https://doi.org/10.33472/AFJBS.6.8.2024.154-165

African Journal of Biological Sciences

Research Paper Open Access

Ulva lactuca ameliorates the Bisphenol A induced neurotoxicity in zebrafish: an experimental approach

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Article History

Volume 6,Issue 8, 2024

Received:20 Feb 2024

Accepted : 28 Mar 2024

doi: 10.33472/AFJBS.6.8.2024.154-165

Abstract

Responsible for inducing carcinogenicity, cognitive impairment, neurotoxicity, oxidative stress, and other adverse effects, the impact of BPA (bisphenol A) on neurotoxicity and its potential mitigation through natural compounds remain poorly understood. This study aimed to investigate the neurotoxic effects of BPA in zebrafish (Danio rerio) through waterborne exposure, as well as the potential protective effects of ulvan co-supplementation. The protective effect of Ulva lactuca against BPA-induced alterations in neurobehavioral response, oxidative stress, and neuromorphological changes was assessed in the zebrafish brain. The results indicate that Ulva lactuca's total extract ameliorated BPA-induced changes in neurobehavioral response. Biochemical analyses suggest that Ulva lactuca has therapeutic potential against BPA-induced oxidative stress in the zebrafish brain. These findings suggest that Ulva lactuca could be an effective intervention against BPA-induced neurotoxicity in zebrafish by reducing oxidative stress.

Keywords: Ulva lactuca, Bisphenol A, neurotoxicity

Introduction:

Consumer demand has boosted the use of artificial polymers in the present production of superior plastic and micro-plastic products. (Abdalla et al. 2013 & Ansari et al. 2009) Their indiscriminate dumping into the environment air, soil and water may represent risk to the development of major healthiness issues in human beings, including neurological illnesses. (Chin-Chan) Bisphenol A (BPA), a chemical moiety with polymeric ability and an analogue of bisphenol (BP), has widely been employed for the manufacturing of plastics while its introduction from 1950s is one among them. BPA is the most common chemical used in manufacture of epoxy and polycarbonate resins (Staples et al., 1998; Kang et al., 2006; Vandenberg et al., 2007; Murata and Kang, 2018). As an anthropogenic xenoestrogen listed as an endocrine-disrupting chemical (EDC), BisphenolA has minimal estrogenic potential. (Inadera, 2015). Being lipophilic, bisphenol A may cross the placenta and the BBB, and it can reach newborns through breast milk. However, multiple studies have found that BPA exposure causes oxidative stress, emotional changes, perceptive impairment, carcinogenicity, and inflammation. Furthermore, elevated oxidative stress is being linked to a number of health issues such as cardiovascular illness, ageing, neurological damage, and inflammation.(Rahal et al 2014). As a further point of comparison, BPA exposure has been found in dust and human urine, demonstrating its ubiquity in the environment. Oxidative stress induced by BPA and its repercussions have been widely studied in *invitro* cell line studies and various organ study, including the liver, pancreas, colon, and testes, but its pathological expression in the brain being a mystery (Wang et al., 2019) Numerous prior studies have also indicated that the increase in reactive oxygen species (ROS) production caused by BPA may be a key factor contributing to its toxicity. Hence, it is imperative to investigate the potential repercussions of the growing exposure to BPA in relation to the development of oxidative stress-induced neurotoxicity (Seachrist et al., 2015). The improper disposal of BPA into water bodies near human-colonised areas presents a noteworthy risk for the emergence of unadorned health issues. Within this context, the aquatic ecosystem is particularly vulnerable to contamination due to sewage discharge from garbage dumps, environmental degradation, and the release of pollutants from industrial facilities. *Danio rerio* (Zebrafish) has become valuable animal model for studying aquatic ecosystems in various preclinical investigations, owing to its distinctive behavioral responses to toxic substances, stressors, and even therapeutic interventions, which closely mirror those observed in mammals.(Cassar et al., 2020). Zebrafish has gained prominence as valuable animal model in the study of various brain disorders, including Alzheimer's disease and Parkinson's disease. A remarkable 82% of genes associated with these diseases have counterparts in the copiously sequenced zebrafish genome. Researchers can tap into zebrafish brain plans and gene expression datasets to investigate the genetic and neuroanatomical facets of brain regions relevant to neuropsychiatric conditions. By mimicking human brain conditions in zebrafish, it becomes feasible to identify novel therapeutic targets and elucidate their molecular connections. Both larval and adult zebrafish can aid as preclinical *invivo* models, allowing for genetic, pharmacological, and experimental manipulations. Zebrafish embryos and larvae, owing to transparency and trivial size, offer an ideal platform for visualizing brain activity, conducting optical manipulations, and conducting high-throughput screenings of molecular-targeted remedies and aspirant genes. (Kalueff et al., 2014, Stewart et al., 2014, Kabashi te al., 2011, Wulliman et al., 2012, Sakai et al., 2018)

The zebrafish has emerged as prominent example in the study of social responses to numerous noxious synthetic substances and stress-inducing scenarios, including those linked to medical interventions. It is now widely recognized as a valuable animal model for investigating aquatic environments in diverse preclinical research contexts, owing to its similarities with mammals. Utilizing natural compounds as a preventive or therapeutic approach to combat neurotoxicity induced by BPA shows considerable promise. Multiple studies involving both animal models and human subjects have provided evidence that natural compounds possessing antioxidant properties may effectively impede advancement of neurodegenerative diseases. (Kawato, 2004).

Ulva lactuca, a green macroalga, is a major contributor to the worldwide occurrence of harmful green tides. These green floods, sometimes known as blooms, are a direct result of human activity. Ulva blooms are mostly found in shallow seas, and their breakdown can produce toxic pollutants. While Ulva lactuca looks like lettuce, genetic investigations have indicated that other green algae with tubular traits belong to the U. lactuca clades, contradicting prior classifications as different species or even genera. Environmental variables such as water salinity levels or symbiotic connections with bacteria may contribute to U. lactuca's capacity to display diverse phenotypes.(Dominguez H & Loret EP, 2019)

Ulva protein hydrolysates are antioxidants (Kazir et al., 2019), inhibit the angiotensin-converting enzyme (ACE) (Paiva et al., 2016), and have immunomodulatory activities (Cian et al., 2018). Ulva includes a little amount of starch (around 1-4 percent) as reserve polysaccharide. Ulva also contains both water-soluble and insoluble cellulose (varying from 38% to 52%), with Ulvan being the most prevalent component (Cardoso et al., 2014). Ulvan polysaccharides are sulfated heteropolysaccharides that contribute to the forte of the cell wall and provide Ulva suppleness, preventing shrivelling during tides. Ulvan polysaccharide is composed of sulfonic acids, sulfated l-rhamnose, xylose and glucose and accounts for nearly 30% of Ulva's dry weight. This polysaccharide and its oligosaccharides have antiviral, anticancer, anticoagulant, lipid-lowering, hepatoprotective, immunostimulating, antidepressant, and anxiolytic properties. As a result, their usage in medicinal and culinary applications is becoming increasingly popular (Sari-Chmayssem et al., 2018). Research on Ulva lactuca on preventive efficacy on BPA-induced neurotoxicity and oxidation changes is unexplored. As a result, zebrafish were employed as an in vivo model in the current study to investigate the adverse effects of BPA on the brain antioxidant defence system and the alleviatory impact of whole extract of ulva lactuca on BPA-induced alterations in the brain of zebrafish.

Methodology

Chemicals and Reagents

All the necessary analytical grade chemicals and standard reagents employed in the current experiments were procured from Sigma-Aldrich and SRL, with the exception of any alternative sources.

Experimental animals

Zebrafish aged 5-6 months were purchased from dealer in Chennai, and were maintained in a 50-L tank at a persistent temperature of 25 °C. The laboratory was kept on a 12–12 h light-dark cycle for zebrafish preservation.

Acute toxicity studies

Dose standardization of BPA

The water-based acute toxicity assessment of BPA adhered to the recommended guidelines established by OECD in 1992. Zebrafish were employed as the test subjects to ascertain the LC50 value for bisphenol A. BPA solution was prepared by dissolving the required quantity in 100% ethanol. The final ethanol concentration in all experimental groups, for both the acute toxicity test and the dose-response investigation of BPA, was 0.003% (volume/volume).

The dose-response investigation uncovered a 100% mortality rate at a concentration of 34.84 μM, establishing the Lethal Concentration 50 for BPA at 24.98 μM. After nintysix hours of the acute toxicity assessment, behavioral changes were observed at a concentration of 17.02 μM. Consequently, for this current study, a concentration of 17 μM of BPA was chosen to explore neurotoxicity mediated by oxidative stress signaling in zebrafish.

Considering the increasing presence of BPA in aquatic environments, we deliberately employed a BPA concentration for water-based exposure that significantly exceeded environmentally relevant levels, enabling a comprehensive examination of its potential impact on zebrafish.

Dose standardization of TEUL

The OECD 203 standards for performing acute toxicity studies on fish were followed, as well as the technique for determining the dose at which half of the fish succumb. All the fish were exposed to test chemical for about 96 hours in accordance to these criteria. The fish's mortality rates were measured at 24, 48, 72, and 96 hour intervals, allowing the LC50 values to be calculated.

To begin the study, zebrafish were obtained and acclimatised to a seven-day acclimatisation phase in the laboratory. Temperatures were rigorously maintained between 21 and 25 degrees Celsius, with a photoperiod of 12-16 hours. In the 24 hours preceding up to the experiment, the fish were fed 2 times a day. Furthermore, every 24 hours, important factors such as liquefied oxygen, pH levels, and the temperature were examined.

A limit test was performed as part of the procedure at a concentration of 100 mg/L (active component) to validate that the LC50 value surpassed this concentration. Each test concentration of 200 mg/L was examined using ten fish for the acute toxicity evaluation, while control group was maintained with the reverse osmosis water. The test system remained inert for the whole observation time, adhering to a static approach for the entire 96-hour duration. Fish with no obvious movement after 24, 48, 72, or 96 hours were reckoned dead.

Any visible anomalies, including but not limited to (a) loss of balance, (b) changes in swimming behaviour, (c) changes in respiratory function, (d) differences in pigmentation, and (e) any other clinical symptoms, were painstakingly noted during the observation period. These findings were critical for detecting toxicity, and LC50 doses were calculated based on relevant exposure periods.

Neuropharmacological evaluation

Novel Tank Dive Test (NTDT) (Bencan et al., 2009)

The Novel Tank Dive Test (NTDT) is a ordinarily employed method for investigating exploratory behavior in zebrafish. Initially, the fish have a tendency to descend to the bottommost of a newly introduced tank and afterwards ascend to top sections, making this challenge designed around that specific behavior pattern. This classic behavioral test was developed based on the observation that zebrafish typically spend the popular of their time in the lower region of a new dive tank, which is often referred to as "bottom-dwelling."

The NTDT was employed to analyze the investigative behavior of zebrafish within the ANY maze video tracking system. Assessments were conducted to compare the fish's positions along the tank's walls to their positions in the center, considering subordinate, central, and high levels.

Light and Dark Preference Test (Serre et al, 1999)

In the study on scototaxis (preference for darkness), the zebrafish were observed favor darker environments using the LDPT (Light-Dark Preference Test). Unlike the novel tank dive test, where the main aversive stimulus is the new environment itself, the scototaxis test focuses on the conflict between the enthusiasms to approach and avoid darkness as its primary driving factor. This test, though seemingly straightforward, relies on zebrafish exploring a black and white tank to reveal their preference, akin to a mouse's behavior in a light & dark box (Magno et al., 2015).

To conduct the light-dark test, a glass tank was employed with dimensions of 18 by 9 by 7 centimeters for length, width, and height, respectively. The tank was divided equally into dark and white sections using a black divider. The water level was maintained at 4 centimeters (approximately 3 liters) to allow the zebrafish to freely move between both sides of the tank. Data analysis was performed using ANYmaze, which tracked and analyzed the fish's behavior.

Biochemical Estimations

Once the behavioural studies were concluded and evaluated, zebrafish were euthanized, and then brains were extracted and kept at a temperature of 4ºC to subsequent biochemical analysis. The brains were utilized for this biochemical estimations, and this process was repeated three times for each experiment, as outlined in the study by Mohanty et al. in 2016. The brain samples were meticulously homogenized at 4ºC using a glass homogenizer in cold RIPA buffer, followed by a 25-minute incubation at 4ºC and subsequent centrifugation at 12,000 RPM for 20 minutes. It was imperative to collect the resulting supernatant, portion it, and store it at -20ºC until it was ready for use.

Lipid Peroxidation

The quantification of lipid peroxidation is typically assessed by measuring the generation of thiobarbituric acid reactive substance (TBARS), as stated in the study by Mohanty et al. (2017). In a brief overview, 100 µL of the brain tissue supernatant was combined with 3.8 mL of a TBAR (thiobarbituric acid reagent) and incubated at 95^0C for roughly 60 minutes in a water bath. Subsequently, it was subjected to centrifugation at 10,000 g for 10 minutes. At this stage, a pink chromophore was produced, which was then analyzed at 532nm utilizing a spectrophotometer. Ultimately, the concentration of TBARS was determined based on its extinction coefficient, and the results were expressed as nMole TBARS formed per milligram of protein.

Superoxide Dismutase

The Total SOD activity was determined using a previously established method with slight adjustments (Beauchamp and Fridovich, 1971). The reacting mixture consisted of 2.9 mL of 50 mM sodium-phosphate buffer, 2 μM riboflavin, 10 μM EDTA, 75 μM NBT (Nitro Blue tetrazolium) 13 mM methionine, and 100 μL of homogenised brain tissue sample. The mixture was then incubated at 30°C for 10 minutes, after which the absorbance at 560 nm was measured using a spectrophotometer. SOD enzyme action was quantified in units, where one unit represented the amount of sample protein required to inhibit 50% of the NBT reduction. SOD activity was expressed as units per milligram of protein.

Catalase activity

The enzyme catalase's performance was evaluated following the procedure outlined in a prior study by Mohanty et al. in 2016. This enzyme facilitated the decomposition of H_2O_2, which was consequently measured using a spectrophotometer at 240 nm at 15-second intervals for a maximum duration of 2 minutes. The catalase's effectiveness was quantified as nkatal/mg of protein, with 1 nano katal (nkatal) corresponding to the consumption of 1 mole of H_2O_2 per second within the reaction mixture.

Glutathione reductase estimation

The zebrafish brain GR activity assay was measured in accord with the previously established procedure (Sarkar et al., 2014). By measuring the NADPH oxidation *uv* spectrophotometrically at 340 nm, the degree of conversion from GSSG to GSH was computed. The molar extinction coefficient of NADPH was used to calculate the glutathione reductase activity, which is expressed as nMole NADPH oxidized/min/mg protein.

Statistical Analysis

The data were presented as the mean value along with the standard error of the mean. To assess the results, a one-way analysis of variance was employed, followed by Dunnet's test, to compare the various groups: naive, control (BPA), BPA+Piracetam, BPA + TEUL 200mg/l groups, and BPA + TEUL 400mg/l groups. We considered a significance level of $p < 0.05$ for all groups.

Results

Light and Dark (Scototoxis) Test

Prolonged exposure to BPA in water had a noticeable impact on the typical light preference behavior of zebrafish. This was evident from the increased tendency of the fish to move toward well-lit areas and their extended time spent in such areas when compared to the untreated and control groups. Additionally, the BPA-exposed fish displayed a considerable delay in entering dark zones during the LDPT test compared to the untreated group. Nevertheless, the altered light preference behavior in zebrafish caused by BPA exposure was significantly improved in the presence of TEUL, as observed in the BPA + TEUL group when compared to the BPA-only group.

Novel Tank Diving (Bottom Dwelling) Test

Moving to the upper zone and the duration spent in that entire zone were notably extended in the groups subjected to BPA in comparison to group 1. Additionally, when contrasted with group 1, the group exposed to BPA exhibited a substantially shorter delay in entering the upper zone. The inclusion of TEUL in the BPA-exposed group resulted in a reduction in the time spent in the upper zone, the frequency of transitions throughout the entire area, and the delay

before entering the upper site. The conclusions drawn from this present study indicate that Ulva lactuca may offer protection against the behavioral alterations induced by BPA.

Invivo antioxidants study

Exposure to BPA over a period of 21 days resulted in a significant surge in lipid peroxidation (LPX) levels in the BPA-exposed group when compared to the control group. Moreover, BPA exposure led to a notable decrease in catalase activity, glutathione (GSH) levels, and superoxide dismutase (SOD) in the zebrafish brain. The primary findings of this study suggest that BPA triggers oxidative stress, which, in turn, causes the degradation of lipid components within the cellular environment of the zebrafish brain, as compared to the control group.

In terms of combating the oxidative stress induced by BPA, the study found that TEUL (the neuroprotective supplement) significantly reduced LPX levels and increased catalase activity, GSH levels, and superoxide dismutase (SOD) in the zebrafish brain.

Discussion

BPA, primarily a man-made toxic substance, is widespread in our environment, posing a potential danger to human health by causing serious neurological illnesses. Our current research not only aims to assess the neurotoxic effects of BPA but also seeks to investigate the protective qualities of the total extract of Ulva lactuca against BPA-induced harm. Our initial study revealed that behavioral changes occurred at a concentration of 17.02 μM of BPA. Consequently, we chose this concentration to assess the neurotoxicity of BPA. In our study, we exposed zebrafish to 17 μM of BPA in water, a level significantly higher than what is typically found in natural water sources, in order to explore the potential impact of the increasing presence of BPA on the zebrafish brain (PK Sahoo et al., 2020).

A neurobehavioral assessment conducted by NTDT revealed that when zebrafish were exposed to BPA through waterborne exposure, co-administration of TEUL significantly improved their bottom-dwelling behavior compared to the untreated and control groups. Furthermore, TEUL supplementation also reversed the alterations in scototaxis behavior induced by BPA in LDPT. This was evidenced by a notable reduction in the number of transitions into the light zone and the time spent in the light zone. These initial findings strongly support the idea that BPA has neurotoxic potential, causing changes in zebrafish scototaxis and bottom-dwelling behavior, and that TEUL serves as a neuroprotective intervention.

To confirm that the rehabilitated neurobehavioral response to BPA is a result of increased oxidative stress in the zebrafish brain, the levels of various antioxidant enzymes and oxidants were examined. We also assessed the neuroprotective effect of TEUL against BPA-induced oxidative stress. Current results demonstrate that co-administration of TEUL during waterborne exposure to BPA led to a significant reduction in ROS levels and LPX in the PGZ region of the zebrafish brain. Subsequent findings indicate that TEUL reversed the BPA-induced decline in antioxidant levels and the activity of the free radical scavenging enzyme system, ultimately resulting in declined oxidative stress in the zebrafish brain.

In summary, this research suggests that Ulva lactuca may have potential neuroprotective benefits in countering the neurotoxic effects of BPA in zebrafish. The changes in neurobehavioral responses were closely linked to increased oxidative stress in the zebrafish brain. Nevertheless, when administered as a therapeutic treatment, the Total extract of ulva lactuca demonstrated a strong ability to remove reactive oxygen species (ROS) and hydroxy

radicals after prolonged exposure to BPA in the water. The neuroprotective potential of this ulva lactuca extract against BPA-induced neurotoxicity appears to be linked to its capacity to restore the redox balance within the cell by enhancing the levels of glutathione (GSH) and antioxidant enzymes. These findings from the current study suggest that TEUL may play a role as a potential intervention to mitigate BPA-induced alterations in neurobehavior, oxidative stress, and neurodegeneration. Future research aimed at understanding the downstream signaling pathways may offer valuable insights into the development of preventive strategies against BPA-induced susceptibility to serious neurodegenerative diseases.

Table:1 Influence of TEUL on antioxidants including Superoxide Dismutase (SOD) and Catalase (CAT), followed by the redox enzyme Glutathione Reductase (GR) and Lipid peroxidation (LPO) of brain tissue of zebrafish

Brain	**Group-I (Sham Control)**	**Group-II (Disease Control; BPA)**	**Group-III (BPA + Standard drug)**	**Group-IV (BPA + Drug [50mg/L])**	**Group –V (BPA + Drug [100 mg/L])**
SOD	5.42 ± 0.63	9.34 ± 1.22	6.73 ± 0.45	7.14 ± 0.27	6.92 ± 0.32
CAT	3.43 ± 0.21	7.34 ± 0.51	5.04 ± 0.16	5. 73 ± 0.14	5 .67 ± 0.53
GR	1.14 ± 0.13	0.57 ± 0.02	1.21 ± 0.04	0.93 ± 0.03	1.32 ± 0.04
LPO	9.54 ± 1.22	16.34 ± 1.30	10.31 ± 0.72	12.04 ± 1.14	10.21 ± 0.36

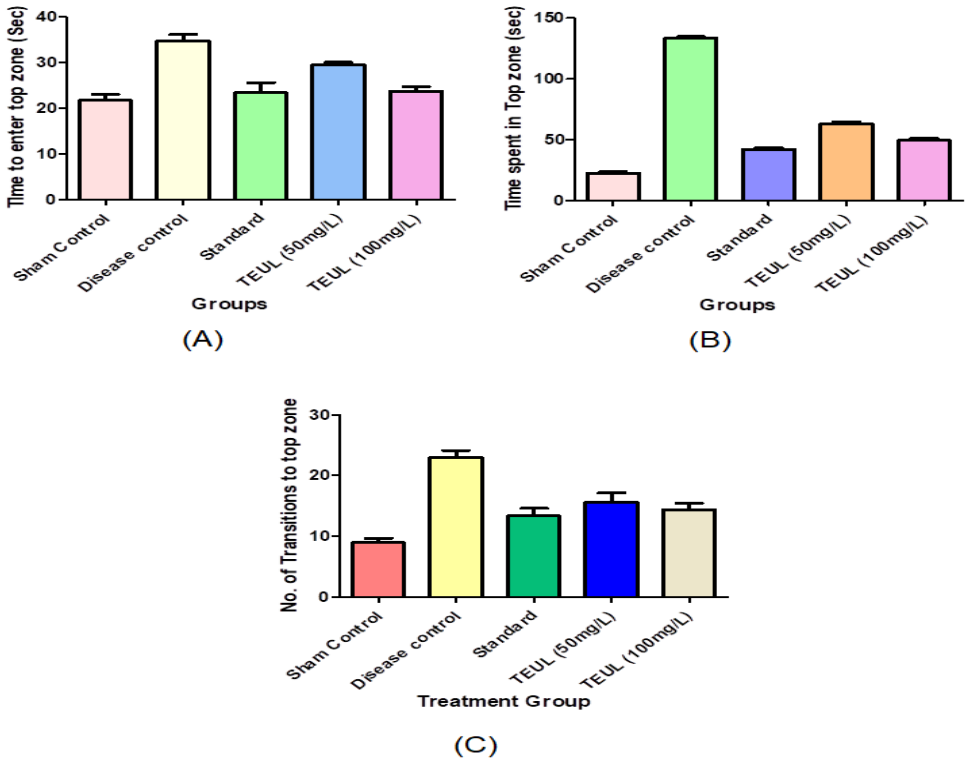

Fig: 1: Novel Tank Diving Test : (A)Time taken to enter Top zone, (B) Time spent in top zone & (C) No. of transitions to top zone

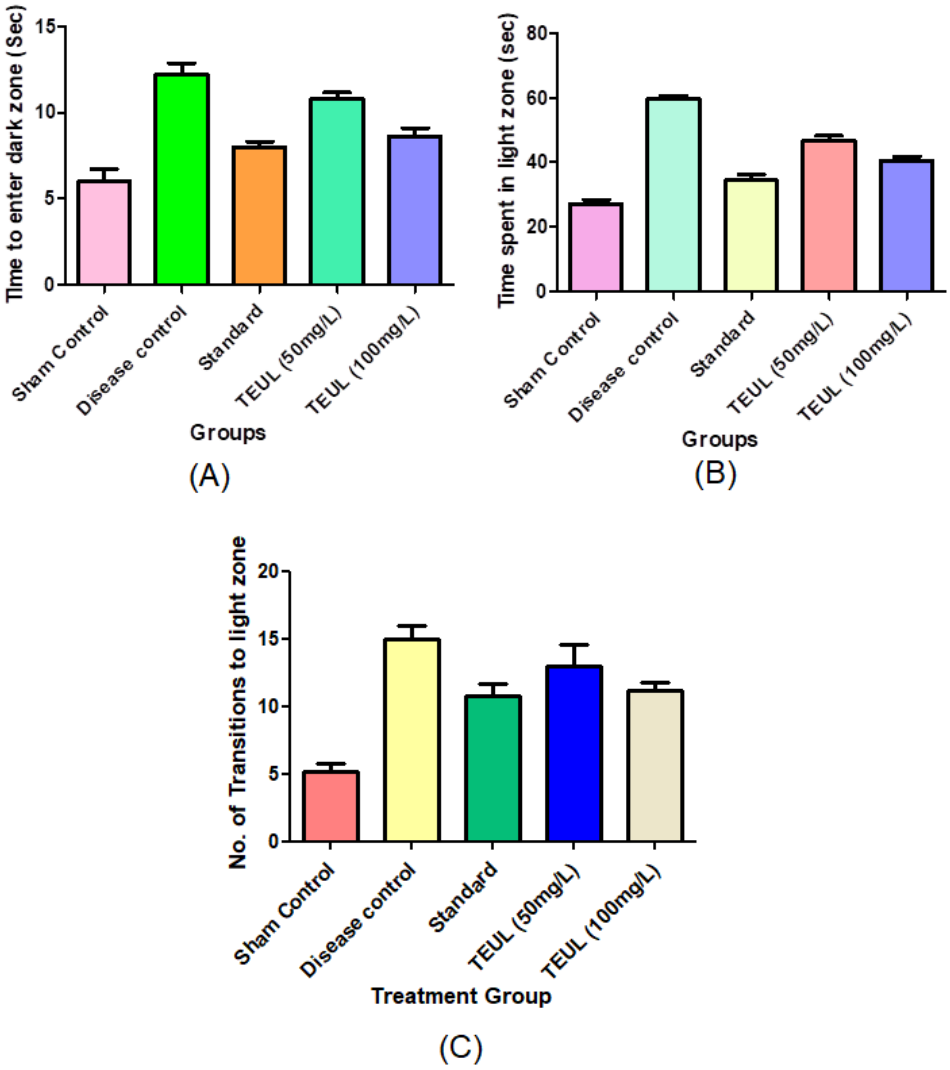

Fig: 2: Light and Dark (Scototoxis) Test : (A)Time taken to enter dark zone, (B) Time spent in light zone & (C) No. of transitions to light zone

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