RESEARCH ARTICLE

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In-vivo Toxicity Evaluation of 3-(2-(3,4 dimethoxyphenyl)-2 oxoethylidene) Indolin-2-one (RAJI) in Zebrafish and Mice Model

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Abstract

Objective: Breast cancer is a global health concern, with millions of cases reported annually worldwide, making it the most common cancer among women. In India, the incidence of breast cancer has been steadily rising, reflecting a growing public health challenge and hence in the development of new drug moieties. Toxicity analysis of such novel drug candidates play a critical role in drug development, ensuring the safety and efficacy of potential therapeutics. Animal models, especially mice and zebrafish in the recent days, have been extensively used for toxicity evaluation owing to their physiological and genetic similarities to humans. This study was hence conducted with an aim to assess the toxicity using animal models, particularly mice and zebrafish. **Methods:** In this study, 3-(2-(3,4-dimethoxyphenyl)-2-oxoethylidene)indolin-2-one (RAJI) - a chemically synthesised novel drug, was assessed for its toxicological potential in both zebrafish and mice models highlighting its survival, hatching, locomotor, neuromotor, behavioural abnormalities in zebrafish model and haematological and biochemical abnormalities in mice model. **Results:** The results obtained emphasise that no significant damages were seen in both zebrafish (survival, hatching, locomotor, neuromotor, behavioural abnormalities) and mice (body weight, haematological and biochemical abnormalities) models when administered in low doses. **Conclusion:** All results obtained signifies that RAJI poses no harmful effects in the model organisms until administered in higher concentrations, thereby emphasising the fact that RAJI is a safe drug.

Keywords: Breast cancer- Toxicity analysis- Drug moiety- Zebrafish- Mice model.

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Introduction

Breast cancer, a heterogeneous and a complex disease has been reported as the most commonly diagnosed and leading cause of cancer-associated deaths in women worldwide. HER-2, luminal A, luminal B, claudin-low, and basal-like are the major subgroups having different metastasis abilities, pathways, and survival rates even after its relapse [1]. Chemotherapy has played a major role in cancer treatment. Chemotherapy drugs frequently exhibit hydrophobicity which necessitates higher dosages to attain therapeutic concentrations due to their diminished biocompatibility [2].Additionally, the limited hydro solubility results in high systemic toxicity, reduced drug bioavailability, minimal specificity and substantial harm to unaffected tissues [3–5], which results in adverse reactions thus highlighting the need for advanced testing to translate preclinical toxicity assessment to the clinical setting with greater accuracy and sensitivity [6].

Toxicity is a prominent problem in the drug development sector these days, making it an important issue for researchers to conduct extensive research. During the past few decades, many reports have declared that a notable percentage of approved drugs have been revoked from the market owing to their severe adverse reactions, including fatalities [7,8]. Because of the prevalence of therapeutic failures attributable to toxicity and clinical safety, toxicity assessment is rapidly gaining importance in the drug development process. Recent data show a significant rise in negative clinical responses linked to newly manufactured medications impacting several organs [6]. These findings highlight the absolute need of developing more sophisticated testing procedures to improve the accuracy and sensitivity of shifting the toxicity evaluations from the lab to the clinical settings [6,8]. Enhanced techniques for assessing toxicity are crucial in order to promptly detect any possible harmful effects during the development phase, hence enhancing the chances of achieving clinical success and ensuring patient safety [8].

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Zebrafish (Danio rerio) have been suggested as a suitable model for assessing toxicity levels due to their physiological, morphological, and histological similarities to mammals [9–12]. There are numerous advantages to using zebrafish as a model organism in biomedical research. It is important to note that they demonstrate a high degree of gene homology to humans, with approximately 70% of human genes having at least one zebrafish ortholog. This genetic similarity facilitates the examination of human gene functions and disease mechanisms within a biological context that is comparable. Furthermore, the thorough sequencing of the zebrafish genome has enabled the development of sophisticated genetic manipulation techniques and has provided a comprehensive understanding of their genetic composition [13]. Additionally, the transparency of zebrafish embryos and larvae facilitates the real-time visualization of physiological responses and developmental processes at the cellular and subcellular levels, further enhancing their utility. Zebrafish are a potent model for the investigation of human genetics, disease pathology, and drug development due to the combination of these attributes [14]. Significant cost convenience, shorter cycles, smaller quantities of test chemicals, and high throughput results prove to be the advantages of using zebrafish in various parameter evaluations. Various toxicity research benefit from the zebrafish model. Developmental toxicity studies determine how poisons cause embryonic abnormalities, while teratogenicity research identifies substances that cause birth deformities [11]. Behavioural toxicity studies use zebrafish to examine how toxicants affect brain function and behaviour. In cardiovascular toxicity studies, their translucent embryos depict heart growth and function. Owing to their similarity to the human liver and well-characterized kidney systems, zebrafish are useful for investigating hepatic and renal damages. Also, other toxicological evaluations include endocrine disruption, neurotoxicity, and immunotoxicity in zebrafish models [12].

Similarly, toxicity analysis using mice models continue to be an invaluable tool in drug development, providing critical insights into the safety profiles of drug molecules. Recent studies have enhanced our understanding of acute toxicity, organ-specific toxicity, and the impact of drugs on haematological and biochemical parameters. Mouse models have long been recognized as invaluable tools for studying human diseases, including cancer. The intricate similarities between mouse and human biology, coupled with the ability to manipulate their genomes, make mice an ideal model organism for anti-cancer drug evaluation.

3-(2-(3,4-dimethoxyphenyl)-2-oxoethylidene) indolin-2-one (RAJI) is a novel drug that has been synthesized in the Department of Pharmacy, 'Indira Gandhi National Tribal University', Madhya Pradesh, India. The components of RAJI includes piperidine, isatin, 3,4-dimethoxy acetophenones and concentrated hydrochloric acid. Piperidine, with the molecular formula (CH2)5NH, is a heterocyclic amine having one amine and five methylene bridges (-CH2-) in its six-membered ring. It is a clear liquid with a pungent odour that is characteristic of amines and is used as a solvent or a base. Isatin is the oxidation product of indigo using nitric and chromic acids. It crystallizes from water, alcohol, or acetic acid in the form of orange-red monoclinic prism crystals melting at 200°C. And, 3′,4′-Dimethoxyacetophenone is a yellowish to beige coloured crystalline powder that is generally used as catalytic agent while synthesizing drugs in the pharmaceutical industry. Since, the drug RAJI is a novel compound, its efficacy was assessed for its behavioural, locomotory and neuro motor responses in zebrafish model followed by a detailed haematological and biochemical evaluation in mice model. The complete assessment procedure was categorized as acute and chronic toxicity which entails exposing animals to the given compound for a period of 14 and 21 days and its activity were analysed.

Materials and Methods

Synthesis of RAJI

The synthesis of 3-(2-(3,4-dimethoxyphenyl)-2-oxoethylidene)indolin-2-one (RAJI) was accomplished through a one pot protocol involving the sequential addition of piperidine and concentrated Hydrochloric acid to a reaction mixture containing isatin and 3,4-dimethoxy acetophenones as depicted in Figure 1.

Toxicity Evaluation In Zebrafish

Zebrafish Maintenance And Embryo Collection

Wild type zebrafish (AB Strain Danio rerio), 6 months of age were used for the study. All fish were acclimated to constant laboratory conditions (14-h light: 10-h dark photoperiod, diet, water, 28°C) for one week in stock aquaria before all experiments were conducted. They were fed with commercial Tetrabit flakes (a complete pet food for Tropical fish from Tetra GmbH, Herrenteich) daily and Artemia salina once a week, until the beginning of the experiments. All fish used in these experiments were random adults from different clutches. 20-09-2024 Housing tanks were cleaned once in every 4 days to keep the fish clean and free from infection.

According to the CPCSEA guidelines, India, zebrafish larvae less than six days were used for developmental toxicology study. The fertilized embryos were observed under the microscope and unfertilized embryos were discarded [15]. Healthy embryos were collected and housed in the embryo medium (E3 medium) containing NaCl (29.4 g/100 mL), KCl (1.27 g/100 mL), CaCl2 2H2O (4.85 g/100 mL), and MgSO4 7H2O (8.13 g/100 mL) and was maintained at a temperature of 28 +/- 1°C and pH between 6.8-7.5.

Developmental Toxicity

Grouping and Dosing

The selected embryos were grouped in a ratio of 10 per well in a 12 well plate. The stock compound RAJI was prepared by dissolving 5 mg of the compound in 5 ml of Phosphate Buffered Saline (PBS). The dosing was initiated within 4 hours post fertilization (hpf). Groups were assigned as 1 control and 7 different dilutions, represented as D1 to D7 constituting 10, 25, 50, 75, 100, 125 & 150 µg of RAJI, which were added into the respective wells.

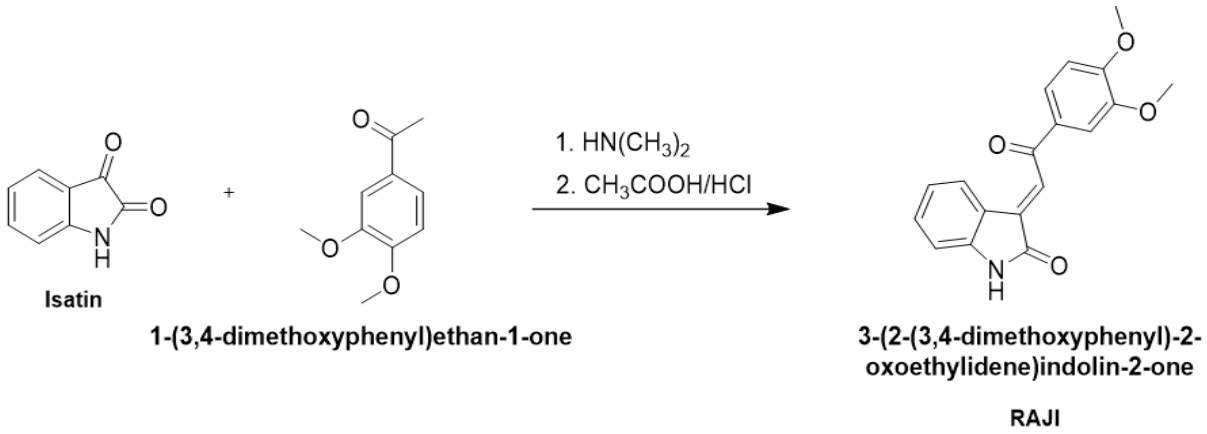


Figure 1. Schematic Representation of the Synthesis of RAJI

The morphological defects such as, heartbeat, spontaneous movement, and hatching rate were all noted since these are crucial developmental endpoints [16,17]. Images of morphological defects were photographed and approximately 90% of the drug solutions were changed daily with fresh dilutions [18].

Toxicity Evaluation In Larvae
Grouping and Dosing

Healthy embryos were collected and maintained in E3 medium. The stock compound RAJI was prepared as 1mg/ml concentration from which 4 further dilutions - D1, D2, D3, & D4 constituting 0.2, 2, 20 & 200 µg of RAJI were prepared. Embryos subjected to only E3 medium served as the control. The dosing was initiated on the 2nd day post fertilization. Washout was done from day 2 to day 5 where the embryo medium was changed every day with the addition of respective dose. Study tanks were filled with system water and the larvae which were picked in random from the different treatment groups were analysed for any locomotor, neuromotor and behavioural abnormality.

a) Locomotion Freezing Response Assay

Changes in the movement pattern in the larvae is used to determine the abnormal locomotion freezing behaviour when treated with the test compounds. The zebrafish larvae are placed in a rectangular tank of about 17cm*14cm*12cm (l*b*h) with water capacity of about 5L and given 1 minute of acclimatization time post which its behaviour is analysed for 3 minutes. The presence of movement is marked as 1 and absence as 0.

b) Burst Movement Assay

Darting pattern of movement is generally observed in the zebrafish larvae under stress, where they move forward (burst) in a single motion and glide (coast) to a slow speed, or stop abruptly and then burst forward again. Such pattern changes were studied in zebrafish larvae using the above-mentioned housing conditions.

c) Stereotyped Movement Assay

The stereotype response of zebrafish larvae to move forward when faced with external stimuli was studied with the housing conditions mentioned previously.

d) C-Bend Response Assay

In a circular drum filled with 30 ml water, larvae were discharged and given 1 minute acclimatization time post which their behaviour were analysed. In response to an external stimulus, the larvae first curves to form a C-shape, and then propel itself away at an angle from its previous position, with a fast swim. The study was observed for 5 touches with capillary tube, and the number of responses per 5 touches is noted.

e) Mortality Assessment

Mortality was counted on an everyday basis to understand mortality curve over therapeutic intervention.

f) Data Processing

The percentage abnormality was calculated from the qualitative analysis. Presence of abnormality was indicated as 1 and absence as 0. Average of these values was used to calculate the percentage abnormality for each of the study group.

Toxicity Evaluation In Adult Fish
Animal Grouping and Dosing

Adult male fish of similar body weight (approximately 0.8 grams per fish) were selected for the study and housed as 8 per group. Study fish were grouped into 5 groups – control and 4 dilutions. Dilution 1, 2, 3 & 4 represented as D1, D2, D3, & D4 constitutes 0.2, 2, 20 & 200 µg of RAJI dissolved in the commercial pellet feed. Control group fishes were fed with the pellet feed without RAJI incorporation. The selected adult fish were transferred to individual study cups containing housing water and were housed in a ratio of 1 fish per study cup for all the 5 groups. The groups were dosed every day with 2 pellets per day. The study was conducted for 2 phases – Acute (14 days) and Chronic (21 days). On days 14 & 21 the adult fish were analysed for locomotor, neuromotor and behavioural abnormalities.

a) Startle Response Assay

A cylindrical drum with a white-coloured backdrop along with a dark visual stimulus strategically positioned

in its centre is used in this study. The drum is positioned outside an experimental bowl. During the rotation of the drum in both clockwise and anticlockwise directions (a total of 20 revolutions each), the zebrafish encounters the stimulus and promptly responds by initiating a spinning motion reflecting an evasion response. The observations of the fish's behaviour throughout this process are recorded.

b) Light and Dark Assay

An acrylic tank (15 cm × 10 cm × 45 cm height × width × length) is divided equally into one-half black and one-half white and filled with water to 3/4th of its capacity. Fish exposed to RAJI is then placed in the tank and allowed for 1 min acclimatization. Subsequently, the fish's preference for a particular-coloured compartment is observed and recorded for a duration of 3 minutes.

c) Memory Maze Test

A 5 L rectangular tank measuring approximately 17cm*14cm*12cm (l*w*h) with three incomplete partitions (hurdles) in the centre is used in this study. The study fish is introduced to one end of the tank and the feed is placed at the other end and the time taken for the fish to recognize the feed after crossing all the hurdles (feed recognition) and to get back to the same start place (learning and memory power).

d) Locomotor Score Test

A rectangular tank with 5 L capacity and approximately 17cm*14cm*12cm (l*w*h) dimensions is used in this study. A study fish exposed to RAJI is then placed in the tank and allowed for 1 min acclimatization post which any abnormalities in its pattern of locomotion is observed for 3 min time period.

e) Abnormal Activity Score Test

A rectangular tank with 5 L capacity and approximately 17cm*14cm*12cm (l*w*h) dimensions is used in this study. A study fish exposed to RAJI is then placed in the tank and allowed for 1 min acclimatization post which any abnormalities in its behaviour is analysed for a period of 3 minutes.

f) Mortality Assessment

Mortality was counted on an everyday basis to understand mortality curve over therapeutic intervention.

g) Data Processing

The percentage abnormality was calculated from the qualitative analysis. Presence of abnormality was indicated as 1 and absence as 0. Average of these values was used to calculate the percentage abnormality for each of the study group.

h) Histopathology

Heart, Liver, Pancreas and Brain tissue samples of control and RAJI treated groups (4 concentrations) from both acute and chronic toxicity experiments were collected from the sacrificed fish and smeared onto a glass slide which were later subjected to Haematoxylin and Eosin (H&E) staining. The slides were visualized under an optical microscope to analyse the presence of any histological alterations.

Toxicity Evaluation in Mice Model

Animal Grouping and Dosing

BALB/c mice with weighing approximately 25 gm were housed separately and Good Animal Practice was followed as per Institutional Animal Ethics Committee (IAEC) with approval no SVCOP/IAEC/014/2021-22 in accordance with the Committee for the Purpose of Control and Supervision of Experiments (CPCSEA), India.

For acute toxicity evaluation in mice, nine mice were divided into three groups of three mice each. The first group of mice were administered with normal saline orally for 14 days, which was treated as the negative control. The other two groups were treated with different doses of RAJI orally (25 and 100 mg/kg) for the same period of 14 days. On the fifteenth day, peripheral blood was collected by cardiac puncture for hematological analysis of liver, kidney and heart function. The mice were euthanized to collect organs like liver, kidney, lung and heart which were subjected to histological evaluation.

a) Body Weight Analysis

The body weight of all the animals were monitored prior and post the experimental period using a sensitive weighing balance and tabulated. Percentage changes in the body weight were calculated by the following formula: Body weight change = (day 1 – day 15) / day 1 x 100%.

b) Haematological Analysis

The concentration of haemoglobin present in the whole blood is measured using colorimetric method and Red Blood Cells, White Blood Cells, Platelets, Lymphocytes, monocytes, Eosinophils, Basophils, Neutrophils and % haematocrit were measured using the coulter principle by fully automated 3-part differential haematology analyser (Mindray BC-2800).

c) Biochemical Analysis

Liver profiles such as Alkaline phosphatase (ALP), Aspartate transaminase (AST) and Alanine transaminase (ALT) and cardio marker including Creatine Kinase (CK) were measured using enzymatic spectrophotometry IFCC method. Kidney profiles including blood urea was measured as blood urea nitrogen and creatinine was measured using modified Jaffe method by fully automated chemistry analyser (Mindray BS-120).

Histopathology

Liver, Kidney, Lung and Heart tissue samples of control and RAJI treated groups were collected. The tissues were washed in graded saline buffer, dehydrated using formalin and subjected to paraffin embedment. The embedded tissue samples were then sectioned using a microtome into 5µm sections and were carefully mounted on glass slides. These slides were then subjected to H & E staining and visualized under an optical microscope for investigating the presence of histological alterations.

Results

Effects of RAJI in Zebrafish

Developmental toxicity of RAJI in Zebrafish Embryos

A total of 10 zebra fish embryos were selected per group and were treated with different concentrations of RAJI (10, 25, 59, 75, 100, 125 & 150 µg/mL). The hatching rate was assessed at 48 hours post fertilization of the embryos. The hatching rate remained 100% till 75 µg/mL but experienced a decline with the increase in concentration at 100, 125 & 150 µg/mL. The survival percentage was least at day 4 at 150 µg/mL, whereas there was not much difference in the survival percentage below 150 µg/mL. Other parameters like growth, heartbeat, tail formation, facial edema and pigmentation were observed to be normal in all the treated concentrations. However, a gradual decrease in its total length was observed at 125 & 150 µg/mL concentrations. Similarly, deformations in the body shape and pericardial sac were found at 125 & 150 µg/mL concentrations as shown in Figure 2.

Toxicity evaluation of RAJI in Zebrafish Larvae

Zebrafish larvae hatch from their chorion between 2-3 days post fertilization (dpf). Hatched larvae initially lie on their side on the bottom of the tank and occasionally dart forward or circle around. The larvae display a startle response when exposed to abrupt stimuli, including touch, sound, water flow, or light. Their sensory and motor systems mature quickly and equip them with the ability to detect and avoid threatening and unpleasant stimuli in their environment. Hence, Stereotype Movement assay, C-Bend Response assay, Abnormal Locomotion Freezing assay and Abnormal Burst and Coast Movement were assessed where abnormalities were observed only in the 20 & 200 ug/mL concentrations of RAJI. Similarly, the mortality rates were observed only at 20 & 200 ug/mL concentrations of RAJI as depicted in Figure 3.

T*oxicity evaluation of RAJI in Adult Zebrafish*

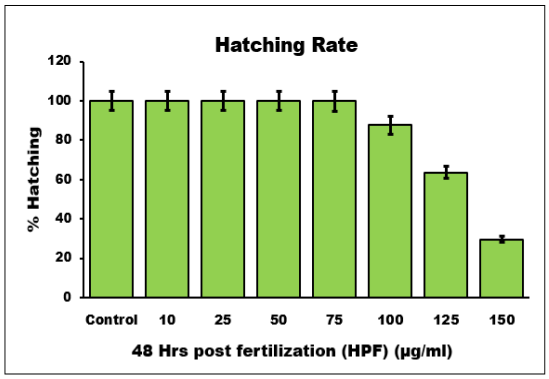
Zebrafish larval locomotor assays can be used as screening tools for testing the behavioural effects of many drugs, chemicals and toxins causing hyper or hypoactivity from a safety pharmacology perspective. In this study, adult zebrafish were used for both acute (14 days) and chronic (21 days) behavioural research analysis. Startle response, Light and Dark assay, Memory Maze test, Burst and Coast movement and Locomotor Activity were assessed during the experimental period where abnormalities were observed only in the 20 & 200 ug/mL concentrations of RAJI as represented in Figure 4a & 4b. Similarly, the mortality rates were observed only at 20 & 200 ug/mL concentrations of RAJI at the end of the experimental period (21st day) as shown in Figure 4c.

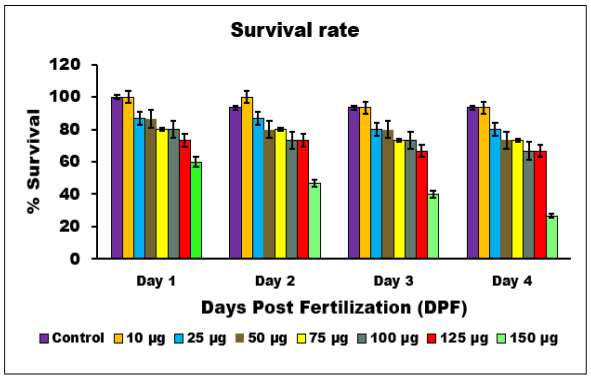
Histopathological Examination of Organ Tissues from Adult Zebrafish

The results of the histopathological examination

Table 1. Effect of RAJI on the Body Weight of Mice Post 14-day Study Period. Data shown as mean ± SD of Triplicate Values.

Day	Control	RAJI (25 mg/ml)	RAJI (100 mg/ml)
Day 1 (Initial)	26.33	35.33	28.03
Day 14 (Final)	21.67	26.06	23.37





Parameters	RAJI Concentration (µg/ml)						
	10	25	50	75	100	125	150
Growth	N	N	N	N	N	N	N
Total length (µm)	496.03	486.92	488.56	435.21	419.68	398.41	360.94
Heartbeat	N	N	N	N	N	N	N
Tail	N	N	N	N	N	N	N
Body shape	N	N	N	N	N	SD	D
Facial edema	NF	NF	NF	NF	NF	NF	NF
Pigmentation	NF	NF	NF	NF	NF	NF	NF
Pericardial Sac	NF	NF	NF	NF	NF	SD	D

N – Normal; SD – Slightly Distorted; D – Distorted; NF – Not Found

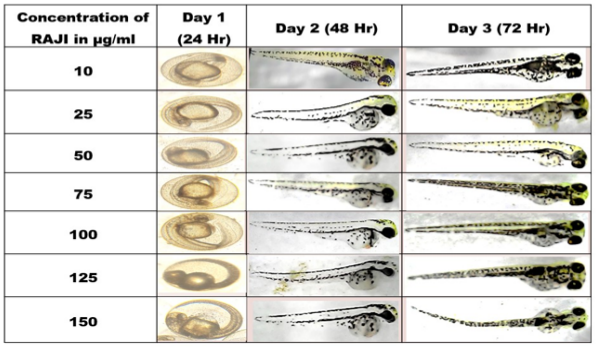


Figure 2. Developmental Toxicity and Morphological Analysis in Zebrafish Embryo Post Different Concentrations of RAJI Treatment

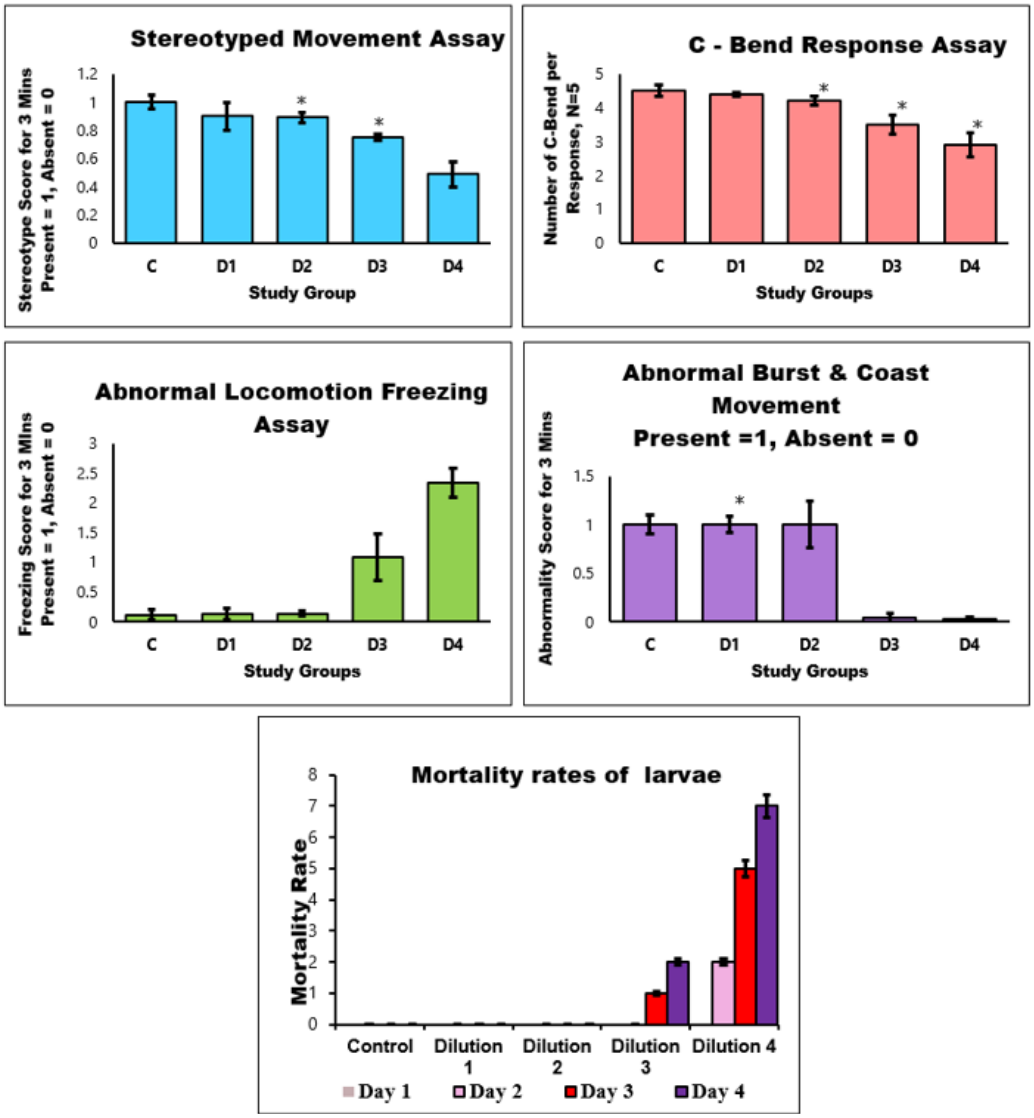


Figure 3. Behavioural Effects of Different Doses of RAJI on Zebrafish Larvae

of tissues samples from both control and RAJI treated groups (4 different concentrations) in two time periods namely Acute (14 days) and Chronic (21 days) were nearly similar and is clearly depicted in Figure 5a & 5b. Smear Pathology of heart in the control group shows well-defined cardiomyocytes with elongated nucleus that are found distributed in the non-eosinophilic cytoplasm along with nucleated erythrocytes that are found distributed throughout at normal count. In dilution D1, D2 & D3 cardiomyocytes with elongated nucleus are scattered throughout the cytoplasm and fewer necrotic and degenerative cells were observed. The dilution D4 displays

Table 2. Concised Haematological Analysis of Control and RAJI Treated Mice Post 14-day Study Period. Data shown as mean ± SD of triplicate values.

Test	Control	RAJI (25 mg/ml)	RAJI (100 mg/ml)
WBC	5.93 ± 0.24	5.84 ± 0.96	4.72 ± 1.00
RBC	7.64 ± 0.39	5.22 ± 0.95	6.89 ± 0.24
Neutrophils	0.87 ± 0.57	0.63 ± 0.29	0.71 ± 0.53
Lymphocytes	4.25 ± 0.80	4.97 ± 0.46	4.11 ± 0.85
Monocytes	0.01 ± 0.02	0.04 ± 0.07	0.01 ± 0.01
Eosinophils	0.03 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
Basophils	0.08 ± 0.06	0.14 ± 0.18	0.10 ± 0.05
Haemoglobin	12.86 ± 0.68	8.80 ± 1.73	13.13 ± 0.90
Platelet	862.33 ± 13.57	650 ± 36.37	352 ± 43.31
Hematocrit (HCT)	38.03 ± 2.83	23.42 ± 1.44	37.2 ± 1.11

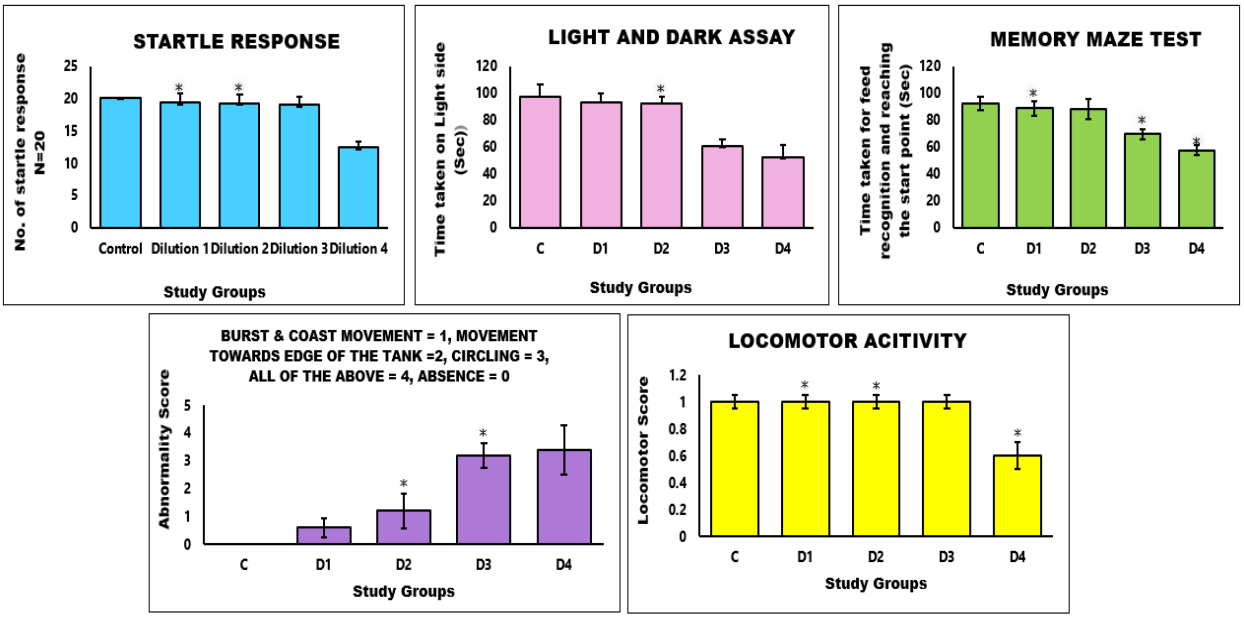


Figure 4a. Behavioural Effects of Different Doses of RAJI on Adult Zebrafish - Acute

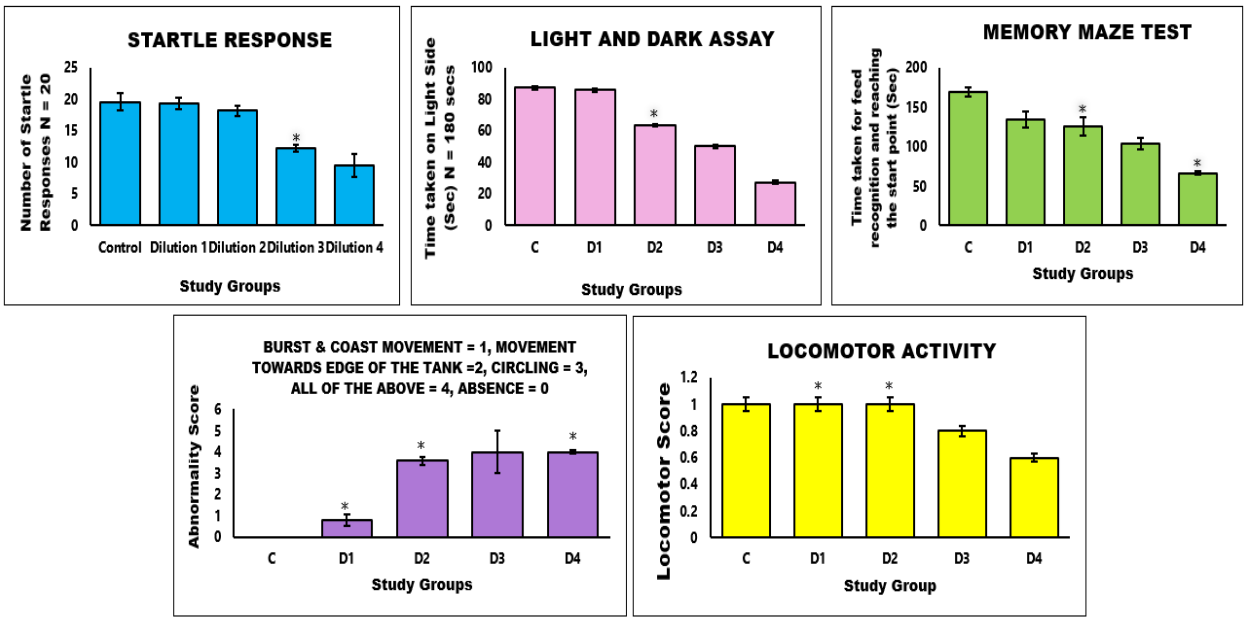


Figure 4b. Behavioural Effects of Different Doses of RAJI on Adult Zebrafish - Chronic

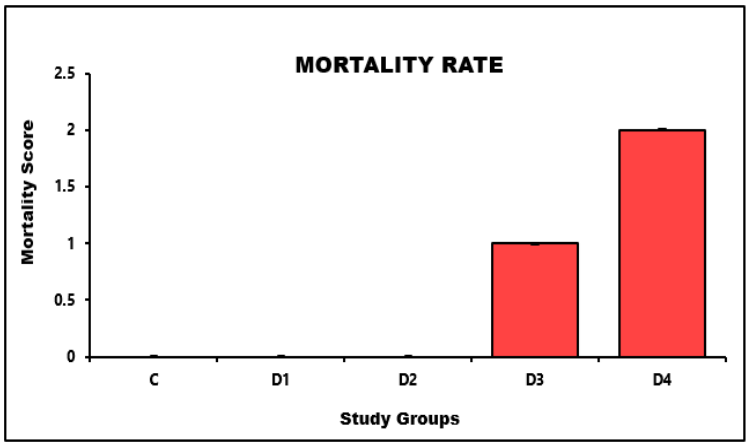


Figure 4c. Mortality in Adult Zebrafish

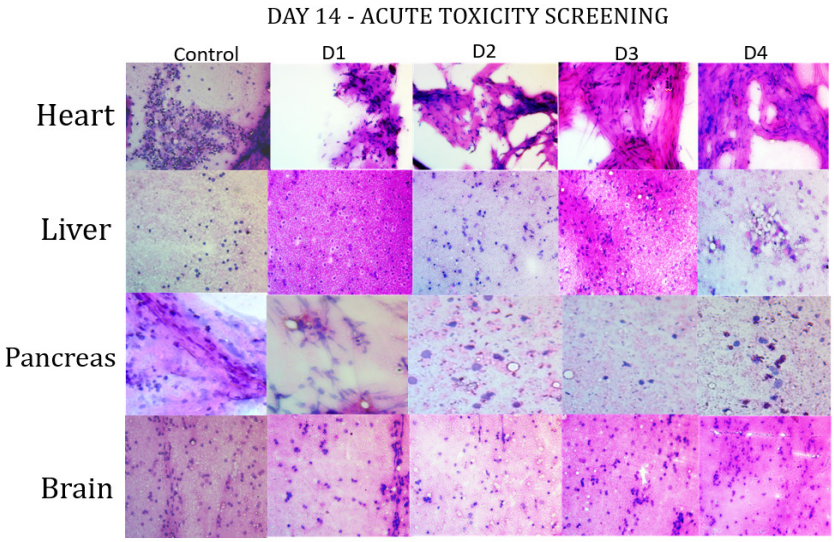


Figure 5a. Histopathological Analysis of Various Organs Obtained from Adult Zebrafish Post Treatment with RAJI - Acute

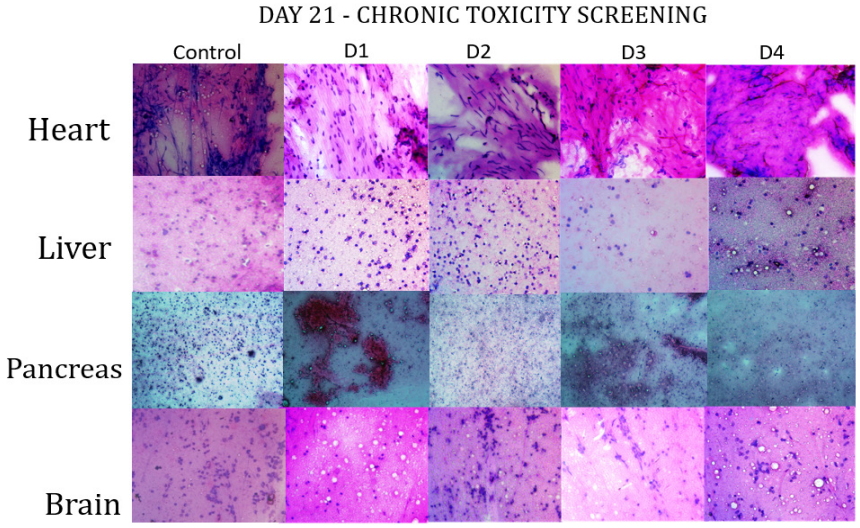


Figure 5b. Histopathological Analysis of Various Organs Obtained from Adult Zebrafish Post Treatment with RAJI - Chronic

fewer cardiomyocytes and nucleus with fewer necrotic and degenerative cells when compared to the control.

Smear pathology of liver in the control group shows well defined hepatocytes, erythrocytes, eosinophilic cytoplasm and vacuoles and has very few degenerative cells. Groups D1, D2 & D3 shows well defined hepatocytes, erythrocytes, eosinophilic cytoplasm and vacuoles and has fewer degenerative cells compared to control. However, the group D4 exhibits well defined hepatocytes, erythrocytes, eosinophilic cytoplasm and vacuoles with higher necrotic and degenerative cells than control.

Smear pathology of pancreas in the control group shows well-defined distribution of endocrine and exocrine

Table 3. Biochemical Evaluation Including Kidney, Liver and Cardiac Function Analysis of RAJI Treated Mice Post 14-day Study Period. Data shown as mean ± SD of triplicate values.

Test	Control	RAJI (25 mg/ml)	RAJI (100 mg/ml)
Blood Urea level	17.4 ± 0.02	33.1 ± 3.67	22.85 ± 3.04
Creatinine	0.105 ± 0.07	0.12 ± 0.014	0.135 ± 0.02
SGOT	464 ± 2.82	206.1 ± 0.42	234.5 ± 6.36
SGPT	87.25 ± 1.76	100.75 ± 1.34	85.9 ± 1.27
Alkaline Phosphatase	124.5 ± 2.12	49 ± 1.41	85.5 ± 0.70
Creatine Kinase	178.33 ± 1.15	155.85 ± 1.87	129.33 ± 1.52

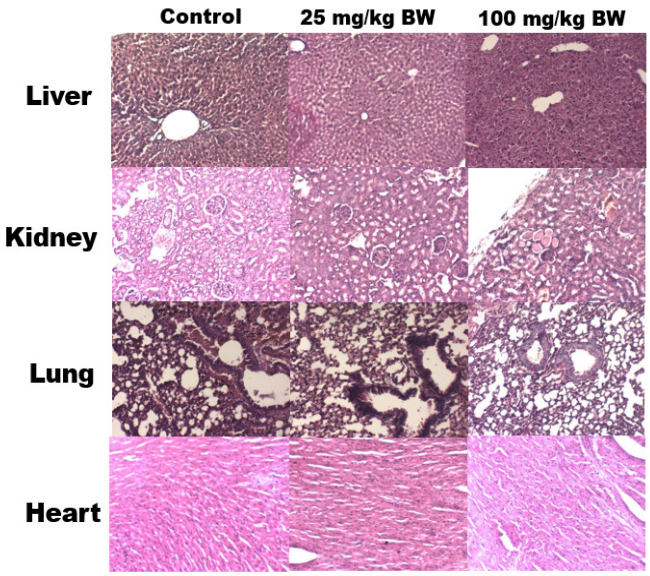


Figure 6. Histopathological Evaluation of Various Organs Obtained from Both Control and RAJI Treated Mice Groups

cells with no degenerative or necrotic cells. But, group D1 shows pale swollen degenerative cells and very few cells were observed in contrast to control. Groups D2 & D3 displayed higher degenerative and necrotic cells with fewer healthy cells. The group D4 displayed fewer necrotic and degenerative cells similar to that seen in the control group with densely packed exocrine and endocrine cells.

Smear Pathology of Brain in the control group shows neuronal cells and Schwann cells with eosinophilic cytoplasm which are uniformly distributed in the parenchyma with no necrotic or degenerative cells. Similarly in the groups D1, D2, D3, & D4 the Schwann cells appear normal with eosinophilic cytoplasm and are uniformly distributed in the parenchyma and very few neuronal cells appear pale and swollen with less stain uptake characterizing degenerative cells.

Toxicity Analysis of RAJI in BALB/c Mice

Body Weight Analysis of RAJI in BALB/c Mice

The effect of RAJI on the body weight of BALB/c mice during the course of a 14-day toxicity study was evaluated. Results displayed a significant loss in the body weight in RAJI-treated mice as compared to the control group with a distinct difference in the 100 mg/ml group which is represented in Table 1.

Haematological Analysis of RAJI in BALB/c Mice

Various components like White Blood Cells, Red Blood Cells, Neutrophils, Lymphocytes, Monocytes, Eosinophils, Basophils, Haemoglobin, Platelets, and % Haematocrit present in the whole blood were measured per 10^3/UL. Not much variations were observed in the parameters between the control and the treated groups, however, there was a significant decline in the platelet count in the 100 mg/ml group alone which is shown in Table 2.

Biochemical Analysis of RAJI in BALB/c Mice

Under Biochemical Analysis, three key parameters were evaluated – Kidney Function, Liver Function and Cardiac Function analysis and the results are tabulated as Table 3. Kidney function analysis comprised of two tests namely – Blood Urea and Creatinine level analysis. Among the two, a distinct increase was observed in the blood Urea level with an increase in the concentration of RAJI however, no significant variation was observed in the Creatinine level. The Liver Function Analysis consisted of three tests namely – Serum Glutamic Oxaloacetic Transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT) and Alkaline Phosphatase ratio in blood. Among the three tests, variations were observed in the SGOT and Alkaline phosphatase levels alone. A significant decrease in the SGOT and Alkaline phosphatase levels were observed in the RAJI treated mice when compared to the control Similarly, the Cardiac Function was evaluated by measuring the Creatine kinase level in the blood. A remarkable decline in its level was observed in the RAJI treated groups when compared to the control group.

Histopathological examination of organ tissues from BALAB/c Mice

The qualitative histopathological examination of tissues sections from both control and RAJI treated groups (2 different concentrations) were evaluated and depicted in Figure 6. The assessment revealed that the slide with the liver section had normal histoarchitecture in all 3 groups. However, slides with kidney sections displayed a normal histoarchitecture in the control and 25mg/kg body weight group but the 100mg/kg group displayed a mild tubular epithelial degeneration with hyaline casts in the lumen. Similarly, slides containing the lung section of both the control and 25mg/kg group displayed a normal histoarchitecture, however, the 100mg/kg group displayed mild degree of emphysema with hyperplasia of bronchiolar epithelium. Slides with the heart sections of all 3 groups displayed a normal histoarchitecture with no visible alterations.

Discussion

Most studies in recent times in the fields of toxicology and developmental biology recourse to employing zebrafish as a model organism because of the similarities it bears with humans in terms of the pathways that control anxiety, its high prolificacy, and its capacity to show physiological stress responses when exposed to unpleasant stimuli [19–22]. When it comes to safety pharmacology perspective, various forms of behavioural tests performed on zebrafish larvae have proven to be a potent way in screening chemicals, poisons, and potential drug candidates that might cause hyperactivity or hypoactivity [23]. Furthermore, it has a high level of genetic conservation, providing additional benefits compared to standard animal models [24].

The developmental toxicity study was aimed to evaluate the toxicological effects of a chemical stress – RAJI on zebrafish embryos and the results highlight the fact that the drug RAJI does not pose as a hazard in the hatching rate, survival aspect and developmental pattern until administered as a high dose. A study with a drug component similar to RAJI demonstrated a dose-dependent developmental toxicity in zebrafish embryos when treated with Anabasine (ANA) in different concentrations (50, 100, 200 & 300 mM). Anabasine is a piperidine alkaloid & structurally similar to nicotine. It has been concluded that this similarity in the results could be due to its ability to cause skeletal deformities and its developmental pattern [25]. The survival rate of the embryos was also assessed and a dose-dependent survival percentage was calculated. A study that employed O-isoproxylacetophenones as a precursor for their synthesis discovered that the survival rates decreased as chalcone and compound concentrations increased, which was consistent with our findings [26]. Another such study proves that Acetophenone toxicity in zebrafish may be caused by producing oxidative stress, altering cellular activities, and interfering with essential developmental pathways, resulting in developmental defects when administered at high doses. The survival percentage was least at day 4 (150 µg/mL concentration), whereas, there was no much difference in the survival percentage in the 10 µg/mL concentration. The growth, heartbeat and its tail pattern were noted to be normal in all the treated concentrations. However, there was a gradual decrease in its total length with the increase in concentration of RAJI. Its pericardial sac was found to be distorted at 150 µg/mL. Similarly, Doxorubicin, Adrenaline, and Terfininadine were used in a study to document the pericardial anomalies in zebrafish embryos. After 72 hours of Terfinadine administration, deformed ventricle was seen. It was reported that Doxorubicin caused serious bleeding which was found to be selectively collected in a high number of micro-vessels [27].

Zebrafish larvae display an intriguing array of traits throughout their first week of development. As their sensory and motor systems develop, they gain the ability to perceive and avoid stimuli that might be harmful or otherwise unpleasant in their environment. Locomotor movements in zebrafish larvae indicate neuromuscular function. They maneuver with slow, purposeful scoots and systematic twisting. The muscles and nervous system in perfect coordination provide smooth, regulated movements. However, the larvae flee quickly and strongly while encountering threats. The larva first creates a tight C-bend and then swims strongly. This burst and coast mechanism helps larvae escape predators by propelling them quickly. The larvae's numerous locomotor methods demonstrate their adaptability to environmental inputs and stresses [28]. Stereotype movement, C-Bend Responsive assay, Abnormal Locomotion Freezing assay, Abnormal Burst and Coast motion and mortality rates were assessed in this study where it was observed that abnormalities were seen only in high concentrations of RAJI. Similar study was conducted with fentanyl at various dosages (1, 10, 50, and 100 µM) which is known to be the parent compound of an exceptionally deadly class of NPS (New Psychoactive Substances). Several morphological defects in larvae were also discovered, mainly at high fentanyl dosages (50 and 100 µM). The findings of the fentanyl study, which found behavioural and morphological defects in zebrafish larvae at high doses, are consistent with our findings of abnormalities primarily at high concentrations of RAJI, implying a potential similarity in the toxicity response of zebrafish to certain compounds when exposed to higher concentrations [29].

Similar to the experiments conducted on zebrafish embryos and larvae, a set of experiments were performed with adult zebrafishes too in two different time periods – 14 days (Acute toxicity) and 21 days (Chronic Toxicity) evaluation. The study assessed various behavioural responses like Startle response, Light and Dark assay, Memory Maze assay, Abnormal Locomotor activity and Burst and Coast movement. The findings illustrate the possible toxicity of RAJI by showing that anomalies were only seen at greater dosages. In general, the startle response shown in zebrafish studies may serve as a guide for future investigations into the cellular and molecular processes behind the behavioural aberrations brought on by novel drug exposure [30]. Recent research found that Aluminium exposure at 11 mg/mL caused behavioural changes such as increased anxiety in adult zebra fish, especially when the exposure lasted more than 15 days, as well as oxidative stress in the fish's brain. The similarity in our findings and the subsequent Aluminium exposure study in adult zebrafish can be attributed to a dose-dependent toxicity principle. Both investigations revealed a trend in which detectable behavioural changes and harmful effects occurred mostly at higher dose levels or concentrations of the relevant drugs. These findings demonstrate that zebrafish display comparable behavioural abnormalities in response to toxic exposures, regardless of the exact chemical composition, indicating a widespread phenomenon of dose-dependent toxicity in zebrafish. This finding emphasises the use of zebrafish as a model organism for examining general toxicity principles that may transcend the chemical identification of the tested chemicals.

BALB/c mice possess physiological reactions similar to those seen in humans, making them a suitable model for predicting drug toxicities [31]. This strain has genetic, immunological, and metabolic resemblances to humans,

enabling accurate extrapolation of toxicity findings to human populations. A recent study shows that BALB/c mice can effectively assess the hepatotoxic effects of Acetaminophen, a common analgesic and antipyretic component [32]. Similarly, another finding demonstrates that BALB/c mice are appropriate for evaluating the cardiotoxic effects of Doxorubicin, a powerful anti-cancer drug [33]. Keeping this in mind the toxic potential of RAJI was evaluated in the BALB/c mice where various parameters like the kidney, liver and cardiac functions were assessed in detail.

The effects of RAJI on the body weight of BALB/c mice during the course of a 14-day toxicity study was investigated. The findings revealed a continuous trend of lower body weight in RAJI-treated mice as compared to the control group. The mice in the control group showed a small rise in body weight, which is indicative of their normal maturation. On the other hand, mice administered RAJI showed a significant decrease in body weight, with the high-dose group (100 mg/ml) suffering a significant reduction. These results underline the significance of body weight as a sensitive indicator of side effects and are in line with other research on the relationship between changes in body weight and pharmaceutical toxicity [34]. A similar study reported in the recent years with the chemical Cinnamonyl piperidine highlights the fact that there were only minor changes in the mice's body weight while they were receiving the medication when compared to the control group [35], thus emphasizing the fact that reductions in body weight could be an indication of systemic toxicity.

The haematological examination of the blood samples obtained from RAJI treat BALB/c mice were assessed for key parameters like WBC, RBC, neutrophils, lymphocytes, monocytes, eosinophiles, basophiles, haemoglobin, platelets and haematocrit (HCT). A decrease in WBC count in both the RAJI-treated groups was noticed, with a more articulated impact in the high-dose group (100 mg/ml). This reveals potential immune suppression [36]. The variation in red blood cell (RBC) count between the two RAJI-treated groups may be indicative of a dose-dependent effect on erythropoiesis. Similarly, a significant reduction in the platelet count was observed in the groups treated with RAJI 100 mg/kg (650 ± 36.37) and RAJI 25 mg/kg(352 ± 43.31), compared to the control group (862.33 ± 13.57), which raises valid concerns regarding the potential haematological toxicity in the animals. These results can be better attributed with a recent finding that highlights the fact that there is a significant decrease in the WBC, RBCs, Hb, and platelets counts followed by a decrease in MCV, MCH, and MCHC levels in rats administered with Doxorubicin [37]. The observed reduction in platelet counts may be due to potential bone marrow toxicity, immune-mediated platelet destruction or interactions with concurrently administered pharmaceutical agents [38]. The observed change in haemoglobin levels is a significant finding in this study. In the control group, the haemoglobin concentration was 12.86 ± 0.68, whereas in the group treated with RAJI at 25 mg/mL, it was 10.80 ± 1.73, and in the group treated with RAJI at 100 mg/mL, it measured 11.13 ± 0.90. The decrease in haemoglobin levels may potentially be indicative of conditions such as anaemia or compromised erythropoiesis stemming from RAJI treatment [39], thereby emphasizing that RAJI may have haematological effects, potentially perturbing erythropoiesis. Haematocrit also termed as packed cell volume, is the proportion of blood volume occupied by red blood cells, is a key parameter in the assessment of haematological health. It reflects the oxygen-carrying capacity of the blood and is tightly interlinked with other crucial parameters, including red blood cell (RBC) count and haemoglobin concentration [40]. The study's control group, displayed a haematocrit level of 38.03 ± 2.83, whereas in the group treated with RAJI at 25 mg/mL, it was 36.42 ± 1.44, and in the group treated with RAJI at 100 mg/mL, it decreased to 32.2 ± 1.11. The reduced haematocrit levels with changes in RBC count and haemoglobin concentration in the RAJI-treated groups strongly suggests that RAJI treatment may have an impact on erythropoiesis. A similar finding revealed that lower haematocrit levels were observed, in Wistar rats with lead exposure highlighting potential risks associated with it [41]. Additionally, the decrease in haematocrit may signify anaemia, which can have profound clinical implications, particularly in terms of oxygen transport and tissue oxygenation [42].

Similarly, the biochemical evaluations included assessment of kidney, liver and cardiac functions. Blood urea and creatinine level in the kidney function analysis are critical for comprehending the possible implications of RAJI therapy on renal health. Notably, the RAJI (100 mg/ml) group had significantly higher blood urea levels than both the control and RAJI (25 mg/ml) groups. This concentration-dependent effect on blood urea levels may reflect changes in renal function, suggesting that RAJI, particularly at higher doses, may have a negative impact on kidney health. Creatinine levels, on the other hand, responded more subtly to RAJI therapy.

The comprehensive evaluation of liver function markers, encompassing SGOT, SGPT, and Alkaline Phosphatase constitutes an indispensable facet in discerning the intricate dynamics of novel compounds upon hepatic health. In the context of the present study, an interesting observation was the significant difference in SGOT levels between the control and test groups, suggesting that RAJI may have caused liver dysfunction or stress in the treated animals. A significant decrease of SGOT levels observed in response to RAJI at a concentration of 25 mg/ml (356.1 ± 0.42) and 100 mg/ml (204.5 ± 6.36) in comparison to the control group can be attributed with kapulaga's possible hepatoprotective properties [43]. Similarly, a significant decrease in the Alkaline phosphatase levels were observed in both the 25 mg/ml (85 ± 1.41) and 100 mg/ml (65.5 ± 0.70) RAJI concentrations that can be corelated with a recent study that documented the ALP level to be lower in hypo-parathyroid patients [38].

In the cardiac function analysis, significant changes in creatine kinase levels were observed. The RAJI-treated groups exhibited dose-dependent effects. The 25 mg/ml group displayed a moderate reduction in creatine kinase levels, while the RAJI 100 mg/ml group exhibited a

more pronounced decrease. These findings suggest that RAJI treatment, particularly at the higher concentration (100 mg/ml), may have a beneficial influence on cardiac function by reducing creatine kinase levels. A recent study suggested that melatonin administration exerts a range of favourable effects on cardiac function. Notably, it leads to a significant improvement in cardiac performance, concurrent with a reduction in levels of creatine kinase (CK) and creatine kinase-MB (CK-MB), both of which are recognized indicators of myocardial damage [44].

In Conclusion, as a result of an attempt to evaluate the toxic potential of RAJI in both Zebrafish and BALB/c mice models, it is confirmed that RAJI does not induce any significant damage when administered at low dosages, however it can cause mild damages when administered at high concentrations thus enabling the conclusion that it can be a potent anti-cancer drug candidate against breast cancer treatment when implemented under combination therapy along with commercially available chemotherapeutic drugs which causes severe side-effects when administered at high concentrations. However, factors such as administration routes and treatment durations need detailed evaluations. It also necessitates to conduct an extensive pharmacokinetic study in order to comprehend the drug's characteristics concerning absorption, distribution, metabolism, and excretion (ADME properties). Furthermore, a comprehensive assessment in xenograft models is necessary to determine its effectiveness in human-like tumour environments, which we plan to execute immediately. Furthermore, elucidating the precise molecular and cellular mechanisms involving transcriptomic, proteomic, and metabolomic analyses to identify key pathways and biomarkers associated with the drug's action underlying RAJI's effects on various biological parameters is crucial and the process is underway. Hence, these phases of study are crucial for determining the drug's therapeutic efficacy and safety, thereby enabling its advancement for clinical use and in order to mobilise RAJI as a potent anti-cancer drug against breast cancer in the near future.

Author Contribution Statement

The drug 3-(2-(3,4 dimethoxyphenyl)-2 oxoethylidene) indolin-2-one (RAJI) was designed and synthesized by Karthikeyan Chandrabose, Department of Pharmacy, Indira Gandhi National Tribal University, Madhya Pradesh, India. The study was designed by Ms. Prathibha and Ashok Kumar Pandurangan, School of Life Sciences, B. S. Abdur Rahman Crescent Institute of Science and Technology, Vandalur, Chennai, India. The experiment execution and the manuscript preparation were carried out by Prathibha Sivaprakasam, School of Life Sciences, B. S. Abdur Rahman Crescent Institute of Science and Technology, Vandalur, Chennai, India.

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Availability of data (if apply to your research)

All the original data presented in the study are included in the manuscript will be shared upon request.

Conflict Of Interest

The authors have no conflict of interest to declare.

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