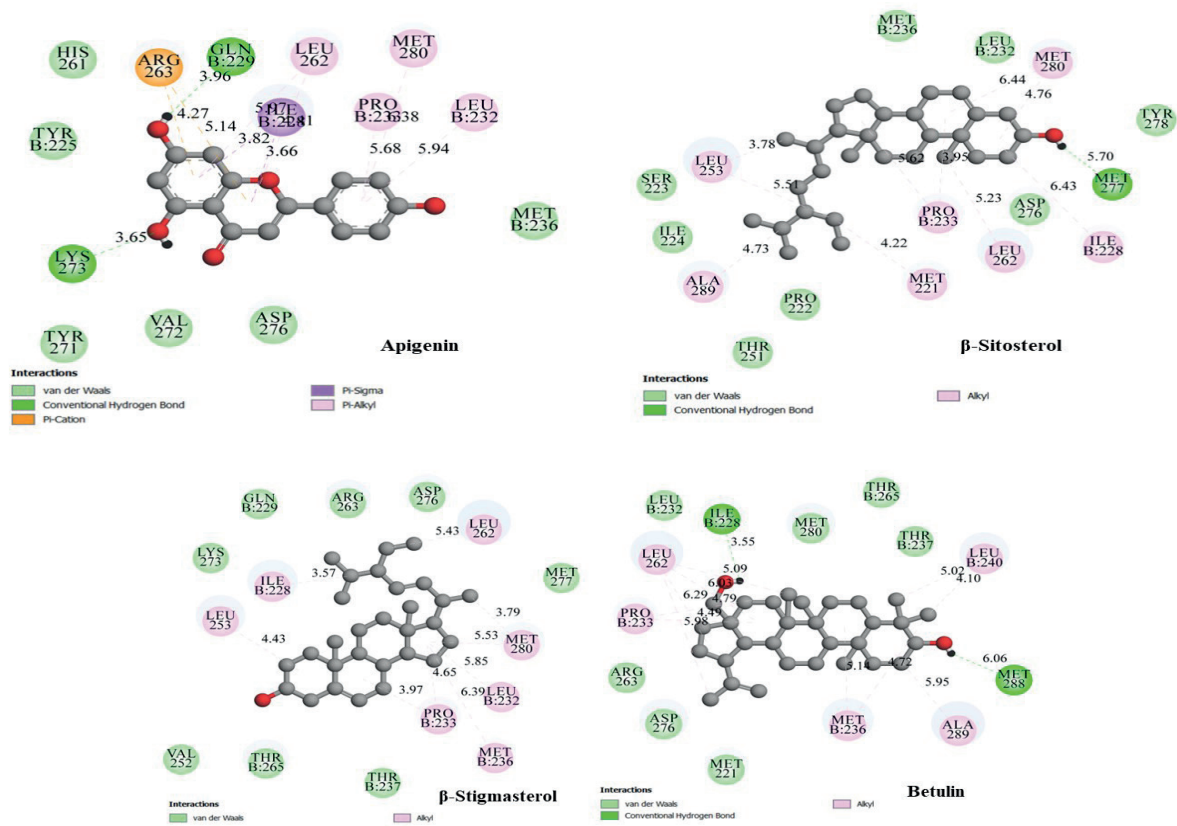
FIGURE 13 - 2D interaction of Drug Diazepam (Before and After Docking) in binding pocket of protein (PDB ID: 6x3x).

Similar dimensions were used for docking other compounds. The Apigenin molecule has hydrogen bonding interactions between Lys273 and Gln229. Ile228 and apigenin were implicated in the formation of pi-sigma bonds. Leu262, Met280, Leu232, and Pro233 were all involved in pi-alkyl interactions, whereas Arg263 was implicated in pi-cation interactions. The Beta-sitosterol molecule and amino acid residue Met277 create a normal H-bond. It formed pi-alkyl bonds with Pro233, Ile228, Leu262, Met280, Leu253, Ala289, and

Met221. In the molecule Beta-stigmasterol, the pi-alkyl interaction was represented by the residues Leu253, Ile228, Pro233, Leu262, Met280, Leu232, and Met236, while the van der Waals interaction was represented by the other residues. Met288 and Ile228 form an H-bond with Betulin. Met236, Ala289, Leu240, Leu262, and Pro233 demonstrated pi-alkyl interactions. Asp276 and Met277 are amino acid residues with Lupeol that form a conventional H-bond. There were pi-alkyl bonds between Leu240, Ala289, Ile228, Pro233, Leu262,

TABLE VIII - Docking score of Diazepam and Phytoconstituents present in *H. auriculata*

S. No.	Class	Phytoconstituents	Dock Score (Kcal/mol)	Number of Hydrogen bond	Number of Hydrophobic bonds
1	Benzodiazepines	Diazepam	-7.44	2	4
2	Triterpenes	Luteolin	-6.98	4	3
3		Lupeol	-9.40	2	8
4		Betulin	-8.95	2	5
5	Sterols	β sitosterol	-9.06	1	7
6		Stigmasterol	-9.46	0	7
7	Flavonoids	Apigenin	-7.05	2	6
8		Quercetin	-6.85	3	5



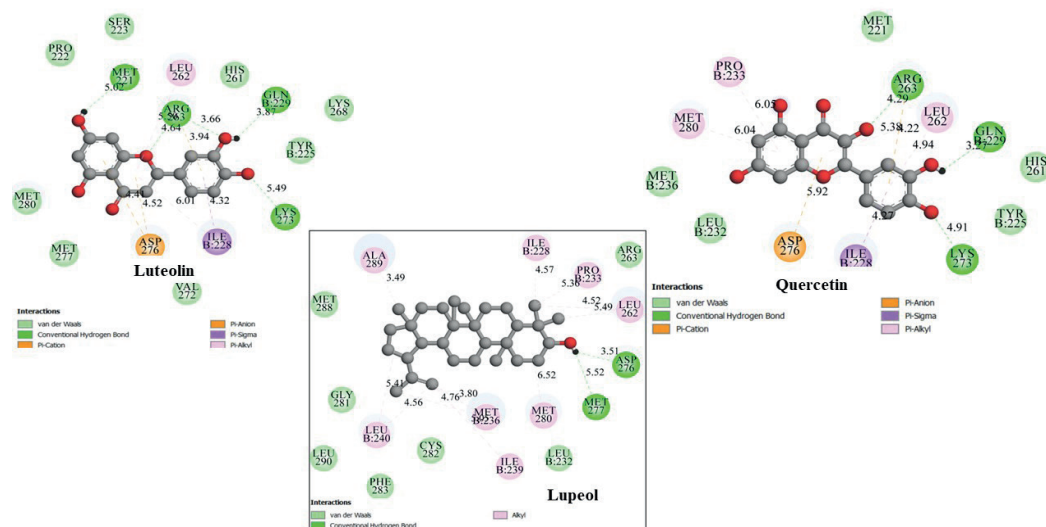


FIGURE 14 - 2D interaction of phytoconstituents in binding pocket of 6x3x protein.

and Ile239. Met221, Arg536, Gln229, and Lys273 interact with Luteolin via H-bonds. Ile228 forms a pi-sigma stacking connection with them. Arg263, Lys273, and Gln229 formed three hydrogen connections with compound quercetin, as well as a pi-cation contact with Asp276. The other significant interactions were pi-alkyl residues Pro233, Met280, and Leu262 (Figure 14).

DISCUSSION

The current research on *Hygrophila auriculata* plant identified the presence of alkaloids, flavonoids, phytosterols, terpenoids, tannins, saponins, and phenolic compounds in the hydroalcoholic extract and ethyl acetate subextract of the leaf. The Unsaponifiable fraction of the seed contains Phytosterols and Terpenoids. The *in-vivo* sedative-hypnotic activity was evaluated through the Hole cross test, Hole board test, open field test, and the thiopental-induced sleep duration test. The activities of the mice were influenced by the administration of either the drug or extract. The animals exhibited high levels of activity prior to treatment; however, their activity decreased, indicating sedation, after receiving either the extract or diazepam. The hypnotized mice exhibited no movement apart

from the contraction of abdominal muscles, suggesting respiratory activity. The mice did not return to their highly active state by the conclusion of the experiment. Additionally, the binding affinities of phytoconstituents were assessed and compared to the standard drug, diazepam, using docking studies. All compounds interact efficiently with amino acids within a range of 5 Å. The alpha1-beta2-gamma2 receptor subtype, in conjunction with GABA and diazepam, was chosen for the docking study. The highest docking scores recorded were -9.46 Kcal/mol for stigmasterol and -9.40 Kcal/mol for lupeol. These results imply that the plant is a viable option for further pharmacological research.

CONCLUSION

The current investigation found that *Hygrophila auriculata* leaves and seeds are effective sources of sedative and hypnotic action. GC-MS analysis of an unsaponifiable fraction of seeds reveals the presence of many phytochemicals, including beta-stigmasterol, lupeol, gamma-sitosterol, betulin aldehyde, and many others. The docking study focused on the receptor alpha1-beta2-gamma2 subtype in conjunction with GABA and diazepam. Lupeol and stigmasterol had the

highest docking scores of -9.40 and -9.46 Kcal/mol, respectively. The oral administration of ethyl acetate subextract and unsaponifiable fraction resulted in a considerable reduction in the number of holes travelled, with a comparable reaction in the open field test. In Hole board test, All the extracts show the number of head insertions fell statistically comparable to the control group. In the thiopental-induced sleeping test, the hydroalcoholic extract, ethyl acetate subextract, and unsaponifiable fraction all improved sleep time when compared to the control group. The drug was deemed safe up to a dosage of 400 mg/kg for leaf and 200mg/kg for seed. These findings indicate that the plant is a viable option for further pharmacological investigation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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