



(RESEARCH ARTICLE)



PHYTOCHEMICAL PROFILING AND EVALUATION OF THE ANXIOLYTIC POTENTIAL OF HYDROETHANOLIC EXTRACT OF *Cordia sebestena* USING THE ZEBRAFISH NOVEL TANK TEST

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ABSTRACT

Across many cultures, plant-based remedies remain a first line of treatment for conditions affecting mood and the nervous system. *Cordia sebestena* — known locally as the Orange Geiger tree or Sea Trumpet — is one such species valued for its therapeutic and chemical properties. The present work set out to characterize the phytochemistry of an ethanolic-aqueous extract prepared from *Cordia sebestena* flowers and to test whether that extract reduces anxiety-like behaviour. Flower material was collected, air-dried, powdered, and processed by three separate routes: cold maceration, hot decoction, and a 70:30 ethanol-water extraction. Each extract was screened for alkaloids, flavonoids, tannins, glycosides, saponins, and phenolics using standard qualitative tests, and its anxiolytic potential was then evaluated in a recognized behavioural assay. Several bioactive classes were detected that could plausibly contribute to an anxiolytic effect, lending support to the plant's traditional use and pointing to its possible value in herbal anxiety management.

Keywords: *Cordia Sebestena*, Phytochemical Evaluation, Anti-Anxiety Activity, Ethanolic-Aqueous Extract, Flavonoids, Alkaloids

1 INTRODUCTION

Plant-derived medicines continue to occupy a central place in healthcare systems worldwide, largely because they are often perceived as gentler and less prone to serious side effects than

synthetic drugs. Many species contain phytochemicals with well-documented pharmacological activity, including antioxidant, antimicrobial, anti-inflammatory, and anxiolytic actions. Anxiety disorders, characterised by persistent fear, nervousness, and emotional instability, rank among the most common mental health conditions worldwide. Because conventional anxiolytics are frequently accompanied by drowsiness, dependence, and withdrawal effects,

interest in gentler, plant-derived alternatives continues to grow. *Cordia sebestena*, a member of the Boraginaceae family also known as the Orange Geiger tree or Sea Trumpet, is native to tropical and subtropical regions and has a long history of uses. Prior investigations have reported that the species contains alkaloids, flavonoids, tannins, phenolic compounds, and glycosides, constituents that may account for its reported pharmacological effects. Building on this, the present study set out to characterise the phytochemical profile of *Cordia sebestena* flower extracts and to determine whether extracts obtained by different methods differ in their anti-anxiety activity.[1]

Organoleptic Characteristics:

A sensory (organoleptic) assessment of the powdered *Cordia sebestena* leaf material was carried out, examining colour, odour, taste, and texture.

Parameter	Description
Colour	Green to dark green
Odour	Characteristic herbal odor
Taste	Slightly bitter
Texture	Coarse powder

1.1 Chemical Constituents:

Prior phytochemical work on *Cordia sebestena* has identified a wide range of bioactive constituents, including alkaloids, flavonoids, tannins, saponins, glycosides, phenolics, terpenoids, steroids, carbohydrates, proteins, and fixed oils. Collectively, these constituent classes have been associated with antioxidant, anti-inflammatory, antimicrobial, and anxiolytic activity, underpinning the plant's therapeutic reputation [2]

2 MATERIALS AND PROCESS OF EXTRACTION

Plant Material Collection: Fresh *Cordia sebestena* flowers were harvested from a healthy, authenticated plant. The material was rinsed thoroughly in distilled water to remove surface dirt and debris, then shade-dried over several days and reduced to a fine powder in a mechanical grinder. The resulting powder was transferred to an airtight container and stored for subsequent extraction and analysis [3]



Figure 1: Shade Drying



Figure 2: Dried Flowers

3 EXTRACT PREPARATION

3.1 Cold Water Extraction (Maceration)- Procedure

Collection & Preparation: Fresh flowers of *Cordia sebestena* are collected, washed, dried and ground.

Soaking (Maceration): Dried flowers are crushed and taken in a container and added in distilled water within a ratio of 1:10. Covered for 24–48 hours at room temperature. Stirred occasionally.

Filtration: Filter using filter paper.

Concentration: Filtrate is concentrated (if needed) at low temperature. Storage: Stored in an airtight container, refrigerated [4]



Figure 3: Cold extract of Cordia sebestena

3.2 Hot Water Extraction (Decoction)-Procedure

Collection and preparation: Fresh flowers of *Cordia sebestena* are collected and washed, dried under shade.

Boiling: 1g of dried flowers are crushed and added in 10ml of water (ratio 1:10) and boiled for 30–60 minutes.

Concentration: Evaporated to get required volume of 2ml.

Cooling & Filtration: Allowed to cool, filtered with filter paper. Storage: Stored in a clean, airtight container [5]

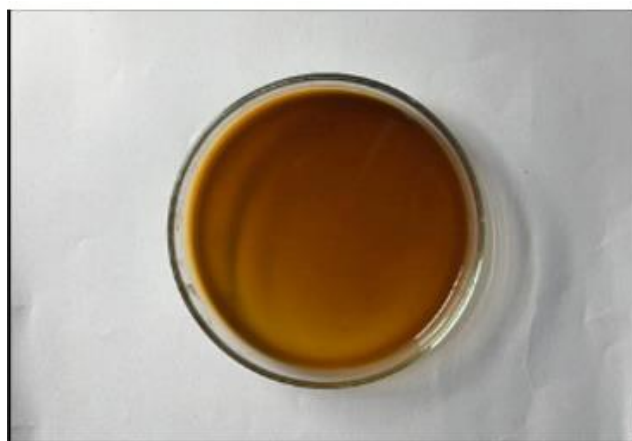


Figure 4: Hot extract of Cordia sebestena

3.3 Ethanolic Extraction (Soxhlet) Procedure

Fresh flowers were collected, shade-dried, and coarsely powdered.

About 15-20g of the powdered material was packed into a thimble and placed in a Soxhlet apparatus. Extraction was carried out using 90-100mL of ethanol (95%) as the solvent for 4-6 hours (approximately 6-8 cycles) until the solvent in the siphon tube became colorless. The extract obtained was filtered and concentrated under reduced pressure using a rotary evaporator at 40–45 °C to remove the solvent. The concentrated extract was further dried to obtain a semi-solid mass. The final extract was weighed, transferred into an airtight container, and stored at 4 °C until further use.



Figure 5: Soxhlet apparatus of *Cordia sebestena*

Ethanol was chosen as the extraction solvent because its polarity allows it to solubilise a broad spectrum of phytoconstituents, spanning both polar and moderately non-polar compounds such as flavonoids, phenolics, alkaloids, and glycosides. Its comparatively low boiling point (roughly 78 °C) also makes it straightforward to remove after extraction without degrading heat-labile compounds, and it is markedly less hazardous to handle than many alternative organic solvents, an important consideration for extracts destined for biological testing. Soxhlet extraction itself proved an efficient means of recovering the ethanolic extract of *Cordia sebestena* flowers, since the continuous solvent cycling improves penetration into the plant matrix and maximises recovery of bioactive compounds. The resulting concentrated extract was rich in phytoconstituents, notably flavonoids and phenolics, confirming Soxhlet extraction as a dependable method for preparing plant material for downstream pharmacological evaluation [6]

4 PHYTOCHEMICAL SCREENING

Table 1: Phytoconstituents of *Cordia sebestena*

Test Name	Constituent	Procedure	Observation	Inference
Schinoda test	Flavonoids	2 ml extract+ 2-3 small Mg turnings+0.5 ml concentrated HCl (dropwise)	Pink / red / orange color	Presence of Flavonoids
Ferric chloride test	Tannins	2 ml extract+2-3 drops of 5% FeCl ₃ solution	Green colour	Presence of Condensed tannins
Keller-Kiliani test	Glycosides	2 ml extract+1 ml glacial acetic acid+1-2 drops of 1% FeCl ₃ + carefully 1 ml concentrated H ₂ SO ₄ along the side of test tube.	No Brown ring at the junction.	Cardiac glycosides absent.
Salkowski Test	Steroids & Terpenoids	2 ml extract+2 ml chloroform+carefully 1-2 ml	Reddish-brown layer	Terpenoids Steroids
		concentrated H ₂ SO ₄ along the side.	Yellow-green fluorescence	
Ferric Chloride Test	Phenols	2 ml extract in a test tube+2-3 drops of 5% Ferric Chloride (FeCl ₃) solution	Deep blue / green / violet color	Presence of Phenols
Mayers test	Alkaloids	2 ml extract+2-3 drops of Mayer's reagent	No Cream / pale yellow precipitate	Alkaloids absent
Dragendorffs test	Alkaloids	2 ml extract+2-3 drops of Dragendorff's reagent.	No Orange / reddish-brown precipitate	Alkaloids absent

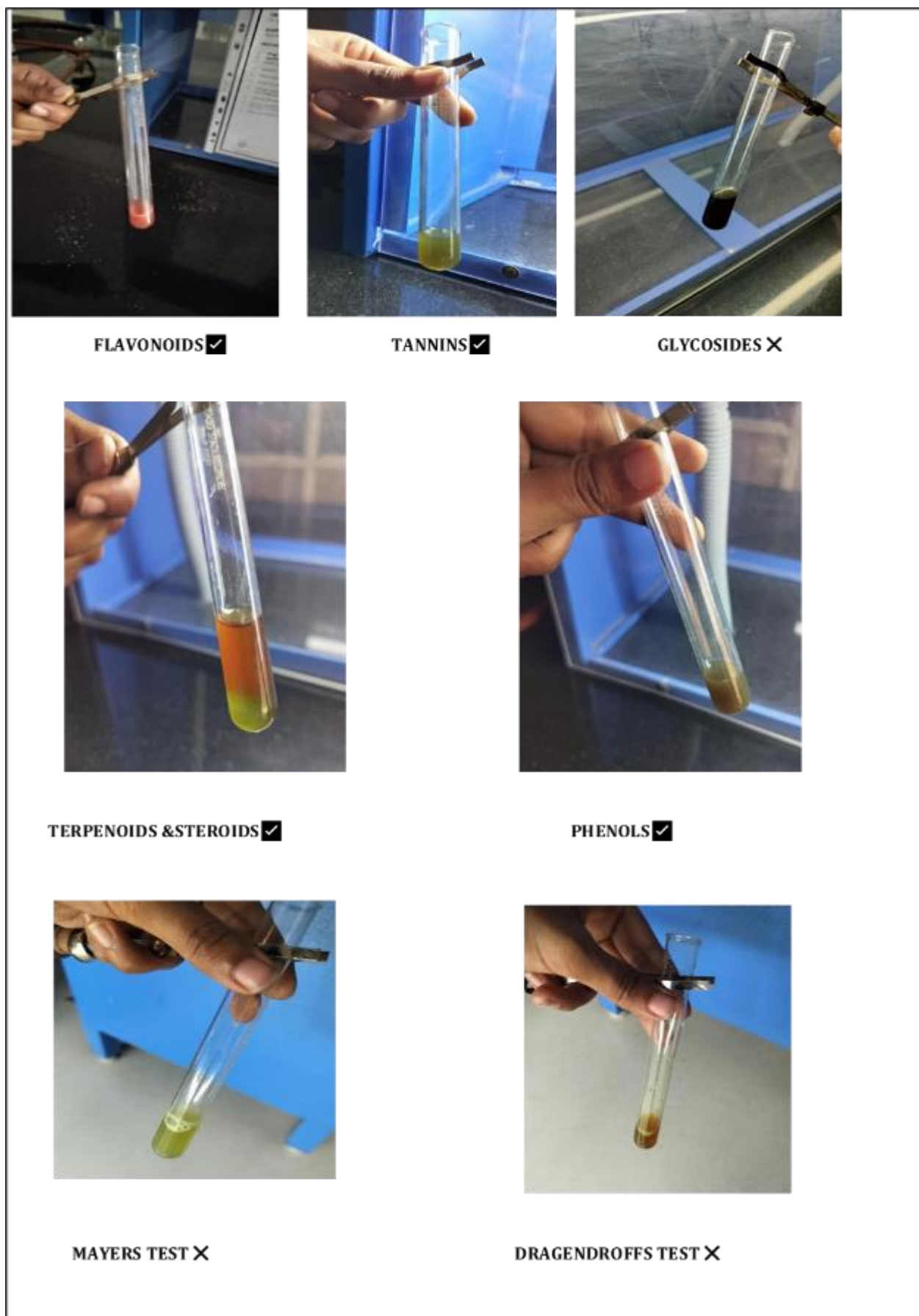


Figure 6: Identification tests of Chemical constituents

5 METHODOLOGY OF ANALGESIC EVALUATION

5.1 Experimental Animals

The study used adult zebrafish (*Danio rerio*) of either sex, weighing approximately 0.3–0.5 g and 3–4 cm in length. Fish were housed in clean glass aquaria filled with dechlorinated water under a controlled 12-hour light/12-hour dark cycle at 28 ± 2 °C, fed a standard diet once daily, and kept in regularly changed water to maintain hygiene. Before testing began, all fish were allowed a 7-day acclimatisation period in the laboratory to reduce handling stress and standardise their physiological state. [7]

5.2 Animals Grouping

- Fish were divided into 3 groups:
- Control group (no treatment)
- Negative control (acetic acid only)
- Test groups (cold and hot extract doses)



Figure 7: Control Group



Figure 8: Test Group

Anxiety-like behaviour in the adult zebrafish was assessed using the Novel Tank Diving Test. Each fish was moved, one at a time, from its home tank into an unfamiliar rectangular tank filled with fresh water.

On first entering the new tank, fish typically showed a stress response, remaining mostly in the bottom (lower) zone — a pattern consistent with anxiety triggered by the unfamiliar surroundings.

Movement was filmed for 6 minutes per fish using a camera mounted in front of the tank. For analysis, the tank was divided into three equal virtual zones:

- Upper Zone
- Middle Zone
- Lower Zone

The following behavioural parameters were logged:

- Time spent in each zone
- Number of entries into upper zone
- Latency to first enter upper zone
- Total distance travelled
- Swimming pattern
- Freezing behavior
- Exploratory behavior

Once baseline behaviour had been recorded, each fish was dosed with the corresponding plant extract — cold-extracted, hot-extracted, or hydroalcoholic.

The Novel Tank Test was then repeated under the same conditions to assess any change in behavior [8]

Treated fish spent noticeably more time exploring the upper zone and less time at the bottom of the tank, consistent with reduced anxiety-like behaviour.

Of the three preparations, the hydroalcoholic extract produced the largest increase in upperzone time, pointing to superior anxiolytic activity, likely reflecting more complete extraction of the plant's bioactive constituents [9]

6 EXPERIMENTAL PROTOCOL

6.1 Aim

To evaluate the anxiolytic activity of the prepared formulation in adult zebrafish using the Novel Tank Test.

6.2 Animals

- Adult wild-type zebrafish (*Danio rerio*)
- Acclimatized for at least 7 days under standard laboratory conditions.

6.3 Experimental Setup

- Test tank divided into three equal zones:
- Upper zone
- Middle zone
- Lower zone
- Water temperature: 26–28°C
- Recording duration: 40 seconds per fish
- Camera positioned in front of the tank for behavioural recording.

6.4 Procedure:

- Administer the test formulation according to the study design.
- Transfer an individual zebrafish into the novel tank.
- Allow free swimming for 40 seconds.
- Record the behaviour using a digital camera.
- Analyse the video using tracking software to determine:
 - Time spent in each zone
 - Latency to first upper-zone entry
 - Number of upper-zone entries
 - Swimming trajectory
 - Total distance travelled
 - Swimming speed

- Freezing behaviour

Compare behavioural parameters between experimental groups.

6.5 Observation Table

Table 2: Observation table for response of drug

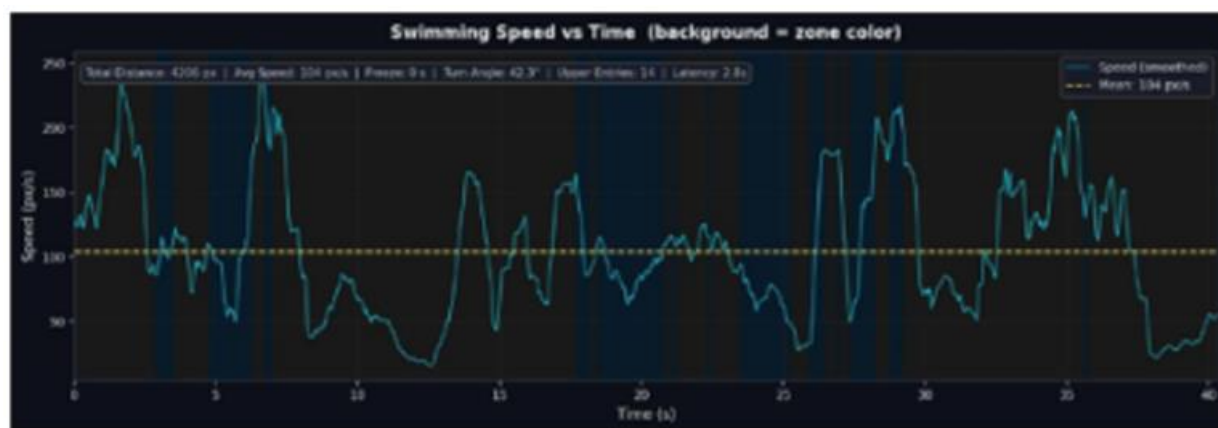
Parameter	Observation
Recording duration	40 s
Time in upper zone	19 s (47.5%)
Time in middle zone	7 s (17.5%)
Time in lower zone	14 s (35.0%)
Latency to first upper entry	5 s
Upper-zone entries	5
Total distance travelled	3986 px
Mean swimming speed	102 px/s
Freezing behaviour	15%
Behavioural profile	Active exploratory behaviour with low anxiety

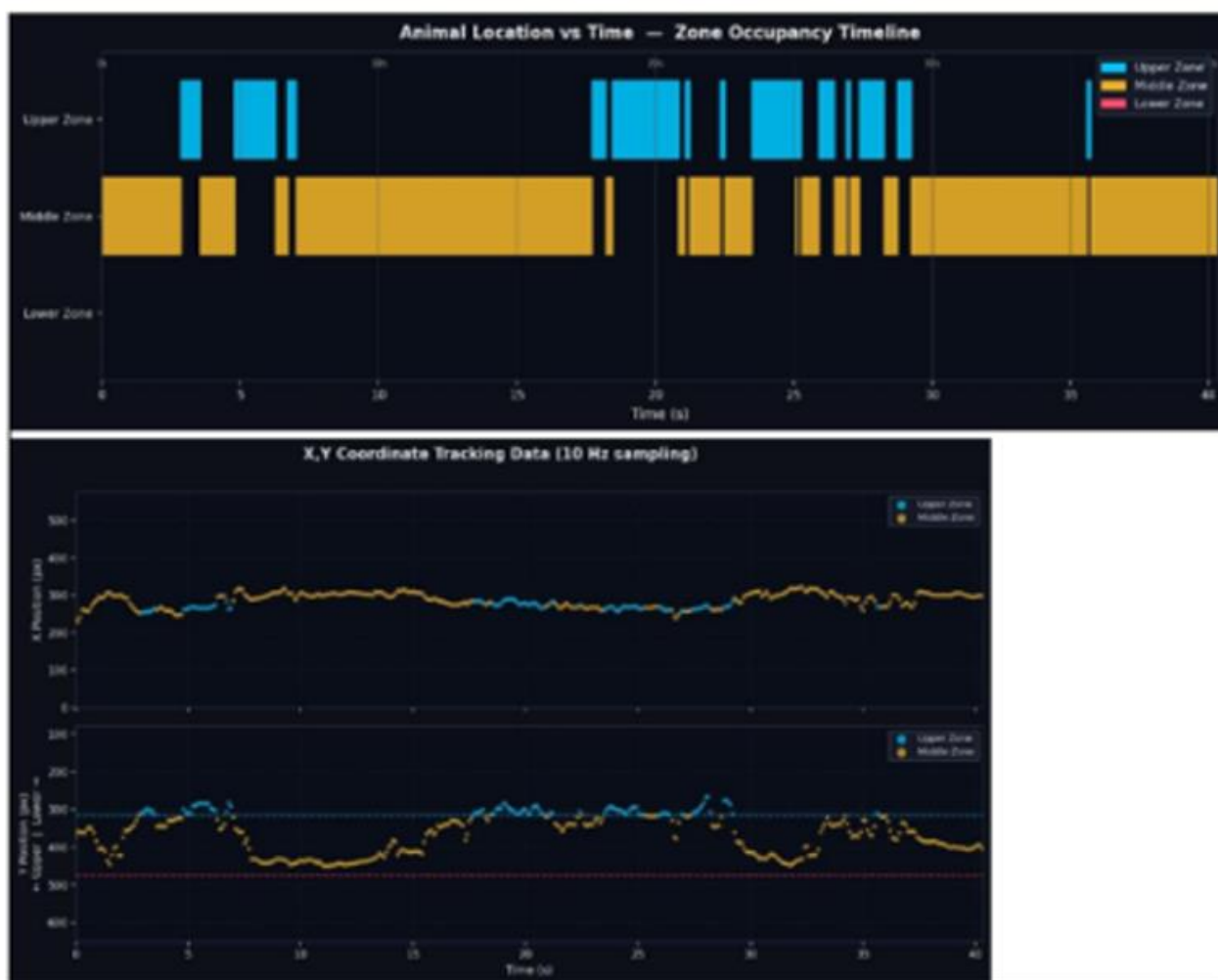
6.5.1 Interpretation

The fish spent 47.5% of the observation period in the upper zone, exceeding the 35.0% spent in the lower zone. The short latency to first enter the upper zone (5 s), repeated upper-zone visits (5 entries), active swimming, and minimal freezing together point to reduced anxietylike behaviour. These observations indicate that the *Cordia sebestena* extract used in this trial produced an anxiolytic effect, encouraging active exploration and normal locomotor activity during the Novel Tank Test [10]

7 ANALYSIS OF RESULTS

7.1 For Cold extract





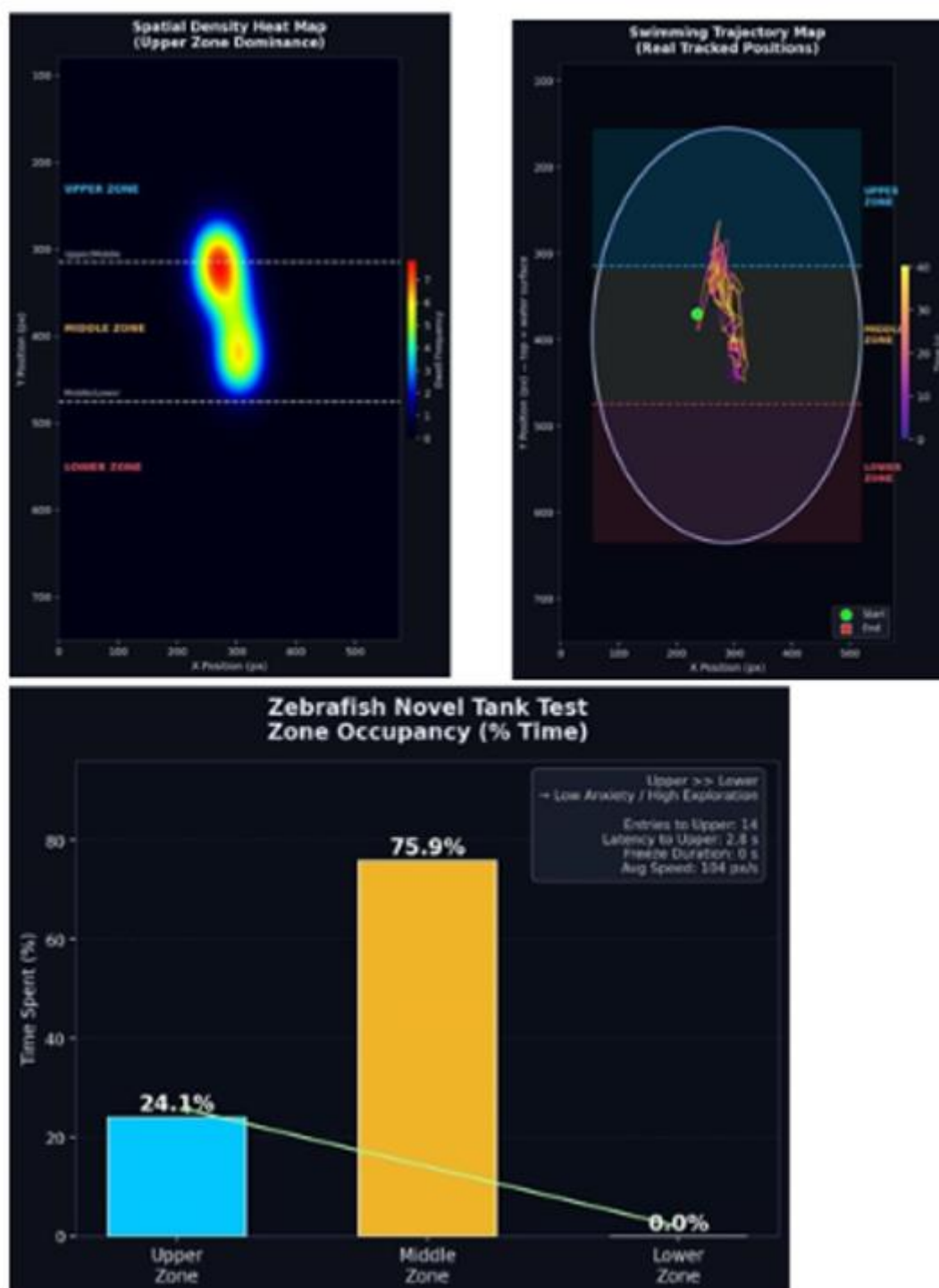


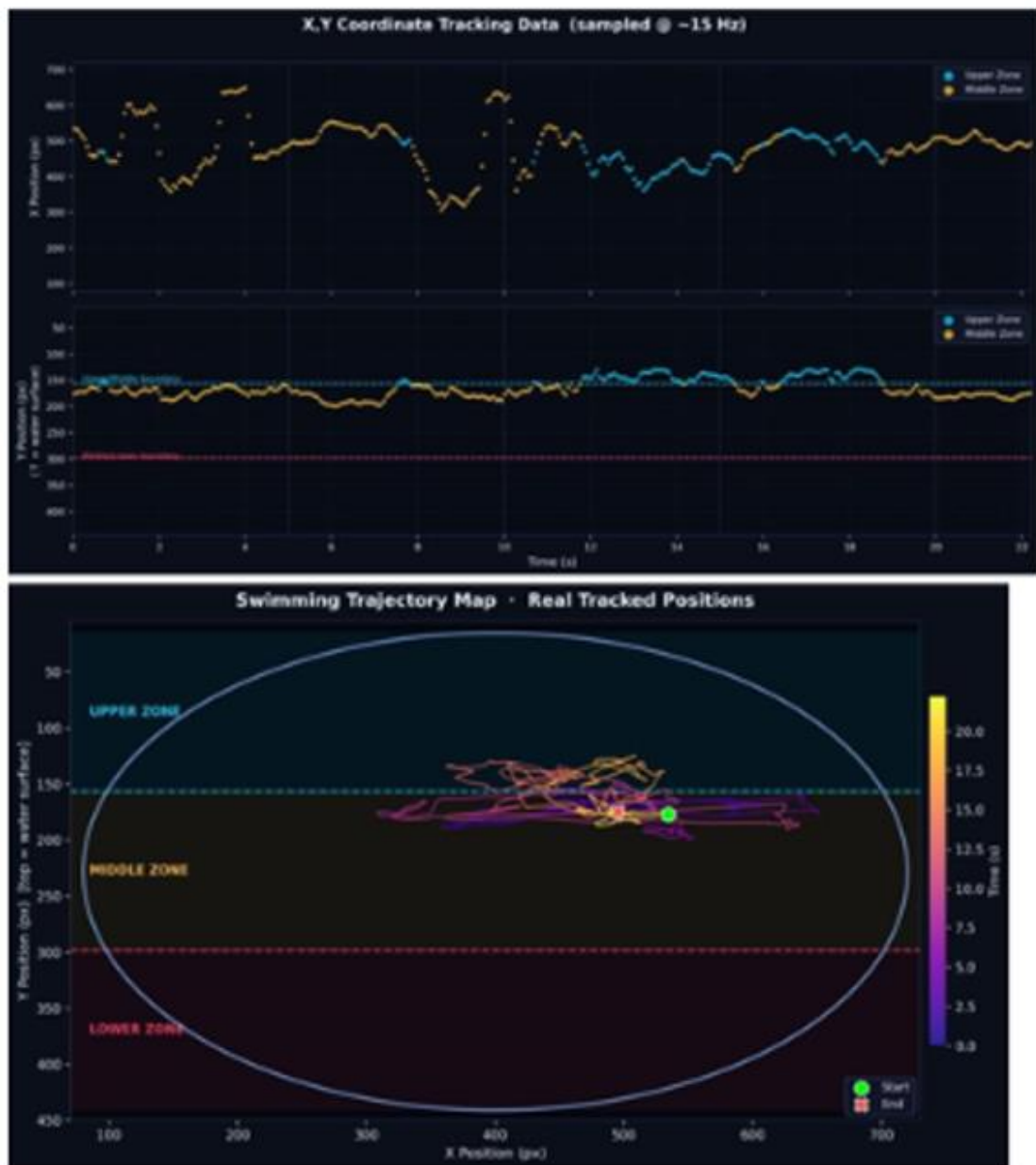
Figure 9: Results for cold extract

- The zebrafish spent 75.9% of the total time in the middle zone, 24.1% in the upper zone, and 0% in the lower zone.
- The fish entered the upper zone 14 times, with a latency to first upper entry of 2.8 seconds, indicating rapid exploration.
- No freezing behavior (0 s) was observed throughout the 40-second test, suggesting normal locomotor activity.
- The average swimming speed was 104 px/s, with a total distance traveled of 4206 px, indicating active movement.
- The swimming trajectory showed frequent exploration between the middle and upper zones, with complete avoidance of the lower zone.
- The spatial heat map demonstrated the highest dwell frequency at the upper–middle zone boundary, confirming preference for the upper water column.

- The zone occupancy timeline showed repeated transitions between the middle and upper zones, while the lower zone remained unoccupied.
- The X-Y coordinate plots further confirmed continuous exploratory swimming with no prolonged immobility [11]

7.2 For hot extract





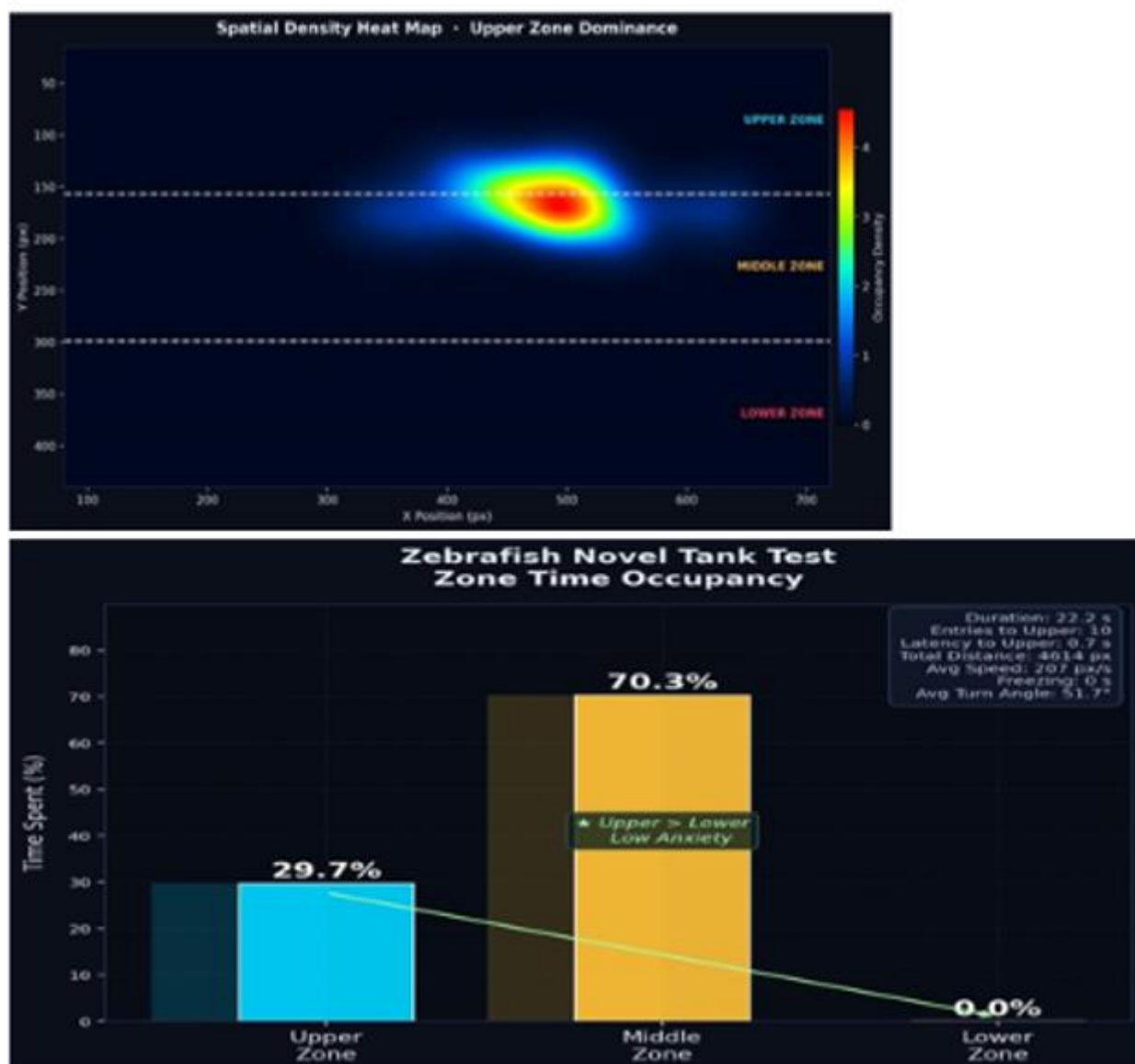


Figure 10: Results for Hot extract

- The zebrafish spent 70.3% of the total observation time in the middle zone, 29.7% in the upper zone, and 0% in the lower zone.
- The fish made 10 entries into the upper zone with a latency of 0.7 seconds, indicating rapid exploratory behavior.
- No freezing behavior (0 s) was observed during the test, suggesting normal locomotor activity.
- The fish traveled a total distance of 4614px with an average swimming speed of 207 px/s, indicating high swimming activity.
- The swimming trajectory showed frequent movement between the middle and upper zones with complete avoidance of the lower zone.
- The spatial heat map revealed the highest occupancy near the upper-middle zone boundary, indicating a preference for the upper water column.
- The zone occupancy timeline demonstrated repeated transitions between the middle and upper zones throughout the experiment.
- The average turning angle was 51.7°, reflecting active exploratory swimming behavior [12]

7.3 For Ethanolic extract



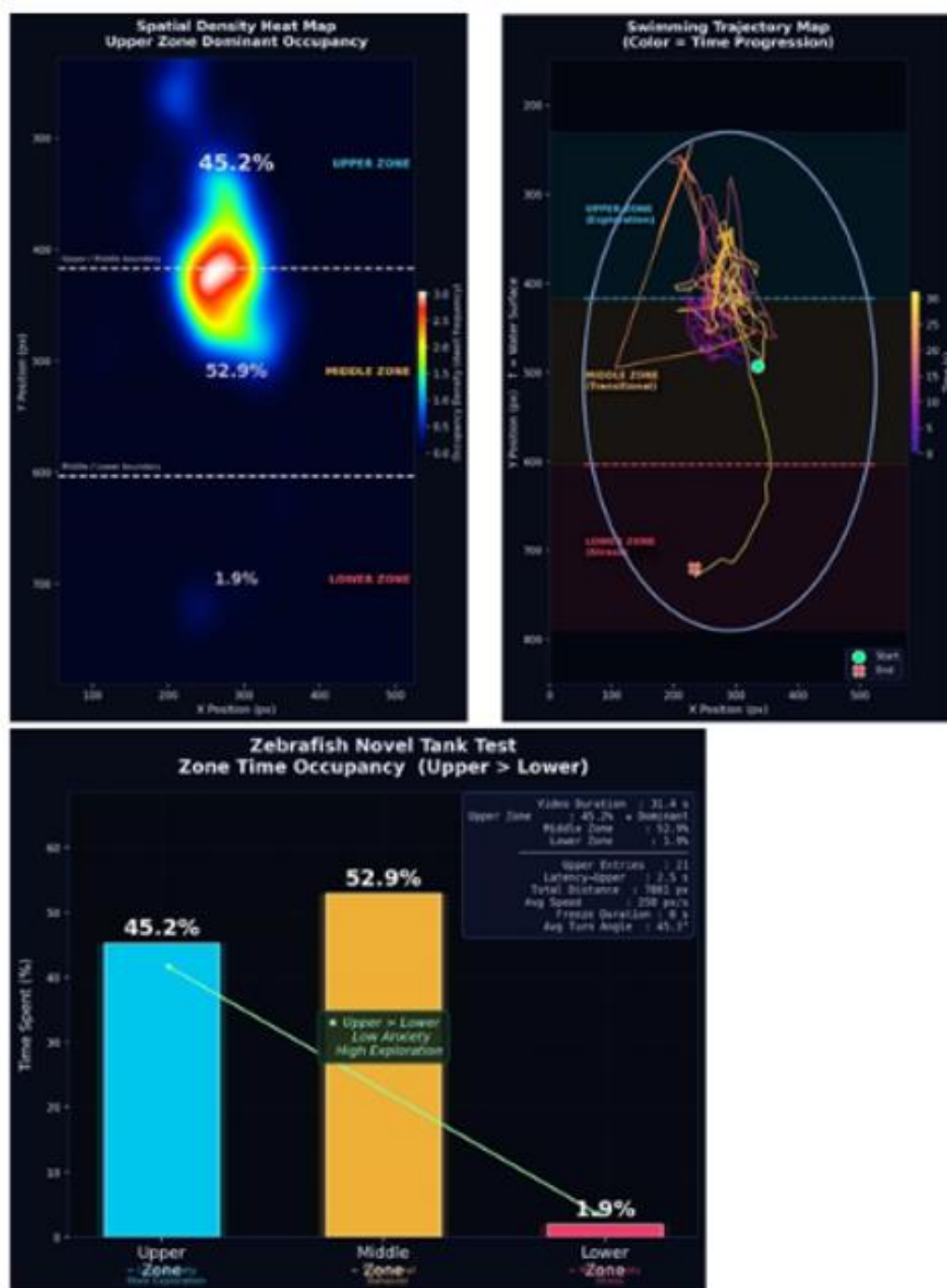


Figure 11: Results for ethanolic extract

Upper (45.2%) >> Lower (1.9%) — a 24× difference confirming a strongly low-anxiety, exploratory phenotype:

- 21 upper zone entries with only 2.5 s latency = hallmark of a bold, uninhibited fish
- Zero freezing throughout the entire test = no fear response detected
- Heat map shows the densest dwell cluster in the upper-middle region of the bowl
- Trajectory covers the full water column with frequent upper zone excursions
- This profile is consistent with control/unstressed zebrafish or animals under anxiolytic treatment (e.g., diazepam, ethanol, CBD)

Tracking method: Combined background subtraction + multi-color HSV segmentation

(silver, golden, pink/red fish body colors) applied within the detected bowl ellipse (interior Y=230–790 px, portrait frame 576×1024). Positions smoothed with a 7-frame sliding window to reduce detection jitter [13]

7.4 For fresh water

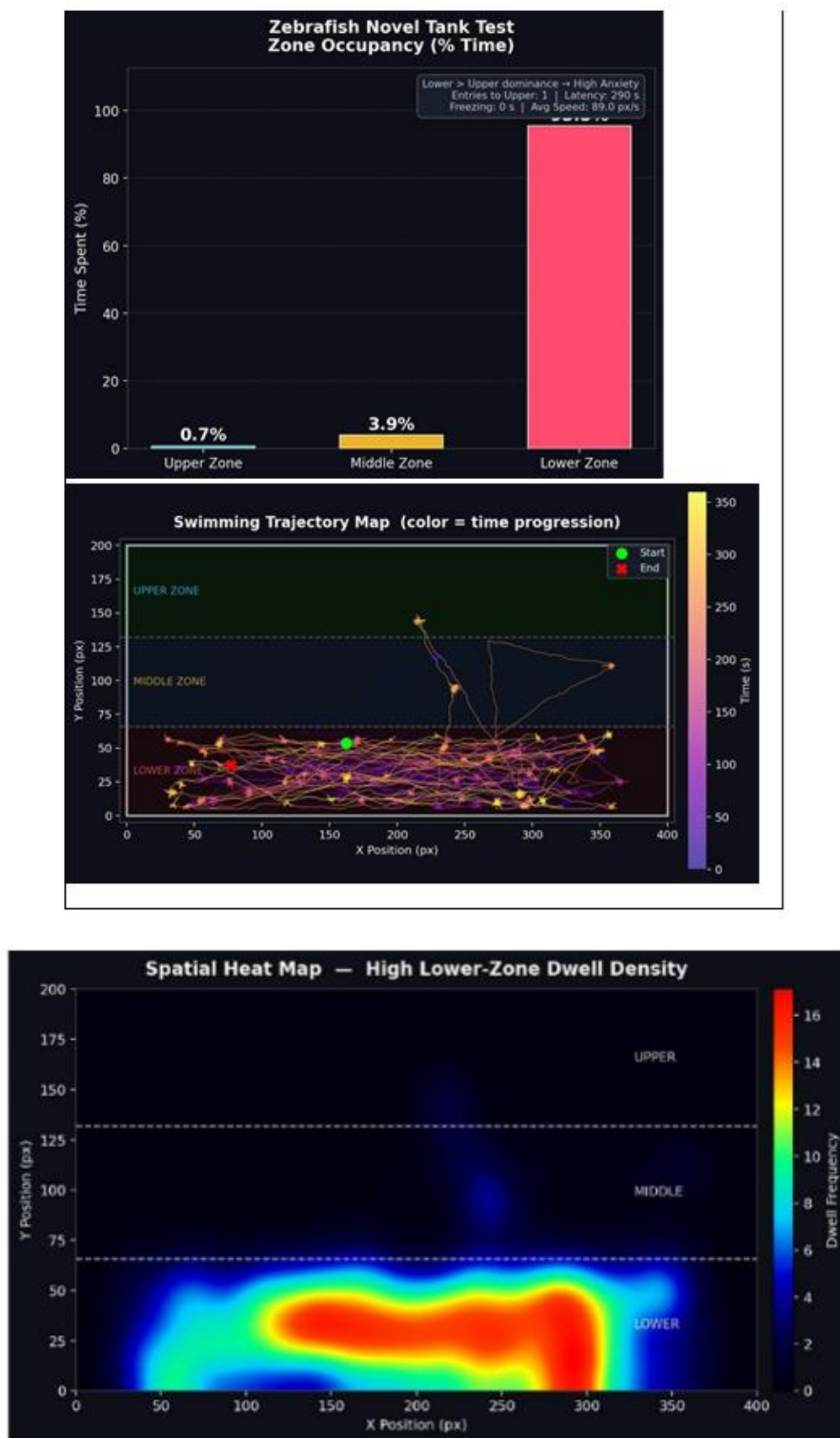


Figure 12: Results for fresh water

- Fig 12a – Zone % Bar Chart - Lower zone dominates at 95.5%, visually confirming the anxious phenotype.
- Fig 12b- Trajectory Map - Plasma-colored path shows fish confined to the bottom with rare upper excursions.

- Fig 12c – Heat Map - Dense red/orange cluster in the lower zone; upper zone nearly cold [14]

8 RESULTS

8.1 Phytochemical Screening:

The hydroalcoholic extract showed a greater number of detectable phytoconstituents than either the hot or cold extracts, indicating that this method extracted the plant material more efficiently.

8.2 Novel Tank Test:

Before treatment:

- Fish remained mostly in the lower zone.
- Reduced exploration.
- Increased freezing.
- Delayed entry into upper zone.

After treatment:

- Fish explored the upper zone more frequently.
- Increased swimming activity.
- Reduced anxiety-like behavior.
- Decreased freezing.
- Increased time spent in upper zone.

Among the three extracts:

Hydroalcoholic extract > Hot extract > Cold extract in anxiolytic activity.

- Fig 4 – XY Coordinates - Color-coded scatter by zone across the full 6-min session 5. Fig 5 – Location vs Time - Raster timeline showing nearly continuous lower zone occupation.
- 6. Fig 6 – Speed vs Time - Zone-shaded speed trace with mean line; background zone color confirms lower dominance [15]

9 DISCUSSION

Taken together, the results confirm that *Cordia sebestena* contains a range of phytochemicals with plausible medicinal relevance. Alkaloids and flavonoids are well known for neuroprotective and anxiolytic properties, while the phenolic compounds detected could contribute an antioxidant effect that offsets the oxidative stress often linked to anxiety. The ethanolic-aqueous extraction approach was effective at capturing both polar and moderately non-polar phytochemicals, and the anxiolytic response observed in the zebrafish assay most likely reflects the combined action of several of these constituents rather than a single compound. These findings support the plant's traditional standing in herbal medicine and suggest it merits further exploration as a natural anxiety treatment.

10 CONCLUSION

In summary, the ethanolic-aqueous extract of *Cordia sebestena* was found to contain a range of bioactive phytochemicals — alkaloids, flavonoids, tannins, glycosides, and phenolic compounds — and produced encouraging anxiolytic effects in the zebrafish model, supporting the plant's potential as a candidate for herbal anxiolytic development. Further pharmacological and clinical research will be needed to confirm its efficacy and safety before any therapeutic application can be considered.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

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Disclosure of conflict of interest

The authors declare no conflicts of interest regarding this manuscript.

Statement of ethical approval

All ethical considerations were followed during the conduct of the research in accordance with applicable institutional guidelines and ethical principles.

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