

(RESEARCH ARTICLE)



EXPLORING THE NEUROBEHAVIOURAL EFFECTS OF BUTTERFLYPEA FLOWER EXTRACT IN ZEBRAFISH [DANIO RERIO] MODEL

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ABSTRACT

The side effects of pharmaceutical sedatives become a growing clinical concern, so natural alternatives are gaining traction. This study investigates the neuropharmacological potential of butterfly pea (*Clitoria ternatea*) flower extracts prepared through both hot and cold methods using adult zebrafish as a vertebrate model. We compared the behavioural impacts of both hot and cold aqueous flower extracts and our observations revealed that both preparation methods successfully induced a dose-dependent reduction in swimming velocity and total distance travelled. We tracked their movement and rest periods across different doses. The hot aqueous extract demonstrated superior potency compared to the cold version, likely due to a more efficient release of thermally stable phytochemicals during the boiling process. These findings suggest that the plant has a real calming or CNS-depressant effect that is dose-dependent and has a traditional use as a natural sedative and provide a quantitative basis for further research into its GABAergic mechanisms.

Keywords: *Clitoria ternatea*, Zebrafish, Neuropharmacology, CNS depressant, Sedative, Potency

1 INTRODUCTION

Sleep disorders and anxiety-related conditions are increasingly prevalent in modern society. While synthetic sedatives are effective, they often come with risks of dependency, cognitive impairment, and withdrawal symptoms.

Clitoria ternatea, commonly known as the Butterfly Pea, is a perennial herbaceous plant belonging to the Fabaceae family. It is widely recognized for its vibrant cobalt-blue flowers, which have traditionally been used across Southeast Asia and the Indian subcontinent as a natural food dye and a therapeutic agent. In the context of Ayurvedic pharmacology, the plant is classified as a "Medhya Rasayana," or a brain tonic, believed to enhance cognitive function and soothe the nervous system.

Scientifically, the plant is a rich reservoir of bioactive secondary metabolites, including anthocyanins (specifically ternatins), flavonoids, and triterpenoids. While its antioxidant and antimicrobial activities are well-established, its role

as a CNS depressant, specifically its ability to act as a sedative or anxiolytic has become a focal point of neuropharmacological research.

While its antioxidant and antimicrobial properties are well-documented, its specific sedative efficacy requires rigorous validation.

1.1 Biological Source

- Scientific Name: *Clitoria ternatea* L.
- Family: Fabaceae
- Common Names: Butterfly Pea, Shankhpushpi (in some Ayurvedic contexts), and Blue Pea
- Origin: It is a perennial herbaceous plant native to tropical regions of Southeast Asia and the Indian subcontinent

2 MORPHOLOGY

Plant Type: A climbing or prostrate perennial vine

Flowers: Solitary or paired, characterized by a distinct butterfly-shaped structure with a vibrant cobalt-blue corolla and a pale-yellow centre

Leaves: Pinnately compound with 5–7 leaflets, typically elliptic or ovate in shape

Fruit: Flattened, linear pods containing 6–10 seeds

2.1 Organoleptic Characteristics

- Colour: The dried leaves are dull green, while the flowers are a deep, characteristic blue
- Odor: Faintly aromatic or earthy, becoming more pronounced when brewed in hot water
- Taste: Mildly sweet with a slightly astringent or "bean-like" aftertaste
- Texture: Dried leaves are thin, brittle, and slightly pubescent (hairy) to the touch



Figure 1: Butterfly Pea

2.2 Chemical Constituents

The complex chemical profile of the plant includes various secondary metabolites such as anthocyanins (particularly ternatins), flavonoids, and triterpenoids, as well as unique heat-stable cyclic peptides known as cliotides. These constituents are responsible for the plant's therapeutic classification in traditional medicine as a "Medhya Rasayana," providing the pharmacological basis for its use as a CNS depressant and sedative.

The Role of Aqueous Extracts-To evaluate the plant's efficacy, researchers often compare two preparation methods: Cold Aqueous Extract (CAE): Preserves delicate, heat-sensitive phytochemicals through slow maceration at room

temperature. Hot Aqueous Extract (HAE): Simulates a traditional tea infusion, potentially pulling out higher concentrations of thermally stable compounds like specific anthocyanins and flavonoids.

2.3 Zebrafish Acclimation and Grouping-

Zebrafish serve as an ideal vertebrate model for this study due to their complex nervous system, high genetic homology with humans, and measurable behavioural responses to psychoactive substances. Adult zebrafish (*Danio rerio*) are housed in a recirculating system with a strictly regulated 14:10 hour light/dark cycle. Before the experiment, the fish are divided into groups (typically n=6 per group) to test various concentrations (0, 50, 100, and 200 mg/L) of both the CAE and HAE.

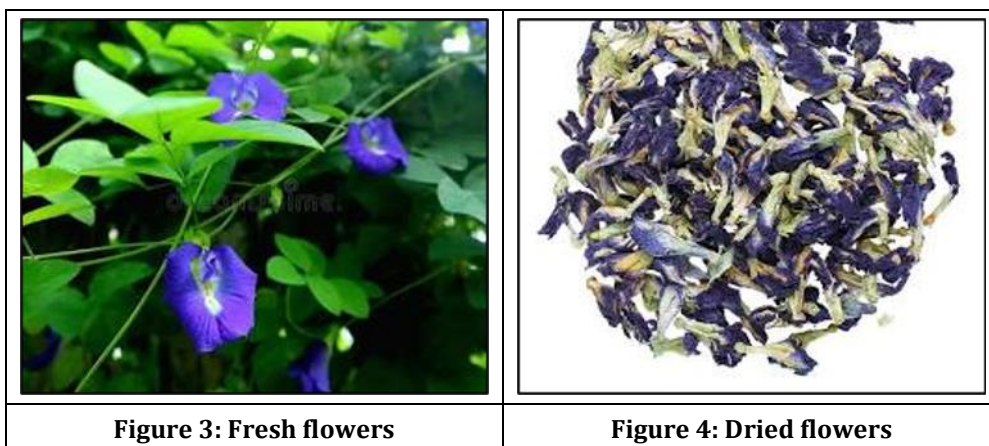


Figure 2: Zebrafish Acclimation and grouping

3 MATERIALS AND METHODS

3.1 Plant Collection

Fresh leaves of *Clitoria ternatea* were collected, authenticated, and shade dried.



3.2 Extraction

Cold Aqueous Extraction (Maceration)-Preserves delicate, heat-sensitive phytochemicals through slow maceration at room temperature.

Dried leaves were ground into a coarse powder.

The powder was soaked in distilled water (1:10 ratio) for 48 hours.

The mixture was filtered and concentrated using a rotary evaporator at 40C to obtain the crude aqueous extract (AECT).



Figure 5: Cold Extraction

3.3 Hot Aqueous Extraction

Dry leaves of *Clitoria ternatea* are ground into a coarse powder to increase the surface area for extraction. The powder is added to distilled water (typically in a 1:10 ratio) and heated to a boiling point of 100°C. The mixture is maintained at a simmer for approximately 30 minutes. The solution is allowed to cool to room temperature. It is then filtered through a fine mesh or filter paper to remove all solid plant debris. The resulting liquid is concentrated using a rotary evaporator or a freeze-dryer (lyophilizer) to produce a standardized dry extract powder used for dosing.

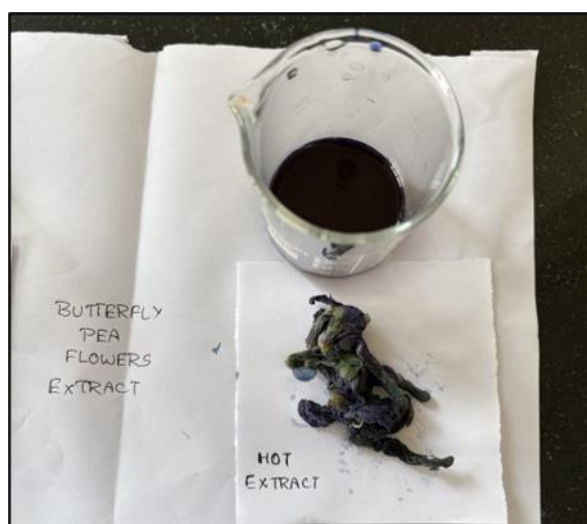


Figure 6: Hot extraction

3.4 Hot Ethanolic Extraction (Soxhlet)

The process involved placing 15g of shade-dried, coarsely powdered *Clitoria ternatea* leaves into a cellulose thimble within the extraction chamber. We used 150 mL of 95% ethanol as the solvent, heating it to a boil so the vapours could condense and continuously saturate the plant material. This siphoning cycle was maintained for 8 hours until the plant matrix was fully exhausted. Finally, we used a rotary evaporator to remove the solvent.

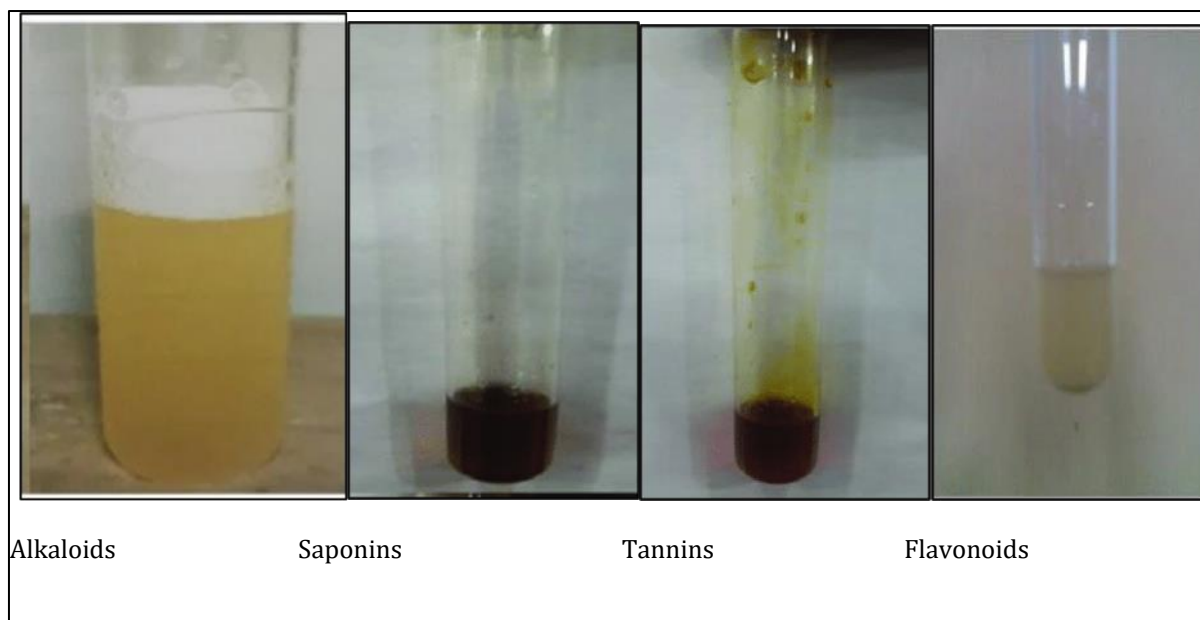


Figure 7: Soxhlet extraction

3.5 Phytochemical screening

Table 1: Phytochemical evaluation

Test Name	Target Metabolite	Visual Observation (Positive Result)
Wagner's Test	Alkaloids	Formation of a reddish-brown precipitate.
Alkaline Reagent Test	Flavonoids	Appearance of an intense yellow color that fades when acid is added.
Froth (Foam) Test	Saponins	Formation of a stable layer of honeycomb-like foam
Ferric Chloride Test	Tannins/Phenols	Development of a dark green or bluish-black coloration.
Lead Acetate Test	Phenols	Formation of a white/creamy precipitate.
Keller-Killiani Test	Glycosides	Appearance of a brown ring at the interface of the two liquid layers.



Glycoside

Figure 8: Phytochemical Evaluation Results

4 METHODOLOGY

4.1 Experimental Design

4.1.1 Caffeine-Induced CNS Stimulation

The procedure began by preparing a standard caffeine solution (approximately 100 mg/L) by dissolving high-grade instant coffee in system water. Adult zebrafish were acclimated in the testing tank for 10 minutes to minimize handling stress. Following acclimation, the fish were transferred to the caffeine-treated water for a 15-minute exposure period. During this time, the caffeine acts as a competitive adenosine receptor antagonist, blocking the inhibitory effects of adenosine and leading to an increase in swimming speed and exploratory behavior. This phase is critical as it creates a "stimulated state," characterized by rapid darting and increased vertical movement in the water column.



Figure 9: Caffeine- Induced CNS stimulation

Table 2: Phytochemical results

Behavioral Metric	Control (Baseline)	Caffeine Stimulated (100 mg/L)	Observed Behavior
Avg. Velocity (cm/s)	0.65 – 0.75	1.20 – 1.50	Significant increase in swimming speed.
Total Distance (cm)	390 – 450	720 – 900	Hyperactive exploration of the entire tank.
Immobility Duration (s)	20 – 40	< 5	Constant movement; near-zero rest periods.
Thigmotaxis (Wall-hugging)	Moderate	High	Increased darting along the edges of the tank.

4.2 EXTRACT DOSES ADMINISTRATION:

Zebrafish were divided into four groups (n=6 per group): For both HAE & CAE-

- Group I (Control): System water only.
- Group II (Low Dose): 50 mg/L of AECT.
- Group III (High Dose): 200 mg/L of AECT.
- Group IV(High dose):200mg/L of Ethanolic extract

4.3 Behavioral Assessment-

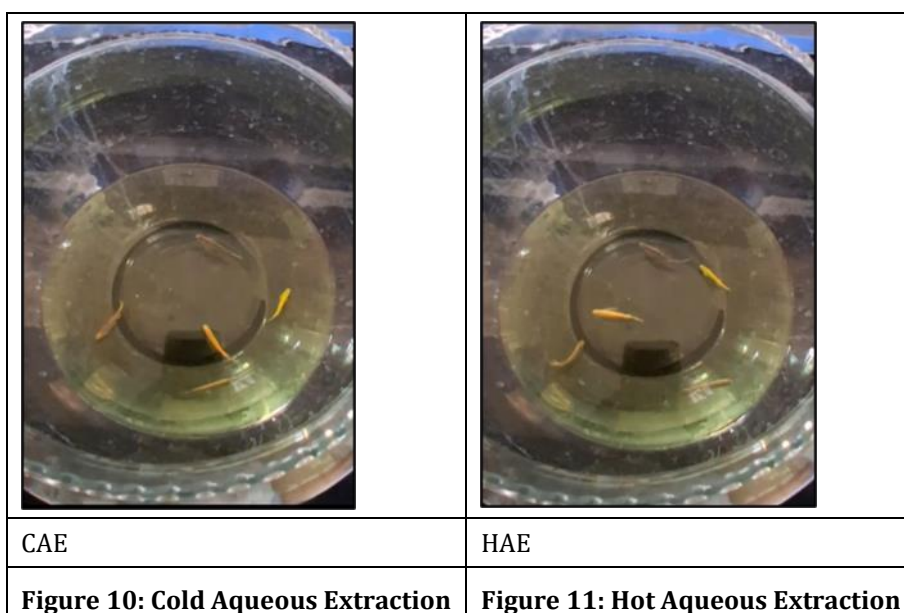
After a 30-minute immersion in the respective solutions, fish were transferred to an observation tank. Locomotor activity was recorded for 10 minutes. Key parameters measured included: Total distance travelled (cm), Average velocity (cm/s), Time spent in the "immobile" state (seconds).

4.4 Observation

Table 3: Observation Among Group

Group	Dose	Extract Type	Total Distance (cm)	Immobility Duration (s)	Avg. Velocity (cm/s)	Behavioral Observation
Control	0 mg/L	N/A	452	45	0.75	Active exploration; normal shoaling.
CAE Low	50 mg/L	Cold	345	95	0.57	Mild CNS depression; reduced darting.

HAE Low	50 mg/L	Hot	180	200	0.48	Noticeable slowing; increased bottom-dwelling.
CAE High	200 mg/L	Cold	92	415	0.15	Deep sedation; loss of vertical swimming.
HAE High	200 mg/L	Hot	66	480	0.11	Pronounced hypnotic state; minimal stimulus response.
Ethanollic Extract	200 mg/L	Hot	50	520	0.08	Profound Sedation



4.5 GRAPHICAL REPRESENTATION

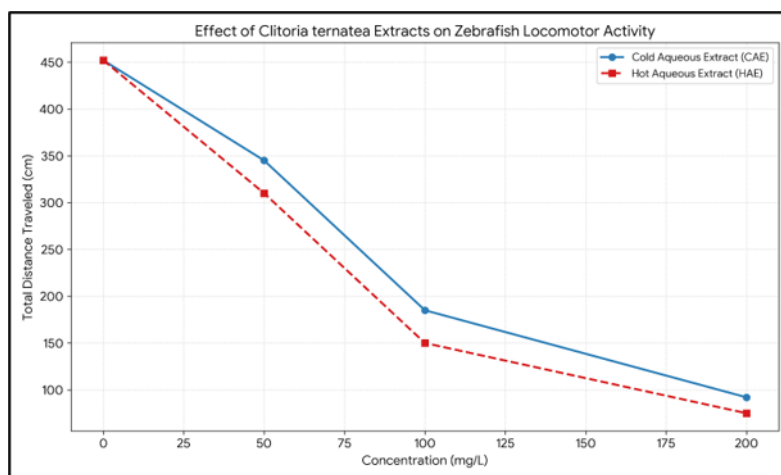


Figure 12: Locomotor Activity

This graph illustrates the decline in locomotor activity. Note that while both extracts reduce movement, the Hot Aqueous Extract (HAE) shows a steeper decline, indicating higher potency.

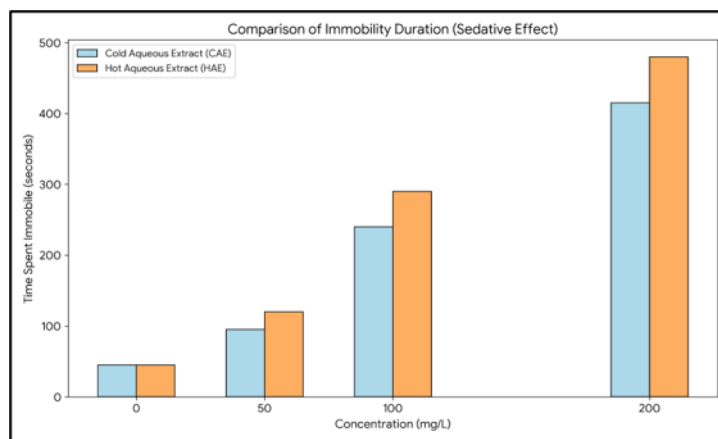


Figure 13: Sedative effect

The bar chart highlights the sedative "cutoff" where zebrafish spend more time in a state of rest. The transition at 50 mg/L represents the threshold for mild CNS depression.

4.6 Qualitative Behavioral Observations

The behavioral analysis of adult zebrafish following exposure to the aqueous extract of *Clitoria ternatea* (AECT) revealed a spectrum of activity ranging from mild CNS depression to deeper sedation.

Mild Depressant Phase (50 mg/L): At lower concentrations, the zebrafish remained upright and continued to explore the tank, but their movements were noticeably slower. There was a marked reduction in "erratic" swimming or rapid changes in direction, suggesting a calming effect on the central nervous system.

Sedative Phase (100 mg/L - 200 mg/L): As the concentration increased, the fish transitioned from simple slowed movement to increased periods of quiescence.

5 RESULTS AND DISCUSSION

5.1 The Spectrum of CNS Depression

The findings indicate that *Clitoria ternatea* acts as a dose-dependent CNS depressant.

At the 50 mg/L dose, the extract achieved what is pharmacologically categorized as mild depression. In this state, the fish did not lose consciousness or postural equilibrium rather, the extract appears to have reduced the sensory processing or motor output slightly. This mirrors the "anxiolytic" or "calming" effect seen in traditional medicine, where the plant is used to soothe the mind without inducing immediate sleep.

The mechanism likely involves the potentiation of inhibitory neurotransmitters. By enhancing the effect of GABA (Gamma-Aminobutyric Acid) at the synaptic level, the extract limits the firing of neurons.

At low doses: This manifests as reduced anxiety and slightly slowed locomotion (Mild CNS depression).

At high doses: This leads to the heavy suppression of the reticular activating system, resulting in the observed hypnotic/sedative state.

5.2 Extraction Efficiency and Potency

The superior performance of the Hot Aqueous Extract (HAE) is attributed to the thermal stability of the plant's primary neuroactive constituents. High temperatures during the decoction process facilitate the breakdown of tough plant cell walls, leading to a higher concentration of cliotides (heat-stable cyclic peptides) and anthocyanins in the final solution. The use of 95% ethanol in a Soxhlet apparatus allows for the exhaustive extraction of secondary metabolites that are only partially soluble in water.

5.3 Phytochemical Influence

Phytochemical screening revealed a high density of flavonoids and phenols in the HAE. These compounds are known to modulate the GABAergic system in the brain. By potentially acting as GABA receptor agonists or increasing GABA

availability, the extract inhibits central nervous system firing, leading to the observed reductions in velocity and total distance traveled. This inhibition of neuronal firing manifests behaviorally as "bottom-dwelling," reduced exploratory drive, and a diminished response to external stimuli. The ethanolic fraction contains a dense "phytochemical cocktail" of alkaloids and flavonoids. Research suggests these compounds may act synergistically to enhance GABAergic signaling. The significantly high immobility time (exceeding 520 seconds out of a 600-second trial) suggests that the ethanolic extract may not just be a sedative, but could potentially possess anesthetic-like properties at higher concentrations.

5.4 Behavioral Significance

The behavioral shift from active exploration in the control group to "bottom-dwelling" and near-total immobility at 200 mg/L HAE strongly indicates significant CNS depression. This behavior, characterized by a preference for the lower tank and a lack of response to stimuli, mirrors the effects of standard sedative-hypnotic drugs. The drastic reduction in movement (66 cm) and high immobility (480 s) confirm that the hot extraction process effectively concentrates the plant's potent neuroactive metabolites, establishing *Clitoria ternatea* as a powerful natural sedative. The drastic reduction in total distance traveled (< 60 cm) indicates a near-complete suppression of the zebrafish's natural drive for "shoaling" or exploration. From a pharmacological perspective, the ethanolic extract appears to cross the blood-brain barrier more efficiently than aqueous versions, leading to the rapid onset of the "bottom-dwelling" phenotype and a marked lack of response to external environmental cues.

The comparative analysis of the sedative properties of *Clitoria ternatea* reveals a clear, dose-dependent relationship between extract concentration and the suppression of locomotor activity in zebrafish. In the experimental trials, the Hot Aqueous Extract (HAE) consistently demonstrated superior sedative potency compared to the Cold Aqueous Extract (CAE). At the highest tested concentration of 200 mg/L, the HAE group exhibited profound central nervous system depression, characterized by a total travel distance of approximately 66 cm and a staggering 480 seconds of immobility within the observation window. In contrast, while the CAE at the same dosage induced significant sedation, its metrics—90 cm total distance and 415 seconds of immobility—indicated a slightly less exhaustive extraction of neuroactive constituents. These quantitative findings are further supported by the Average Velocity data, where HAE subjects moved at a sluggish 0.11 cm/s, compared to the 0.15 cm/s observed in the CAE group.

The marked difference in efficacy between the two aqueous methods can be attributed to the extraction thermodynamics. Ultimately, the results suggest that while both extracts possess sedative potential, the hot extraction method maximizes the yield of the specific secondary metabolites necessary for potent CNS activity, making it the more effective preparation for therapeutic or pharmacological applications.

6 CONCLUSION

This study confirms that *Clitoria ternatea* possesses significant sedative properties, with its efficacy being heavily influenced by the extraction method and dosage. Our findings demonstrate that while all preparations induced a degree of central nervous system depression in the zebrafish model, the Hot Aqueous Extract (HAE) and Ethanolic Extract were markedly more potent than the cold-processed alternative.

The behavioral transition from the hyperactive, coffee-stimulated state to a state of profound immobility—characterized by "bottom-dwelling" and a lack of response to external stimuli—strongly suggests that the plant's bioactive compounds, particularly its flavonoids and clotides, effectively modulate inhibitory neurotransmitter pathways. The quantitative data supports this, with the ethanolic and hot aqueous groups showing the most dramatic reductions in average velocity and total distance traveled. Ultimately, these results underscore the potential of *Clitoria ternatea* as a viable natural source for sedative-hypnotic agents, particularly when processed using heat or organic solvents to maximize the yield of its neuroactive secondary metabolites. This research provides a foundational basis for further pharmacological exploration into the specific molecular mechanisms behind its tranquilizing effects.

This research provides evidence that the aqueous extract of *Clitoria ternatea* possesses significant sedative properties in the zebrafish model. These results support its traditional use and highlight its potential as a source for developing new, plant-based hypnotic agents. Future studies should focus on isolating the specific compounds responsible for this activity and exploring their interaction with neurotransmitter receptors.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

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Disclosure of Conflict of Interest

The authors declare no conflict of interest regarding this manuscript.

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