Int. J. Pharm. Investigation, 2026; 16(1):302-308.
https://www.jpionline.org

Original Article

Effects of Limonin-Induced Hepatotoxicity and Anxiety in Adult Zebrafish

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ABSTRACT

Background: The study explores the impact of limonin-induced hepatotoxicity and anxiety in adult zebrafish. Limonin is a chemical compound found in Citrus fruits and seeds, known for its biological properties. **Materials and Methods:** The zebrafish model was used to study the effects of limonin on liver tissue and neurological function. The fish were exposed to different concentrations of limonin, and their behaviour, biochemical and histochemical responses were studied. **Results and Discussion:** The behavioural assay (Novel tank test) showed that the exposed groups spent 50 times more time in the deeper section of the tank, indicating increased anxiety-like behaviour, which was proportional to the dosage of exposure. The biochemical analysis showed that the exposed fish had increased liver enzyme profiles, which increased with time and dosage of exposure. The enzyme activity of AST, ALT, and ALP increased by 2.8, 1.5, and 1.3 folds, respectively, when compared to the positive control. **Conclusion:** Overall, the study suggests that high concentrations of limonin can cause both neurological and hepatic damage in zebrafish. These findings could be significant for further research on the effects of limonin on potential impact on human health.

Keywords: Limonin, Zebrafish, Phytotoxicity, Behavioral studies.

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Received: 02-05-2025;
Revised: 30-06-2025;
Accepted: 18-08-2025.

INTRODUCTION

Phytochemicals are plant derived compounds known for their extensive biological properties which are reported to be both therapeutic and toxic to mammalian system. Limonin is one such compound with proven anti-oxidant, anti-cancer and neuroprotective activity. Limonin is a tetracyclic triterpenoid usually extracted from citrus fruits and seeds (Fan *et al*., 2019).

Limonin exhibits antioxidant properties reported to be greater than that of Vitamin C. Limonin is found to suppress the generation of free radicals with a simultaneous reduction in the accumulation of fatty acid oxidation products. The antioxidant behavior of limonin is likely to be due to the absence of hydroxyl groups and its hydrophobic nature (Sun *et al*., 2005; Yu *et al*., 2009).

Limonin also ameliorates the oxidative stress in neuronal tissue by neutralizing ROS, which can account for its neuroprotective activity. Behavioral studies investigating the learning and memory abilities demonstrated that exposure to limonin had a positive effect on rat behavior, thus improving the learning and memory ability (Yoon *et al*., 2010; Lin *et al*., 2016).

Limonin exhibited anti-cancer activity by suppressing the proliferation and improving the pro-apoptotic action in cells exposed to known carcinogen. Hence it elicited a therapeutic action in response to carcinogenic insults, thus inhibiting the progression of colon cancer in rat models (Chidambara *et al*., 2013). In genetic studies involving pancreatic adenocarcinoma cell lines, limonin was found to trigger cancer cell apoptosis by downregulating the expression of certain oncogenes and consequently activating the intrinsic pathways for cell death (Patil *et al*., 2010). Apart from the pancreatic cancer model, similar experiments have confirmed the antineoplastic activities of limonin in human liver, breast and cervical cancer models. However, the mechanisms behind such actions have not been completely understood and hence remain largely argumentative.

Certain phytocompounds can be toxic to biological systems when present in high concentrations. Research has shown that limonin can induce oxidative damage to mitochondria in rats. The primary hepatotoxic mechanism involves the disruption of mitochondrial permeability transition, resulting in ATP depletion and the release of cytochrome C, ultimately leading to

DOI: 10.5530/ijpi.20260475

apoptosis (Cai *et al.*, 2014). Additionally, limonin decreases the activity of antioxidant enzymes like superoxide dismutase and glutathione, which normally protect against free radical damage, while increasing nitric oxide synthase levels, contributing to liver fibrosis in mice liver tissue (Liang *et al.*, 2017).

Zebrafish (*Danio rerio*) is a model organism with 70% genetic similarity with humans, widely used for toxicity studies as well as behavioral assays hence making it an ideal model for this study. Recent research has revealed that zebrafish have mechanisms similar to mammals, such as enzyme induction and oxidative stress due to the presence of homologs of lipid metabolizing enzymes to humans. Furthermore, the zebrafish complete primary morphogenesis by 48 hpf and the liver is fully developed and functional by 72 hpf, making it an ideal model to examine hepatotoxicity. Adult zebrafish hepatotoxicity protocols have recently been developed. According to this, test substances can be delivered through injection or oral intubation. Following the administration of drugs, liver histopathology and liver function enzymes such as ALT and AST should be measured (Mcgrath and Li, 2008).

In addition to this, zebrafish has emerged as a potential *in vivo* pre-clinical model organism for studying the behavioural and molecular factors behind brain diseases such as anxiety. In zebrafish, various environmental stressors can induce anxiety, including exposure to novel situations, predators, alarm pheromones, anxiogenic medications, and the withdrawal from drugs. Hence, both the larval and adult zebrafish models are appropriate for researching acute and chronic anxiety-like states (Collier *et al.*, 2017).

The aim of this study is to investigate limonin-induced hepatotoxicity and anxiety in adult zebrafish. The level of liver damage was assessed using histochemical staining methods (H&E staining), the hepatotoxicity was examined by determining the altered liver enzyme profile (ALP, AST and ALT assays) and behavioral assays were used to examine the limonin-induced anxiety in the developed model.

MATERIALS AND METHODS

Zebrafish Husbandry and Maintenance

Healthy wild-type zebrafish (*Danio rerio*), 5-6 months old, sourced from a local animal nursery in Kolathur, were used for the study. Fish were kept in a glass tank with filtered water, ambient temperature (26±2°C), and pH (7.8±0.2) under regulated photoperiod conditions (14 h light and 10 h dark). Overcrowding was avoided in the tanks and oxygen pumps were fit in the tanks to maintain proper aeration. Fish were fed with commercially available Multinutrient micro pellet and freeze-dried tubifex worms, twice daily. Waste was regularly removed, and the tank was rinsed with clean water. The experimental groups had a random selection of male and female fish. Fish care and toxicity testing were carried out in accordance with the Organization for Economic Cooperation and Development's recommendations (OECD) (Willemsen *et al.*, 2011). All experimental protocols involving Zebrafish were reviewed and approved by the Institutional Animal Ethics Committee.

Grouping of animals and study design

Fishes were separated into six groups consisting of 22 fish per group. Four groups of fish were treated with varying concentration of limonin (250 µg, 500 µg, 750 µg and 1000 µg) for 24 hr. One group of fish were treated with 10 mM of acetaminophen for induction of liver damage as a positive control for 24 hr of time and one group was healthy control group. The fish were observed for behavioral changes and samples were collected for biochemical analysis at same intervals in all groups.

Behavioral Assay-Novel Tank Test

Traditionally, animal models of anxiety have been observed for behavioural changes in response to novel environments. Zebrafish are an excellent animal model for researching anxiety-related traits. Zebrafish were individually placed in a tank holding 2 L of water after pre-treatment. Zebrafish swimming behaviour was observed when they were transferred to new tanks. Time spent in the upper region of the tank, time spent in the deeper section of the tank, number of erratic movements, number of freezing bouts, and freezing duration were all recorded. A noticeable decrease in upper section exploration or higher freezing spells are signs of severe anxiety and stress. These endpoints were selected based on prior zebrafish research using the novel tank diving paradigm (Egan *et al.*, 2009).

Biochemical assays

Preparation of liver homogenate

The fish from various experimental and control groups were dissected, according to the observation time (vide Study Design). At appropriate time point of observation, liver was dissected to get the homogenate. The huge size, lobed structure, tannish colour, and profuse vascularization distinguished the liver. The livers were stored on ice in a microcentrifuge tube. The livers were homogenised using a tissue homogeniser with 0.25M sucrose solution after which it was subjected to centrifugation at 900 xg for 10 min. The final supernatant was collected and centrifuged for 20 min at 10000 rpm. The final homogenate was used as the enzyme source (Nagaraj *et al.*, 2021).

Estimation of Alkaline Phosphatase (ALP)

In the experiment, 150 mL of a carbonate-bicarbonate buffer at pH 10.4 were added to both the 'test' and 'control' tubes. Next, 150 µL of disodium phenyl phosphate substrate and 20 µL of magnesium chloride were incorporated into each tube. The mixtures were thoroughly mixed and pre-incubated at 37°C for 5 min. To the 'test' tube, 10 µL of enzyme was added and mixed

well, followed by a 20-min incubation at the same temperature. 100 µL of 10% trichloroacetic acid was introduced to all test tubes, with an additional 10 µL of enzyme added to the 'control' tube. The tubes were then centrifuged for 10 min at 3000 rpm. From the supernatant, 200 µL was collected, to which 100 µL of Folin's phenol reagent and 200 µL of 15% sodium carbonate were added. The mixtures were well mixed, and the intensity of the blue color was measured at 620 nm using a 96-well plate reader. Enzyme activity was quantified as micromoles of phenol released per milliliter of the sample (Deebiga and Vaidyanathan, 2021).

Estimation of Aspartate Aminotransferase (AST)

100 mL of phosphate buffer substrate were pipetted into both the 'test' and 'control' tubes. To the 'test' tube, 10 µL of the enzyme source was added, and the tubes were pre-incubated at 37°C for 60 min. Following this incubation, another 10 µL of enzyme was added to the 'control' tube. Then, 50 µL of 2,4-DNPH reagent was introduced to both tubes, which were kept at 37°C for 10 min. The reaction was halted by adding 500 µL of 0.4 N NaOH, followed by vortexing, and the mixtures were allowed to sit at room temperature for 5 min. The absorbance was then read at 540 nm using a 96-well plate reader, and enzyme activity was calculated as micromoles of pyruvate released per milliliter of the sample (Deebiga and Vaidyanathan, 2021).

Estimation of Alanine Aminotransferase (ALT)

One hundred microliters of phosphate buffer substrate were dispensed into both 'test' and 'control' tubes. To the 'test' tube, 10 µL of the enzyme source was added, and the tubes were pre-incubated at 37°C for 30 min. After this incubation, an additional 10 µL of enzyme was added to the 'control' tube. Then, 50 µL of the 2,4-DNPH reagent was added to both tubes, which were maintained at 37°C for 10 min. The reaction was terminated by adding 500 µL of 0.4 N NaOH. The mixtures were vortexed and left at room temperature for 5 min. Finally, the absorbance was measured at 540 nm using a 96-well plate reader, and the enzyme activity was expressed as micromoles of pyruvate released per milliliter of the sample (Deebiga and Vaidyanathan, 2021).

RESULTS

Behavioral Assay - Novel Tank Test

An increase in time spent in the deeper sections was observed which indicated the increase in anxiety levels. A dose-dependent anxiety trigger was observed across the limonin-exposed groups, the maximum at 1000 µg, equivalent to the positive control. The group exposed to 1000 µg showed a 50-fold increase in the time spent in the deeper section, when compared to the untreated negative control group (Figure 1).

Biochemical Assays

The levels of the enzymes Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) were measured to study hepatotoxicity. An increase in these enzyme levels reciprocated liver damage.

AST assay

The enzymatic activity of aspartate aminotransferase across the limonin-exposed groups over the time period of 3-6 days post exposure was measured. The data collected showed that the enzyme levels increased with the increase in time of exposure. The enzyme activity across the groups (250 µg, 500 µg, 750 µg, 1000 µg) on day 3 and day 4 post exposure showed a negligible difference in comparison to the positive control (Acetaminophen). Whereas, on day 5 and day 6 post exposure, the enzyme activity superseded the activity of the positive control as evidenced by a 2.8-fold increase (Figure 2). Hence, indicating that limonin increases the activity of aspartate transferase in a time dependent manner.

ALT Assay

The enzyme activity of alanine aminotransferase in limonin-exposed groups 3-6 days after exposure was measured. The obtained data revealed that enzyme levels developed in a time-dependent way. On day 3 and day 4 after exposure, the enzyme activity across the groups (250 µg, 500 µg, 750 µg, 1000 µg) showed minimal variation when compared to the positive control (Acetaminophen). On day 5 and day 6 after exposure, however, the enzyme activity exceeded that of the positive control, as evidenced by a 1.5-fold increase (Figure 3). As a result, limonin was found to cause a rise in the activity of alanine transferase with increasing exposure length rather than dosage.

ALP Assay

The enzyme activity of Alkaline Phosphatase in limonin-exposed groups 3-6 days after exposure was measured. The obtained data revealed that enzyme levels developed in a time-dependent way. On days 3, 4 and 5 after exposure, the enzyme activity across the groups (250 µg, 500 µg, 750 µg, 1000 µg) remained lesser than or equal to that of the positive control (Acetaminophen). However, on day 6 after exposure there was a 1.3-fold increase in the enzyme activity exceeding the positive control (Figure 4). As a result, limonin was found to cause a rise in the activity of Alkaline Phosphatase with increasing exposure length rather than dosage.

In conclusion, the novel tank test showed a dose-dependent increase in the time spent in the deeper section and the biochemical assays showed alterations in the liver enzyme profile. It demonstrated a time-dependent increase in enzyme activity when compared to the positive control (Acetaminophen). It showed that for AST and ALT on day 3 and 4 the enzyme activity was relatively normal whereas, on day 5 and 6 post exposure the

enzyme activity was more than that of the positive control thus indicating a rise in the enzyme levels. On the other hand, for ALP assay the rise in enzyme activity was very minimal on days 3, 4 and 5 post exposure but increased drastically on day 6, exceeding the positive control.

DISCUSSION

Phytocompounds have lately sparked the interest of researchers due to their numerous health advantages. Limonin has been demonstrated to have anti-cancer, anti-obesity, anti-osteoporosis, neuroprotection, anti-inflammatory, and anti-bacterial properties. The purpose of this study was to look at the effects of limonin-induced hepatotoxicity and anxiety using adult zebrafish model to identify the non-toxic concentrations that could be used for therapeutic interventions.

To study the anxiety induced, the novel tank test was performed. In natural habitat Zebrafish are used to dive deep to avoid predators when introduced in a new environment. A study investigating nicotine's influence on novelty-elicited deep diving response concluded that Zebrafish can be exploited as a model organism for observing stress reactivity and anxiety (Bencan *et al*., 2009). Willson *et al.*, (2021) developed a unique tank diving test to investigate the anxiolytic impact of *Calocybe indica*. Following ethanol withdrawal, there was a significant reduction in the number of entries into the upper section of the tank as well as an increase in freezing bouts. This demonstrated ethanol's anxiogenic impact (Wilson *et al*., 2021).

On that note, we observed that there was a dose-dependent increase in the time spent in the deeper section across the limonin-exposed groups. The maximum at 1000 µg showing a 50-fold increase in comparison to the normal untreated zebrafish. Hence, indicating that higher doses of limonin can have anxiogenic effects.

To study the hepatotoxic effects of limonin the liver enzyme activity was studied. The enzymes ALP, AST, and ALT were investigated using zebrafish liver homogenate, according to Nagaraj *et al*., (2021), who employed the liver homogenate for biochemical analysis to assess the anti-obesity benefits of *Calocybe indica*. Obesity was generated using corn oil and brine shrimp, and AST and ALT levels were reported to increase in the liver homogenate from the obese models. After being exposed to the substance tested, typically the enzyme levels were reduced (Nagaraj *et al*., 2021). According to research performed by Martins *et al*., (2021), hepatic lesions and liver damage are associated with a considerable rise in ALP, AST, and ALT levels. Their research's objective was to determine whether the 2,4-D herbicide caused hepatotoxicity. Their results suggested that the enzyme levels significantly increased, which led to the conclusion that the drug was hepatotoxic (Martins *et al*., 2021).

Studies that try to detect hepatotoxic effects in zebrafish usually indicate liver tissue damage connected to increased activity of liver biomarkers (mainly ALT/AST ratio). Zhang *et al*., (2017) exposed zebrafish to recognised mammalian hepatotoxic substances as carbaryl and pyrazinamide, the liver area index levels dramatically dropped, suggesting liver damage and correlating with greater AST and ALT activity (Zhang *et al*., 2017).

In the current study, ALP, AST and ALT were studied in Limonin-exposed adult Zebrafish; the enzyme activity was studied

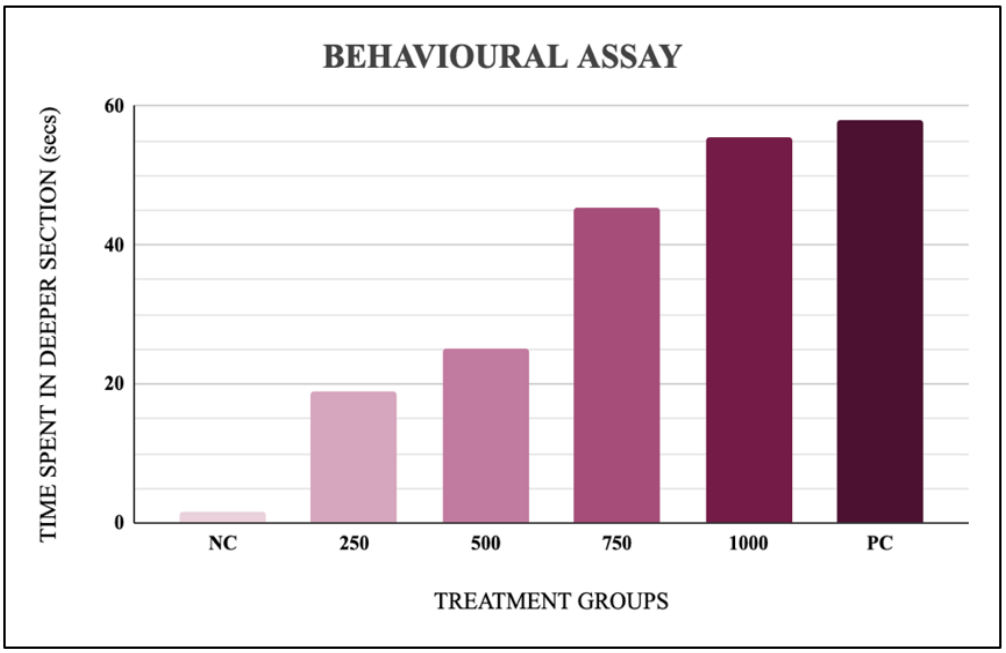

Figure 1: Study on anxiety trigger in adult Zebrafish. Dose dependent increase in the time spent by the adult zebrafish in the deeper section was observed across the different limonin-exposed groups.

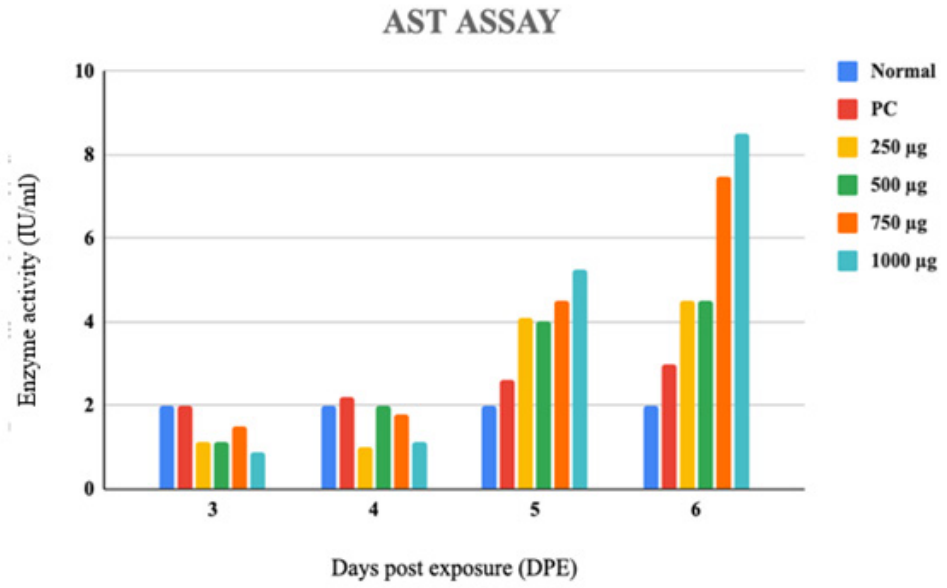

Figure 2: Enzymatic activity of Aspartate Aminotransferase (AST) of adult Zebrafish exposed to different concentrations of limonin across varied time periods.

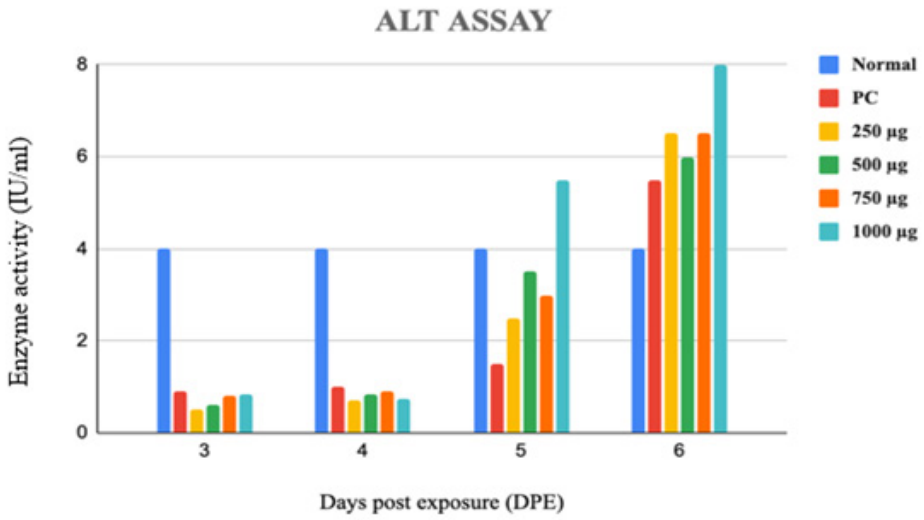

Figure 3: Enzymatic activity of Alanine Aminotransferase (ALT) of adult Zebrafish exposed to different concentrations of Limonin across varied time periods.

in terms of amount of product liberated. The coloured solution was read spectrophotometrically and the values were interpreted. We observed that there was a time-dependent increase in enzyme activity in comparison to the positive control (Acetaminophen). The enzyme activity was monitored over a span of 4 days (3-6 days post exposure) and there was a 2.8-fold increase in AST activity, 1.5-fold increase in ALT activity and 1.3-fold increase in ALP activity. This indicates that long exposure to high concentrations of limonin can be toxic to the liver. Research has also shown that hepatotoxicity and anxiety are related. Cohort research by Labenz *et al*., (2020) sought to examine the prevalence of anxiety and depression as well as the effects of Non-Alcoholic Fatty Liver Disease (NAFLD) over a period of ten years. The outcome was that, even after accounting for confounding comorbidities, NAFLD still represents an independent risk factor for developing depression and anxiety (Labenz *et al*., 2020).

Another common method used to study liver damage is histochemical staining. A study which aimed to evaluate the effects of ethanol on the liver of zebrafish was conducted by Schneider *et al*., (2017). The zebrafish were exposed to 0.5% of ethanol for 2-4 weeks and later the fish were dissected, liver samples were collected and H & E staining was performed. The samples were

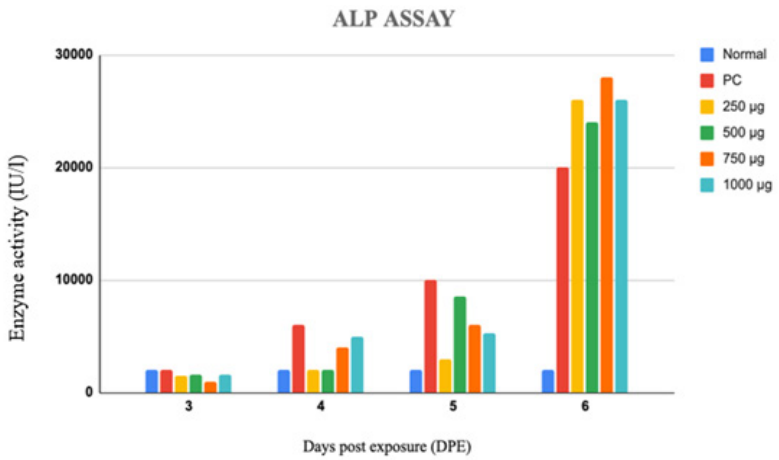

Figure 4: Enzymatic activity of Alkaline Phosphatase (ALP) of adult Zebrafish exposed to different concentrations of Limonin across varied time periods.

fixed, mounted and stained following which they were studied for the extent of liver damage. Post 4 weeks of exposure the liver tissue showed enlargement of cells and fatty infiltration that was denoted by the shift in position of the nuclei. Hence indicating liver damage (Schneider *et al.*, 2017). From the reports of the current study, we observed a dose-dependent increase in anxiety levels as well a time-dependent increase in liver enzyme activity, indicating hepatotoxic effects of limonin. We expect to observe morphological changes in the histochemical stained tissue sections that suggest liver damage has occurred across the limonin-exposed groups.

Apart from the psychological disturbances created directly by the limonin there might be a possible correlation between anxiety and hepatotoxicity according to some studies (Suh *et al.*, 2013). One possible mechanism explaining the occurrence of anxiety in liver injury patients is reduction in serotonin levels. Alterations in thyroid hormone levels and varying gut microbiome population can be some of the possible mechanisms by which liver injury might cause anxiety symptoms (Alekseeva *et al.*, 2008; Wu *et al.*, 2023; Zimbrean and Jakab, 2024).

CONCLUSION

Limonin was found to be hepatotoxic in the animal model used for the study. The model presented behavioral changes (anxiety-like escape behavior) and an increase in liver enzyme activity after extended exposure to high doses. With further preclinical testing in higher animal models, appropriate non-toxic biological concentrations may be used to get the maximum bioactivity out of the natural compound.

ACKNOWLEDGEMENT

The authors would like to thank the Department of Biomedical Sciences, Sri Ramachandra Institute of Higher Education and Research Chennai, Tamil Nadu for providing all necessary infrastructure.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AST: Aspartate aminotransferase; **ALT:** Alanine aminotransferase; **ALP:** Alkaline phosphatase; **H&E:** Hematoxylin and Eosin; **NAFLD:** Non-alcoholic fatty liver disease; **DPE:** Days post exposure.

ETHICAL STATEMENT

This study was performed after approval by the Institutional Animal Ethics Committee (IAEC/69/SRIHER829/20323), Sri Ramachandra Institute of Higher Education and Research, Chennai, India."

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Cite this article: Reddipalli S, Srinivasan MK, Vaidyanathan L. Effects of Limonin-Induced Hepatotoxicity and Anxiety in Adult Zebrafish. Int. J. Pharm. Investigation. 2026;16(1):302-8.