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ACUTE ORAL TOXICITY STUDY OF ETHANOLIC EXTRACT OF *PUNICA GRANATUM (L.)* LEAVES IN ZEBRA FISH (*DANIO RERIO*)

[1]Sreedevi P. and [2]Vijayalakshmi K.*

[1]Research Scholar, Department of Biochemistry, Bharathi Women's College.

[2]Associate Professor, Department of Biochemistry, Bharathi Women's College.

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***Corresponding Author**
Vijayalakshmi K.
Associate Professor,
Department of
Biochemistry, Bharathi
Women's College.

ABSTRACT

The safety of *Punica granatum (L.)* leaf is important in relation to its medicinal application. In this study acute toxicity of ethanolic extract of *Punica granatum (L.)* leaf was investigated in Zebra fish (*Danio rerio*). The acute toxicity was performed as per OECD guidelines. A total of 40 Zebra fish were randomly divided into 4 groups. Group I (control) was administered orally with 5 µl of 1% dimethyl sulphoxide, Group II, III and IV were administered orally with a single dose of ethanolic extract of *Punica granatum* leaves at various concentrations, 200 mg, 600 mg and 1000 mg /kg bw respectively. The mortality rate was recorded every 24 hours up to 96 hours after administration of the extract. Body weight was noted before and after administration of the extract. After 4 days, blood was collected from anesthetized fish for hematological assay and organs (Liver, Kidney, Gills and Heart) were excised from sacrificed fish for histopathological studies. No fish was died during the entire study period. The median Lethal dose (LD_{50}) of the ethanolic extract of the *Punica granatum* leaf may be above 1000 mg/kg body weight of Zebra fish. Increased in body weight was less in all treated groups when compared to control. Hematological parameters (differential count, red blood cell count, haematocrit and platelet count) and histopathological observation of Liver were significantly altered in Group IV fed with 1000 mg/kg bw when compared to control.

KEYWORDS: EPGL, Zebra fish,Mortality rate, Haematology and Histopathology study.

INTRODUCTION

The plants can synthesize a variety of chemical compounds which have physiological and pharmacological effects and hence they are popularly used as drug and food.[1] Moreover, a

large number of drugs are derived from plant sources and even 25% of all prescription contains one or more active ingredients of plants. In addition, plant derived substances are the basis for the many commercial drugs used today for the treatment of ailments like heart disease, high blood pressure, pain, asthma and other illnesses.[2] There is also an emerging increase in the consumption of herbal derived substances by the public because there is strong belief that these substances are natural and therefore they are safe for the treatment of ailments.[3] In India, over 3,000 plants were officially recognized for their medicinal value, but it is generally estimated that over 6,000 plants are being used in traditional, folklore, and herbal medicines. Although the scientific study of some medicinal plants clearly validates the effectiveness and reliability of ethno-medical knowledge and traditional use in managing diseases, however herbal medicines are complex mixtures of many bioactive phytochemicals which may differ in different mechanisms.[4] Some level of toxicity arises from the potent toxic compounds present in it, and nontoxic compounds can also behave like a toxic compound even at a lower dose, and can produce an adverse effect by interacting with human or animal cells. Therefore, not all medicinal plants are safe for consumption in the crude form. Thus, such plants should be investigated to better understand their properties, safety and efficiency.

Punica granatum, generally called as Pomegranate, is a deciduous tree belonging to the family Punicaceae. In Ayurveda, *Punica granatum* is considered as "a pharmacy into itself" and is used as an antiparasitic, antiviral, antifungal, antibacterial, hemostatic, anticarcinogenic agent and as a blood tonic. The most therapeutically beneficial pomegranate constituents are found to be ellagic acid, ellagitannins (punicalagins, punicalin, punicafolin), punicic acid, flavonoids, anthocyanidins, anthocyanins, flavonols, flavone glycosides and flavones.[5] The leaves of *Punica granatum* are known for their anti-inflammatory, anti-cholinesterase and cytotoxic activities.[6]

The *Danio rerio* (Zebrafish) is a tropical freshwater fish belonging to the minnow family (Cyprinidae) of the order Cypriniformes. Since it has the physiological and anatomical characteristics similar to that of the higher vertebrates, it is widely used as an important model organism. They breed rapidly and yield more embryos, which are transparent and thus it can be used for the experimental studies.[7]

Despite the widespread use of the *Punica granatum* leaves in herbal medicine, its toxicity studies have not been reported so far. This study is based on the acute oral toxicity of

ethanolic extract of *Punica granatum* leaf (EPGL) in adult zebra fish, with the aim to obtain information on the safety of this plant and provide guidance for selecting a safe dose in its use in traditional medicine.

MATERIALS AND METHODS

Extract preparation

The fresh leaves of *Punica granatum* were collected from around the Sathyavedu village, Andhra Pradesh on August 2016. The taxonomical identification was carried out by Dr. P. Jayaraman, Director of National Institute of Herbal science, Plant anatomy research centre, Chennai (Voucher number: PARC/2017/3381).

The fresh leaves of *Punica granatum* were washed; air dried and ground using electric blender to form a fine powder. The powdered material was then extracted using a solvent ethanol in the ratio 1:10 using a Soxhlet apparatus. After extracting all coloring material, the solvent was removed by evaporating in a water bath, which gives rise to a solid mass of the extract. The yield of the extract was 12% based on the dry weight.

Fish maintenance

Wild type Zebra fish (*Danio rerio*) with similar length and age (2 $\pm$ 1 cm, 3 months old) were purchased from a local pet supplier (Kolathur, Chennai) and were raised in Sathyabama University. The experiments were performed according to the OECD guidelines.[8] Before the experiment, fishes were acclimatized for 2 weeks in aerated glass containers filled with tap water free from chlorine with 14 hrs light and 10 hrs dark photoperiod. They were fed daily with commercial pellets. During the acclimation period, neither mortality nor abnormal behaviour was reported thereby meeting the batch validity criteria for further experiments. The pH (7.4 $\pm$ 0.2) and temperature (24 $\pm$ 2^0 C) of the water tanks were kept constant and checked every day. Other parameters were additionally checked in both acclimatization and experimental tanks, including total hardness, calcium hardness, magnesium hardness, water conductivity, dissolved oxygen, total solids, total dissolved solids, total suspended solids, total alkalinity and chloride having concentrations like 95 mg/l, 61.6 mg/l, 35 mg/l, 431 µS/cm, 7.37 ppm, 321 mg/l, 221 mg/l, 100 mg/l, 163.3 mg/l and 20.3 mg/l respectively indicating that all of these parameter concentrations were in normal range.

Acute toxicity testing

After 2 weeks of acclimation, an experiment was conducted for a period of 96 hours in static conditions and median lethal dose (LD_{50}) was determined with three different concentrations of extract. Forty healthy fishes were selected from the stock and were transferred into the 4 water tanks (10 in each tank). Prior to the start of the experiment, it was assured that all aquariums were properly washed with distilled water to remove any sort of impurities and dust particles. The crude extract was suspended in 1% DMSO. Fish were randomly assigned into four groups: Group1 (control) was given orally 5 µl of 1% DMSO and Group 2, 3 & 4 (treatment groups) were given orally 5 µl of EPGL in 1% DMSO with their respective concentration (200 mg/kg bw, 600 mg/kg bw and 1000 mg/kg bw) on day 1.

Oral administration method

All fishes were fasted overnight and weighed prior to study. On the day of dosing, fresh EPGL in 1% DMSO were administered orally at the required dose with the dose volume of 5 µl / fish. The extract is administered gently into the mouth of zebra fish by inserting small tip of micropipette. The extract solution is then gently released into the fish, ensuring that the administered solution does not regurgitate.[9]

LD_{50} determination

Mortality of the fish was recorded after every 24 hours up to 96 hours of extract administered and LD_{50} value was determined by the use of Finney's Probit Analysis method.[10] A fish was considered dead when its gill movements ceased and it did not respond to gentle prodding. Dead fish were removed from the aquaria to avoid deterioration. After 96 hrs, number of live fish and its weight was noted.

Body weight measurement

The body weight of all experimental fish was taken by using digital electronic balance before administration of extract orally and then after 96 hrs of extract administration.

Haematological examination

On 5th day, the fish was anesthetized by immersion in ice water (4^0 C). Once it no longer responds to external stimuli, an incision was made between the anal and caudal fin by small blade. Blood oozes out of the incision was collected by heparin coated microtip and quickly transferred to eppendrof tubes containing heparin.[11] The collected samples were immersed immediately in an ice bath to preserve the morphology and cell number. The Automated

Hematology Analyzer (Mindray, BC-2800) was used to analyze the hematological parameters such as red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), white blood cell (WBC), platelets (PLT), Procalcitonin (PCT), lymphocytes, monocytes and granulocytes.

Histopathological examination

After collecting the blood, control and experimental fish were sacrificed by decapitation. After sacrificing the fish by decapitation, parts of the liver, kidney, heart and gill tissues were collected for histological studies. The tissues were washed with normal saline and fixed immediately in 10 % formalin for a period of at least 24 h, dehydrated with alcohol, embedded in paraffin Wax, cut into 4-5µm thick sections, and stained. The sections were examined and photographed by electronic microscope.[12] The microscopic features of the organs of fish were compared with the control group.

Statistical analysis

Values were expressed as a mean $\pm$ standard deviation. SPSS (IBM, 19) software was used to calculate paired T test to detect the significant (p value) difference between control and experimental means, and represented as $p \leq 0.05$ = significant, $p \leq 0.01$= highly significant and $p \leq 0.001$ = maximum highly significant and result reported above these values are represented as non-significant.

RESULTS

Table 1: Mortality rate of Zebra fish 96 hours post administration of EPGL.

Groups	Dosage	Mortality rate
Group 1	5 µl of 1 % DMSO	0/10
Group 2	5 µl of 200 mg/kg bw EPGL in 1 % DMSO	0/10
Group 3	5 µl of 600 mg/kg bw EPGL in 1 % DMSO	0/10
Group 4	5 µl of 1000 mg/kg bw EPGL in 1 % DMSO	0/10

mg/kg bw: milligrams per kilogram body weight DMSO : Dimethyl sulphoxide

EPGL: Ethanolic extract of Punica granatum (L.) leaf

Mortality rate

Table 1 Showed that the oral administration of EPGL at different doses (200, 600 and 1000 mg/kg bw) did not produce any mortality in male Zebra fish. This indicated that the mean lethal dose (LD_{50}) of EPGL was above 1000 mg/kg bw.

Body weight determination

The body weights of control and experimental fish were presented in table 2. All the fish gained weight in the course of study, however the weight gain of the experimental fish were lesser than control significantly ($p < 0.001$).

Table 2: Body weight of Zebra fish before and after 96 hours administration of EPGL.

S.No	Initial body weight (g)	Final body weight (g)	Change in body weight (g)
Group 1	3 ± 0.150997	3.973333 ± 0.120139	0.973333 ± 0.032146
Group 2	4.603333 ± 0.325781	5.353333 ± 0.297377	0.75 ± 0.043589***
Group 3	4.133333 ± 0.080208	4.566667 ± 0.051316	0.433333 ± 0.030551***
Group 4	4.17667 ± 0.196554	4.353333± 0.185831	0.176667 ± 0.020817***

Values were given as mean ± SD (n=10). Values were statistically significant at * p< 0.05, at ** p< 0.01 and at *** p< 0.001. Group 2, 3 and 4 were compared with group 1 NS: Non-significant.

Table 3 showed the total and differential White blood cell (WBC) count of all groups. There was no significant variation in total WBC count between control and experimental groups. But there was significant variation in Group 4 (receiving the extract at a dosage of 1000 mg/kg of bw) in lymphocyte ($p < 0.01$), Monocyte ($p < 0.05$) and Granulocyte ($p < 0.001$). Group 2 and 3 do not show any significant variation in differential WBC count.

Table 3 also depicted the results of red blood cell (RBC) count, Hemoglobin (Hb), haematocrit (HCT), platelet count (PLT) and Procalcitonin (PCT). There was no significant Variation in Hb and PCT values between control and experimental groups. However, in Group 4 (receiving the extract at a dosage of 1000 mg/kg bw), there was a significant increase in RBC ($p < 0.05$), HCT ($p < 0.001$) and PLT ($p < 0.001$). Group 3 (receiving the extract at a dosage of 600 mg/kg bw) also showed significant increase in HCT ($p < 0.05$) respectively. Group 2 and 3 (except in HCT) did not show any significant variation in this respect while compared to control.

Figure 1 indicated the RBC indices of control and experimental groups. There was no significant variation in Mean corpuscular volume (MCV), Mean corpuscular hemoglobin

(MCH) and Mean corpuscular hemoglobin concentration (MCHC) among experimental and control groups.

Table 3: Haematology of zebra fish 96 hours post administration of EPGL.

Haematology	Group 1	Group 2	Group 3	Group 4
WBC (X 10^6 cells / 3 µl)	11.9 ± 0.36	11.43 ± 0.25 ^{NS}	11.5 ± 0.43 ^{NS}	10.9 ± 0.26 ^{NS}
Lymphocyte %	79.3 ± 0.83	78.2 ± 0.4 ^{NS}	78.43 ± 0.8 ^{NS}	76.6 ± 0.72 **
Monocyte %	3.23 ± 0.4	3.43 ± 0.251 ^{NS}	3.23 ± 0.32 ^{NS}	2.5 ± 0.43 *
Granulocyte %	17.43 ± 0.96	18.4 ± 0.264 ^{NS}	18.3 ± 1.1 ^{NS}	20.9 ± 0.458 ***
RBC (X 10^6 cells / 6 µl)	7.51 ± 0.27	7.53 ± 0.32 ^{NS}	7.63 ± 0.378 ^{NS}	8.3 ± 0.458 *
Hb (g/dl)	14.8 ± 0.7	15.4 ± 0.6 ^{NS}	15.2 ± 0.37 ^{NS}	15.5 ± 0.37 ^{NS}
Haematocrit (%)	44.5 ± 1.37	45.63 ± 0.75 ^{NS}	47.2 ± 1.16 *	49.7 ± 1.34 ***
Platelet count (X 10^6 cells / 3 µl)	1166 ± 5.68	1152 ± 6.24 ^{NS}	1160 ± 8.5 ^{NS}	1203 ±12.1 ***
Procalcitonin (%)	0.617 ± 0.0055	0.609 ± 0.0036 ^{NS}	0.609 ± 0.0053 ^{NS}	0.613 ± 0.0046 ^{NS}

Values were given as mean ± SD (n=10). Values were statistically significant at * p< 0.05, at **p< 0.01 and at ***p< 0.001. Group 2, 3 and 4 were compared with group 1 NS: Non-significant.

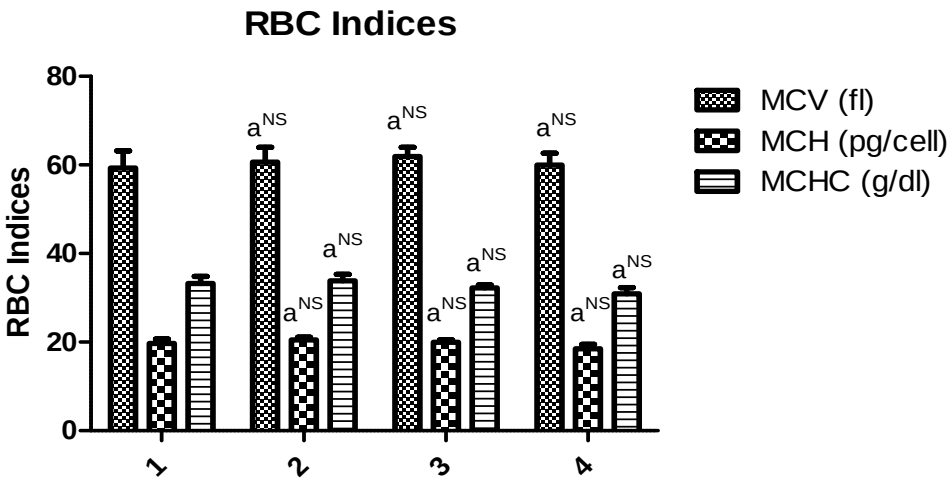

Figure 1: Values were given as mean ± SD (n=10). Values were statistically non-significant. Group 2, 3 and 4 were compared with group 1 NS: Non-significant.

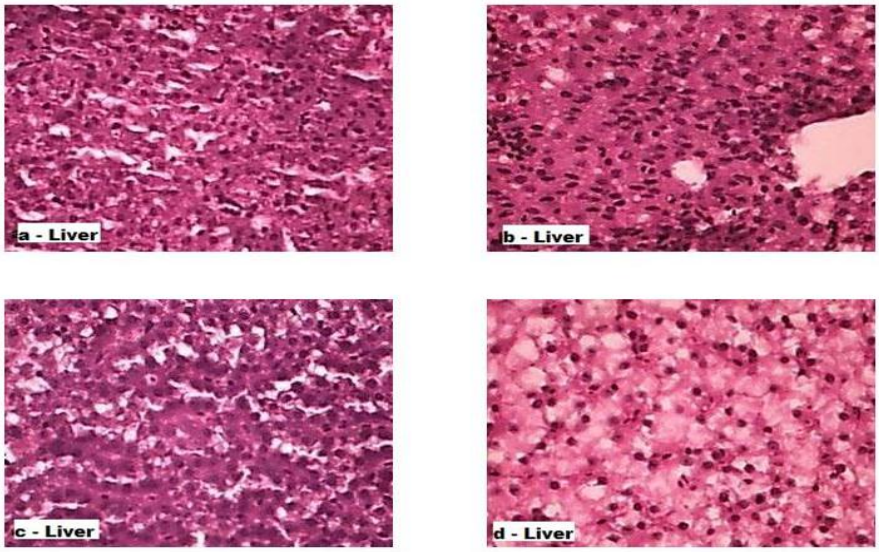

Figure 2: Photomicrograph of the liver section of Zebra fish after 4 days administered with 5µl of 1% DMSO and EPGL in 1 % DMSO. a: (control), B: treated with EPGL (200 mg / kg body weight), c: treated with EPGL (600 mg/kg body weight) d: treated with EPGL (1000 mg/kg body weight) (H&E, 100 X magnification).

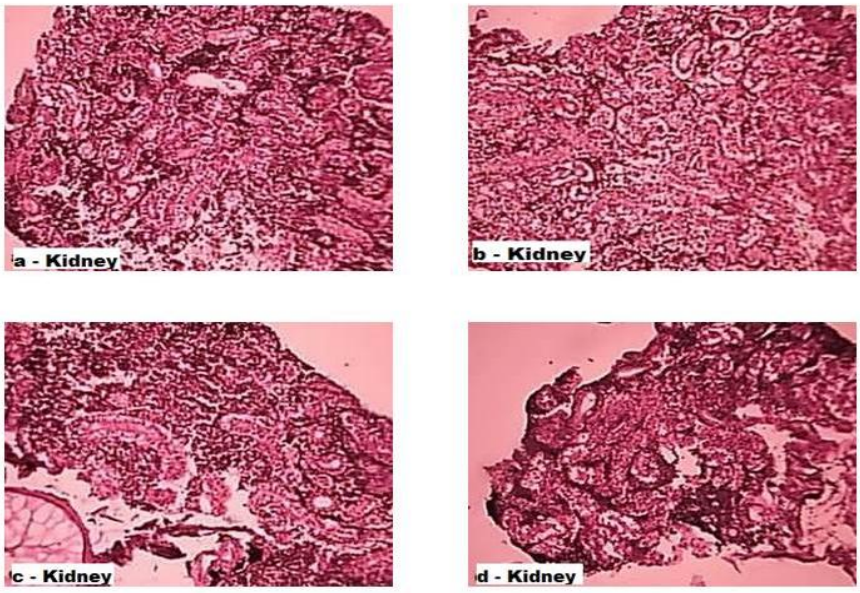

Figure 3: Photomicrograph of the kidney section of Zebra fish after 4 days administrated with 5 µl of 1% DMSO and EPGL in 1 % DMSO. a: (control), B: treated with EPGL (200 mg / kg body weight) c: treated with EPGL (600 mg/kg body weight) and d: treated with EPGL (1000 mg/kg body weight) (H&E, 100 X magnification) .

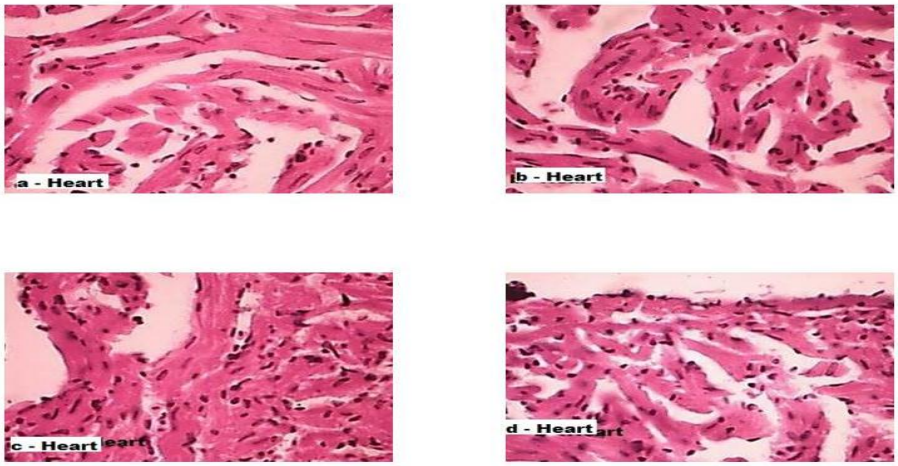

Figure 4: Photomicrograph of the heart section of Zebra fish after 4 days administered with 5 µl of 1% DMSO and EPGL in 1 % DMSO. a: (control) B: treated with EPGL (200 mg / kg body weight), c: treated with EPGL (600 mg/kg body weight) and d: treated with EPGL (1000 mg/kg body weight) (H&E, 100 X magnification).

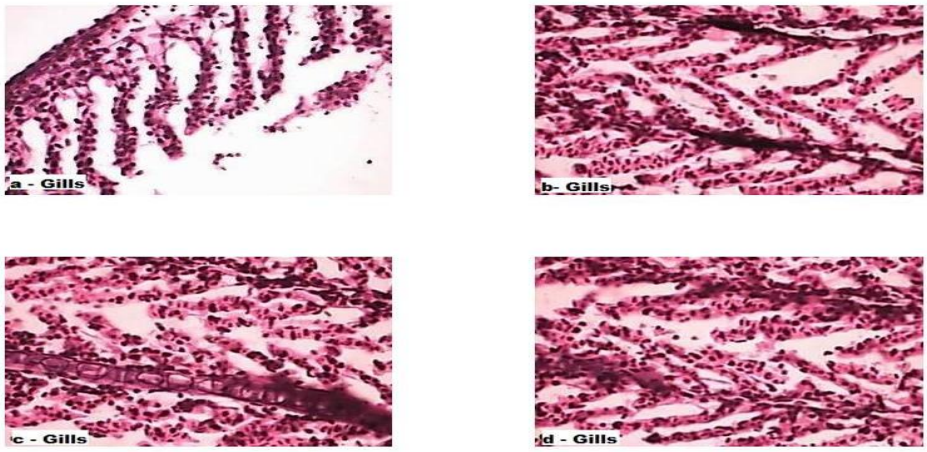

Figure 5: Photomicrograph of the gills section of Zebra fish after 4 days administered with 5 µl of 1% DMSO and EPGL in 1 % DMSO. a: (control) B: treated with EPGL (200 mg / kg body weight), c: treated with EPGL (600 mg/kg body weight) and d: treated with EPGL (1000 mg/kg body weight) (H&E, 100 X magnification).

Figure 2 showed the histopathological examination of liver sections of zebra fish treated with 1% DMSO and EPGL. The liver in Zebra fish administered with 5 µl of 1% DMSO, 200 mg and 600 mg EPGL / kg bw showed the normal hepatic plates and portal vein, but Group 4 administered with 1000 mg EPGL / kg bw showed mild degeneration of hepatocytes.

Figure 3 showed the histopathological examination of kidney sections of zebra fish treated with 1% DMSO and EPGL. The all experimental groups showed the normal glomeruli and tubuli structure when compared to control.

Figure 4 showed the histopathological examination of heart section of zebra fish treated with 1% DMSO and EPGL. The all experimental groups showed the normal ventricle structure when compared to control.

Figure 5 showed the histopathological examination of gill sections of zebra fish treated with 1% DMSO and EPGL. The all experimental groups showed the normal lamellar structure when compared to control.

DISCUSSION

For many centuries, medicinal plants in the form of extracts or fraction or compounds have been used for the treatment of various diseases. For screening the medicinal plants for its pharmacological activity, assessment of its toxicity is usually an initial step.[13] Regardless of the pharmacological beneficial effects of ethanolic extract of *Punica granatum* leaf (EPGL), detailed knowledge about its acute toxicity was lacking. Hence, the current study was undertaken to evaluate the acute toxicity of EPGL in a zebra fish model.

Survival of the Zebra fish after 4 days of administration with EPGL at different doses such as 200, 600 and 1000 mg/kg body weight showed that the median lethal dose (LD50) of EPGL was beyond 1000 mg/kg body weight. Any material with toxicity beyond 1000 mg/kg body weight may be considered to be of low toxic and safe to use.[14] The findings on EPGL suggested that it was nontoxic and safe for oral use.

Body weight gain in experimental fish was lesser than control, it may be due to the decreased appetite and thereby lower calorie intake by fish. Miaffo *et al.*,[15] suggested that the poor body weight of rat might partially be due to the effect of saponins and tannins present in the extract. Nevertheless, these significant increase and decrease in body weight could be the result of variation in size of internal organs,[16] but not as a result of toxicity induced by drug. The assessment of hematological parameters could be used to reveal the deleterious effect of foreign compounds including herbal extracts on the blood constituents of animals. They are also used to determine possible alterations in the levels of biomolecules such as enzymes,

metabolic products and the normal functioning of the organs.[17] Therefore, the extent of toxic effect of EPGL can be determined by assessment of hematological parameters.

In herbal toxicity studies, an increase in WBC may indicate the impact of plant extracts in stimulating the immune response of treated animals. On the other hand, significant decrease in the WBC of the blood indicates that body showed less immunity. However, the hematological analysis in this study demonstrated that the estimated total WBC count after oral administration was not significantly changed in response to the administered EPGL at doses of 200 mg/kg, 600 mg/kg and 1000 mg/kg bw compared to the control. Similarly, there was no significant change in Lymphocyte, Monocyte and Granulocyte in all treatment groups except group 4. This result may indicate that the EPGL in this study does not possess chemicals capable of inducing leukocytosis, which is an abnormally high number of WBC in the blood circulation or in suppression of normal production of WBC.[18] Debelo et al., 2016[19] also found no change in WBC count in treatment groups when compared to control.

There was non-significant increase in Hb in all treatment groups when compared to control, but there was a significant increase in RBC in group 4 but not in other groups. Similarly, there was a significant increase in haematocrit (HCT) in group 3 and 4 except group 2. The observed increase in RBC count, Hb and HCT may therefore be assumed to be associated with normal haemopoeisis. In addition, oxygen carrying capacity of the blood and the amount of oxygen delivered to the tissues were not affected following the extract administration since RBC and Hb are very important in transferring respiratory gases.[20] These results suggested that acute administration of EPGL may not induce anemia.

The increase in PLT count in all treatment groups in this study suggested that the EPGL contained some compounds that stimulated the release of thrombopoietin which enhanced the synthesis of clotting factors that helped to precipitate blood coagulation or clotting, especially during severe bleeding or haemorrhage.[21] This showed that EPGL has thrombopoietin activity.

PCT is a 116-amino acid prohormone of Calcitionin and is produced mainly by C-cells of the thyroid gland. It has a molecular weight of 13 kDa. In healthy state or in the absence of infections, its blood level is very low (<0.1 ng/ml). In case of infection, this protein is synthesized in the liver, lung, kidney, intestine, and almost all other tissues throughout the body.[22] We observed non-significant decrease in Procalcitonin in all treatment groups when

compared to control. This showed that EPGL does not cause infection in all experimental groups.

Red blood indices such as the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) are the most useful indicators in the diagnosis of anemia in most animals.[18] Fig 2 indicated the effect of the EPGL on MCV, MCH and MCHC was insignificant in the treated group compared to the control. These observations demonstrate that the EPGL in this study did not cause significant toxic effects on the levels of red blood cell (RBC) indices at all the doses.

Histopathological examination of vital organs like kidney, heart and gills (except liver) from both treated and control fish showed a normal architecture, suggesting no microscopic changes and morphological disturbances were caused due to oral administration of EPGL up to 1000 mg/kg bw dosage. The mild degeneration of hepatocytes was observed in group 4 administered with EPGL at the dosage of 1000 mg/kg bw. Many chemicals produces zonal necrosis, i.e. necrosis confined to a specific zone of the hepatic acinus. However, the remarkable ability of the liver to regenerate itself makes it to withstand moderate zonal / diffuse necrosis. Over a period of several days, necrotic cells are removed and replaced with new cells; and normal hepatic architecture and function are restored.[23]

CONCLUSION

EPGL was found to have a high safe margin validating its wide use. However, caution should be excised when using this extract as was indicated by the altered hematological parameters and Liver structure in Group 4; it is therefore recommended that doses lower than 1000 mg/kg bw should be used for treatment.

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