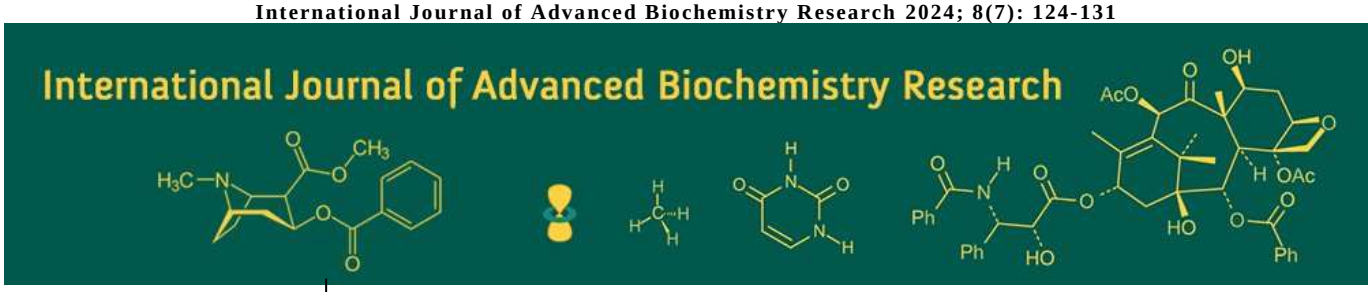

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Dr. Astha Moolchandani
M.V.Sc. Scholar, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, Madhya Pradesh, India

Dr. Vidhi Gautam
Associate Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, Madhya Pradesh, India

Dr. Sachin Jain
Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, Madhya Pradesh, India

Dr. Rakhi Vaish
Professor and Head, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, Madhya Pradesh, India

Dr. Anil Gattani
Associate Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, Madhya Pradesh, India

Dr. Prateek Mishra
PhD Scholar, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, Madhya Pradesh, India

Dr. Shivangi Jain
M.V.Sc. Scholar, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, Madhya Pradesh, India

Corresponding Author:
Dr. Astha Moolchandani
M.V.Sc. Scholar, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, Madhya Pradesh, India

Evaluation of protective efficacy of quercetin on microplastic induced behavioural toxicity in zebrafish (*Danio rerio*)

Dr. Astha Moolchandani, Dr. Vidhi Gautam, Dr. Sachin Jain, Dr. Rakhi Vaish, Dr. Anil Gattani, Dr. Prateek Mishra and Dr. Shivangi Jain

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Abstract
The study was conducted to evaluate the protective effect of quercetin on microplastic induced behavioral abnormalities in zebrafish. Total 240 zebrafish were divided into six groups containing 40 fish in each. Group I served as control and other groups received microplastic and different dose of quercetin either alone or in combination. Research was conducted for the period of 21 days. Results of the study revealed behavioral abnormalities in microplastic alone treated group, quercetin (75 µg/L) alone treated group and in the group where microplastic was given with quercetin (75 µg/L).

Keywords: Quercetin, microplastic, behavioural toxicity, zebrafish (*Danio rerio*)

Introduction
Microplastics are recognized to be a global contaminant of concern and can persist in the environment for centuries causing harm to marine life and entering the human food chain through seafood production [1]. In addition, as microplastics exist in micro-level to nano-level sizes, they are virtually impossible to remove once released into the environment. Due to these characteristics, microplastics pose potential hazards to human beings and the environment.
Zebrafish is a fresh-water fish and non-mammalian vertebrate model belonging to the family Cyprinidae. Zebrafish of distinct developmental stages has become a popular model for toxicity test of environmental pollutants. Adult zebrafish has the complete intestinal structure and has been used as a powerful model to study intestinal functions and diseases [2, 3].
Quercetin is widely found in plants including apples, berries, brassica vegetables, capers, grapes, onions, spring onions, tea and tomatoes as well as in many seeds, nuts, flowers, bark and leaves [4]. Found that quercetin is the most effective free radical scavenger in the flavonoid family. Mechanistically, quercetin has been shown to exert antioxidant, anti-inflammatory and anticancer activities in a number of cellular and animal models, as well as in humans through modulating the signalling pathways and gene expression involved in these processes [5].

Materials and Methods
Experimental animals
The research work was conducted on adult zebrafish (*Danio rerio*) of 4-5 months age. The healthy adult zebrafish (*Danio rerio*) of either sex were used. The experiment was approved by the Institutional Animal Ethics Committee (IAEC) of College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University (NDVSU), Jabalpur.

Housing of fish
The zebrafish were housed in standard glass aquarium of 20-liter capacity filled with RO water and proper aeration. The temperature of water in tank was 26°C - 28°C. The pH of water was in the range of 6.8 - 7.4. Tanks were cleaned daily in order to remove debris and maintain fish under hygienic condition.

Acclimatization of animals
All fish wfere acclimatized to laboratory conditions under constant observation for 15 days before start of the experiment.

Experimental design: A total of 240 healthy adult zebrafish (*Danio rerio*) of either sex were randomly divided into six groups with 40 fish in each group. The fish of different groups were kept separately in standard glass aquarium and treated with different concentrations of microplastic and quercetin, as given in table 01:

Table 1: Design of experiment

Group	No. of animals	Treatment
I	40	Normal control
II	40	Polystyrene microplastic (100 µg/L; in water for 21 days)
III	40	Quercetin (50 µg /L; dissolved in water for 21 days)
IV	40	Quercetin (75 µg /L; dissolved in water for 21 days)
V	40	Polystyrene microplastic (100 µg/L; in water for 21 days) + Quercetin (50 µg /L; dissolved in water for 21 days)
VI	40	Polystyrene microplastic (100 µg/L; in water for 21 days) + Quercetin (75 µg /L; dissolved in water for 21 days)

The study involved exposing fish to various chemicals, including polystyrene microplastic and quercetin over a period of 21 days. The control group was fish from group I, while group II, group III, group IV, group V and group VI were exposed to different concentrations of chemicals.

Exposure to microplastic
Fish were exposed to polystyrene microplastic at the dose of 100 µg/L in water, daily for 21 days.
Water from fish tank was drained off every morning and then replaced with fresh water and then polystyrene microplastic was added.

Exposure to quercetin
A standard stock solution of 10 mg/ml quercetin was prepared in Dimethyl sulphoxide (DMSO) and distilled water at 1:4 ratio. Fish were exposed to quercetin in two concentrations i.e., at 50 µg /L and 75 µg /L in water, daily for 21 days and water was replaced every morning.

Behavioural parameters
Behavioural responses of zebrafish in different treatment groups were analyzed on day 0 (pre-treatment) and day 21 (post- treatment) of the experiment. Behavioural parameters i.e., number of line crossings, novel tank diving paradigm, light-dark preference and mirror aggression were evaluated.

a) Number of line crossing
For behavioural analysis, fish were placed in a glass petri dish (20 cm diameter), divided into quadrants and the the stress response was quantified in terms of locomotor activity i.e., number of line crossings during 0-10 minutes [6].

b) Novel tank diving paradigm
Zebrafish exhibit behavioral responses to stress and anxiety, as demonstrated in a novel tank diving test. Initially diving to the bottom, they gradually increase vertical swimming, reducing anxiety. The test is simple and sensitive to manipulations. The effect of microplastic and quercetin was noted through observation of behaviour in a novel tank and following movements were recorded [7].

i. Time spent in upper portion of tank
ii. Number of entries into upper portion of tank
iii. Average entry duration
iv. Number of erratic movements

c) Light dark preference test
In zebrafish, scototaxis (i.e., the avoidance of bright places) is a behavioural correlate for anxiety, with increased time in dark being associated with increased anxiety. A light/dark box model is used to study anxiety-like behavior in zebrafish. The anxiety level in zebrafish after exposure to microplastic and protective efficacy of quercetin was recorded through following activities for the next 10 minutes [8]:

i. Time spent in white compartment
ii. Total number of crossings between the dark and light compartment
iii. Freezing behaviour in white

d) Mirror aggression test
To assess zebrafish aggressiveness, the mirror-biting test was performed. The mirror-biting test involves a slight modification of the novel tank test, the mirror image stimulation induced aggressive behavioural responses in zebrafish. The behaviour of zebrafish was recorded for 5 minutes, scoring freezing, swimming, rapid time movement, mirror biting time, and longest duration on the mirror side [9].

Results and Discussion
Number of Line crossing by zebrafish
The study evaluated the anxiety behavior response of zebrafish to a toxic substance, microplastic and its combinations with different doses of quercetin. Results of the present investigation indicates that microplastic reduced the locomotor activity of zebrafish in terms of number of line crossings which indicate anxiety in zebrafish. Quercetin reduced the anxiety level produced by microplastic and increased locomotor activity. The mean values of number of line crossings by zebrafish of different groups are given in table 02. In agreement with the present findings, a study reported that the treatment of nanoquercetin (5 and 10 mg/kg I/P) shown ameliorative effect against Streptozotocin induced behavioural abnormality and was shown by increased number of line crossings in dose-dependent manner [10].

Table 2: Efficacy of quercetin on number of line crossings by zebrafish in microplastic induced toxicity

Group	Treatment	Number of line crossings (Mean±SE)	
		Day-0	Day-21
I	Normal control	134.00±1.93	140.16^{a}±3.78
II	Polystyrene microplastic (100 µg/L of water; for 21 days)	135.50±1.60	45.16^{c}±1.74
III	Quercetin (50 µg/L of water; for 21 days)	136.66±1.89	141.66^{a}±3.01
IV	Quercetin (75 µg/L of water; for 21 days)	132.16±2.56	80.00^{b}±1.46
V	Polystyrene microplastic (100 µg/L of water; for 21 days) + Quercetin (50 µg/L of water; for 21 days)	132.50±2.06	150.80^{a}±6.44
VI	Polystyrene microplastic (100 µg/L of water; for 21 days) + Quercetin (75 µg/L of water; for 21 days)	138.66±7.05	50.16^{c}±2.32

*Values are mean of six observations

*Means bearing different superscript in the same column differ significantly (p<0.05)

Novel tank diving paradigm

The study conducted a novel tank diving test to assess various behavioral parameters of zebrafish in response to microplastic and quercetin. The mean values of various behavioural parameters are presented in table 03. Results of the present investigation indicate that microplastic produced behavioural abnormalities in novel tank diving paradigm whereas quercetin ameliorates the behavioural abnormalities induced by microplastic. In agreement with the present findings, a study also reported abnormal behaviour of zebrafish in novel tank after exposure of different size and dose of microplastic [11]. In another study, they reported that quercetin significantly increased the time spent in the top zone and the number of entries in the top zone as compared to time spent in the bottom zone in novel tank diving test [12].

Light dark preference test

The study examined zebrafish's scototaxic behavior after exposure to microplastic for 13 minutes, revealing that microplastic reduced locomotor activity and caused abnormal behavior in light-dark preference tests. Quercetin, an antioxidant, improved locomotor activity and ameliorated these abnormalities when given at the dose of 50. The mean values of various scototaxic behavioural parameters of zebrafish in response to microplastic and its combination with quercetin have been presented in table 04. Results of the investigation indicates that microplastic as toxicant reduced the locomotor activity between white and black compartment and produced abnormal behaviour in light dark preference test whereas quercetin as an antioxidant agent improves the behaviour of zebrafish in terms of locomotor activity between white and black compartment and ameliorates the behavioural abnormalities induced by microplastic

In agreement with the present findings, A study reported that in toxicity of cadmium chloride, mercury chloride and their combination, time spent in dark side by zebrafish was significantly higher and numbers of entries in the light side by zebrafish was significantly lower as compared to control in light dark preference test [13]. These findings align with a study in which they found that quercetin increased time spent in the light compartment and entries into the light compartment as compared to the dark compartment [12].

Mirror aggression test

In present study, mirror aggression test was carried out to evaluate the aggressive response of zebrafish by observing its own image in the mirror placed vertically inside the tank for the duration of 5 minutes. The mean values of various aggressive behavioural parameters of zebrafish in response to microplastic and its combination with quercetin have been presented in table 05.

The results of the present study indicates that microplastic produced behavioural abnormalities in mirror aggression test in terms of increased freezing time, rapid movement and mirror biting time and decreased swimming time. Quercetin in lower dose improved the behaviour of zebrafish in terms of aggressiveness while in higher dose increased the abnormality in mirror aggression test.

In agreement with the present findings, a study reported that antibiotic exposure altered the aggressive behaviour measured by the time spent in the mirror biting zone. Ciprofloxacin-treated animals spent more time in the segment nearest to the mirror than control zebrafish. Ceftazidime treatment produced a similar effect. Moreover, the number of bites against the mirror was significantly increased in Ciprofloxacin and Chlortetracycline-treated animals [14]

Table 5: Efficacy of quercetin on novel tank diving by zebrafish in microplastic induced toxicity

Group	Treatment	Time spent in upper portion of tank (sec) (Mean±SE)		Entries into upper portion of tank (no.) (Mean±SE)		Average entry duration (sec) (Mean±SE)		Erratic movement (no.) (Mean±SE)		Freezing bouts (no.) (Mean±SE)		Freezing duration (sec) (Mean±SE)	
		Day 0	Day 21	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21	Day 1	Day21
I	Normal control	317.50±3.90	328.16^{b}±9.50	27.66±1.28	30.16^{b}±1.27	11.59±0.51	10.89^{a}±0.41	0.83±0.30	1.00^{de}±0.25	0.83±0.30	1.16^{d}±0.30	1.83±0.79	2.66^{d}±0.95
II	Polystyrene microplastic (100 µg/L of water; for 21 days)	320.83±11.88	91.50$^{e±}$3.38	28.83±1.40	13.50^{d}±0.95	11.24±0.69	6.93^{b}±0.53	0.66±0.33	8.83^{b}±0.30	1.00±0.25	11.33^{b}±0.95	2.00±0.51	141.66^{b}±7.38
III	Quercetin (50 µg/L of water; for 21 days)	335.33±11.03	380.16^{a}±5.70	29.66±1.22	37.66^{a}±0.66	11.42±0.71	10.11^{a}±0.28	0.83±0.30	0.50^{e}±0.22	0.50±0.22	0.66$^{d±}$0.33	1.33±0.66	2.00^{d}±1.03
IV	Quercetin (75 µg/L of water; for 21 days)	322.00±6.41	147.66^{d}±15.71	26.83±0.60	17.16^{d}±0.47	12.04±0.49	8.64^{a}±0.99	0.33±0.21	5.50^{c}±0.42	0.66±0.33	13.50^{b}±0.76	2.16±1.10	159.66^{b}±11.27
V	Polystyrene microplastic (100 µg/L of water; for 21 days) + quercetin (50 µg/L of water; for 21 days)	324.16±7.15	243.66^{c}±5.85	30.50±1.23	22.50^{c}±0.76	10.75±0.62	10.87^{a}±0.38	1.16±0.16	2.16^{d}±0.30	0.33±0.21	4.66^{c}±0.21	1.33±0.84	17.66^{c}±2.26
VI	Polystyrene microplastic (100 µg/L of water; for 21 days) + quercetin (75 µg/L of water; for 21 days)	344.66±9.00	66.83^{e}±4.74	31.50±1.05	10.83^{d}±1.13	10.99±0.41	6.56^{b}±0.80	1.16±0.30	11.00^{a}±0.73	0.66±0.33	17.16^{a}±0.60	2.5±1.20	195.33^{a}±4.81

*Values are mean of six observations

*Means bearing different superscript in the same column differ significantly (p<0.05)

Table 6: Efficacy of quercetin on light dark preference by zebrafish in microplastic induced toxicity

Group	Treatment	Total time spent in white compartment (seconds) (Mean±SE)		Number of crossings between black and white compartment (Mean±SE)		Freezing in white compartment (Mean±SE)	
		Day-0	Day-21	Day-0	Day-21	Day-0	Day-21
I	Normal control	115.50±2.50	118.50$^{b±}$5.96	25.00±1.48	26.66^{a}±1.62	0.33±0.33	0±0
II	Polystyrene microplastic (100 µg/L of water; for 21 days)	120.66±2.48	52.83^{de}±1.74	25.83±1.88	13.33^{b}±0.80	0.66±0.66	0±0
III	Quercetin (50 µg/L of water; for 21 days)	117.00±3.84	142.50^{a}±2.60	24.33±1.97	30.16^{a}±1.07	0.66±0.66	0.33±0.33
IV	Quercetin (75 µg/L of water; for 21 days)	115.16±3.80	70.16^{d}±0.98	22.00±1.41	15.00^{b}±0.57	0±0	0±0
V	Polystyrene microplastic (100 µg/L of water; for 21 days) + Quercetin (50 µg/L of water; for 21 days)	114.66±2.91	89.83^{c}±0.94	22.66±1.60	15.16^{b}±0.79	0±0	0±0
VI	Polystyrene microplastic (100 µg/L of water; for 21 days) + Quercetin (75 µg/L of water; for 21 days)	121.00±2.11	48.5^{e}±1.87	23.16±1.13	8.50^{c}±0.42	0.50±0.50	0±0

*****Values are mean of six observations

*Means bearing different superscript in the same column differ significantly (p<0.05

Table 5: Efficacy of quercetin on mirror aggression by zebrafish in microplastic induced toxicity

Group	Treatment	Freezing time percent (Mean±SE)		Swimming time percent (Mean±SE)		Rapid time movement Percent (Mean±SE)		Mirror biting time percent (Mean±SE)	
		Day 0	Day 21	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21
I	Normal control	18.70±0.55	16.41^{d}±0.93	76.50±0.38	79.38^{b}±0.86	4.48±0.24	4.53^{a}±0.21	21.23±0.58	20.06^{b}±0.47
II	Microplastic (100 µg/L of water; for 21 days)	18.46±0.44	30.06^{a}±0.42	76.80±0.32	67.80^{d}±0.43	4.63±0.38	2.68^{b}±0.16	22.56±0.53	12.01^{c}±0.25
III	Quercetin (50 µg/L of water; for 21 days)	19.08±0.48	11.91^{e}±0.28	76.11±0.57	82.90^{a}±0.33	4.80±0.20	5.01^{a}±0.19	23.43±0.38	25.60^{a}±0.70
IV	Quercetin (75 µg/L of water; for 21 days)	18.25±o.70	23.51^{c}±0.29	77.28±0.96	72.73^{c}±0.30	4.46±0.30	3.75^{b}±0.16	24.08±0.29	15.61^{b}±0.19
V	Polystyrene microplastic (100 µg/L of water, for 21 days) + Quercetin (50 µg/L of water; for 21 days)	19.75±0.27	24.41^{b}±0.36	75.23±0.40	71.66^{d}±0.35	5.01±0.21	4.18^{a}±0.22	22.60±0.60	18.03^{b}±0.39
VI	Polystyrene microplastic (100 µg/L of water; for 21 days) + Quercetin (75 µg/L of water; for 21 days)	18.41±0.57	28.75^{a}±0.37	76.45±0.68	68.60^{d}±0.46	5.13±0.25	2.80^{b}±0.16	25.30±0.35	9.80^{c}±0.44

*Values are mean of six observations
*Means bearing different superscript in the same column differ significantly (p<0.05)

Plate 1: Polystyrene microplastic

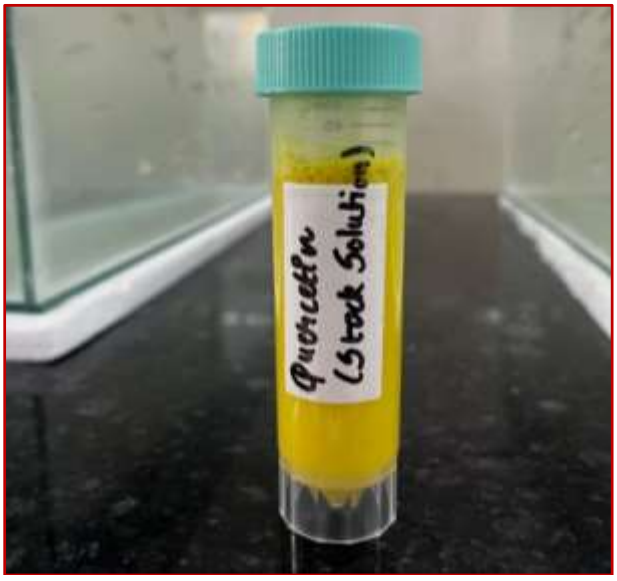

Plate 2: Stock solution of quercetin

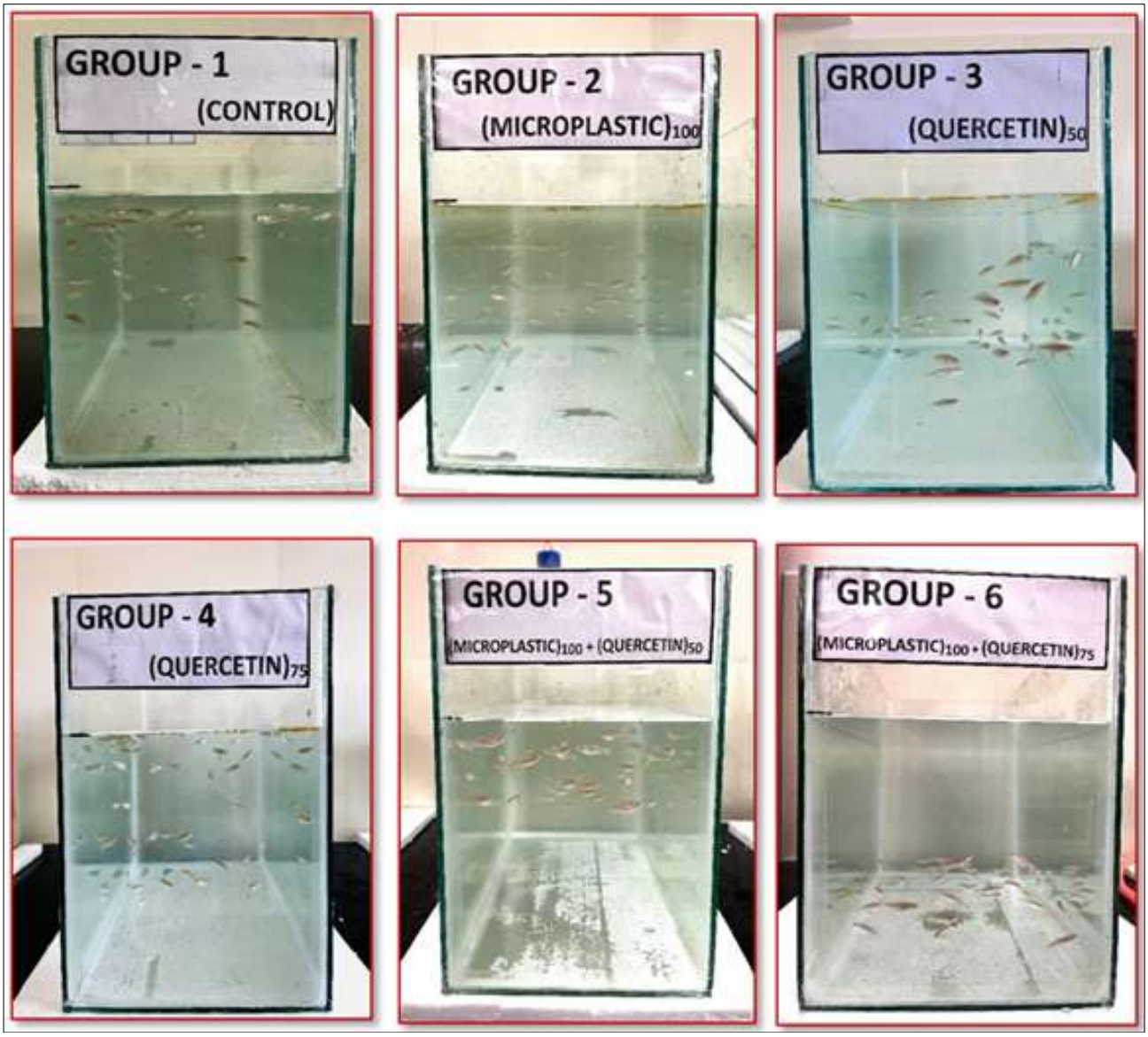

Plate 3: Experimental groups

A. Novel tank diving paradigm

B. Number of line crossings

Plate 4: Evaluation of behavioural alterations

A. Dark light preference test

B. Mirror aggression test apparatus

Plate 5: Evaluation of behavioural alterations

Conclusions

- Microplastic at the dose of 100 µg/L of water, daily for 21 days in zebrafish showed significant alterations in behaviour of zebrafish.
- Quercetin at the dose of 50 µg/L of water, daily for 21 days, induced behavioural modifications as indicated by improved behavioural parameters.
- Quercetin at the dose of 75 µg/L of water, daily for 21 days, with or without microplastic produced abnormal behaviour in zebrafish.

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