Research Paper

Acute Toxicity of Epigallocatechin Gallate and C-Phycocyanin in Zebrafish (*Danio rerio*): Behavioral and Histopathological Studies

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Epigallocatechin gallate and C-phycocyanin are natural food supplements, gaining a lot of popularity in biomedical researches. The lethal concentration 50 value for the zebrafish model was determined using a 96 h semi-static acute toxicity test. Zebrafish were transferred into a 1 l water tank starting with a minimal dose of 8 mg of epigallocatechin gallate and 1 g of C-phycocyanin, which further increased to the concentration of 30 mg for epigallocatechin gallate and 5 g for C-phycocyanin. Lethal concentration 50 of epigallocatechin gallate and C-phycocyanin was calculated to be 21.37 mg/l and 2.45 g/l respectively at 96 h in the zebrafish model. At high doses above 12 mg of epigallocatechin gallate and 1 g of C-phycocyanin, the test fish displayed various behavior responses like erratic and jerky swimming, attempts to jump out of the water, frequent surfacing and swallowing of air, normal skin color change, decreased opercular movement and finally leading to death. Histological observations showed hypertrophy, atrophy and vacuolar degeneration in the liver whereas tubular degeneration, glomerular hyper cellularity, dilation of Bowman's space, hemorrhage, and necrosis were observed in the kidneys exposed to the high doses of epigallocatechin gallate and C-phycocyanin treated fish. The gills of the test animals also expressed lamellar synechiae, hyperplasia, curling of lamellae, mucus secretion and shortening of the secondary lamella. The results demonstrated that doses above 30 mg/l and 5 g/l in the case of epigallocatechin gallate and C-phycocyanin respectively gave 100 % mortality and expressed organ-specific toxicity. Overall results revealed that exposure to high concentrations of epigallocatechin gallate and C-phycocyanin uninterruptedly may lead to severe alterations in the tissue of experimental animals.

Key words: Acute toxicity, behavior response, C-phycocyanin, zebrafish model, epigallocatechin gallate, histological observations

Toxicity is the leading cause of drug development failure and it is a key concentration and issue for current drug discovery[1]. People assume that all-natural products are safe to consume and that no more research is required on toxicity. Off late natural food supplements are gaining a lot of popularity; two such products are Epigallocatechin Gallate (EGCG) and C-Phycocyanin (C-PC). EGCG is one of the most studied and abundant catechins found in green tea extract. It is well known to exhibit antioxidant and anti-inflammatory properties and is marketed as a dietary supplement[2]. Studies in model animals have shown that it inhibits the development and growth of tongue cancer cells in Keratin 14 (KRT14)/ERT-Kirsten Rat Sarcoma Virus (Kras) transgenic mice[3]. It induces apoptosis and inhibits the Phosphatidylinositol-3-Kinase (PI3K)/Protein Kinase B (Akt)/mammalian Target of Rapamycin (mTOR) pathway progression in precancerous lesions of gastric carcinoma treated rats[4]. It protects and improves the colon from chemically induced agents like N, N′-Dimethylhydrazine (DMH) in Wistar rats and protects the lung against fluoride-induced oxidative injury[5,6]. However, findings suggest the use of EGCG as a pro-oxidant; a high dose of EGCG causes events of hepatotoxicity as well as suppresses the antioxidant enzyme in the liver[7].

C-PC is a light-harvesting, water-soluble, protein-bound pigment obtained from some microalgae

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Accepted 19 March 2024
Revised 16 April 2023
Received 04 September 2022
Indian J Pharm Sci 2024;86(2):546-555

such as *Spirulina platensis* that has been attracting a lot of importance as a nutritious supplement with its high protein content[8]. It is the primary pigment constituent component of *Spirulina platensis*, making up approximately 15 % of its total dry weight. They are extensively used as a dietary supplement in several countries due to their nutritional asset[9]. It exhibits a vast array of therapeutic potentials such as anti-inflammatory, neuroprotective, hepatoprotective, immunomodulatory, antimutagenic, antiviral, antimalarial, anticancer, antioxidant and free radical scavenging agent and diabetic nephroprotective agent[10]. Thus, with EGCG and CPC exhibiting such strong and diverse potential for their application in various biomedical studies the need to understand the toxicity and nature of this compound arises.

Zebrafish (*Danio rerio*) is used as an excellent animal model to inspect samples of the bioactive compounds through toxicity assay. Commonly used mammalian models have some drawbacks with higher cost and a prolonged time for results, yet ethically questionable. It has been acknowledged as an effective animal model for assessing drug targets for toxicity and safety risks because of their sharing of common physiological, morphological and histological characteristics with mammals. It has short cycle, utilizing smaller amounts of test substance and high throughput over the classic *in vivo* model. Multiple organs may be monitored and pharmacodynamic, pharmacokinetic and metabolite activities can be studied at the same time. The toxic effect of drugs/compounds on the heart, liver, kidney, developmental stages, and behavior can be effectively studied using the zebrafish model system[11,12].

A toxicology test is necessary for allopathic treatment and alternative medicine to uncover any possible side effects that may not be apparent until they manifest. Currently, the use of natural products such as EGCG and C-PC is on the rise and their application in various biomedical researches. Although many findings show the capability of EGCG and C-PC to suppress the effect caused by different carcinogens and mutagens still only a few reports regarding the safe use of these compounds have been recorded[13,14]. As such, the need for proper knowledge of the toxicity of these two compounds is to be studied carefully. The assessment of toxicity using acute toxicity bioassay can prove the safety of traditional medicine using EGCG and C-PC and promote its consumption and other research activities. The purpose of this study was to examine the acute toxicity of EGCG and C-PC in the zebrafish models and observe the histological damages cause in the tissue of kidney, liver and gills of the adult zebrafish.

MATERIALS AND METHODS

Chemicals:

EGCG (>95 %), hematoxylin-eosin stain, xylene and absolute alcohol were purchased from Sigma Aldrich. Commercially available purified C-PC was procured from Algallio Biotech, India. All other chemicals were of reagent grade. Solutions were prepared with deionized water. All glassware and aquariums used in the experiment were washed and thoroughly rinsed with distilled water before use.

Acclimatization of zebrafish:

Zebrafish (5-7 mo; 3-5 cm long; weighing about 300-500 mg) with an equal gender ratio were purchased from Aqua fish and pets, Jorhat Assam, India. Fishes were purchased and then treated with 0.05 % Potassium permanganate solution ($KMnO_4$) for 5 min to avoid any cutaneous infection. It was cultured in a zebrafish housing system (Model-NT-ZB-11; Make-Narshi Technologies) to ensure constant temperature (28°±2°), persistent chemical, biological and mechanical water filtration and aeration (7.20 mg Oxygen (O_2)/l). Polycarbonate fish tanks were maintained under a 14 h per day and 10 h per night photoperiod cycle. Adult zebrafish were fed three times a day with feed that is commercially available containing protein 28 %, crude fat 3 %, fiber 4 % and moisture 10 %.

Acute toxicity study and behavioral study:

The 96 h semi-static acute toxicity test of zebrafish exposed to EGCG and C-PC was performed according to guideline 203 of Organisation for Economic Co-operation and Development[15]. Adult zebrafish were randomly exposed to 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 and 30 mg of EGCG and 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 3.25, 3.5, 3.75, 4, 4.25, 4.5, 4.75 and 5 g of C-PC. The different concentrations of EGCG and C-PC were dissolved in 1 l dechlorinated water. The test performed consists of ten adult zebrafish. The fresh solution was prepared and changed at 48 h after the first exposure. The test animals

were fed with commercially available feed and were not fed 24 h before the experiment. Test animals were observed periodically for 4 d. Dead animals and feces were timely removed from the experimental jar to ensure non-interference in the system. Behavioral changes were recorded such as gulping activity where the zebrafish were observed according to the piping and gasping movement of their opercular at the surface of the experimental solution, hyperactivity like skittering and erratic swimming movements, and hypo activity such as lethargy, inactivity, weakness, quiescent, ceased swimming and immobility were recorded individually in the experimental subjects. In order to observe the morphological change, the whole-body image of the experimental zebrafish was captured by using a Nikon D3500 digital camera.

Histopathological study:

Histopathological techniques were followed based on the method of Abdelhamid *et al.*[16]. The organs were dissected and immediately rinsed with the physiological saline solution to remove blood and debris adhering to the tissue then; they were then fixed in 10 % buffer formalin for 24 h. The fixative was removed by washing through running tap water overnight. After dehydrating through an ascending graded series of ethyl alcohols, the fixative was then cleared using two change of xylene and embedded in paraffin wax. The tissues were cut at the 4-5 µ thickness and stained with Harris-hematoxylin, and counterstained with eosin, dissolved in 95 % alcohol. After dehydration and clearing, sections were mounted in Canada balsam. The prepared slides were observed and photographed under a CXL microscope attached to a Sony digital camera (model E31SPM20000KPA; USB 2.0) and analyzed using image J software.

Statistical analysis:

The recorded mortality data were corrected for natural response by Abbott's formula which was further subjected to probit analysis for calculating Lethal Concentration 50 (LC_{50}) and other statistics at 95 % confidence limits of upper confidence limits[17], and lower confidence limits were calculated using Statistical Package for the Social Sciences (SPSS) 22.0 software (SPSS Inc., United States of America (USA)). Mortality percentage graph of the test animal was prepared using graph pad prism version 5.0. The result of the behavioral responses for the adult zebrafish were presented as mean±standard error of mean and represented using an error bar.

RESULTS AND DISCUSSION

The experimental zebrafish displayed mortality in a dose- and time-dependent manner (fig. 1). The LC_{50} was calculated to be 26.91 mg/l, 25.70 mg/l, 23.44 mg/l and 21.37 mg/l for EGCG whereas the LC_{50} value for C-PC was 3.71 g/l, 3.31 g/l, 2.88 g/l and 2.69 g/l at 24 h, 48 h, 72 h and 96 h respectively (Table 1). The linear curve between mortality of test fish against the different concentrations of EGCG and C-PC treated for 96 h showed a highly positive correlation (r=0.932 for EGCG and r=0.977 for C-PC) (fig. 2). The mortality was found to be 100 % at doses above 30 mg/l in the case of EGCG and above 5 g/l for C-PC. Test animals responded to the treatment with rapid movement swimming, swallowing, jumping activity and immobile behavior as the concentration increased (fig. 3). At higher doses, fins became harder and stretched. A large amount of mucus was present over the fish body. The dead zebrafish showed reddish skin coloration in the opercular region due to hemorrhage. There was a decrease in the pigments of the zebrafish body as exposure to time and dose were increased. Severe necrosis and edema were observed in the chest area of the fishes exposed to EGCG and C-PC (fig. 4a-fig. 4f). The magnitude of damage caused to the organs was found to be a time- and dose-dependent factor. Longer duration and higher doses were found to inflict significantly more damage.

A comparative study was done to further understand the histopathological changes of the control group, with the minimum dose (12 mg of EGCG and 1 g of C-PC) where the test animal started showing lethargy and the highest dose (30 mg of EGCG and 5 g of C-PC) in which the degree of toxicity showed to be harmful, resulting in increased mortality. The test zebrafish showed changes in the liver tissue compared to the control group depending on the dose incorporated. Exposure to 12 mg/l of EGCG displayed cell dead or necrosis, atrophy and vacuolar degeneration, whereas higher vacuolar degeneration in different areas of the liver, atrophy, necrosis, hypertrophy, and sinusoids space was noticed at 30 mg/l of EGCG. Hepatic tissue exposed to 1 g/l of C-PC showed vacuolar degeneration, hepatocyte necrosis and atrophy, whereas severe vacuolar degeneration was observed when exposed to 5 g/l C-PC (fig.

5a-fig. 5f).

12 mg of EGCG exposure showed histoarchitectural changes in the kidney such as focal renal tubular degeneration, glomerular shrinkage, and dilation of Bowman's space whereas exposure to 30 mg EGCG showed glomerular shrinkage, acute cellular degeneration, vacuolar degeneration, hemorrhage, and Bowman's space dilation. Test zebrafish exposed to 1 g of C-PC showed vacuolation and hyper cellularity of glomerulus whereas a dose of 5 g showed necrosis, tubular degeneration, vacuolation, hemorrhage and edematous fluid (fig. 6a-fig. 6f).

In the experimental group treated with 30 mg/l of EGCG and 5 g/l of C-PC, gills exhibited hypertrophy, shortening of secondary lamellae, edema, curling of lamellae, epithelial hyperplasia, epithelial lifting, and lamellar synechiae. Further, with a higher dosage of EGCG 30 mg/l caused mucus secretion and fusion of secondary lamellae as well as fusion with the adjacent sides can be clearly seen. A lower concentration of EGCG 12 mg/l and 1 g/l of C-PC showed lesser lamellar fusion and hyperplasia compared to the high dosage, slight epithelial lifting was observed, and curling of the lamellae was observed in EGCG as well as in C-PC (fig. 7a-fig. 7f).

In recent years, numerous researches have shown the effects of nutraceuticals on animal models however very little researches have been done on the LC_{50} activity of EGCG and C-PC in zebrafish model. The toxicity of test compounds was determined by immersing adult zebrafish in the solution of EGCG and C-PC and observing their behavior as well as undergoing histological changes. Doses above the LC_{50} of EGCG and C-PC have been found to be harmful to different target organs. Study on several exposure routes of pyraclostrobin in adult zebrafish, mortality was found to be increased in a dose- and time-dependent way[18]. The assessment of behavioral response is a useful tool for determining the toxicological influence of the test compounds on the test animals[19]. When animals are exposed to a stressful environment, such as through chemical compounds, their defense mechanisms are activated, as their homeostasis is disrupted, resulting in behavioral changes such as escape behavior, settling down at the bottom of the tank, and rapid movement to avoid hazardous substances.

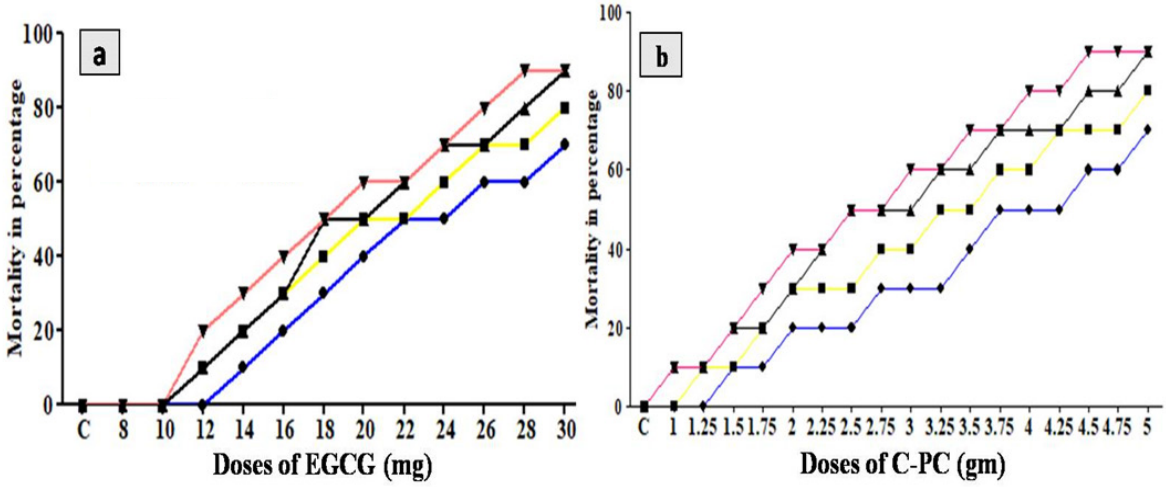

Fig. 1: Dose response curve of (a): EGCG (mg/l) and (b): C-PC (g/l) after exposure of various time periods (24 h, 48 h, 72 h and 96 h) in zebrafish model
Note: ( ): 24 h; ( ): 48 h; ( ): 72 h and ( ): 96

TABLE 1: LC_{50} OF EGCG AND C-PC FOR VARIOUS DURATION (24 h, 48 h, 72 h AND 96 h) IN ZEBRAFISH MODEL

Duration	LC_{50} of EGCG (mg/l)	LC_{50} of C-PC (g/l)
24 h	26.91	3.71
48 h	25.7	3.31
72 h	23.44	2.88
96 h	21.37	2.69

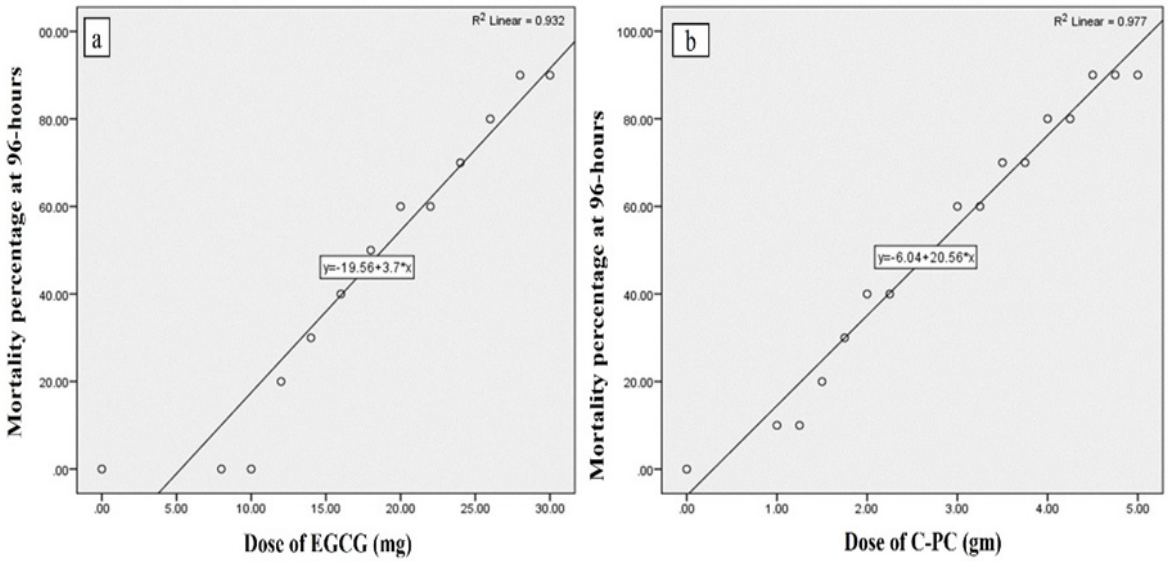

Fig. 2: Mortality percentage at 96 h of (a): EGCG (mg/l) and (b): C-PC (g/l)

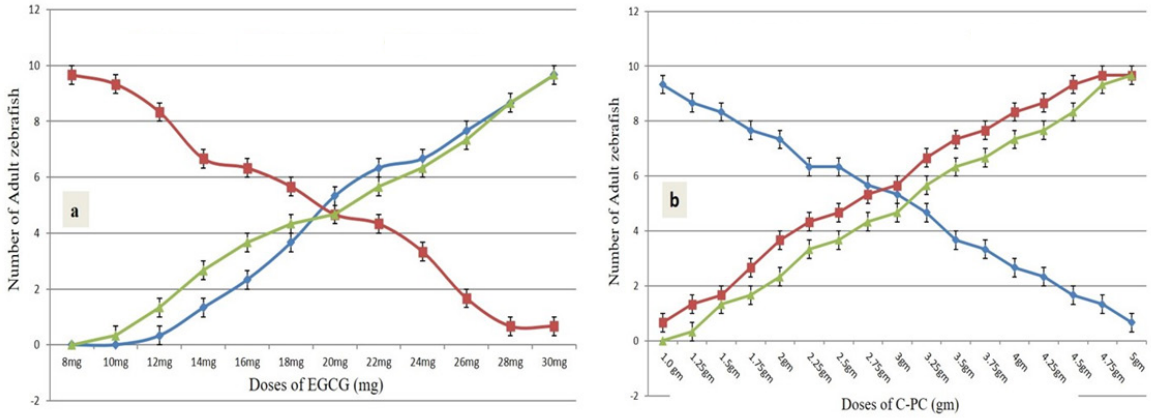

Fig. 3: Behavioral changes in zebrafish model of (a): Adult zebrafish showing gulping behavior, hypo activity and hyperactivity EGCG (mg/l) and (b): Adult zebrafish showing hyperactivity, hypo activity and gulping behavior C-PC (g/l)

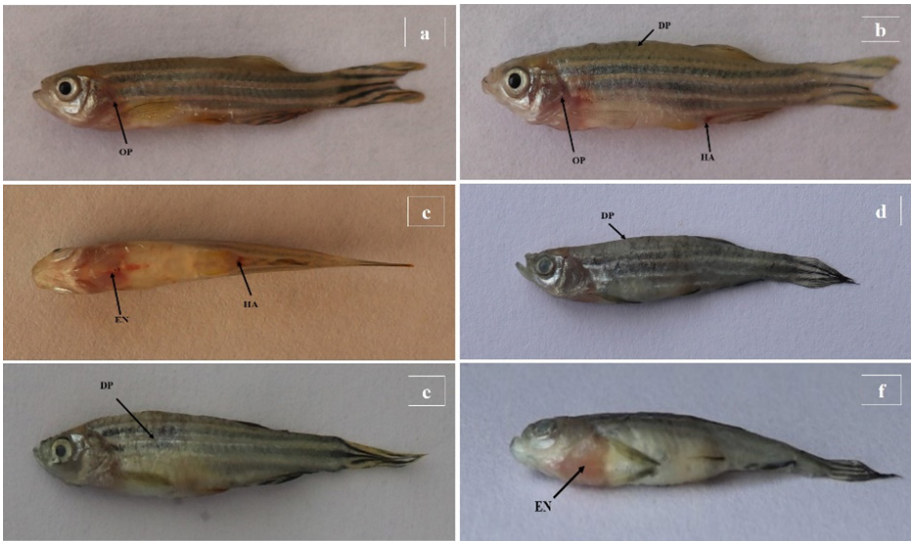

Fig. 4: Morphological changes in zebrafish exposed to EGCG and C-PC, (a): Adult zebrafish exposed to 12 mg/l EGCG exhibiting blood aggregation resulting in red coloration in the Opercular Area (OP); (b and c): Adult zebrafish exposed to 30 mg/l EGCG exhibited hyperaemia towards the anal fin (HA) , increased red coloration in the opercular region (OP), serious edema and necrosis in the thorax area (EN), highly depigmentation of the body (DP); (d): Adult zebrafish exposed to 1 g/l C-PC adult zebrafish exhibiting Depigmentation (DP) and (e and f): Adult zebrafish exposed to 5 g/l C-PC exhibiting DP, serious Edema and Necrosis (EN)

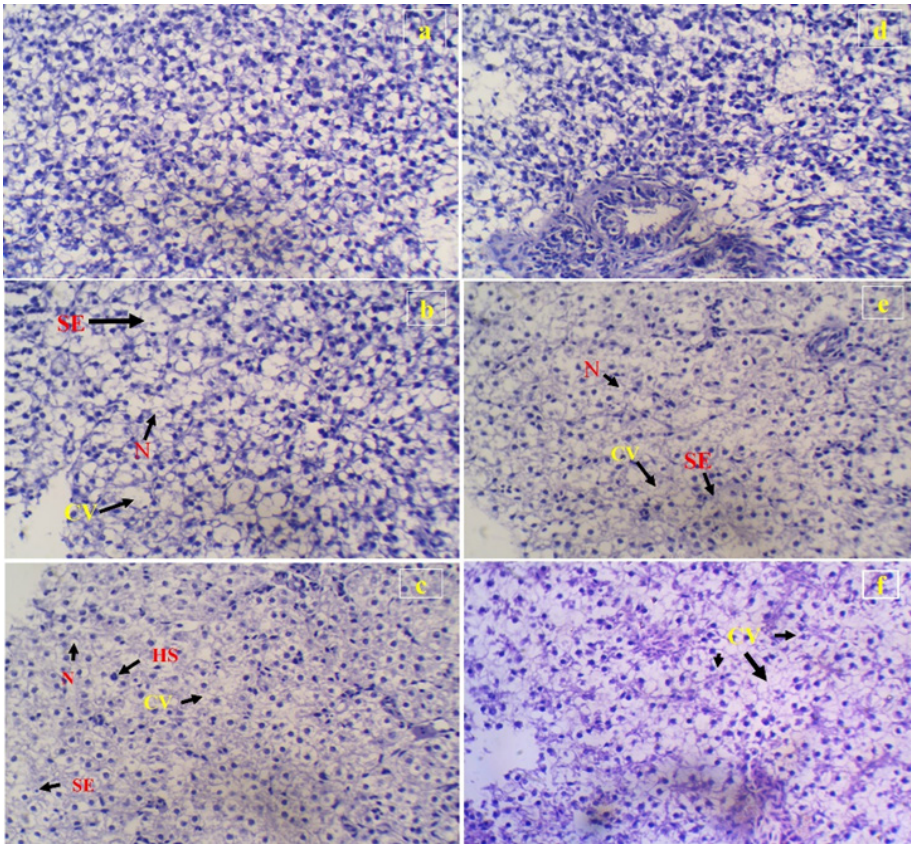

Fig. 5: Histological changes in zebrafish model exposed to EGCG and C-PC, (a): Control liver (0.0 mg/l EGCG); (b): Liver exposed to 12 mg/l EGCG exhibiting atrophy (SE), necrosis (N), vacuolar degeneration (CV); (c): Liver exposed to 30 mg/l EGCG exhibited cell hypertrophy (HS), atrophy (SE), vacuolar degeneration (CV), necrosis (N); (d): Control liver (0.0 mg/l C-PC); (e): Liver exposed to 1 g/l C-PC exhibiting necrosis (N), cell atrophy (SE), vacuolation (CV) and (f): Liver exposed to 5 g/l C-PC exhibiting high vacoular degeneration (CV)

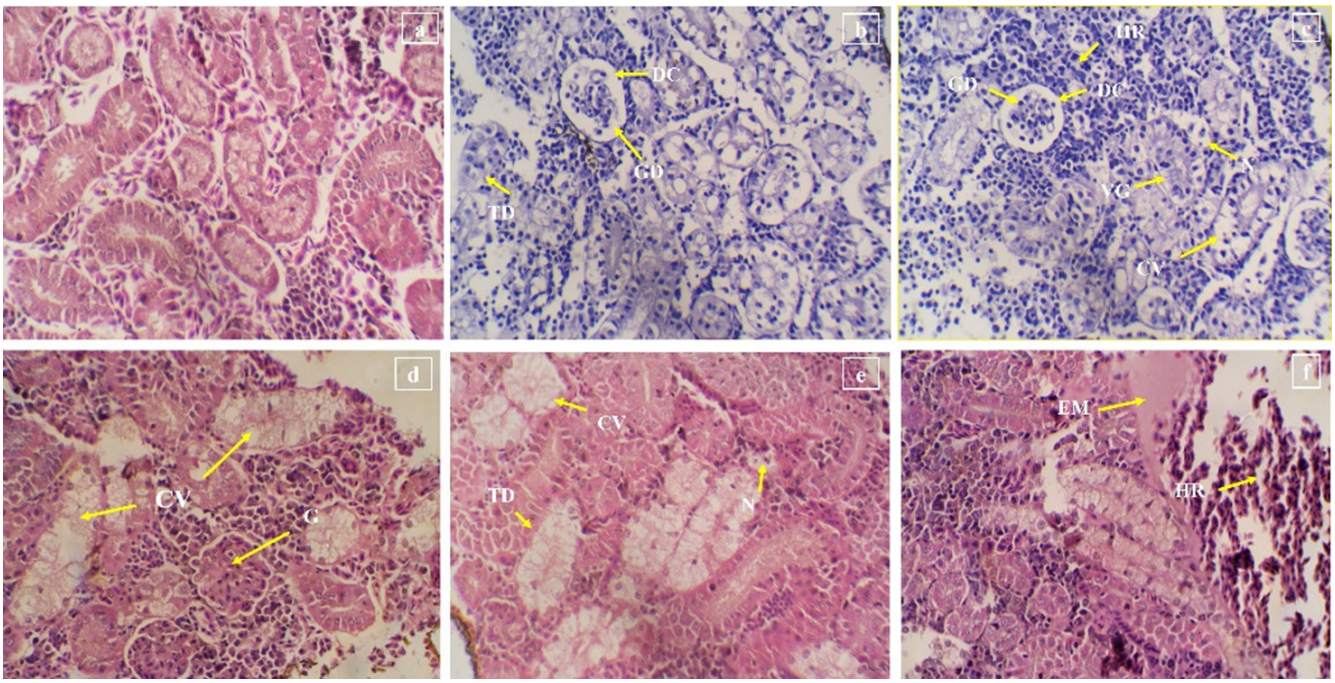

Fig. 6: Histological changes in zebrafish model exposed to EGCG and C-PC, (a): Control liver (0.0 mg/l EGCG); (b): Liver exposed to 12 mg/l EGCG exhibiting atrophy (SE), necrosis (N), vacuolar degeneration (CV); (c): Liver exposed to 30 mg/l EGCG exhibited cell hypertrophy (HS), atrophy (SE), vacuolar degeneration (CV), necrosis (N); (d): Control liver (0.0 mg/l C-PC); (e): Liver exposed to 1 g/l C-PC exhibiting necrosis (N), cell atrophy (SE), vacuolation (CV) and (f): Liver exposed to 5 g/l C-PC exhibiting high vacoular degeneration (CV)

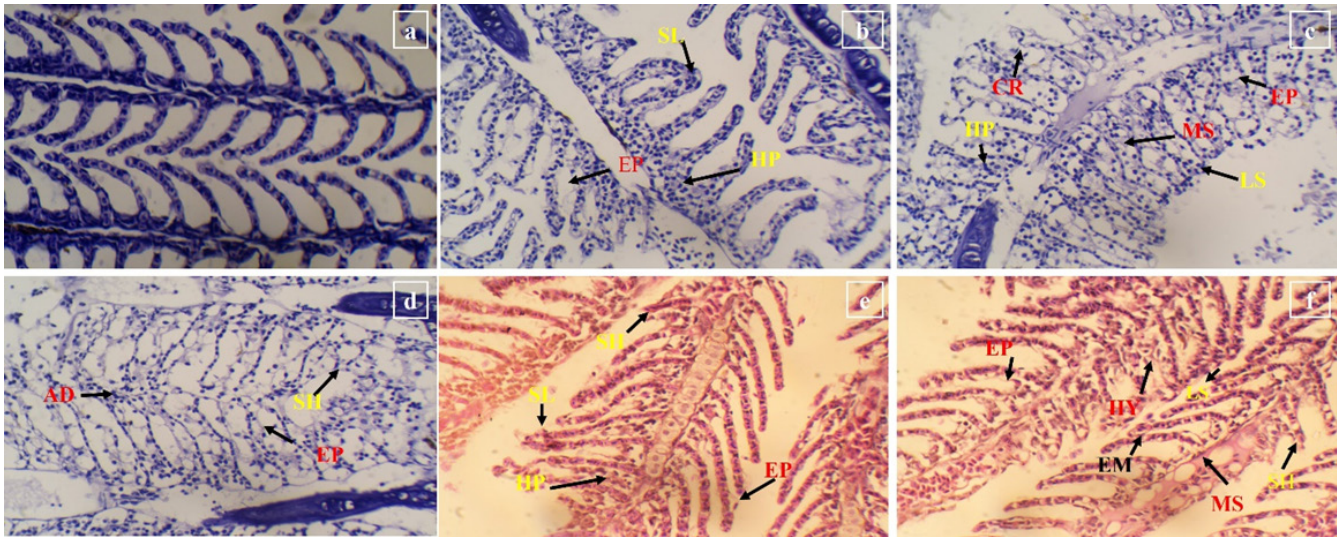

Fig. 7: Histological changes in zebrafish model exposed to EGCG and C-PC, (a): Control gills (0 mg/g/l); (b): Gills exposed to 12 mg/l EGCG exhibiting epithelial lifting, hyperplasia (HP), fusion of secondary lamella; (c-d): Gills exposed to 30 mg/l EGCG exhibiting epithelial lifting, epithelial hyperplasia, curling of lamella, mucus secretion, lamellar synechieae, fusion with secondary and adjacent lamella, and shortening of secondary lamella; (e): Gills exposed to 1 g/l C-PC exhibiting hyperplasia, epithelial lifting, shortening of secondary lamella and fusion of secondary lamella and (f): Gills exposed to 5 g/l C-PC exhibiting shortening of secondary lamella, lamellar synechieae, edema, mucus, epithelial lifting and hypertrophy

The treatment group displayed behavioral changes similar to those described by other researchers, where zebrafish exposed to Kandhamal haladi demonstrated irregular, erratic, and aberrant swimming movements[20]. Fin hardening occurs as a result of muscular stretching, making it difficult for the fish to swim. When zebrafish are exposed to a harmful environment, inflammation of the skin causes excessive mucus secretion. Mucus establishes a barrier between harmful surroundings and lowers irritation caused by the agents, which is a common defense strategy of fishes[21]. The red coloration in the test animal which is considered to be a result of massive hemorrhage was also recorded when studying the zebrafish response after exposure to propylthiouracil[22]. Further, when the toxic substances in the environment are high the test fishes exhibit hyperemia on various parts of the body similar results were seen when treating Nile tilapia with deltamethrin[23]. Disruption of the endocrine gland causes depigmentation, which alters the number of chromatophores in the thoracic area of zebrafish under toxic stress, including necrosis and edema when exposed to the venom of *Bothrops alternatus*[24].

The liver is the organ that detoxifies fatal chemical enzymes, but it is negatively affected when exposed to a higher toxicity dose. The extent of organ damage was in a time- and dose-dependent manner, with longer duration and larger dosages inflicting much greater harm. Cytoplasmic vacuole and necrosis in the cell in juvenile fish *Colossoma macropomum* were reported when testing the acute toxicity of a hot aqueous extract from *Terminalia catappa* leaves similarly cellular hypertrophy[25], atrophy, cytoplasmic vacuolation, and necrosis were found in common carp subjected to chlorpyrifos[26]. Cytoplasmic vacuolation and dilation of the sinusoid space were observed in zebrafish when subjected to Silver oxide (Ag_2O) and Silver carbonate (Ag_2CO_3) doped Titanium dioxide (TiO_2) nanoparticles and pure TiO_2 particles[27]. Hypertrophy in the liver tissue is the result of a biological reaction to stress in the body environment. Poor hepatocyte metabolism caused by an obstruction of sinusoidal blood flow eventually results in cell atrophy. The creation of vacuoles in the liver cell was thought to signal an early stage of necrosis[28,29]. Hepatocyte necrosis is a probable cause of lethal compounds invading the cell and building up a toxic material inside the cell over time[30].

The zebrafish kidney is found in the ventral section of the vertebral column and has a distinct head and trunk area. It has nephrons with glomeruli, proximal and distal tubules and a collecting duct, similar to mammalian kidneys (fig. 6a). It helps filtration of blood residues, as well as salt and water absorption and maintains tissue homeostasis. The occurrence of degeneration in the tubules,

vacuolation of tubules, change in the glomerulus and the Bowman's capsule are considered to be the most consequent alterations in the kidney due to contamination in the environment[31]. Aluminum concentrations caused histological changes in the kidney, including cellular degeneration, hemorrhage and edematous effusion in *Tilapia zillii*[32]. Tubular degeneration in adults and embryos of zebrafish was reported while investigating the acute toxicity of *Libidibia ferrea*, a traditional anti-inflammatory source[33]. Tubular degeneration, vacuolation, glomerular shrinkage with dilated Bowman's space, and necrosis were observed in freshwater fish *Catla catla* when treated with cypermethrin[34]. The striped catfish *Pangasianodon hypophthalmus* exposed to chromium also caused structural changes such as renal corpuscle shrinkage, Bowman's space dilation, and necrosis[35]. Tubular alteration in the kidney is thought to be an indirect cause of metabolic failure in zebrafish caused by toxic chemical exposure. These modifications are supposed to lead to necrosis in the long run[36].

Fish gills are crucial in determining the impact of toxicants since they are easily impacted by foreign compounds in the water[37]. The chemicals had a considerable impact on the gills of treated groups, causing hypertrophy, hyperplasia, secondary lamellae shortening and fusion, edema, lamellae curling, and epithelial lifting. Tissue-specific toxicity of clothianidin on rainbow trout caused fusion and shortening of secondary lamellae, hypertrophy of secondary lamellae, and necrosis of secondary lamellae[38]. Changes in the structure including secondary lamellae curling, epithelial lifting, hyperplasia, and lamella fusing were reported in *Sparus aurata* subjected to erythromycin and oxytetracycline[39]. As a defense mechanism, this alteration in the structure of the gills occurs to minimize the interaction between the blood and the environment, so inhibiting and reducing the impact of the hazardous material. An animal protects its underlying tissue from damaging toxicants; hyperplasia increases the number of cells that are typically suited[30]. Edema and epithelial cell lifting in the gills are also thought to be a defense mechanism used by the fish to defend itself from xenobiotics in the environment[40]. Lamellar synechiae and mucus secretion was observed after short-term coexposure to TiO_2 and CuO in the gills of *Cyprinus carpio*. Cell proliferation happens quickly, increasing the size of the epithelial layer and causing fusion between adjacent lamellae, maximizing the oxygen diffusion distance between blood and water[41]. Furthermore, the area available for gas exchange is substantially reduced due to the fusion of the respiratory epithelia, resulting in hypoxia and stress[42]. The preceding actions harm the physiology of fish, resulting in death. In conclusion, we observed that zebrafish were very sensitive to EGCG and C-PC as evidenced from the behavioral responses, demonstrating the utility of this animal as a model for studying the effects of exposure to EGCG and C-PC. A high dose of EGCG and C-PC clearly showed to be very toxic causing severe damage to the gills, kidneys and liver, thus harming the normal physiological function of the experimental zebrafish. The result demonstrated that a higher concentration of EGCG above 30 mg/l and C-PC above 5 g/l gave 100 % mortality and expressed organ-specific toxicity but lower doses showed the least toxic effect. Therefore, using EGCG and C-PC in relatively high quantities is not safe considering the number of tissue damage caused to the animal.

Acknowledgments:

The authors gratefully acknowledge the financial support from DST-SERB, New Delhi, India, for an early career research award (ECR/2016/001398).

Authors contributions:

Ajungla Jamir and Sentiyanger Longkumer executed all the experimental work and drafted the manuscript. Pranay Punj Pankaj planned the concept of the work, prepared the final manuscript and supervised the work.

Conflict of interests:

The authors declared no conflict of interests.

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