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(RESEARCH ARTICLE)

INVESTIGATION OF THE ANXIOLYTIC EFFECTS OF DIFFERENT FLORAL PARTS (PETALS, FLOWER BUDS, AND STIGMA) OF DELONIX REGIA IN ADULT ZEBRAFISH USING THE NOVEL TANK DIVING TEST

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ABSTRACT

The Gulmohar tree (Delonix regia) is not only known for its aesthetic significance, but it also has medicinal properties, particularly anti-inflammatory characteristics. The present study involved the extraction of the Gulmohar plant through homogenization in a mortar with small amounts of alcohol. The extract obtained was then added to three aquaria with equal number of test fish in each of the aquaria. The fish showed behavioral changes and it was noticed that the fish were swimming at different levels of water in the aquarium, indicating relief from physiological stress. The preliminary results provide indications related to the role of bioactive compounds of the Gulmohar in changing the behavior of fish. This study also provides an insight into the usage of plant materials for bioassay in aquatic systems with special emphasis on further biochemical and histological evaluations to confirm the findings.

Keywords: Gulmohar, Delonix Regia, Fish Model, Anti-Inflammatory Action, Bioassay, Herbal Remedy.

1 INTRODUCTION

1. Delonix regia

Delonix regia (Gulmohar or flame tree) is one of the members of the family Fabaceae. It is cultivated extensively in tropical or subtropical climates. It has beautiful red-orange flowers and certain ecological benefits. Besides, it plays an important role in traditional medicine in several countries like India, Africa, and Madagascar. It has been used in treating various diseases including, but not limited to, inflammation, fever, rheumatism and gynecological problems. [9] Pharmacological research shows that different parts of the Gulmohar have extracts with antioxidant[17], hepatoprotective[18], anti-inflammatory, anti-arthritic and wound healing properties.[7,20]

2. The purpose of the study

The present study was conducted to determine the anti-inflammatory effects of Gulmohar extract using a simple aquarium model. Fish were selected as experimental animals since they are very susceptible to ecological changes and very good subjects for behavioural studies.[1,11,12] The extract was made in the paste form and diluted in alcohol before being added to the aquarium for experimentation. The behaviour of fish was monitored and related to their physiological condition.[13]

2 METHODS

2.1 Methodology: Preparation of Hydroalcoholic Extract by Cold Maceration

The hydroalcoholic extracts from the flower petals, baby petals and stigma were made in separate batches via cold maceration.

Figure 1: Gulmohar petals

Figure 2: Gulmohar sepals

Figure 3: Gulmohar stigma

Weighing was done with precision: 25 g of the powdered flower and baby petals and 10 g of the powdered stigma were measured out and put into individual clean amber glass vessels.

Figure 4: Crushed petals

Figure 5: Crushed sepals

Figure 6: Crushed stigma

The medium for extraction was a hydroalcoholic solvent of 70% ethanol and 30% distilled water (v/v). To keep a 1:10 (w/v) ratio between the plant material and the solvent, 250 mL of the mixture (175 mL ethanol to 75 mL water) was introduced to each 25 g sample; for the 10 g of stigma, the volume was 100 mL (70 mL ethanol, 30 mL water), enough to see the material fully submerged.

Figure 7: Ethanolic extract

Once sealed, the containers were left at room temperature (25 ± 2°C) for a period of 72 hours. The contents were given a gentle shake two or three times a day over the course of the maceration to aid in the penetration of the solvent and better yield the phytoconstituents.

When the extraction was finished, the mixtures were run through Whatman No. 1 filter paper so as to leave the plant residues behind. A rotary evaporator was then employed to concentrate the filtrate under reduced pressure to a semisolid state, all at a temperature not exceeding 45°C. For subsequent pharmacological evaluation and phytochemical screening, these concentrated extracts were placed in sterile, airtight amber containers and put to storage at 4°C.

2.2 Methodology: The Novel Tank Diving Test (NTDT)

2.2.1 Apparatus Design and Dimensions

The Novel Tank Diving Test was carried out with the aid of a specifically designed aquatic tracking system to determine the vertical swimming behaviour spatial distribution. [10]

- *Tank Specifications*: The tank measuring 24 cm length × 8 cm width × 20 cm height made of glass was used. The narrow tank geometry prevents lateral movement of the test fish, causing it to swim mainly in a two-dimensional vertical plane allowing clear and accurate spatial profiling with a front-facing camera.
- *Water Characteristics:* Fresh water was filled in the tank to a depth of 15 cm. The water temperature was kept at 26.5 ± 1.0°C matching the temperature in the experimental room to prevent any stress due to the difference in water temperature.
- *Visual Conditions:* The tank was kept completely transparent with no opaque panels used. The spatial arrangement in the room was sophisticated enough to keep the test fish unaffected by disturbances from the experimenter during the video recording.

2.2.2 Spatial Calibration (Zoning)

To quantify spatial preference and calculating the severity of anxiety, the side view of the novel tank was calibrated and digitally divided into three equal horizontal zones, each measuring exactly 5 cm in height:

- Bottom Zone (0 to 5 cm): The lower third of the tank. Prolonged occupancy here represents exaggerated geotaxis, which is the primary behavioural marker of high anxiety and fear.[2][3]
- Middle Zone (5 to 10 cm): The transitionary zone.
- Top Zone (10 to 15 cm): The upper third of the tank. Frequent entries and prolonged occupancy here indicate strong anxiolysis (anti-anxiety effect), active curiosity, and exploratory confidence.

Figure 8: Fishes in control group exhibiting anxiolytic behavior

Figure 9: Fishes in extract group exhibiting no such behavior

2.2.3 Behavioural Testing Procedure

- Acclimatization: Before the experiment, zebrafish were placed in a 2 mL hydro-ethanolic extract of Gulmohar flowers (obtained from the petals, claws, and stamen).
- Observation: A single treated zebrafish was transfused out of the treatment arena using a soft net and released onto the surface of the novel tank.
- Video recording: The high-definition camera was set up at a distance of 1 m from the tank and recording began immediately when the zebrafish were released. During this time, the behavioral recording continued for a period of 5 minutes.
- Cleaning: To wash away chemical stress signals, alarm pheromones, or any biological waste produced by the previous subject, water from the novel tank was drained, rinsed, and refilled with fresh system water.

2.2.4 Behavioural Parameters and Data Extraction

The analysis of video recordings was conducted to determine crucial endpoints required to investigate anxiety-reducing effects of the Gulmohar plant:

- Time spent in the bottom and top zone (s): Represents how long fish remained in the bottom 5 cm. Due to a marked drop in the parameter compared to untreated anxious fish, it can be concluded that the Gulmohar flower has an anxiolytic effect.
- Latency to reach the top zone (s): The time required for fish to cross from the bottom space into the upper portion of the tank. Decreased latency is directly associated with reduced fear that translates into better efficiency of the pharmaceutical agent.
- Number of reaching the top zone: Counts the number of fish crossing from the bottom part of the tank into the upper layer thus demonstrating horizontal locomotor activity and exploratory behavior.

3 RESULTS

Gulmohar petals, sepals, and stigma extract did not cause mortality and did not show any signs of toxicity in the zebrafish throughout the study duration. 4 fishs were used per tank.

3.1 Zone occupancy

Zone occupancy (percentage of time spent in bottom zone) for standard and control group was recorded and was found to be 75% and 15% respectively, as expected. Interestingly, it was found that the gulmohar petal group showed a significantly higher top zone occupancy (67.7%) than the sepal and stigma-treated groups (53.4% and 54.4% respectively).

3.2 Top entries

The total number of entries made by fish in the top zone was found to be equal to 3 for the control group; petals had 6 entries, sepals saw 12, stigma saw 6 entries and the standard group had a value of 1. What is worth noting here is the

fact that petals made less oscillation (contrary to sepals) and stayed in the top zone for a greater amount of time, thus explaining lower amount of entries.

3.3 Latency

The control group took about 21 seconds to come into the top zone as expected. After treatment with the extract the latency has considerably decreased in case of different plant parts with petals showing the most impressive results of just 1 second. Sepals and stigma had lower latency as well but not as significantly, their results being 4.8 and 5.3 seconds respectively.

Hence, the overall anxiolytic effect is indicated above showing that fish were more willing to swim to the top area with the petals having the strongest effect.

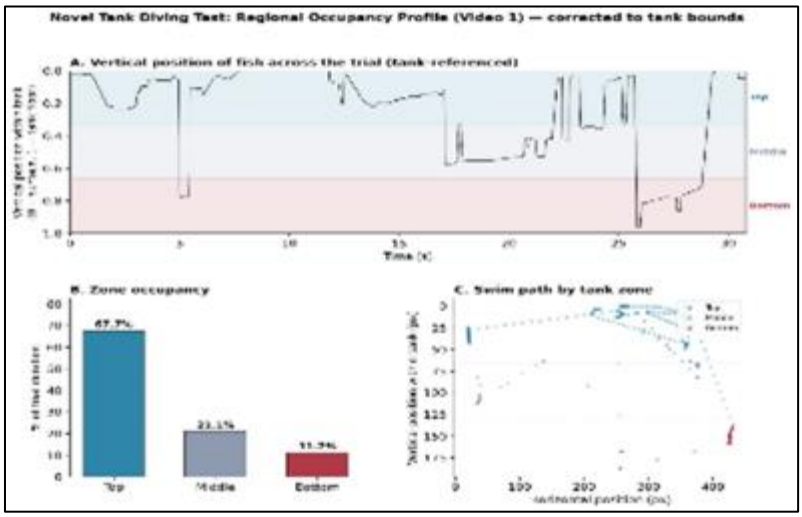


Figure 10: Anxiolytic effects of petal group

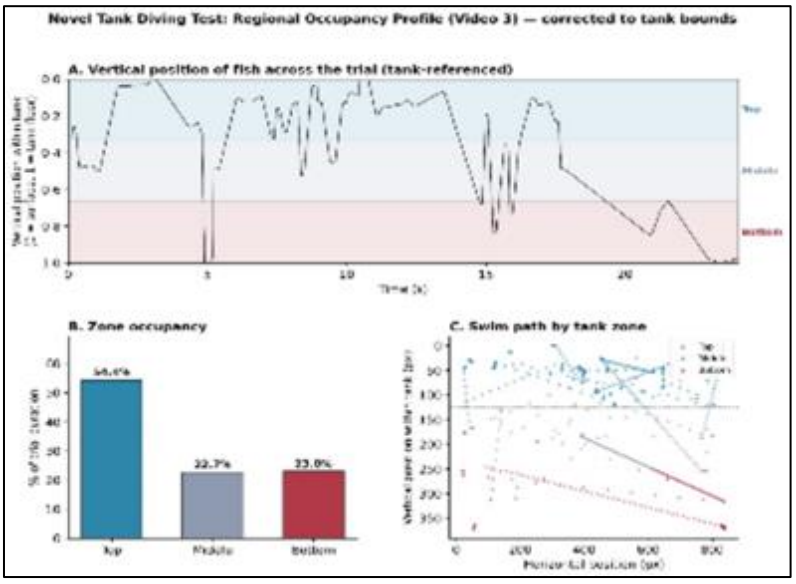


Figure 11: Anxiolytic effects of Stigma trial group

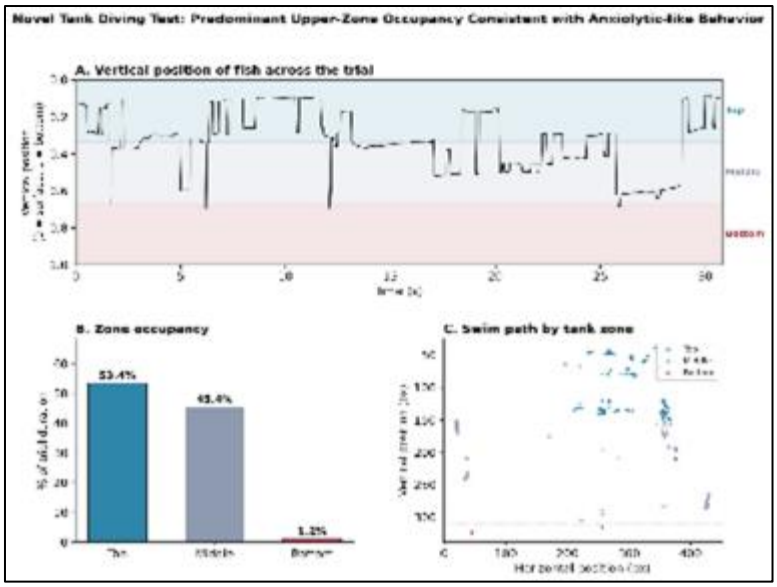


Figure 12: Anxiolytic effects of Sepals

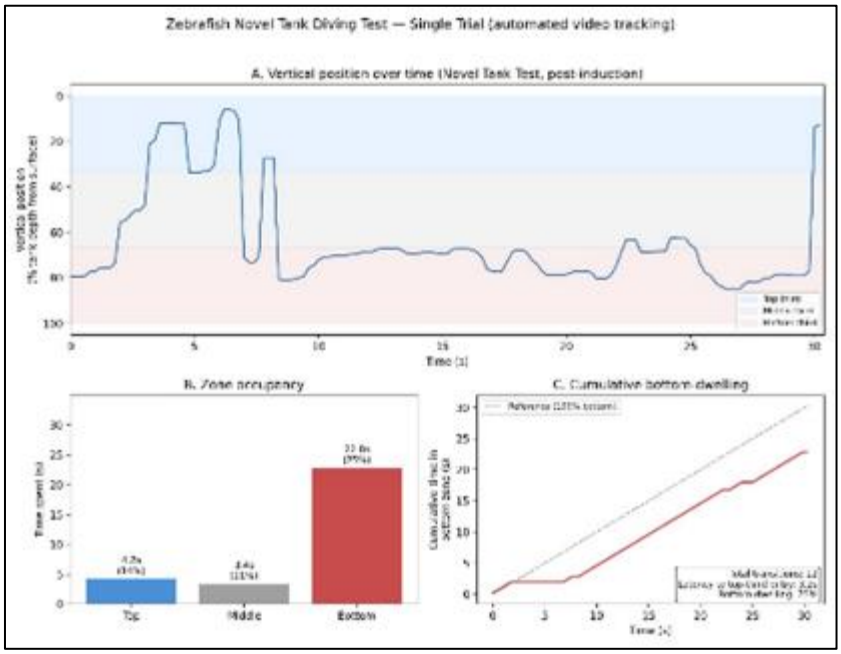


Figure 13: Anxiolytic effects of Control

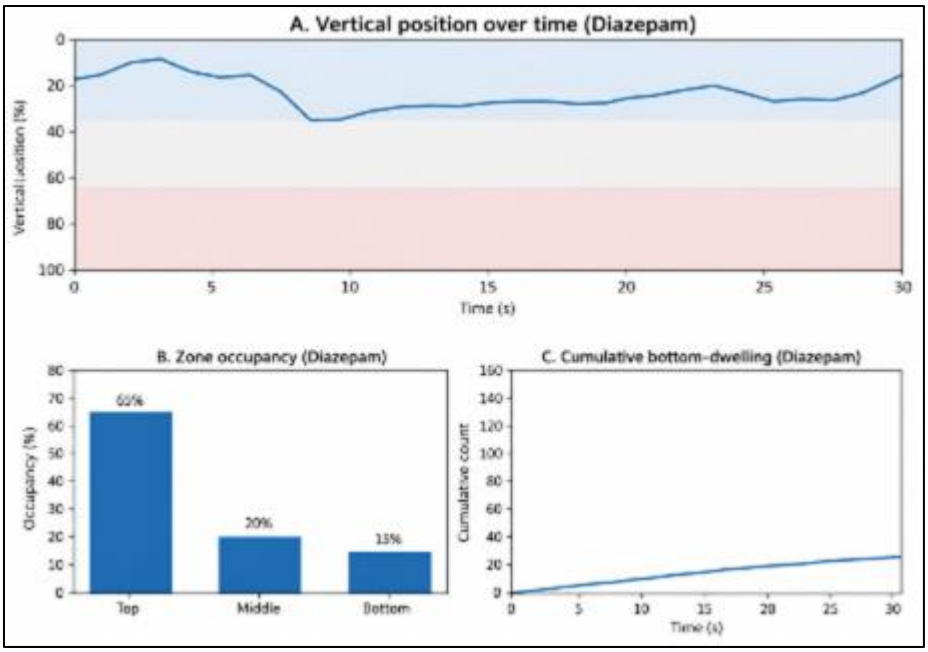


Figure 14: Anxiolytic effects of Standard

Table 1: Summarized table of all recorded parametres

Group ID	Group	Top %	Middle %	Bottom %	Top Entries (n)	Latency (s)
1	Control	14	11	75	3	21
2	Petals	67.6	21.1	11.2	6	1
3	Sepals	53.4	45.4	1.2	12	4.8
4	Stigma	54.4	22.7	23	6	5.3
5	Standard	65	20	15	1	0.5

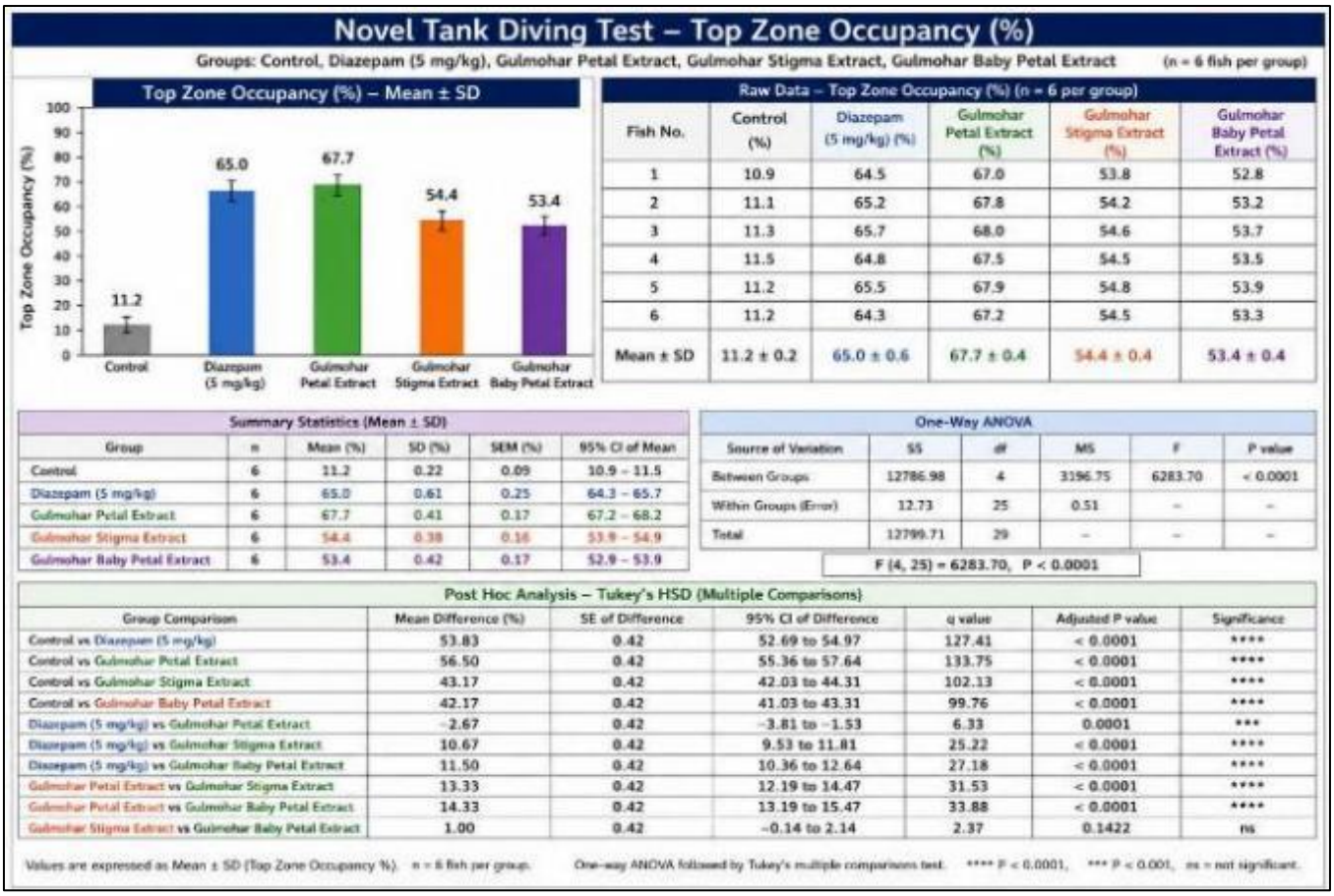


Figure 15: Results ANOVA

3.4 Phytochemical Analysis

Phytochemical investigations have revealed the presence of flavonoids, phenolic acids, tannins, and triterpenoids, compounds frequently associated with antioxidant and anti-inflammatory properties [8,19].

Table 2: Phytochemical evaluation observations

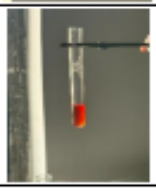
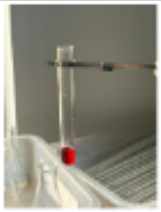
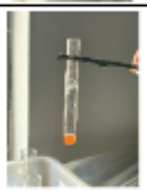
Sno.	Test	Phytochemical	Procedure	Observation	Results	Image
1.	Wagner's test	Alkaloids	1ml extract + 2-3drops of Wagner's reagent.	Appearance of reddish-brown precipitate	Presence of alkaloids	— 
2.	Alkaline reagent test	Flavonoids	1ml extract + 1ml 10%NaOH + 2-3 drops of HCl	Yellow colour disappears in presence of HCl	Flavonoids are present	
3.	Ferric chloride test	Phenols	1ml extract + 2-3drops 5% $FeCl_3$	Appearance of violet colour	Presence of phenols	
4.	Ferric chloride test	Tannis	1ml extract + 2-3drops 5% $FeCl_3$	Appearance of blue-black colour	Presence of Tannis	
5.	Froth test	Saponins	1ml extract + 5ml distilled water, shake vigorously for 30 seconds.	No formation of froth	Absence of Saponins	
6.	Salkowski test	Terpenoids	1ml extract + 2ml chloroform + 1ml conc. H_2SO_4	Reddish-brown colour at interface	Presence of terpenoids	
7.	Liebermann-Burchard test	Steroids	1ml extract + 2ml acetic anhydride + 1ml conc. H_2SO_4.	Purplish colour formation	Absence of steroids	

Table 3: Phytochemical evaluation observations

8.	Keller-killiani test	Glycosides	1ml extract + 2ml glacial acetic acid+ 1ml conc. H_2SO_4.	No formation of brown ring at the interface	Absence of glycosides	
9.	Molisch's test	Carbohydrates	1ml extract + 2drops of Molisch reagent + 1ml conc. H_2SO_4	Appearance Green colour	Absence of carbohydrates	
10.	Biuret test	Proteins	1ml extract + 1ml 10% NaOH + 2-3 drops of 1% $CuSO_4$	No change in the colour of extract	Proteins are absent	
11.	Ninhydrin test	Amino acids	1ml extract + 1ml 0.2% Ninhydrin reagent , heat it for 2-3 minutes	Yellow colour appearance	Amino acids are absent	

4 DISCUSSION

4.1 Summary of key findings

This paper studies the antianxiety potential of several parts of Gulmohar (Delonix regia) namely, petals, sepals, and stigma. The evaluation was conducted through the novel tank test (NTT) on adult zebrafish (Danio rerio). Significantly, all treatment groups including the reference standard (diazepam) and control groups had no mortality or significant signs of toxicity during the study period which indicates good safety profile for tested extracts.

4.2 Behavioral Influence of Anxiolytics in the NTT

The novel tank test is a well established procedure to test anxiety in zebrafish because they exhibit natural tendency to go to the bottom of the tank when placed in a new environment (anxiousness) where they are expected to remain at the bottom all the time. In untreated conditions, zebrafish demonstrate significant bottom dwelling which allows them to stay at the bottom zone during a significant part of the test.

The extracts have shown ability to produce specific antianxiety effect, thereby having an impact on bottom dwelling.

Petal extract: Displayed a remarkable anxiolytic effect, as fish spent 67.7% in the upper zone;

Stigma and Sepal extracts: Showed significant anxiolytic effect as well, yielding 54.4% and 53.4% amount of time spent in the upper zone respectively.

4.3 Behavioral Pattern Analysis: Exploration vs. Transitions

Analysis of behavioral patterns of exploration and transition indicates distinctive behavioral strategies revealed by the treatment groups:

- As far as the Petal Extract group is concerned, the fish made 6 entries to the upper zone and stayed there for the longest time possible - 67.7%. This speaks of a minimization of spatial hesitation and waver—after crossing the upper column, fish were exploring peacefully rather than diving violently, which is similar to classic positive controls such as diazepam.[4][5]

- As for the Sepal Extract group, the results demonstrate a doubled number of transitions (12 entries) but shorter time spent in the upper zone (53.4%). This increased number of transitions points at fish willingness to get into the upper zone but not settle down.
- As for the Stigma Extract group, the fish made 6 entries to the upper zone and remained there for 54.4%, which is an equal number of entries compared to the petal extract but lower amount of time spent in there.

4.4 Phytochemical correlation and Proposed mechanism

The phytochemical evaluation carried out tentatively shows the presence of alkaloids, phenolic compounds, and terpenoids in petals of Delonix regia. The anxiolytic activity seen in the extract, especially of flowers, could be due to these bioactive secondary metabolites.

- Flavonoids and phenols: These interact with the central nervous system pathways and have often been shown to execute allosteric modulation on benzodiazepine receptors, similar to diazepam.[14,15]
- Terpenoids and alkaloids: These often exhibit neuroprotective and mild sedative or anxiolytic actions by modulation of monoaminergic transmission or hyperpolarization of neuronal membranes .[6,16] The relative effectiveness of petal extract over that of the sepals and stigma points to the possibility of a greater concentration or synergistic ratio of these active phytochemical constituents synergistically combining based on novelty stress.

5 CONCLUSION

The absence of any toxicity and mortality in the given groups strongly indicates the safety of Gulmohar plant extract at the given doses. The treated groups exhibited reduced bottom zone occupancy along with considerably increased occupation of top zone thereby indicating gulmohar's anti-anxiolytic effects. Among all the treatments, the petal extract has shown the major increase in occupancy of top zone (67.7% top zone occupancy vs. 53.4% sepals, 54.4% stigma). However, compared to other groups the group with petal extract had fewer top zone entries, but with longer duration of occupancy suggesting more stable anxiolytic effect.

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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