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Research Article

Sedative Activity of Ethanolic Extract of Enhydra fluctuans Lour in Hole-Board and Rota-Rod Models in Mice

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Abstract

Objectives: Prolonged benzodiazepine use is limited by tolerance, dependence and withdrawal effects, renewing interest in medicinal plants as safer sedative options. *Enhydra fluctuans* Lour (Asteraceae), a semi-aquatic vegetable traditionally used across North-East India for nervous system complaints, has not previously been assessed for sedative activity in validated behavioural models. This study assessed the sedative activity of the ethanolic extract of the whole plant of *E. fluctuans* Lour (EEEFL) in albino mice using the hole-board and rota-rod tests.

Materials and Methods: EEEFL was prepared by percolation with 90% ethanol; acute oral toxicity was assessed by OECD guideline 425, and three doses (100, 200 and 400 mg/kg, p.o.) were selected. In the hole-board test, mice (n=5/group) received EEEFL, diazepam (3 mg/kg, i.p.) or vehicle, and head-pokes (number and duration) were recorded over 5 minutes. In the rota-rod test, mice (n=5/group) received EEEFL, diazepam (4 mg/kg, i.p.) or vehicle, with fall-off time from a rotating rod (25 rpm) recorded before and after treatment. Data were analysed by one-way ANOVA with Bonferroni's test.

Results: EEEFL produced a significant, dose-related decrease in both the number and duration of head-pokes in the hole-board test ($p<0.05$ vs control at all doses). In the rota-rod test, EEEFL dose-dependently shortened fall-off time, by 25%, 44% and 56% at 100, 200 and 400 mg/kg, versus an 87% decrease with diazepam.

Conclusion: The ethanolic extract of *Enhydra fluctuans* Lour shows dose-dependent sedative activity, reflected in reduced exploratory behaviour and impaired motor coordination, supporting its traditional use and further evaluation as a candidate sedative phytomedicine.

Keywords: Enhydra fluctuans; Hypnotics and Sedatives; Diazepam; Motor Activity; Plant Extracts.
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Introduction

Sleep disorders and states of pathological central nervous system overactivity are among the most frequent presentations in clinical practice, with insomnia alone thought to affect a large proportion of adults at some point in their lives.[1] Benzodiazepines such as diazepam remain the most commonly prescribed sedative-hypnotics owing to their rapid onset of action and wide safety margin, acting via an allosteric site on the GABA*A* receptor-chloride channel complex to enhance GABAergic inhibitory transmission.[2,3] Prolonged benzodiazepine use, however, is constrained by tolerance, psychological and physical dependence, rebound insomnia on discontinuation, and residual daytime sedation with impaired psychomotor function, which has kept alive interest in medicinal plants as potentially safer alternatives or adjuncts for managing sleep and anxiety-related disorders.[1,4]

A comprehensive review of hypnotic herbal medicines by Ghasemzadeh Rahbardar and Hosseinzadeh (2024)[1] catalogued numerous plants, including *Valeriana officinalis*, *Crocus sativus*, *Rosa damascena* and *Melissa officinalis*, which improve sleep latency, duration and quality mainly by acting on the GABAergic and serotonergic systems of the central nervous system. The authors noted that, notwithstanding this encouraging evidence, rigorous mechanistic and dose-ranging studies of many traditionally used sedative plants are still lacking. Corroborating evidence from binding and behavioural studies on other Asian medicinal plants has likewise linked sedative-hypnotic activity to modulation of the GABA*A*-benzodiazepine receptor complex and, in certain cases, to melatonin-synthesis pathways. GABAergic potentiation and impaired psychomotor performance remain the main,

complementary endpoints used to screen candidate sedative agents.[2,3]

Behavioural assays such as the hole-board test, which measures exploratory head-dipping as a marker of central nervous system depression, and the rota-rod test, which measures loss of motor coordination as a marker of muscle relaxation and sedation, remain among the simplest and most widely used first-line screens for sedative activity in rodents, and are still applied alongside newer mechanistic studies of plant extracts when characterising candidate sedative-hypnotic agents.[2,3]

Medicinal plants have long been an important source of sedative and anticonvulsant lead compounds, and numerous plant extracts have demonstrated combined sedative and anticonvulsant effects in rodent models, among them decoctions of *Passiflora edulis* leaves, *Bridelia micrantha* and *Croton macrostachyus*, *Erythrophleum ivorense* stem bark, *Securidaca longepedunculata* root, and *Spondias mombin*, illustrating the recurring overlap of sedative and anticonvulsant activity within these phytochemical classes.[5-9]

Enhydra fluctuans Lour (family Asteraceae), called marsh herb or water cress in English and known locally as Helechi (Assamese) or Helencha (Bengali), is an edible semi-aquatic herb that grows abundantly in ditches, fish ponds and rice fields across North-East India, Bangladesh, Malaysia and other areas of South and South-East Asia.[10] Traditional applications include use as an analgesic, antipyretic, antioxidant, anti-inflammatory, antimicrobial, hypotensive, hepatoprotective and anti-diarrhoeal agent, with the leaf juice taken orally for nervous system and liver disorders.[10,11] Recent ethnobotanical surveys from North-East India, including a systematic review of the Barak Valley of Assam and a wider review of aquatic and semi-aquatic medicinal plants, continue to record the regional use of *E. fluctuans* for gastrointestinal, hepatic and nervous system complaints, respectively.[12,13]

Phytochemical investigations of different solvent extracts of *E. fluctuans* have repeatedly identified flavonoids, saponins, tannins and terpenoids, and a recent survey of medicinal weeds from the lower Gangetic delta has additionally drawn attention to the antioxidant potential of this and related species.[14-17] Flavonoids, saponins and tannins have each been independently linked to sedative and anxiolytic effects, largely via interaction with the benzodiazepine site of the GABA*A* receptor complex, or through non-specific central nervous system depression, giving *E. fluctuans* a phytochemical basis on which sedative activity might reasonably be anticipated.[1,4]

Despite this pharmacological rationale, its continued traditional use, and the anticonvulsant activity of the ethanolic extract of this plant that our group demonstrated in a companion study (unpublished data) using the maximal electroshock seizure and pentylenetetrazole models, the sedative potential of *E. fluctuans* has not, to our knowledge, previously been examined using validated behavioural screens. This study was accordingly undertaken to evaluate the sedative activity of the ethanolic extract of the whole plant of *E. fluctuans* Lour (EEEFL) in albino mice, using the hole-board and rota-rod tests.

Aims and Objectives: To determine the sedative activity of the ethanolic extract of the whole plant of *Enhydra fluctuans* Lour in albino mice using the rota-rod and hole-board tests, with diazepam employed as the reference standard, and to examine whether any effect observed is dose-dependent at 100, 200 and 400 mg/kg.

Materials and Methods

Plant Material and Authentication: Mature specimens of *Enhydra fluctuans* Lour were gathered from Dibrugarh, Assam, between December and January, and identified by a taxonomist at the Department of Life Sciences, Dibrugarh University, Assam (voucher specimen no. DULSc 459).

Preparation of the Ethanolic Extract: The harvested plant material was cleaned, dried in shade, and ground to powder in an electric grinder. This powder was moistened with 90% ethanol, packed into a percolator, allowed to macerate for 24 hours, and percolated following the previously described standard method[18]; the marc was then pressed, and the pooled percolate was concentrated to yield the crude ethanolic extract (EEEFL), which was stored in an airtight container at room temperature until required.

Preliminary Phytochemical Screening: EEEFL was subjected to qualitative phytochemical testing for alkaloids, flavonoids, glycosides, saponins, tannins, sesquiterpenes and triterpenes, following standard chemical procedures described previously.

Experimental Animals: Healthy albino mice (Mus musculus) of either sex, weighing 20–35 g, were obtained from a CPCSEA-registered breeder and allowed to acclimatise in the Central Animal House under standard conditions of temperature, a natural light–dark cycle, and a standard pelleted diet with free access to water. The Institutional Animal Ethics Committee approved the study protocol, which was conducted in accordance with CPCSEA guidelines governing the care and use of laboratory animals.

Acute Oral Toxicity Study: The acute oral toxicity of EEEFL was assessed using the limit-test

procedure of OECD guideline 425[20] at a limit dose of 2000 mg/kg body weight in albino mice, with sequential dosing and a 14-day observation period for mortality and signs of toxicity. As no mortality or overt toxicity occurred up to 2000 mg/kg, three sub-toxic doses (100, 200 and 400 mg/kg) were chosen for the subsequent sedative activity experiments.

Drugs and Dose Preparation: Diazepam served as the reference standard, at 3 mg/kg for the hole-board test and 4 mg/kg for the rota-rod test; it was prepared as a suspension in 1% w/v gum acacia and given intraperitoneally. EEEFL, at 100, 200 and 400 mg/kg, was suspended in normal saline and given orally, while control animals received normal saline (10 mL/kg, p.o.).

Hole-board Test: Sedative activity was assessed indirectly in this test by scoring head-dipping, an index of the animals' willingness to explore a novel environment. The apparatus comprised a wooden box (40 × 40 × 25 cm) fitted with sixteen evenly spaced holes (3 cm diameter) in the floor, raised to a height of 25 cm. Mice were given EEEFL or vehicle orally, or diazepam (3 mg/kg) intraperitoneally, and placed individually at the centre of the board 30 minutes (intraperitoneal) or 1 hour (oral) later. The number of head-pokes and the total duration of head-dipping were recorded over a 5-minute observation period, with a decrease in either measure taken as evidence of sedative effect.

Group	Number (n)	Treatment
A (Control)	5	Normal saline 10 mL/kg, p.o.
B (Test 1)	5	EEEFL 100 mg/kg, p.o.
C (Test 2)	5	EEEFL 200 mg/kg, p.o.
D (Test 3)	5	EEEFL 400 mg/kg, p.o.
E (Standard)	5	Diazepam 3 mg/kg, i.p.

Rota-rod Test: Motor coordination on the rotating rod served here as the muscle-relaxant, sedation-related endpoint. Mice were first trained to stay on the rotating rod (25 rpm) for at least 3–5 minutes, and only those meeting this criterion were included. Baseline fall-off time was recorded before mice received EEEFL or vehicle orally, or diazepam (4 mg/kg) intraperitoneally. Fall-off time was recorded again 30 minutes (intraperitoneal) or 1 hour (oral) later, over a 5-minute observation period, and the percentage decrease relative to the pre-treatment value was calculated for each animal.

Statistical Analysis: Data are presented as mean ± standard error of the mean (SEM) for groups of five animals. Between-group differences were tested by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test; the pre- and post-treatment fall-off times within each group were further compared using a paired t-test. All analyses were carried out with GraphPad Prism software, and $p<0.05$ was taken to indicate statistical significance.

Results

Yield and Phytochemical Screening: Percolating 425 g of shade-dried, powdered plant material with 90% ethanol gave 11.997 g of dry extract (a yield of approximately 2.8% w/w). Preliminary phytochemical analysis of EEEFL indicated the presence of flavonoids, sesquiterpenes, tannins, saponins and triterpenes, whereas alkaloids and glycosides were not detected (Table 1).

Group	Number (n)	Treatment
A (Control)	5	Normal saline 10 mL/kg, p.o.
B (Test 1)	5	EEEFL 100 mg/kg, p.o.
C (Test 2)	5	EEEFL 200 mg/kg, p.o.
D (Test 3)	5	EEEFL 400 mg/kg, p.o.
E (Standard)	5	Diazepam 4 mg/kg, i.p.

Table 1: Preliminary phytochemical screening of the ethanolic extract of *Enhydra fluctuans* Lour (EEEFL)

Phytochemical constituent	EEEFL
Alkaloids	Absent
Flavonoids	Present
Sesquiterpenes	Present
Tannins	Present
Saponins	Present
Glycosides	Absent
Triterpenes	Present

Effect of EEEFL on exploratory behaviour in the hole-board test

EEEFL brought about a significant, dose-related reduction in the number of head-pokes relative to control (36.20 ± 0.58), decreasing to 23.40 ± 0.98, 20.00 ± 0.71 and 14.00 ± 0.89 at 100, 200 and 400 mg/kg respectively ($p<0.05$ vs control at every dose), with a further significant reduction at 400 mg/kg relative to the 100 and 200 mg/kg groups. A corresponding, significant, dose-dependent decrease was observed in the duration of head-poking, from 36.60 ± 0.68 seconds in control to 24.60 ± 0.77, 21.80 ± 0.66 and 15.00 ± 0.71 seconds at the respective doses. Diazepam (3 mg/kg) produced the largest reduction in both measures, significantly exceeding that seen with any dose of EEEFL (Table 2).

Table 2: Effect of EEEFL on exploratory head-poking behaviour in the hole-board test in albino mice (mean ± SEM)

Group / Treatment	Number (n)	Pre-treatment	Number of head pokes (mean ± SEM)	Duration of head pokes, seconds (mean ± SEM)
Group A – Control	5	Normal saline 10 mL/kg, p.o.	36.20 ± 0.58	36.60 ± 0.68
Group B – EEEFL 100 mg/kg, p.o.	5	EEEFL 100 mg/kg	23.40 ± 0.98 [a]	24.60 ± 0.77 [a]
Group C – EEEFL 200 mg/kg, p.o.	5	EEEFL 200 mg/kg	20.00 ± 0.71 [a]	21.80 ± 0.66 [a]
Group D – EEEFL 400 mg/kg, p.o.	5	EEEFL 400 mg/kg	14.00 ± 0.89 [a,b,c]	15.00 ± 0.71 [a,b,c]
Group E – Diazepam 3 mg/kg, i.p.	5	Diazepam 3 mg/kg	9.80 ± 0.73 [a,d]	9.00 ± 0.71 [a,d]
One-way ANOVA (F, df, p)			F=163.9, df=4,20, p<0.05	F=231.0, df=4,20, p<0.05

One-way ANOVA followed by Bonferroni's multiple comparison test. [a]$p<0.05$ vs Group A (control); [b]$p<0.05$ vs Group B; [c]$p<0.05$ vs Group C; [d]$p<0.05$ vs Groups B, C and D.

Effect of EEEFL on motor coordination in the rota-rod test.

Baseline fall-off times were similar across all five groups before treatment ($F=0.39$, $p>0.05$), confirming that pre-training was adequate. Following treatment, EEEFL produced a significant, dose-dependent reduction in fall-off time at all three doses relative to control ($p<0.05$), decreasing from a pre-treatment value of 40.80 ± 1.24 s to 30.20 ± 0.86 s at 100 mg/kg (25% decrease), from 40.20 ± 1.16 s to 22.60 ± 0.68 s at 200 mg/kg (44% decrease),

and from 41.20 ± 1.66 s to 18.00 ± 0.71 s at 400 mg/kg (56% decrease). Diazepam (4 mg/kg) produced the largest reduction, from 39.20 ± 1.36 s to 4.80 ± 0.66 s (87% decrease). The reduction at 400 mg/kg did not differ significantly from that at 200 mg/kg, although both doses differed significantly from the 100 mg/kg group and from control. The pre- to post-treatment reduction in fall-off time was statistically significant within each treated group (paired t-test, $p<0.05$), while control animals showed no change (Table 3).

Table 3: Effect of EEEFL on fall-off time in the rota-rod test in albino mice (mean ± SEM)

Group / Treatment	Number (n)	Fall-off time before drug (s)	Fall-off time after drug (s)	% decrease in fall-off time
Group A – Control (normal saline 10 mL/kg, p.o.)	5	41.60 ± 1.97	41.60 ± 1.97	N/A
Group B – EEEFL 100 mg/kg, p.o.	5	40.80 ± 1.24	30.20 ± 0.86 [a,e]	25
Group C – EEEFL 200 mg/kg, p.o.	5	40.20 ± 1.16	22.60 ± 0.68 [a,b,e]	44
Group D – EEEFL 400 mg/kg, p.o.	5	41.20 ± 1.66	18.00 ± 0.71 [a,b,e]	56
Group E – Diazepam 4 mg/kg, i.p.	5	39.20 ± 1.36	4.80 ± 0.66 [a,d,e]	87
One-way ANOVA (F, df, p)		F=0.39, df=4,20, p>0.05	F=156.9, df=4,20, p<0.05	N/A

One-way ANOVA followed by Bonferroni's multiple comparison test (post-treatment values); paired t-test for pre- vs post-treatment comparison within groups. [a]$p<0.05$ vs Group A (control); [b]$p<0.05$ vs Group B; [d]$p<0.05$ vs Groups B, C and D; [e]$p<0.05$, paired t-test, pre- vs post-treatment within group.

All animals stayed healthy throughout the experiments, with no mortality recorded. Overall, EEEFL showed significant, dose-dependent sedative activity in both the hole-board and rota-rod tests, with the greatest effect consistently seen at 400 mg/kg, although diazepam remained more potent than the extract at the doses examined.

Discussion

As noted above, the GABA*A*-mediated potentiation underlying benzodiazepine action reduces spontaneous activity, dampens excitement, and lowers exploratory behaviour.[2,3] The hole-board test capitalises on this last property: head-dipping reflects an animal's willingness to explore an unfamiliar environment, and a decrease in the number and duration of head-pokes is read as a depressant, sedative-like effect.[21] In this study, EEEFL brought about a dose-related fall in both measures, following the same direction as diazepam though at substantially lower potency, a pattern consistent with genuine central nervous system depressant activity rather than a non-specific reduction in overall motor output.

The rota-rod test offers a complementary, motor-coordination-based measure of sedation, since benzodiazepines are well known to impair performance on this test through muscle relaxation and psychomotor slowing.[21] EEEFL significantly shortened fall-off time on the rotating rod in a dose-dependent manner without altering baseline performance, confirming that the effect reflected drug action rather than inherent variability between groups. The agreement between these two mechanistically distinct behavioural measures, reduced exploration and impaired motor coordination, supports a sedative, CNS-depressant profile for EEEFL, although confirmation at the receptor level was beyond the scope of this study.

The same review by Ghasemzadeh Rahbardar and Hosseinzadeh[1] (2024) concluded that most clinically promising sedative plant extracts act mainly via potentiation of GABAergic transmission and, to a lesser degree, serotonergic modulation, and stressed the ongoing need for such extracts to be tested in more than one behavioural paradigm before mechanistic conclusions are drawn. The present two-model design (hole-board and rota-rod) meets this recommendation directly, and the pattern observed for EEEFL, namely parallel, dose-dependent depression of exploratory and motor measures, closely resembles that described for other recently studied plant sedatives acting through GABAergic mechanisms, including water extracts of Coptidis Rhizoma and related traditional medicinal plants assessed using pentobarbital-sleep and GABA*A*-benzodiazepine receptor-binding assays.[3] Herbal remedies that act on the GABAergic system have likewise been proposed to affect sleep architecture through comparable receptor mechanisms.[4]

Preliminary phytochemical screening identified flavonoids, saponins, tannins, sesquiterpenes and triterpenes in EEEFL. Flavonoids are known to bind the benzodiazepine site of the GABA*A* receptor complex, while saponins and terpenoids have been shown to possess sedative and anxiolytic properties in several plant extracts, and tannins may add further, non-specific central nervous system depression.[1,4] Similar links between phytochemistry and sedative activity have been proposed for other plant extracts tested in comparable models: Hossain et al. (2016) described sedative activity in the methanolic extract of *Sterculia villosa* leaves, Bhadoriya et al.[23] (2009) in essential oil and methanolic extract of *Zanthoxylum budrunga* fruits, Akindele and Adeyemi[24] (2010) in aqueous leaf extract of *Byrsocarpus coccineus*, and Jiang et al. (2007) in flavonoid- and saponin-rich fractions of Semen *Ziziphus jujube*. Given this pattern, it seems plausible that the sedative activity of EEEFL observed here derives, at least partly, from this shared phytochemical composition, though the present data do not permit identification of the specific constituent(s) responsible.

Ethnobotanical surveys from North-East India continue to record the traditional use of *E. fluctuans* for nervous system complaints, and the present findings, taken together with the anticonvulsant activity of the same extract reported in our companion study (unpublished data) using the maximal electroshock seizure and pentylenetetrazole models, offer converging experimental support for this traditional use. This is consistent with the recurring observation that sedative and anticonvulsant activities frequently co-occur within the same plant extracts, as documented for a range of other species, including *Erythrophleum ivorense*, *Securidaca longepedunculata* and *Spondias mombin*.

This study has several limitations. The extract tested was a crude ethanolic preparation, so the specific phytochemical constituent(s) responsible for the observed sedative activity remain unidentified; bioassay-guided fractionation and isolation work is needed. Only acute, single-dose effects at a single post-dosing time-point were examined; the duration of action, the potential for tolerance with repeated dosing, and possible anxiolytic, muscle-relaxant, hypnotic (pentobarbital-potentiation) or withdrawal effects were not assessed and merit dedicated future investigation. Formal receptor-binding assays (e.g., GABAA-benzodiazepine binding) fell outside the scope of the present work and would be needed to confirm the proposed mechanism.

Conclusion

The ethanolic extract of the whole plant of *Enhydra fluctuans* Lour produced dose-related sedation across both behavioural screens in albino mice, evident as reduced exploratory head-poking and impaired motor coordination, with the strongest effect at 400 mg/kg, although it remained less potent than diazepam. These findings offer

experimental support for the traditional use of this plant specifically for sedative or calming indications and, combined with its demonstrated anticonvulsant activity, justify bioassay-guided fractionation to isolate the active constituent(s) and further clarify the precise mechanism underlying its central nervous system depressant activity.

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Conflicts of Interest: There are no conflicts of interest.

Ethical Approval: The study was approved by the Institutional Animal Ethics Committee, Assam Medical College, Dibrugarh (approval no. IAEC/AMC/03, dated 29 November 2016), and was conducted in accordance with CPCSEA guidelines.

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