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Toxicological screening of jambolan hydroalcoholic extract (*Syzygium cumini* (L.) Skeels) in zebrafish (*Danio rerio*)

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ABSTRACT

Jambolan (*Syzygium cumini* (L.) Skeels) is an important source of phenolic compounds, especially anthocyanins, known for their biological properties. This study investigated the acute toxicity of jambolan hydroalcoholic extract (JE) in zebrafish (*Danio rerio*) at different life stages. JE, obtained from freeze-dried fruits, was analyzed by high-performance liquid chromatography (HPLC) and found to be rich in total phenolic compounds (TPC). A total of 15 phenolic compounds were identified in the HPLC extracts, mainly anthocyanins (≈ 82 % of TPC), and JE presented relevant antioxidant properties in *in vitro* tests. Exposure to concentrations between 50 and 200 μg/ml resulted in increased malformations and mortality in both embryos and adult zebrafish, and doses of 300 and 400 μg/ml were lethal to the animals. Lethal concentrations (LC50) were estimated at 118.4 μg/ml for embryos and 68.86 μg/ml for adults. Despite no significant cardiovascular or neurological toxicities, behavioral impacts were observed at lower concentrations (10 μg/ml), indicating a nonmonotonic concentration-response curve. Our findings suggest that moderate JE doses (around 25 μg/ml) are safe for zebrafish; however, further studies are needed to ensure its safety and efficacy under different health conditions and exposure regimes.

1. Introduction

Jambolan (*Syzygium cumini* (L.) Skeels), also known as Java plum, Indian blackberry, and purple olive, is a species native to South Asia [49] very well adapted to Brazil's climate [52]. Its chemical composition includes various compounds with nutritional and biological value such as oxalic acid, gallic acid, tannins, and certain alkaloids which contribute to its astringent taste. The fruit's dark purple color is due to anthocyanins which contribute to its bioactive properties [26].

Anthocyanins are natural water-soluble flavonoids abundantly found in fruits, leaves, roots, and other parts of plants [39]. Studies show that the color of anthocyanins is closely related to pH levels, varying between red and purple in acidic conditions (pH < 7.0), and becoming blue or colorless in more basic pH levels (pH > 7.0) [36]. These flavonoids have been associated with various biological activities, including gut microbiota modulation, oxidative stress reduction, and aid in the control of non-communicable chronic diseases [46].

Given the numerous benefits of anthocyanins, recent research has focused on finding better sources of this pigment and optimizing its extraction and stabilization [50]. Producing extracts with high levels of anthocyanins has been a key research focus in the food, pharmaceutical, and cosmetic industries [21]; however, conducting studies to evaluate the toxicological potential of these natural products and their phytoconstituents is a necessary step to ensure their efficacy and safety before their application for any purpose [43].

From a regulatory perspective, agencies like the Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the Brazilian Health Regulatory Agency (ANVISA) require *in vitro* and *in vivo* tests to assess the toxicological potential of molecules at different doses, concentrations, and exposure times, following guidelines from the Organisation for Economic Co-operation and Development (OECD) [42]. These experiments shall guide future studies investigating

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biological activities.

In recent decades, the zebrafish (*Danio rerio*) has emerged as a prominent animal model for experimental studies seeking to test new drugs and substances due to its anatomical, genetic, and functional characteristics similar to mammals [22]. This versatile, easy-to-handle, and low-cost organism has a high reproductive rate, rapid life cycle and a high genetic similarity with humans (70 %), offering a feasible and reliable translational model to biomedical research [15]. Given this context, this study characterized the jambolan hydroalcoholic extract (JE), investigated its antioxidant potential, and evaluated its toxicological profile in zebrafish (*Danio rerio*) at two life stages (embryo/larva and adult).

2. Material and methods

2.1. Jambolan Extract (JE)

2.1.1. Sample processing and extract preparation

Jambolan fruits (*Syzygium cumini* (L.) Skeels), genetic registration ADAE032 on the National System of Genetic Heritage (SISGEN), were obtained from domestic cultivations in Natal, Rio Grande do Norte, Brazil, during the harvest period between December 2022 and January 2023. Fully ripe fruits (dark purple, no deterioration) were sanitized, manually pulped, and lyophilized at −50°C under 0.6 Pa for 48 h. The dried material was ground, sieved (20 mesh), and frozen (-20°C). For the jambolan extract (JE), 1 g of lyophilized pulp was extracted with ethanol-water (70:30 v/v) via cold extraction. After three centrifugation cycles, the supernatant was filtered, concentrated by rotary evaporation (40°C), lyophilized (96 h), and stored (-20°C) for analysis.

2.2. Extract characterization

2.2.1. Total phenolic compounds

Total phenolic compounds (TPC) were determined following da Silva et al. [17] with modifications, using distilled water as a blank. JE diluted in distilled water reacted with Folin-Ciocalteu reagent and saturated sodium carbonate solution. Gallic acid solutions (320–1000 μL) were used as a calibration curve. TPC was expressed as gallic acid equivalents (mg GAE) per gram of dry fruit.

2.2.2. Monomeric anthocyanins by pH differential method

Total monomeric anthocyanins (TMA) were determined following Lee et al. [34] with modifications, using distilled water as a blank. Absorbance (A) was measured at 520 and 700 nm and calculated using Eq. 1. TMA concentration was determined with Eq. 2 and expressed as cyanidin-3-glucoside equivalents per gram of dry fruit.

$$A = [(A520\ nm - A700\ nm)\ pH = 1{,}0] - [(A520\ nm - A700\ nm)\ pH = 4.5] \quad (1)$$

$$TMA\ (cyd\text{-}3\text{-}glu\ eq.,\ mg/L) = A\ x\ Mw\ x\ FD\ x\ V\ x\ 1000\ /\ Ma\ x\ L\ x\ m \quad (2)$$

In which Mw is the molecular weight of cyanidin-3-glucoside = 449.2 g/mol; FD is the dilution factor of the sample in buffer; V is the volume of the buffer in ml; Ma is the molar extinction coefficient = 26,900 mol/L x cm; L is the optical path length of the cuvette (1 cm); m is the sample mass.

2.3. High-performance liquid chromatography (HPLC)

Individual phenolic compounds in JE were determined following Padilha et al. [44] with an Agilent 1260 Infinity LC System and diode-array detector (DAD). Data were processed using OpenLAB CDS software. A Zorbax Eclipse Plus RP-C18 column with a Zorbax C18 pre-column was used, with separation at 35°C and a 20 μL injection of extract filtered through a 0.45 μm membrane. Flow rate was 0.8 ml minˆ-1 and a gradient of solvents (A: 0.52 % phosphoric acid; B: methanol with 0.52 % phosphoric acid) achieved separation. Phenolics were identified and quantified using external standards with calibration curves ($R^2 > 0.998$) and evaluated for spectral purity (purity factor > 950).

2.4. In Vitro antioxidant activity

Antioxidant capacity was tested using the ABTS+ radical (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) scavenging method modified according to Rufino et al. [51], and the DPPH method (2, 2-difenil-1-picrilhidrazil) following Chen et al. [12] and Fia et al. [25], respectively, with some modifications. For ABTS+ , the solution was adjusted to an absorbance of 0.80 ± 0.02 at 734 nm. After adding diluted JE (80x) or trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standard, absorbance was measured after 6 min to determine IC50 values. Results were expressed as mmol Trolox/g dry fruit.

For DPPH, a stock solution (0.236 mg in 100 ml methanol) was mixed with 0.1 ml of the sample and 3.9 ml of DPPH or ethanol. Absorbance at 515 nm was measured after 30 min in the dark using a UV–visible spectrophotometer. Values were expressed in μM trolox/g dry fruit. DPPH free radical scavenging activity was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [1 - (A\ i\ /A\ 0)] \times 100\%$$

in which A i is the absorbance of samples or standards, A 0 the absorbance of the blank control.

2.5. In Vivo toxicity evaluation

2.5.1. Ethical procedures

Male and female zebrafish (*Danio rerio*), wild-type Tübingen strain (TU), were kept according to CONCEA (National Council for Animal Experimentation Control) normative resolution No. 34. All maintenance procedures and experimental protocols were approved by the Animal Use Ethics Committee of the Federal University of Rio Grande do Norte (CEUA, institutional certificate No. 301.035/2022) and followed ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Health and well-being of the animals were monitored daily throughout all experimental phases.

2.5.2. Animal stocking and reproduction

Adult zebrafish (~6 months old) were housed in the FishLab at UFRN and fed twice daily with Artemia sp. nauplii and commercial flake feed (45 % protein, 55 % fat). The animals were maintained in an automated rack system (ZebTEC) at 28°C, 600 μS/cm conductivity, < 0.001 mg/L ammonia/nitrite, pH 7.2, and a 14 h/10 h light/dark photoperiod. Weekly, zebrafish from four tanks were placed in breeding tanks (3 males: 2 females), separated overnight by a divider allowing visual and chemical contact. During the morning light cycle, the divider was removed for 60 min of mating. Fertilized eggs were then collected, observed under magnification, and assessed for fertilization and blastula formation at 3 hpf.

2.5.3. Embryotoxicity

Embryotoxicity testing followed [41] and Westerfield [62] protocols. Zebrafish embryos were analyzed at 24, 48, 72, and 96 hpf, with dead embryos removed at each observation to prevent contamination. Development was examined under a binocular stereoscopic microscope (80x magnification), with normal and abnormal development classified according to Kimmel et al. [32]. LC50 was calculated from mortality at 96 hpf. JE doses were based on Chensom et al. [13] and ranged from 10 to 400 μg of TMA/ml, with negative (system water, i.e., water from zebrafish maintenance system) and positive controls (3,4-dichloroaniline). Fertilized eggs (n = 192) were distributed in eight plates, with 1 egg per well.

2.5.4. Larvae cardiotoxicity

Cardiotoxicity was evaluated by altering cardiac parameters in 96 hpf larvae. Heart rate was determined by counting the heartbeats of 20 animals per group (control and treated groups) using a binocular stereoscopic microscope (80x magnification) that allows an enlarged view of the atrium and ventricle movements in the pericardium. Heart rate count was performed for 60 sec in triplicate for each individual, and the mean value was used [29]. Cardiac functioning often precedes other physiological dysfunctions, making heart rate a key parameter in safety evaluations of chemical substances.

2.5.5. Larvae neurotoxicity

At 7 days post-fertilization (dpf), 12 larvae from each treatment (negative control and JE-exposed groups) underwent optomotor and avoidance response tests, as per Creton [14], performed in triplicate. Imaging analysis was conducted using ImageJ, extracting centroid values and X-Y coordinates for larval orientation. Coordinates were exported to Excel and standardized to quantify the number of larvae showing alignment (OMR+) or misalignment (OMR-) with stripe movement, and those evading the aggressive object.

2.5.6. Adult acute toxicity

JE doses were based on Chensom et al. [13]. Adult TU zebrafish (6–7 months old) were divided into seven groups (25 fish/group in 15 L tanks). Six groups were exposed to JE at concentrations of 10, 25, 50, 100, 200, and 300 μg TMA/ml, with one negative control group (system water). Acute toxicity testing lasted 96 h, following OECD guideline 203. The solution was renewed daily, and fish were observed at 15, 30, 60, 120, 180, 300, 1440, 2880, 4320, and 5760 min for mortality and abnormalities. LC50 was determined based on the results.

2.5.7. Adult neurotoxicity

Neurotoxicity was evaluated by behavioral analyses, including Novel Tank, Open Field, Sociability, Aggressiveness, and Avoidance Response (n = 15 for each group).

Novel Tank: Individuals from each treatment were placed individually in a 2 L tank (20 cm×12 cm x 15 cm). Each animal's behavior was recorded for 6 min by a video camera positioned in front of the tank [40].

Open Field: Animals from each group were placed individually in the center of a circular tank (24 cm diameter) and filmed for 10 min by a video camera positioned above the tank, after which they were returned to their home tank [58].

Sociability: Animals were placed individually in a 2 L tank (20 cm×12 cm x 15 cm) between two 5 L tanks (15 cm×10 cm x 10 cm) with a shoal track and only water, with barriers to prevent visibility between them. Animal behavior was filmed for 11 min. After the first minute of filming, the barriers were simultaneously removed so the fish could see the shoal and the tank with only water, and filming continued for another 10 min, after which the animal was returned to its home tank. After filming every three focal animals, the water in the central tank was replaced to avoid accumulation of signaling substances, and the shoal was switched sides [24,6].

Aggressiveness (Mirror Test): One animal from each treatment was individually placed in the filming tank, where it remained 3 min for adaptation. It was then filmed for 5 min without the mirror, after which the mirror barrier was removed, and the fish was filmed for another 5 min. After filming every three animals, the water in the tank was replaced to avoid accumulation of signaling substances [64].

Avoidance Response: Each animal was individually placed in a circular tank (24 cm diameter) and left to adapt for 10 min. Then, an object simulating a natural predator (a bird model) was brought close to one of the tank quadrants (the tank was divided into four identical quadrants). Fish behavior was recorded by a camera located above the tank for 6 min, 3 min before and 3 min after the predator's approach, to analyze its avoidance behavior [27].

Behavioral records were analyzed using the ANY-Maze™ tracking software (Stoelting, CO, USA), which allows for precise and comprehensive behavioral data analysis and provides detailed information on swimming and other behavioral patterns under various experimental conditions.

2.6. Statistical analysis

Data in graphs are presented as mean ± standard deviation. Log-rank (Mantel-Cox) test was used for survival and hatching rate analysis. Embryotoxicity and acute toxicity analyzed up to 96 hpf had a Kaplan-Meier curve and LC50 estimation calculated. Occurrence of malformations was compared by one-way ANOVA followed by Tukey's post-test. One-way ANOVA was used for neurotoxicity analyses. Tukey's post-test evaluated the interaction between x and y coordinates as a dependent quantitative variable, with treatments as fixed-effect variables and observation as a random-effect variable. Behavioral analysis of adult animals employed one-way ANOVA followed by Tukey's test. For sociability, aggressiveness, and avoidance test data, indices were used to evaluate changes in animal behavior after the stimulus. Behavior before the stimulus was subtracted from behavior after the stimulus, with negative values indicating a decrease in behavior, and positive values indicating an increase. All analyses were performed using GraphPad Prism 9.5.1 software. A 95 % confidence interval was adopted, and differences were considered significant if $p < 0.05$.

3. Results

3.1. Jambolan extract (JE)

Jambolan extract (JE) showed a total phenolic content of 2180.4 mg ± 0.06 of gallic acid equivalents (GAE) per 100 g of dried fruit. For anthocyanins, the skin and pulp of freeze-dried jambolan had a total anthocyanin content of 1139.12 ± 91.49 mg per 100 g of dried fruit.

HPLC identified 15 phenolic compounds (Table 1). Total phenolic content of freeze-dried jambolan pulp and skin, as determined by HPLC analysis, was 4532.23 ± 158.03 mg/kg. Anthocyanins were the predominant compounds in the extracts, representing approximately 82 % of the total phenolic compounds quantified. Anthocyanin profile detected 3 of the 6 most common anthocyanins in nature in the

Table 1
Compounds identified in JE by HPLC.

Phenolic Compounds	Quantity (mg/kg of dried fruit) ± SD
Gallic Acid	114.06 ± 16.93
Caftaric Acid	151.33 ± 2.65
Chlorogenic Acid	4.43 ± 0.34
Caffeic Acid	20.05 ± 0.31
Quercetin 3-glucoside	3.98 ± 0.00
Rutin	2.63 ± 0.00
Kaempferol 3-glucoside	3.99 ± 2.46
Procyanidin B1	10.42 ± 1.09
Catechin	36.67 ± 0.71
Procyanidin B2	457.26 ± 3.04
Epicatechin	20.03 ± 0.86
Cyanidin 3,5-diglucoside	295.03 ± 6.27
Delphinidin 3-galactoside	559.45 ± 0.99
Malvidin 3,5-diglucoside	2833.44 ± 120.98
Malvidin 3-glucoside	19.46 ± 1.40
Total Anthocyanins	**3708.18 ± 129.64**
Total Phenolic Compounds	**4532.23 ± 158.03**

The analyses were performed in triplicate. Phenolic compounds in JE were determined through High-Performance Liquid Chromatography with an Agilent 1260 Infinity LC System. Separation was achieved using a Zorbax Eclipse Plus RP-C18 column with a gradient of solvents (0.52 % phosphoric acid and methanol) at 35°C. Phenolics were identified and quantified with external standards and calibration curves ($R^2 > 0.998$), ensuring spectral purity (purity factor > 950).

following proportions: Malvidin 3,5-diglucoside (76 %), Delphinidin 3-galactoside (15 %), and Cyanidin 3,5-diglucoside (8 %). Besides anthocyanins, other phenolic compounds were identified in JE, including Procyanidin B2 (457.26 mg/kg), Caffeic acid (151.33 mg/kg), and Gallic acid (114.06 mg/kg).

3.2. In Vitro antioxidant activity

JE showed an ABTS value of 323.88 ± 11.05 mmol Trolox equivalents/g fruit. Regarding the ABTS+ radical inhibitory activity curve, JE inhibited ABTS+ radical generation in a concentration-dependent manner, with maximum inhibition observed at 1.0 mg/ml (86.2 %). IC50 was 0.606 mg/ml for JE. For DPPH, JE presented a value of 844.70 ± 58.8 mmol Trolox Eq./g fruit. Fig. 1

3.3. In vivo toxicity evaluation

3.3.1. Embryotoxicity

At the end of the 96-hour exposure period, embryos exposed to 10 and 25 μg/ml of JE showed 100 % survival rate. Starting at 50 μg/ml, higher JE concentrations resulted in increased mortality, with 400 μg/ml (the highest concentration) causing 100 % mortality. Statistical differences were observed starting from 100 μg/ml of JE compared with the negative control (system water), $p < 0.0001$ (Fig. 2A). Based on the survival percentages of animals exposed to JE at both life stages, LC50 values were calculated as 118.4 ± 11.7 μg/ml for embryos, indicating the JE concentration required to cause 50 % mortality in each group.

Regarding hatching, we found no statistical difference between 10, 25, and 50 μg/ml of JE and the negative control, with most larvae hatching at 72 hpf. Starting at 100 μg/ml concentration, the hatching rate decreased in a dose-dependent manner, with statistical differences observed compared with the negative control (Log-rank test χ^2=359.3, df=6, $p < 0.0001$). At 400 μg/ml of JE, no hatching occurred within the 96 hpf observed (Fig. 2B).

As for the total number of morphological abnormalities, embryos exposed to JE showed no significant changes between the 10–100 μg/ml concentrations. However, embryos in the 200 μg/ml group presented statistically significant differences (Dunnett's multiple comparisons test, F (6, 294) = 1074; $p < 0.0001$) in the number of malformations compared with other extract concentrations and the positive control (3,4-DCA) (One-way ANOVA, F (6, 294) = 1074; $p < 0.0001$). Notable morphological abnormalities included yolk sac edema, pericardial edema, and malformations of the eyes, head, tail, and spine (Fig. 2D). Yolk sac and pericardial edemas were the most frequently observed abnormalities, but without statistical difference between the JE-treated groups and the negative control, only in relation to the positive control (One-way ANOVA, F (6, 30) = 111.9; $p < 0.0001$) (Fig. 2C and D). Assessing malformations in the group exposed to 400 μg/ml was unfeasible, as this dose resulted in 100 % embryo mortality within the first 48–72 h of the experiment.

By the end of the 96 h, larval size was compared with the negative control group. Larvae exposed to 10, 25, and 50 μg/ml of JE showed no significant changes; however, larvae in the 100 μg/ml group presented statistically significant differences (One-way ANOVA, F (4, 278) = 14.49; $p < 0.0001$), showing larger size compared with the negative control and other extract concentrations probably be due to edema. When compared with each other, larvae exposed to 10 and 50 μg/ml differed statistically, with a p-value of 0.0325 also possibly related to edema (Fig. 2E). Larvae exposed to 200 and 400 μg/ml, as well as those in the positive control group, were not analyzed due to high number of malformations and mortality.

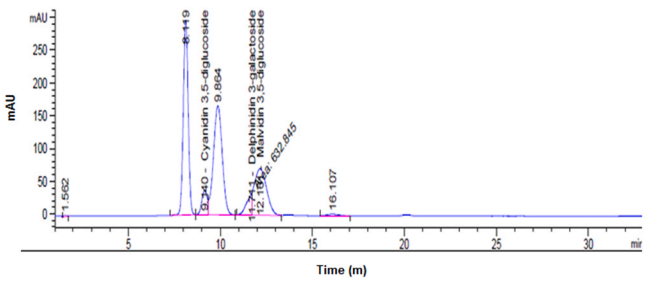

Fig. 1. Chromatogram of jambolan hydroalcoholic extract (JE).

3.3.2. Larvae cardiotoxicity

Cardiotoxicity assessment was performed in animals from the control group and those exposed to 10 μg/ml up to 100 μg/ml of JE, as the remaining groups presented high numbers of mortality and malformation. We observed no significant alterations in heart rate in 96hpf embryos (One-way ANOVA, F (4, 295) = 2.273; $p = 0.0614$). Heart rate values ranged between 138 and 168 bpm (Fig. 3).

3.3.3. Larvae neurotoxicity

All evaluated groups showed positive optomotor responses (OMR+) above 50 %. Average OMR+ of the control group was 81.64 %, 59.78 % for 10 μg/ml of JE, 75.92 % for 25 μg/ml, 84.86 % for 50 μg/ml, and 72.17 % for 100 μg/ml. One-way ANOVA identified statistical significance between groups (F (4, 175) = 15.94; $p < 0.0001$). Dunnett's multiple comparison test indicated that the 10 μg/ml group had the lowest OMR+ response, followed by the 100 μg/ml group, whereas the 25 μg/ml and 50 μg/ml groups were not significantly different from control (Fig. 4A).

Results from the avoidance response assessment showed high positive response for all groups. Three different values were assigned for larval positions in relation to the aversive stimulus (bouncing ball): 0 – did not avoid the stimulus, 1 – avoided the stimulus but remained near its location, and 2 – avoided the stimulus and moved as far away as possible. The values obtained for each treatment were 1.558 for the negative control, 0.9167 for 10 μg/ml, 1.506 for 25 μg/ml, and 1.519 for both 50 μg/ml and 100 μg/ml. One-way ANOVA identified statistical significance between groups (F (4, 145) = 23.82; $p < 0.0001$). Dunnett's multiple comparison test indicated that the 10 μg/ml group presented a statistically lower avoidance response compared with all other groups (Fig. 4B).

3.3.4. Adult acute toxicity

Adult fish exposed to JE showed similar response to that observed in embryos but were more sensitive to the extract. The 10 μg/ml group had 100 % survival rate, whereas those exposed to 25 μg/ml of JE had a survival rate of 96.3 %. Starting from 50 μg/ml, survival rates were dose-dependent with 300 μg/ml causing 100 % mortality. Log-rank testing revealed a statistical difference between the survival curves compared with the negative control starting from 50 μg/ml ($p < 0.0001$) (Fig. 5).

Based on the survival percentages of animals exposed to JE at both life stages, LC50 values were calculated as 118.4 ± 11.7 μg/ml for embryos/larvae and 68.86 ± 4.45 μg/ml for adult zebrafish, indicating the concentration required to cause 50 % mortality in each group.

3.3.5. Adult neurotoxicity

After 96 h of JE exposure, the surviving animals (control groups and 10 – 100 μg/ml of JE) were evaluated for neurological damage by the following behavioral tests: Novel Tank (Fig. 6), Open Field (Fig. 7), Sociability and Aggressiveness (Fig. 8), and Avoidance Response (Fig. 9).

For the novel tank test, one-way ANOVA indicated statistical significance between groups for total distance traveled (F (4, 70) = 4008; $p = 0.005$) and speed while moving (F (4, 70) = 3.982; $p = 0.005$). Tukey's post hoc test showed that animals exposed to 10 μg/ml of JE had lower distance traveled and lower speed compared with control (Fig. 6). For the other tests conducted, the behavior did not differ between

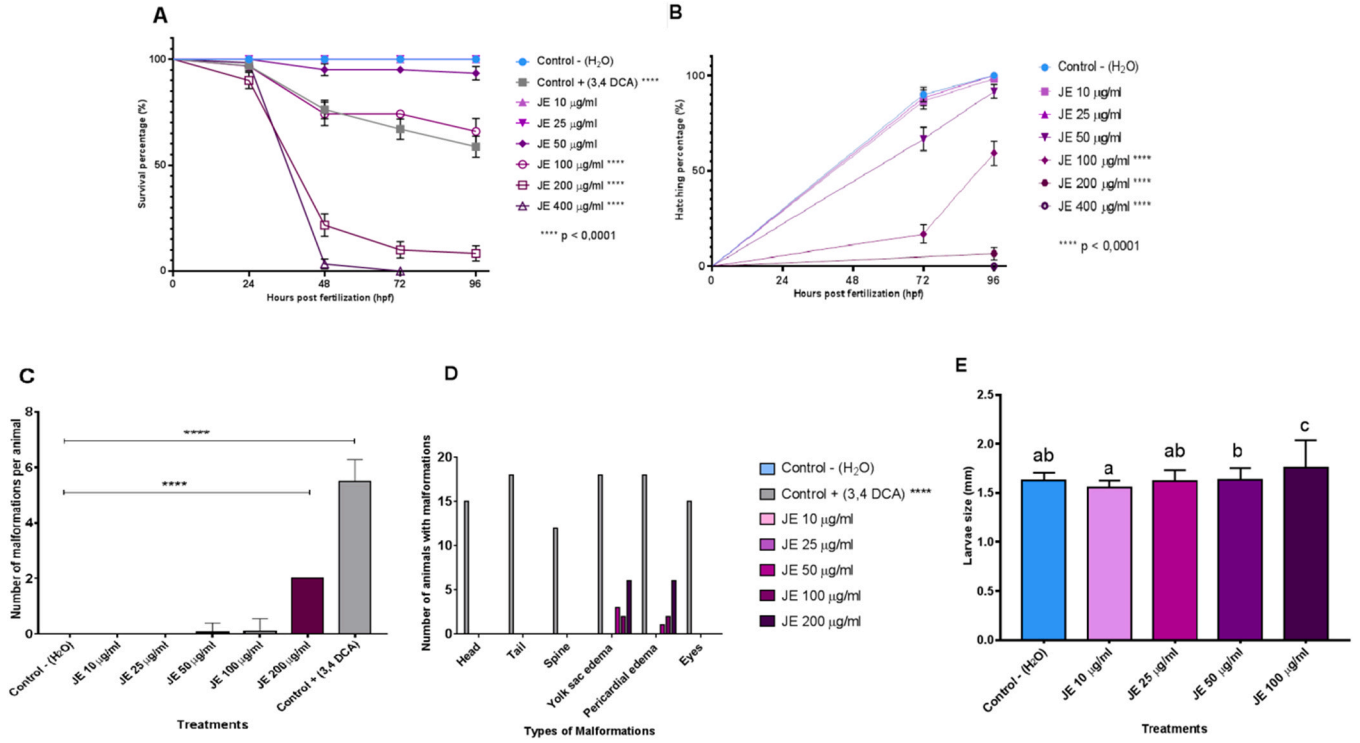

Fig. 2. Embryotoxicity endpoints in zebrafish exposed to JE for 96 h. Exposed animals were compared with control groups: Control - (water), Control + (3,4 Dichloroaniline). Six JE concentrations were tested (10, 25, 50, 100, 200, and 400 µg/ml, n = 20 for each concentration), with survival (A), hatching (B), and malformations (C – total malformations, D – types of malformation) assessed daily over 96 h. Larvae body size was measured at 96hpf. Survival (Log-rank test $p < 0.0001$), hatching (Log-rank test $p < 0.0001$), malformations (* indicates statistical significance by Anova, $p < 0.05$) and body size (different letters indicate significance between groups by Anova, $p < 0.05$) showed statistical significance between groups.

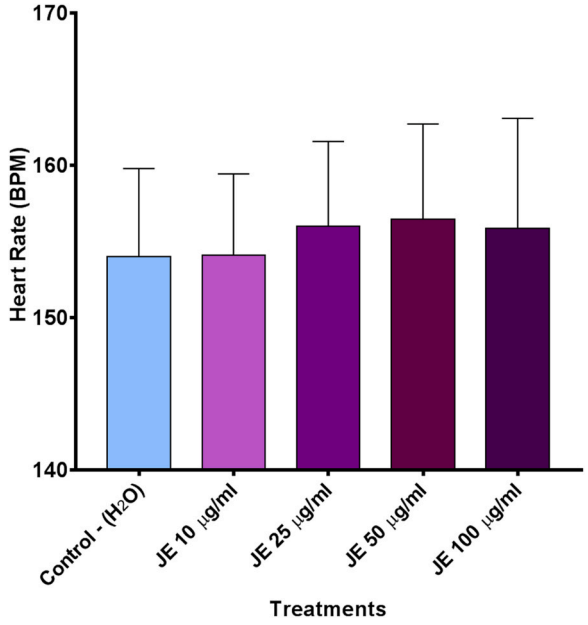

Fig. 3. Number of heartbeats per minute (bpm) of 96hpf larvae exposed to different JE concentrations. Animals were exposed to JE for 96 h (from egg to larvae). Control - (water, n = 20), JE (Jambolan extract) 10–100 µg/ml (n = 20). Bars represent mean ± SD. Results were not statistically significant between groups.

groups: distance from the bottom ($F(4, 70) = 1.201$; $p = 0.3182$), time at the bottom ($F(4, 70) = 1.051$; $p = 0.3873$) and latency to the top area ($F(4, 59) = 1.071$; $p = 0.3789$).

In the Open Field test, fish could individually explore a circular tank. Locomotion and anxiety-like behavior, measured by the time close to the tank border, were assessed. One-way ANOVA indicated statistical significance for average speed ($F(4, 70) = 5.428$, $p = 0.0007$) and Tukey's post hoc test showed that 50 µg/ml of JE caused an increase in speed compared with the other groups (Fig. 7A). For total distance traveled, one-way ANOVA showed statistical significance ($F(4, 70) = 5.412$, $p = 0.0007$) between groups and, again, Tukey's test indicated 50 µg/ml as the group presenting the highest distance traveled (Fig. 7B). Time spent at the tank periphery, which suggests anxiety-like behavior, did not differ between groups ($F(4, 70) = 1.64$; $p = 0.1739$; Fig. 7C). Regarding thigmotaxis index, animals exposed to JE at all concentrations showed a typical thigmotaxis response seen in the control fish (One-way ANOVA, $F(4, 64) = 1.082$, $p = 0.3730$; Fig. 7D).

For the sociability test, one-way ANOVA indicated a statistical significance for average swimming speed ($F(4, 68) = 3.427$, $p = 0.0130$) and total distance traveled ($F(4, 68) = 2.811$, $p = 0.0320$). In both tests, Tukey's post hoc showed that 100 µg/ml of JE reduced locomotion compared with control (Fig. 8A and B). However, there was no statistical significance for distance from the group between experimental groups ($F(4, 70) = 1.292$, $p = 0.2814$; Fig. 8C).

In the aggressiveness test, one-way ANOVA indicated a statistical significance between groups for average speed during movement ($F(4, 70) = 2.569$, $p = 0.0454$) and total distance traveled ($F(4, 70) = 2.603$, $p = 0.0431$). Tukey's post hoc test showed increased locomotor response for the 50 µg/ml group compared with 25 µg/ml (Fig. 8D and E). As for the distance fish kept from the mirror, there was no statistical significance between groups (One-way ANOVA, $F(4, 70) = 0.9814$, $p = 0.4234$; Fig. 8F).

Lastly, for the response to the predator threat, one-way ANOVA indicated a statistical significance for overall average speed ($F(4, 70) = 3.08$, $p = 0.0214$) and average speed within the predator quadrant (F

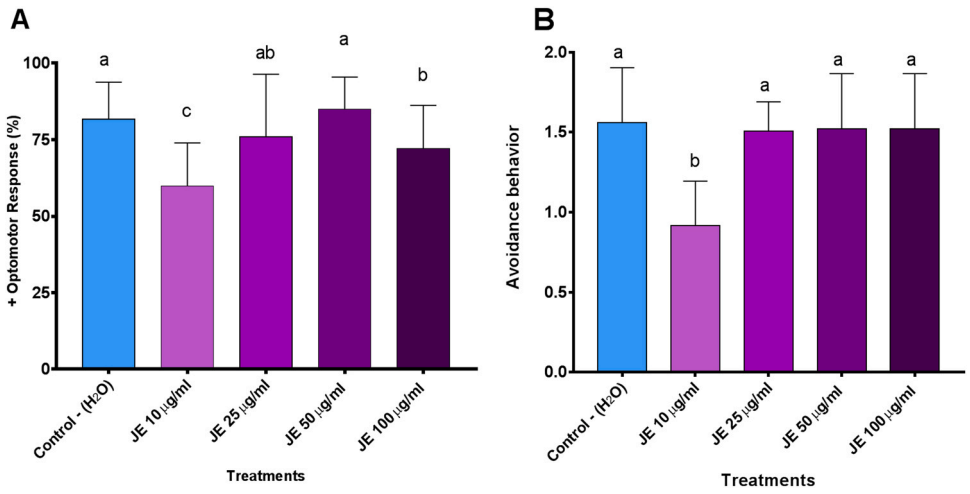

Fig. 4. A – Optomotor Response and B – Avoidance Response of 7dpf zebrafish larvae. Animals were exposed to JE for 96 h (from egg to larvae). Tested groups: Control - (water, n = 12), JE 10–100 µg/ml (n = 12). Bars represent mean ± SD. Different letters above bars indicate statistical significance (One-way ANOVA followed by Dunnett's test ($p < 0.0001$).

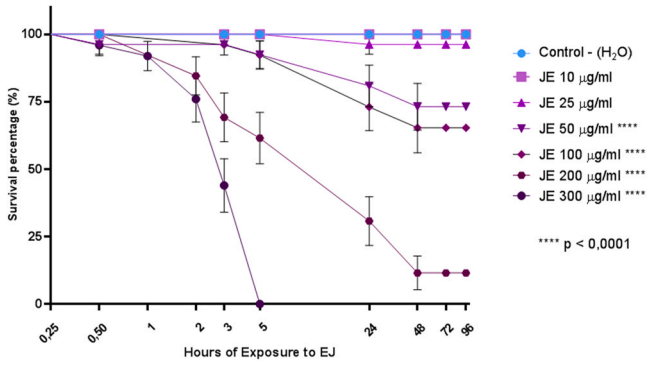

Fig. 5. Adult zebrafish (6–7 months) survival rate during 96 h exposure to JE. Animals (n = 25 each group) were exposed to water (control group) and different JE concentrations (10, 25, 50, 100, 200, and 300 µg/ml). Survival was checked every 24 h and dead animals removed from the tanks. Bars represent mean ± SD. Statistical significance was observed between groups (Log-rank test $p < 0.0001$).

(4, 66) = 3.317, p = 0.0155). In both cases, only the 10 µg/ml group presented reduced locomotion compared with control (Fig. 9 B and D). No effect was observed between groups for total distance traveled in the whole tank (F (4, 70) = 0.5053, p = 0.7319; Fig. 9A) but the distance traveled within the predator quadrant was statistically significant (F (4, 70) = 4.207, p = 0.0474), with the 25 µg/ml group showing increased distance compared with control (Fig. 9C). Time spent in the predator quadrant was statistically significant between groups (One-way ANOVA, F (4, 69) = 2.93, p = 0.0277). Tukey's test indicated that the 50 µg/ml group stayed longer in the threat area compared with control (Fig. 9E). There was no statistical significance in the number of entries into the predator quadrant between groups (F (4, 70) = 2.489, p = 0.0510; Fig. 9F).

4. Discussion

This study showed that jambolan extract is rich in phenolic compounds, including anthocyanins, with antioxidant capacity. Notably, a concentration of 25 µg/ml of TMA was deemed safe, showing no morphological, cardiac, or neurological toxicity in zebrafish embryos and no toxicity in adult zebrafish. Toxic effects were only observed at concentrations above 50 µg/ml, with LC50 values of 118.4 µg/ml for embryos and 68.86 µg/ml for adults. To our knowledge, this is the first study to evaluate the biosafety of jambolan extract in an in vivo model.

Phenolic compounds, concentrated in seeds and outer plant tissues, are key bioactive phytochemicals [56]. We found jambolan to have a phenolic content of 2180.4 mg GAE/100 g, higher than previous reports [23,28,51], likely due to differences in extraction methods, including solvent ratios and techniques. Such variations also stem from factors like fruit maturity, cultivation location, and climate.

Anthocyanins, major phenolic compounds in jambolan, were measured at 1139.12 mg/100 g in this study, surpassing prior findings [35,52,59] and other anthocyanin-rich fruits like açaí and blueberry. Environmental factors like solar radiation influence anthocyanin content, as reported by [38], who found that reduced light exposure lowered anthocyanin synthesis. Such high anthocyanin levels reflect effective extraction and optimal fruit maturation, with anthocyanin accumulation peaking during ripening.

HPLC analysis identified high levels of Malvidin 3,5-diglucoside (2683 mg/kg) and Cyanidin 3,5-diglucoside (356 mg/kg), aligning with Lestario et al. [35] but surpassing results from de Brito et al. [18] and Faria et al. [23]. Despite not using acidified solvents, our extraction efficiency was comparable or superior, influenced by factors such as extraction time, temperature, and fruit maturation.

Two unidentified peaks at 8.1 and 9.8 min may correspond to Delphinidin 3,5-diglucoside and Petunidin 3,5-diglucoside, suggesting potential underestimation of anthocyanin content. JE extract also contained Procyanidin B2 (457.26 mg/kg), Caffeic acid (151.33 mg/kg), and Gallic acid (114.06 mg/kg), known for their antioxidant, anti-inflammatory, and health-promoting properties [3,53]. Ethanol-water extraction (70:30, v/v) at room temperature effectively preserved phenolic compounds, performing on par with non-conventional methods [52,54].

JE exhibited ABTS values thrice as higher than previous reports [51, 55,61], inhibiting ABTS+ radicals in a concentration-dependent manner, with maximum inhibition (86.2 %) at 1.0 mg/ml. IC50 (0.606 mg/ml) was higher than reported in other studies [10,2], indicating lower antioxidant activity probably due to differences in ABTS volume used (260 µl vs. 180–4000 µl in other studies).

For DPPH, JE showed antioxidant activity of 844.70 ± 58.8 mmol Trolox Eq./g, comparable to red fruits [12] and surpassing popular fruits like strawberries, blueberries, and blackberries. This value also exceeded those for purple carrots, which range from 12.3 to 727.2 mmol Trolox Eq./kg [47].

Assessing the *in vivo* safety of JE is essential for potential food and

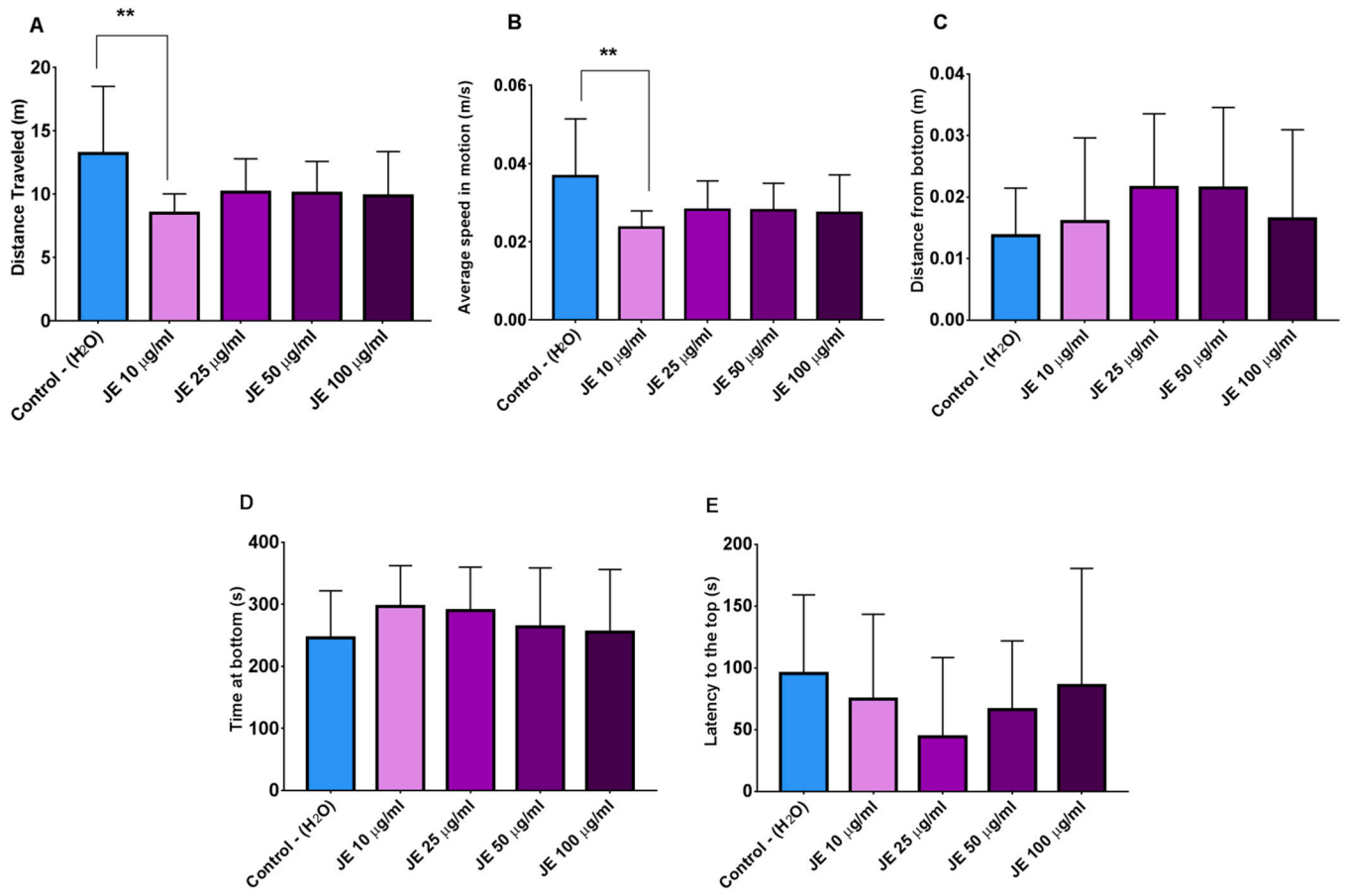

Fig. 6. Locomotor activity and anxiety-like behavior of zebrafish in the Novel Tank Test over 6 min. Zebrafish (6–7 months, n = 15 each group) were exposed to different JE concentrations (0 – control, 10 µg/ml, 25 µg/ml, 50 µg/ml, and 100 µg/ml) for 96 h and then tested for behavioral response in the novel tank. Data include A) Total distance traveled, B) Average moving speed (m/s), C) Distance from the bottom (m), D) Time spent at the bottom (s), and E) Latency to the top area (s). Data are expressed as mean ± SD. Asterisks indicate statistical significance between JE exposures (One way ANOVA, p = 0.005).

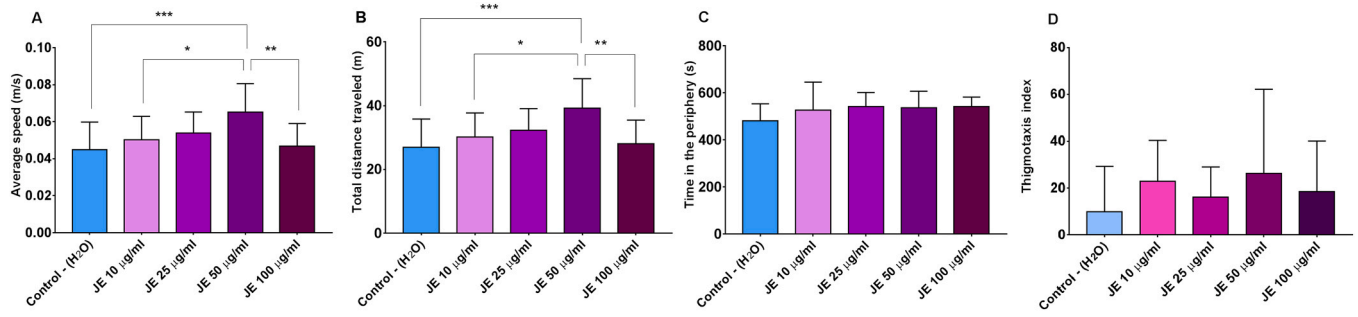

Fig. 7. Locomotor and anxiety-like behavior of zebrafish in the Open Field test over 10 min. Zebrafish (6–7 months, n = 15 for each group) were exposed to different JE concentrations (0 – control, 10 µg/ml, 25 µg/ml, 50 µg/ml, and 100 µg/ml) for 96 h and then tested for behavioral response in the open field. Data include A) Average speed (m/s), B) Total distance traveled (m), C) Time spent in the periphery (s), and D) Thigmotaxis index. Data are expressed as mean ± SD. Asterisks indicate statistical significance between JE exposures (*p ≤ 0.05), (**p ≤ 0.005), (***p = 0.0008).

medicine applications. Here, zebrafish embryos and adults exposed to JE presented LC50 values of 118.4 µg/ml and 68.86 µg/ml, respectively, with higher toxicity observed in adults. This difference may be due to the protective role of chorion in embryos which filters compounds larger than 3 kDa [4], whereas adults directly absorb compounds through their gills, bypassing digestion and increasing the risk of organ accumulation [48]. [5] found a strong correlation (r = 0.9) between fish embryo tests (FET) and traditional adult fish toxicity tests. This supports FET (OECD TG 236) as a reliable alternative for predicting acute fish toxicity, aligning with the results of conventional adult tests (OECD TG 203). Moreover, sensitivity to toxicants varies depending on their mode of action, with certain chemicals like neurotoxins and endocrine disruptors affecting embryos and adults differently due to variations in receptor expression and physiological processes. Adult fish possess active biotransformation enzymes that can alter the toxicity of certain compounds, leading to different outcomes compared with embryos. While FETs are valuable tools for predicting acute toxicity, these differences must be carefully considered when extrapolating results to adult fish.

Flavonoids can act as antimutagens or pro-mutagens and antioxidants or pro-oxidants, depending on concentration and physiological

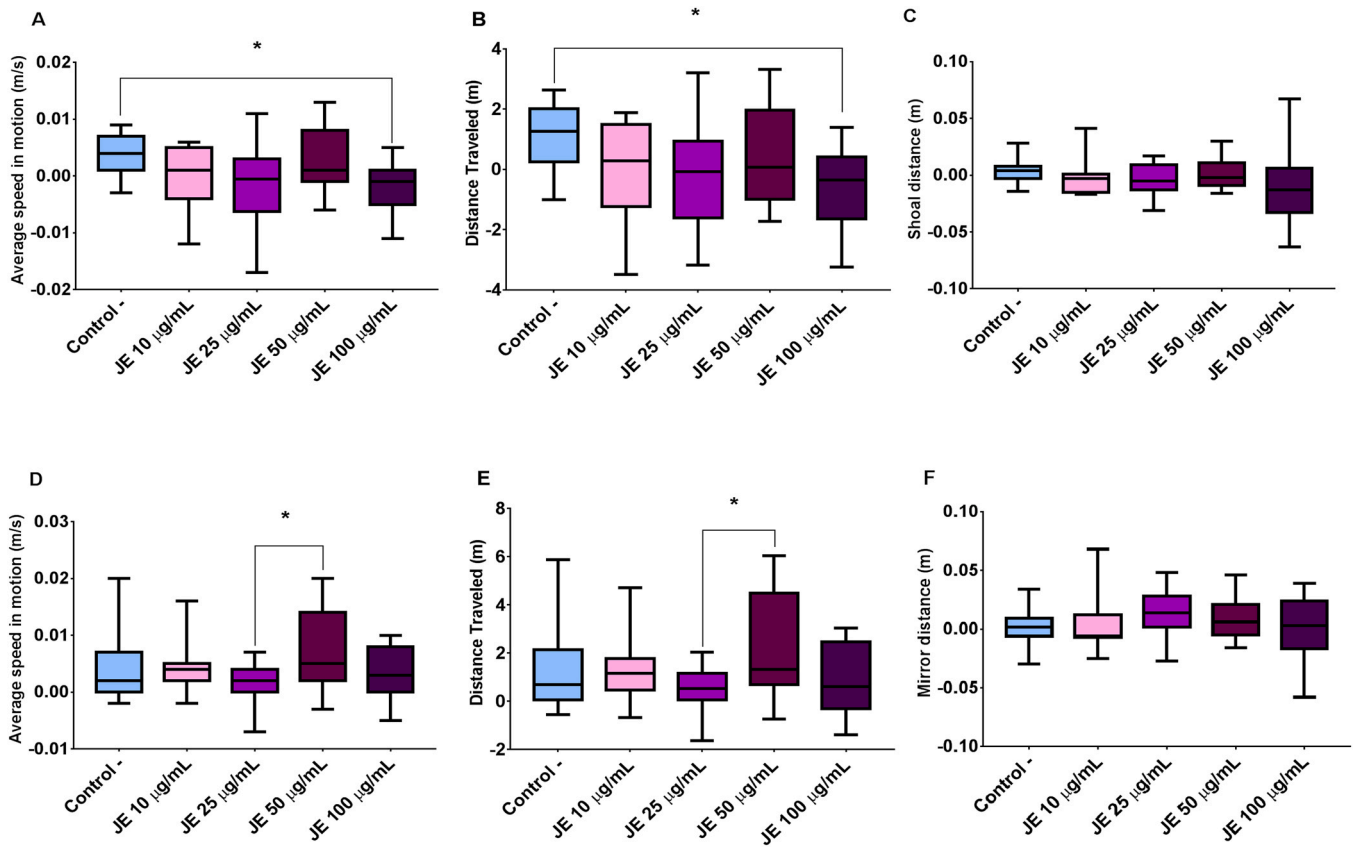

Fig. 8. Behavioral response of adult zebrafish in the sociability (A, B, C) and aggressiveness tests (D, E, F). Zebrafish (6–7 months, n = 15 each group) were exposed to different JE concentrations (0 – control, 10 µg/ml, 25 µg/ml, 50 µg/ml, and 100 µg/ml) for 96 h and then tested for behavioral response to the social group (sociability) and to the mirror image (aggressiveness). Data include A) Average speed (m/s), B) Total distance traveled (m), and C) Distance to the shoal (m) for the sociability test, D) Average speed (m/s), E) Total distance traveled (m), and F) Distance to the mirror (m) for the aggressiveness test. Data are expressed as median ± quartiles of the difference between fish behavior before and after the stimulus. Stimulus was the shoal for the sociability test and the mirror for the aggressiveness test. Fish behavior was recorded 5 min before and 5 min after the stimulus. Asterisk indicates statistical significance between JE exposures (*$p \leq 0.05$).

conditions. High levels may generate reactive oxygen species and cause DNA damage [57]. Here, JE concentrations above 50 µg/ml caused malformations such as pericardial and yolk sac edema, aligning with findings from Chensom et al. [13] and Kundap et al. [33], who reported similar effects at high anthocyanin concentrations.

Ali et al. [1] observed lower toxicity for Davidson's plum (54 % anthocyanin) compared with JE (≈82 % anthocyanin), suggesting varying toxicity limits among phenolic compounds. Other studies [19, 30] confirm that toxicity depends on extract type, concentration, and phenolic interactions which can amplify effects. Based on OECD and ECHA classifications, JE is non-toxic to embryos but harmful to adults. Safe application requires concentrations around 10 % of the LC50 to minimize adverse effects.

Other levels of toxicity should be considered when searching for a compound biosafety. Here, zebrafish embryos exposed to 10 and 25 µg/ml of JE showed no cardiac malformations and heart rates of 138–168 bpm, suggesting no cardiotoxicity. Normal embryonic heart rates in zebrafish range from 120 to 180 bpm, a value much closer to that of humans than more traditional models, like rats [20].

Response (OMR) and avoidance behavior reflect nervous system integrity and are valuable tools for neurotoxicity research. Most larvae exposed to JE showed positive visual responses, indicating no significant neurological damage. At 10 µg/ml, however, larvae performed poorly in both OMR and avoidance tests. This may be due to hormesis, a biphasic dose-response phenomenon where low concentrations of a given substance can have detrimental effects, whereas higher concentrations may trigger protective or adaptive responses [7,8]. While JE presented no overt neurological defects, the observed poor performance at lower concentrations might result from a specific compound in the extract affecting cell differentiation during the embryonic phase, potentially causing cognitive delays. The exact compound responsible, however, remains undetermined as JE is a crude extract composed of multiple phenolic compounds. Moreover, no existing research has documented deleterious effects of anthocyanins on the nervous system during embryonic development.

Zebrafish are increasingly used in biomedical research due to their complex behavior which help investigate mood and affective disorders [16]. They exhibit anxiety-like behaviors such as increased latency to explore and preference for tank edges when exposed to stressors like new environments or chemicals (K. M. [31]). Anxiety is also indicated by reduced exploratory activity and slow habituation [37]. Flavonoids, particularly anthocyanins, are known for their antioxidant properties and potential neuroprotective effects, with studies highlighting their potential to influence neuroinflammation and disorders like depression and anxiety [63].

Here, the novel tank test showed that 10 µg/ml of JE reduced zebrafish locomotion, but this effect was not observed in other behavioral tests. In the open field, 50 µg/ml of JE increased locomotion whereas 100 µg/ml reduced it in the sociability test. In the aggressiveness test, 50 µg/ml of JE increased locomotion compared with 25 µg/ml, but none of the experimental groups differed from control. For predator avoidance, 10 µg/ml of JE reduced locomotion whereas 25 µg/ml increased it. Overall, anxiety, social, aggressive, and predator avoidance behaviors were not affected by JE exposure. While it may influence locomotion, the effects were inconsistent and likely due to specific testing conditions, including animal isolation.

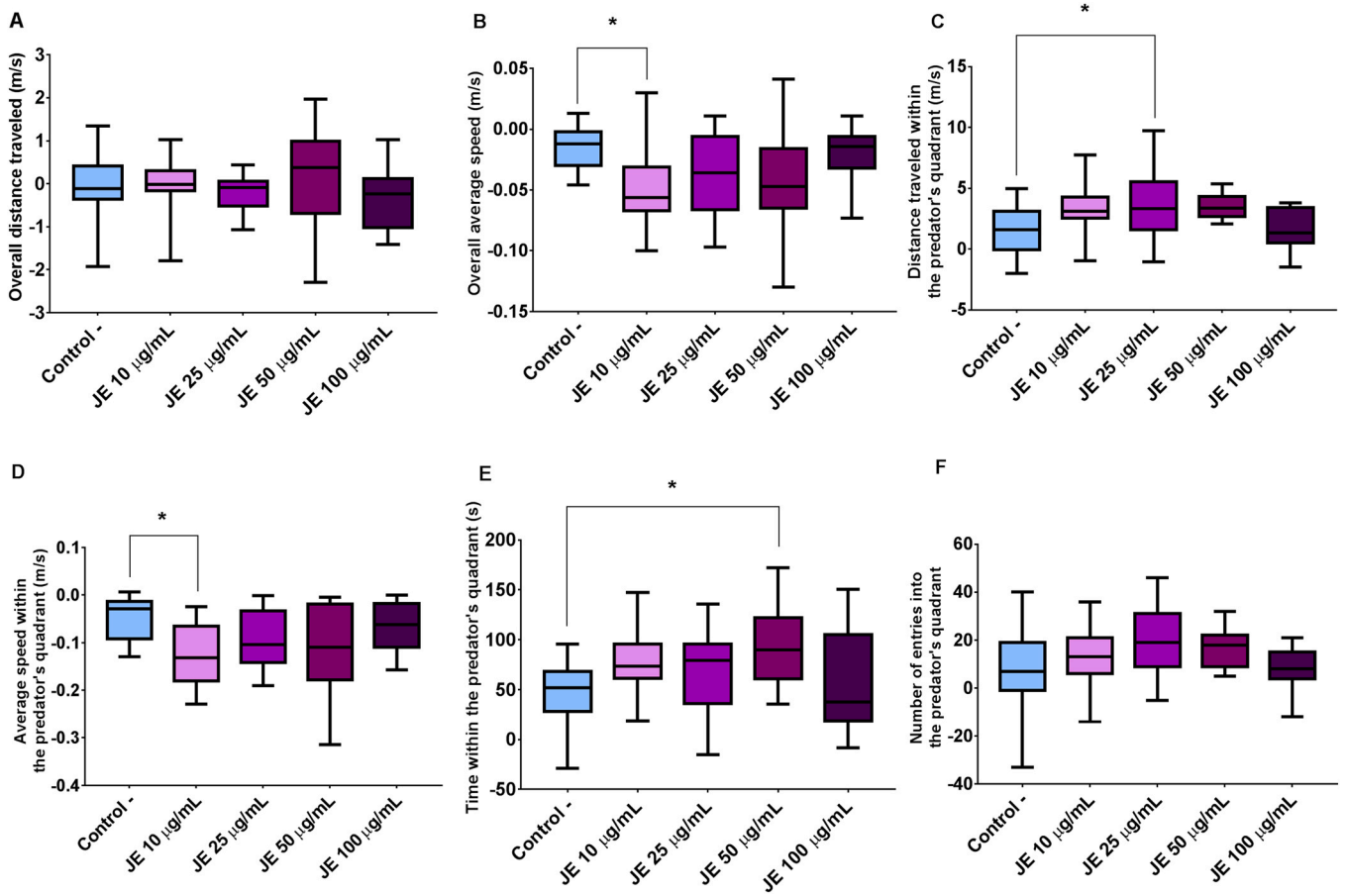

Fig. 9. Behavioral response of adult zebrafish in the avoidance test. Zebrafish (6–7 months, n = 15 each group) were exposed to different JE concentrations (0 – control, 10 μg/ml, 25 μg/ml, 50 μg/ml, and 100 μg/ml) for 96 h and then tested for behavioral response in a circular tank before (3 min) and after (3 min) being threatened by a predator (bird model). Data include A) Distance traveled (m), B) Swimming speed (m/s), C) Distance traveled in the area where the predator appeared (m/s), D) Average speed in the area where the predator appeared (m/s), E) Total time within in the area where the predator appeared (s), and F) number of entries in the area where the predator appeared. Data are expressed as median ± quartiles of the difference between fish behavior before and after predator presentation. Asterisk indicates statistical significance between JE exposures (*$p \leq 0.05$).

Comparative studies on phenolic compounds show varied behavioral effects. Capatina et al. [9] found that Origanum vulgare essential oil prevented scopolamine-induced hypolocomotion and presented anxiolytic effects at higher doses, whereas Thayumanavan et al. [60] reported that flavonoids like silibinin and naringenin mitigated bisphenol-A-induced behavioral changes in zebrafish. Research on cantaloupe melon extracts and melamine exposure found no significant behavioral changes [11,45].

Despite observed behavioral differences, JE did not increase anxiety or cause cardio and neurotoxicity at the tested concentrations. Further studies are needed to assess its effects on morphological, biochemical, hormonal, and genetic parameters to ensure JE safety and efficacy, and investigate the chronic effects of sublethal concentrations (10 % of LC50). The toxicological potential of flavonoids and phenolic compounds, particularly at chronic doses, warrants further exploration as the current literature focuses primarily on their benefits, with little known about potential harmful effects from excessive acute or chronic intake.

The doses of jambolan extract used in this study were based on the work of Chensom et al. [13], who analyzed Titanbicus (TB), a hybrid hibiscus species with potential as an edible flower. This flower is rich in bioactive compounds, particularly anthocyanins, the main compound found in jambolan extract. In this study, concentrations ranging from 50 to 200 μg/ml were used to assess the acute toxicity of the extract in zebrafish embryos. The 50 μg/ml dose was found to be safe for 100 % of the animals, a result very similar to ours for both embryos and adult fish. Additionally, Chensom et al. conducted acute toxicity tests in rats using the same concentrations, estimating an LD50 above 2000 mg/kg of body weight. According to the authors, these findings indicate that TB has low toxicity and is suitable for human consumption. Although our study did not include toxicological tests in other animal models, the similarity in concentrations and compounds between both studies suggests that our extract may also be safe for human consumption. However, we acknowledge the need for further research on this topic and will continue conducting toxicity studies on jambolan extract in other animal models to ensure its safety and feasibility for human consumption in the future.

5. Conclusions

Our results suggest that when administered at concentrations below 50 μg/ml, JE does not cause toxic effects at either life stage analyzed. Despite no major signs of neurotoxicity, the study cautions for low and medium JE concentrations as it seems to present a nonmonotonic concentration-response curve. Thus, this study provides a comprehensive and multifaceted evaluation of JE's toxicological potential, offering new insights into its safety and efficacy and paving the way for further research into this compound's biological properties.

Credit author statement

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manuscript.

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CRediT authorship contribution statement

Silva-Maia Juliana Kelly: Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Luchiari Ana Carolina:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **dos Santos Lima Marcos:** Methodology, Investigation. **de Araújo Morais Ana Heloneida:** Writing – original draft, Conceptualization. **dos Santos Jéssica Anarellis Barbosa:** Investigation. **de Sousa Teixeira Júlia Robert:** Investigation. **dos Santos Melo Yohanna Layssa:** Investigation. **Lopes Beatriz Silva:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT in order to improve language and readability, with caution. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Declarations of interest: none

Data availability

Data will be made available on request.

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