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Sub Acute Toxicity Studies of Punicaflavone from the Methanolic Flower Extract of *Punica granatum* (LINN.) in Rats

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Abstract: In the present study, the isolated compound Punicaflavone (PF) from the methanolic flower extract of *Punica granatum* (Linn.) was subjected to toxicity assessment in Wistar albino rats. Sub-acute toxicity studies were carried out by oral administration of the PF from *Punica granatum* (Linn.) for a period of 28 days at different dose levels of 50, 100 and 200mg/kg body weight. No major changes in body and organ weight, behavioural, haematology and histology were observed at the end of 28 days of daily oral administration. Biochemical parameters such as the levels of Glucose, Protein, Urea and Creatinine in serum were found to be well within the normal limits. Histopathological examination of the major vital organs (heart, liver and kidney) revealed no significant pathological alterations in the treated group of rats. The sub-acute toxicity studies of the PF showed no mortality and no symptoms of toxicity or behavioral changes at the maximum dose (1000mg/kg). The results indicate that the PF is safe up to a dose of 1000 mg/kg.
Keywords: *Punica granatum* (Linn.), Methanol extract, Punicaflavone and Sub acute toxicity.

INTRODUCTION

Herbs are alternative medicines for treatment of various diseases due to their assumed acceptability, effectiveness, affordability, safety and low cost [1]. There is also an emerging increase in the consumption of herbal formulations by the public because of the strong belief that these products are natural; hence, they are safe for the treatment of ailments [2].

As the use of medicinal plants increases, experimental screening of their toxicity is crucial to guarantee the safety of users [3]. Natural products are the cornerstone of health care delivery especially in resource poor settings. Present estimates indicate that about eighty percent of the world's population relies on traditional medicine for health care delivery [4]. So much has been done in screening medicinal plants for efficacy based on traditional claims while less emphasis is placed on the issue of safety, as reports of efficacy far outnumber those of toxicity, probably as a result of the greater demands for resources and time such exercise warrant. Pharmacological and toxicological evaluations of medicinal plants are essential for drug development [5].

Alternative approach to drug discovery is through the medicinal plants. Many number of people seeking remedies and health approaches free from side effects caused by synthetic chemicals. Recently, attention has been paid to utilize eco-friendly and bio-friendly plant based products for the prevention and cure of different human diseases. Most of the people have faith in traditional medicine, particularly plant drugs for their primary healthcare [6].

Punica granatum L. (Punicaceae), known as pomegranate, is a deciduous small tree, up to 8 m in height with attractive reddish scarlet edible fruits. The species originated in Iran, Afghanistan and Baluchistan, found wild in the warm valleys of the Himalayas and is cultivated throughout India [7]. The dried flowers, known as Gulnar, are efficacious to treat haematuria, haemoptysis, diarrhoea, dysentery, nasal hemorrhage [8] and in Unani literature as a remedy for diabetes [9,10]. Flower juice is recommended as a gargle for sore throat, in leucorrhoea, hemorrhages and ulcers of the uterus and rectum. The root bark and stem bark of the plant are astringent and used as anthelmintic especially against tapeworms. Fruit rind is valued as an astringent in diarrhea and dysentery. The powdered flower buds are useful in bronchitis. The seeds are reputed as stomachic and the pulp as cardiac and stomachic. The green leaf paste is applied to relieve

conjunctivitis [11]. In Chinese medicine these flower are also used for the treatment of injuries from falls and grey hair of young man [12]. In addition *Punica granatum* L. is considered as "a pharmacy unto itself" in ayurvedic medicine and is used as an antiparasitic agent, a blood tonic, and to ulcers [13].

MATERIALS AND METHODS

Collection and authentication of plant material

The flowers of *Punica granatum* L. were collected from in and around the Mannargudi, Thiruvarur District, Tamilnadu, India. They were identified and authenticated by Dr. S. John Britto, Department of Botany, RAPINAT Herbarium and Center for Modular Systematics, St. Joseph's College, Trichurappalli, Tamilnadu, India.

Fig-1: *Punica granatum* L.

Preparation of plant material

Collected plant material were thoroughly washed with distilled water and then dried under shade at room temperature for few days. The dried plant samples were ground well into a fine powder using blender. The powdered samples were then stored in airtight containers for further use at room temperature.

Preparation of extract

For sample preparation, 100 g of powdered plant material (flower) of *Punica granatum* L. was filled in a thimble and extracted exhaustively by Soxhlet apparatus (72h) using 1.0 L of 95% methanol solvent at 25°C. The extract obtained was collected and passed through Whatman no.1 filter paper to remove all debris and unextractable matter, including cellular materials and other constitutions that are insoluble in the extraction solvent. The solvent from extract was removed under reduced pressure and controlled temperature (40-50 ºC). The yield of the extract was 12.28% w/w. The dry extract was kept in tightly closed container in refrigerator for further analysis.

Screening for phytochemicals

The Phytochemical tests were carried out on the methanol extract of the flowers of *Punica granatum* (Linn.) using standard procedures to identify the constituents as described by Harborne 1983 [14].

Isolation, purification and characterization of compound

The isolation, purification and characterization of bioactive compounds were carried out using repeated silica gel column chromatography and thin layer chromatography (TLC). The purified bioactive compound was characterized by subjected to UV, IR, NMR and MS spectroscopy studies.

Sub-acute toxicity studies

Experimental Animals

Healthy young albino rats weighing between 120 to 130gms were obtained from Tamil Nadu university of Veterinary and Animal Science [TANUVAS], Madhavaram, Chennai, India and were used for the sub acute toxicity profiling. They were housed in a clean polypropylene cage and maintained under standard laboratory conditions of humidity, temperature (25 ± 2^0C), and 12 hr light/dark cycle. They were fed ad libitum with standard feed, and had free access to water. The animals were acclimatized for a week before the commencement of the study. A standard protocol was drawn up in accordance with current guidelines for the care for laboratory animals and ethical guidelines for investigations of experiments in conscious animals were strictly adhered. All the procedures described were reviewed and approved by Institutional Animal Ethical Committee (IAEC) prior to the initiation of the experiment and the care of the laboratory animals was taken as per the rules of CPCSEA (SSN/IAEC/BC/2016/22).

Grouping of Animals

The isolated compound (PF) of the selected plant specimen (flowers of *Punica granatum* L.) was initially tested for sub acute oral toxicity as per the

OECD guidelines. Healthy young albino rats of either sex weighing between 120 to 130gms were used for sub acute toxicity study and tested up to 1000 mg/kg body weight.

The sub acute oral toxicity test of the PF was evaluated in albino rats as reported by Muhammad *et al.* [15] with little modifications which involved two phases. The first phase was conducted as follows. In the first phase, animals were divided into 4 groups of six rats.

Group I: Animals in this group served as control & were fed with pellet food & water ad libitum
Group II: Animals were treated with PF (250mg / kg body weight) orally for 28 days
Group III: Animals were treated with PF (500mg / kg body weight) orally for 28 days
Group IV: Animals were treated with PF (1000mg / kg body weight) orally for 28 days

Mortality and clinical signs (General behaviour)

The animals were observed keenly for about 30 min for any signs of toxicity or mortality, and further observations were made every 8 h for 24 h after administration of the PF. Further observation of all the rats was made for a period of 28 days. During the four-week dosing period, all the animals were observed daily for clinical signs and mortality patterns. Signs of toxicity, body weight, feed and water consumption of each animal was also observed every day for 28 days. The absence of death of any animals in this phase necessitated the conduct of the second phase. The second phase was conducted as follows. In the second phase, animals were divided into 4 groups of six rats.

Group I: Animals in this group served as control & were fed with pellet food & water ad libitum
Group II: Animals were treated with PF (50mg / kg body weight) orally for 28 days
Group III: Animals were treated with PF (100mg / kg body weight) orally for 28 days
Group IV: Animals were treated with PF (200mg / kg body weight) orally for 28 days

The first day of dosing was taken as day 0 and blood was collected on day 28 respectively and used for biochemical, haematological and histopathological analysis. The LD50 was calculated as the geometric mean of the highest non-lethal dose (with no death) and the lowest lethal dose (where death occurred).
LD50 = √minimum toxic dose × maximum tolerated dose

Weekly body weight measurement

The body weight of each rat was expressed using a sensitive balance during the acclimatisation period, once before commencement of dosing, once weekly during the period and once on the day of sacrifice.

Collection of Rat Blood and Tissues

On the 28thday (i.e.) at the end of the experimental period for sub acute studies, blood samples were collected from jugular vein of sacrificed animal by giving overdose of sodium pentabarbitone. The sample bottle was shaken gently to mix up the blood with EDTA and prevent clothing. The values of the red blood cells (RBCs) count, total white blood cells (WBCs) count, platelet count and hemoglobin (Hb) content were determined.

The blood sample was kept at room temperature for 30 min to clot. Afterwards, the test tube containing the clotted blood sample was centrifuged at 3000 r/min for 10 min using a table centrifuge to enable a complete separation of the serum from the clotted blood. The clear serum supernatant was then carefully aspirated with syringe and needle and stored in a clean sample bottle for the biochemical tests. Organs such as liver, heart, kidney and brain were excised and washed with ice cold saline and weighed. These organs were preserved in a fixation medium of 10% buffered formalin for histopathological study.

Organ weight

The heart, liver and kidney of rats in the various groups were excised on the day 28 immediately after blood collection. Following excision, the organs were trimmed of extraneous tissues, placed on a saline soaked gauze pad to retard desiccation and were immediately weighed (paired organs were weighed together).

The relative organ weight of each animal was calculated as follows:
Relative organ weight = (Organ weight (g) /Body weight of the animal on sacrifice day (g) × 100

Histological examination

Organs such as heart, liver and kidney that were excised from the animals were immersed in 10% formalin solution and after formal processing, they were embedded in paraffin wax and thin sections of 5µm thickness were cut down and stained using Hematoxylin and Eosin (H&E) for microscopic examinations. The photograph was taken using light microscope at 100x magnification [16].

STATISTICAL ANALYSIS

Data collected from the biochemical and haematological analyses were expressed as mean ± standard error of mean (S.E.M.) for 6 animals in each group. The statistical analysis was performed by one way ANOVA followed by Dunnett's T3 multiple comparison tests for all parameters using SPSS package. The values were considered significant at the levels of $p< 0,001$, $p < 0.05$ and $p < 0.01$.

RESULTS

Sub-acute toxicity study

To establish the effective utilization of medicinal plants as therapeutic agents, the safety aspects have to be scientifically documented. The aim of performing sub acute toxicity studies is for establishing the therapeutic index of a particular drug and to ensure the safety in-vivo. Acute toxicity study is generally carried out for the determination of LD50 value in experimental animals. The sub acute toxicity test was executed as per OECD guidelines adoption 423 in overnight fasted Wistar albino rats at 120-130mg/kg body weight. Oral administration of different concentration of PF showed neither any sign of clinical abnormality nor any mortality. Hence the sealing doses were considered safe for the drug. One tenth and 1/20th of the safe dose was selected for lipid lowering activity.

Mortality and toxicity signs

In the toxicity study, the rats were treated with different doses of PF orally from the range of 250 – 1000 mg/kg body weight which did not produce significant signs of toxicity, behavioral responses, physiological changes, physical observations (skin, fur, eyes mucous membrane, behavior patterns, tremors, salivation, and diarrhea of the rats) and mortality in the test groups when compared to the controls, observed during the entire 28 days of the sub acute toxicity experimental study period. There was neither mortality (observed at the tested dose) nor any weight loss in this study group rats. All the animals were free of any intoxicating signs and physical changes even in the highest dose (1000 mg/kg) group throughout the drug dosing period of 28 days. No mortality and no clinical signs in any group were observed throughout the experimental period (Table 1).

Table-1: Mortality rate & toxicity sign for the dosing period of 28 days in rats

Groups	Dose(mg/Kg)	Number	Toxicity sign	Mortality rate
Group I	Saline 1ml	6	NT	ND
Group II	250	6	NT	ND
Group III	500	6	NT	ND
Group IV	1000	6	NT	ND

NT-No toxicity sign; ND- None detected

Body weight and Relative organ weight

All the rats showed significant increase in body weight compared to their initial values. However there was no significant difference between the different treatment groups and the control, indicating that the drug did not possess any potent effect on the body weight, which is used to assess the response to the therapy of drug (Table 2 &3) and (Fig 2 &3). No signs of toxicity or mortality were observed during the whole 50 days experimental period.

Table-2: Body weight of rats over for the dosing period of 28 days in rats

		Body weight		
Groups	Dose(mg/Kg)	Day 0	Day 14	Day 28
Group I	Control	125.2 ± 8.4	148.2 ±7.1	161.5 ± 6.5
Group II	50	129.1 ± 7.3	145.3 ±5.5	168.3 ± 6.9
Group III	100	128.1 ± 8.7	139.6 ± 4.7	173.8 ± 10.1*
Group IV	200	125.6 ± 1.5	140.1 ± 3.6	179.6 ± 8.8*

Data represent the means ± SEM, n=6. *P<0.05, statistically significant as comparedwith control rats.

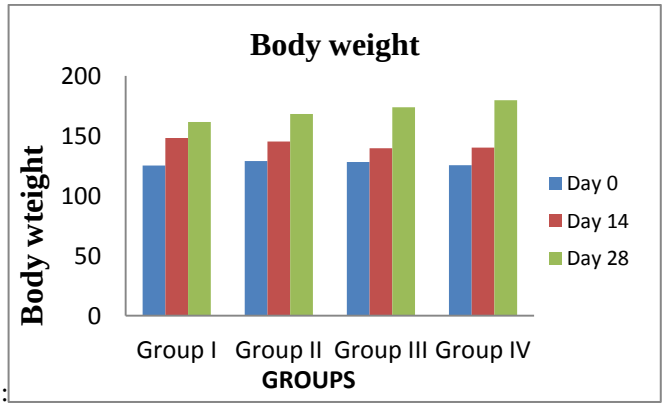


Fig-2: Body weight of rats over for the dosing period of 28 days in rats

Table-3: Effect of flavones on organ weights of rats after the dosing period of 28 days

Parameters (g)	Group I	Group II	Group III	Group IV
Heart	0.59 ± 0.03	0.61 ± 0.04	0.70 ± 0.06	0.73 ± 0.06*
Liver	3.65 ± 0.12	3.98 ± 0.29	4.12 ± 0.28	4.43 ± 0.35*
Kidney	0.91 ± 0.07	1.02 ± 0.99	1.11 ± 0.74	1.17 ± 0.10
Brain	0.94 ± 0.04	0.96 ± 0.08	0.93 ± 0.08	0.97 ± 0.05

Data represent the means ± SEM, n=6. *P<0.05, statistically significant as compared with control rats

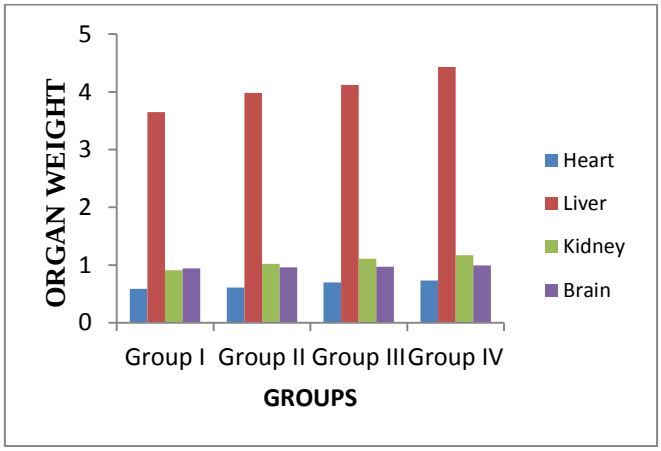


Fig-3: Effect of flavones on organ weights of rats after the dosing period of 28 days

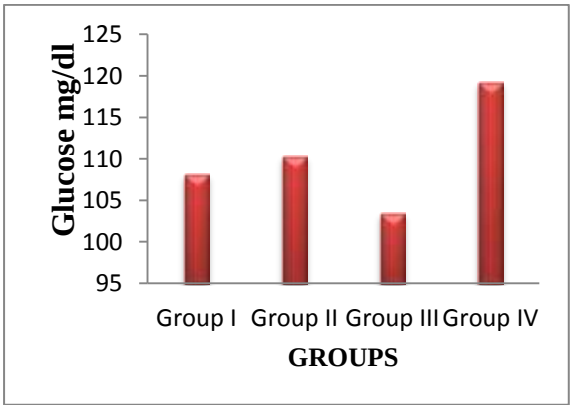
Biochemical parameters

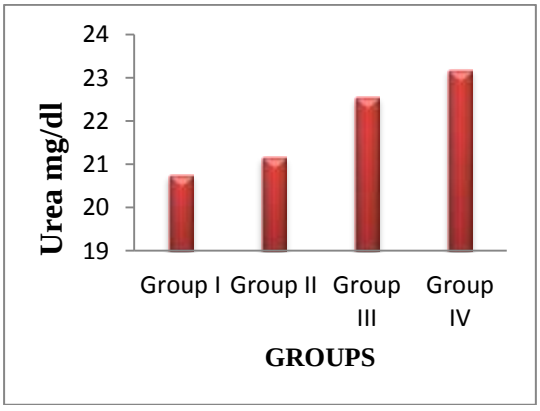
Table 5 and Fig 4 showed the effect of the PF on biochemical parameters including glucose, urea, creatinine and total protein in serum of control and experimental rats. The kidney function parameters (urea and creatinine) did not reveal any relevant changes following administration of PF. These results clearly exhibited that the PF at different levels tested did not produce any considerable changes in the levels of the different biochemical parameters tested.

Table-5: Clinical blood chemistry values of experimental rats

Parameters	Group I	Group II	Group III	Group IV
Glucose (mg/dl)	108.1 ± 6.3	110.3 ± 5.9	103.4 ± 8.6	119.2 ± 6.1
Urea (mg/dl)	20.75 ± 1.3	21.15 ± 1.7	22.56 ±1.8	23.18 ± 2.1
Creatinine (mg/dl)	0.48 ± 0.03	0.50 ± 0.04	0.51 ± 0.04	0.49 ± 0.02
Total Protein (g/dl)	6.08 ± 0.51	6.43 ± 0.47	7.09 ± 0.65	7.12 ± 0.54

Data represent the means ± SEM, n=6. The drug treated rats showed non-significant as compared with control rats.





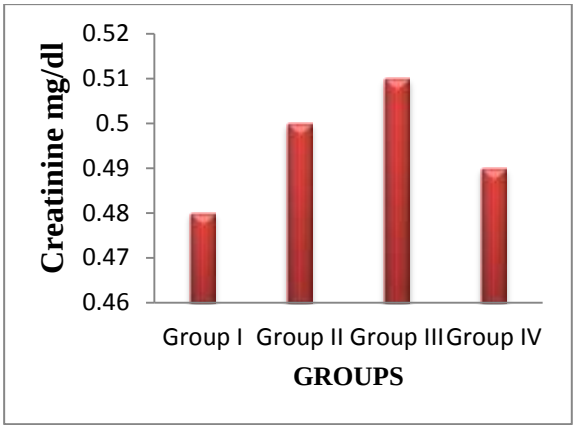


Fig-4: Clinical blood chemistry values of experimental rats

Haematology Parameter

The effects of sub acute administration of PF on haematological parameters were studied. Most haematology measures (haemoglobin (Hb), red blood cells (RBC), white blood cells (WBC) and platelet count in treated rats were not significantly different from the controls, with the exception of border line marginal variations in certain parameters (Table 6 Figure 5 respectively). From the present study it was clearly seen that there was no significant change in the haematological parameters in the PF treated group compared to the normal control group.

Table-6: Hematological values of experimental rats

Parameters	Group I	Group II	Group III	Group IV
RBC (millions/cu.mm)	4.12 ± 0.35	4.75 ± 0.28	4.89 ±0.34	4.99 ± 0.18
Hb (g/dl)	13.80 ± 1.1	14.22 ± 0.93	15.13 ± 1.0	15.64 ± 1.3
WBC (cells / cu.mm	7485 ± 51.9	7561 ± 39.8	7689 ± 24.6	7745 ± 20.9
Platelet count (cells/cu.mm)	1.89 ± 0.11	1.73 ± 0.1	1.92 ± 0.16	2.01 ± 0.18

Data represent the means ± SEM, n=6. *P<0.05, statistically significant as compared with control rats.

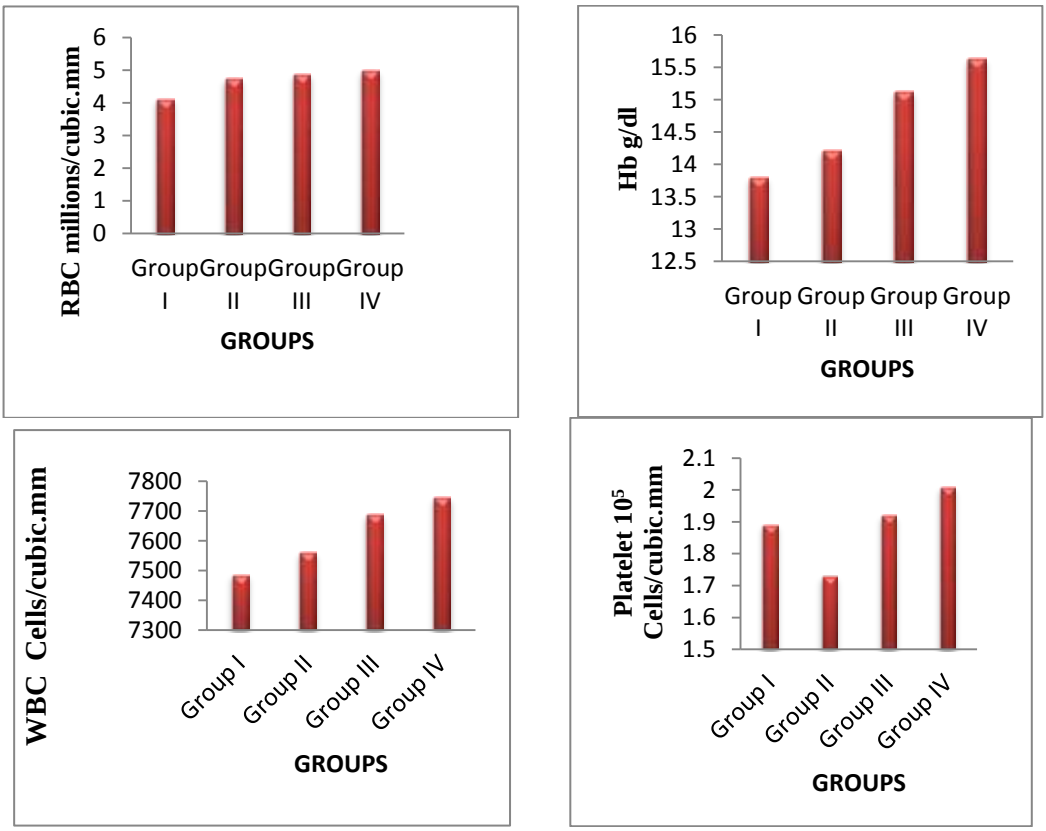
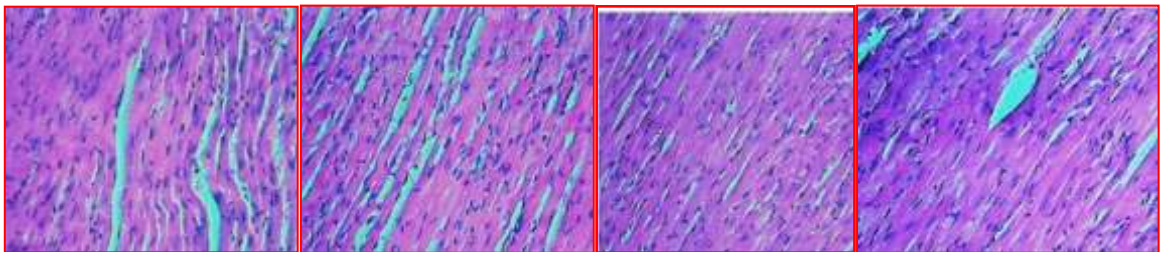


Fig-5: Hematological values of experimental rats

Histological examination

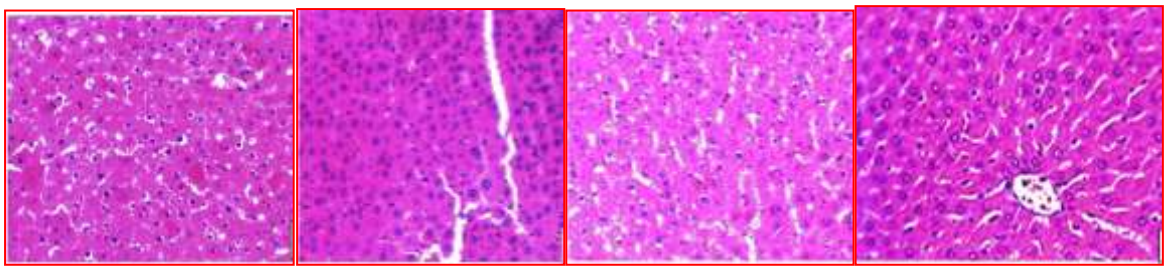
The knowledge of histopathology is useful to distinguish normal cells from abnormal or diseased one and helps in diagnosis of many diseases [17]. The isolated compound (PF) at a dose of 200 mg/kg produced no toxic effect on the behavioural responses of the treated rats (dosed once) and observed for 28 days. Histological studies with heart, liver and kidneys did not reveal any pathological changes after treatment even with the highest dose of 200 mg dose of PF. No remarkable structural alterations were seen in the histopathology of liver, heart and kidneys in high dose (200 mg/kg) and control group. The results of histopathological examination of heart, liver and kidneys section in rats treated with normal saline and PF are shown in Figure 6. The LD_{50} of this plant was, therefore, estimated to be more than 1000 mg/kg.

Fig-6: **Histological sections of rat's Heart, Liver and Kidneys from the control and different dose treated groups' 100x magnification**



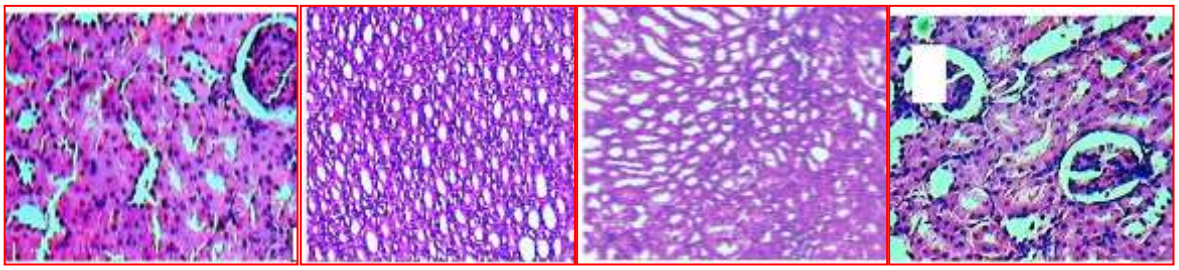
A B C D

Photomicrograph of rat heart treated with different doses of PF for 28 days. A: Control Group; B: PF (50 mg/kg b.wt per day) Group; C: PF (100 mg/kg b.wt per day) Group; D: PF (200 mg/kg b.wt per day) Group showing normal heart histology.



A B C D

Photomicrograph of rat liver treated with different doses of PF for 28 days. A: Control Group (0.5 mL normal saline); B: PF (50 mg/kg b.wt per day) Group; C: PF (100 mg/kg b.wt per day) Group; D: PF (200 mg/kg b.wt per day) Group showing normal liver histology.



A B C D

Photomicrograph of rat kidney treated with different doses of PF for 28 days. A: Control Group (0.5mL normal saline); B: PF (50 mg/kg b.wt per day) Group; C: PF (100 mg/kg b.wt per day) Group; D: PF (200 mg/kg b.wt per day) Group showing normal kidney histology.

DISCUSSION

Phyto-therapeutic using medicinal plants or plant products has become universally popular in primary healthcare, particularly in developing countries. The medicinal plants and plant products are presumed to be safe. Nevertheless, there is a lack of proven scientific studies on the toxicity and adverse and / or

undesirable effect of these remedies. Therefore, oral sub acute toxicity study of PF from the flower extract from *Punica granatum* (Linn.) was investigated.

Toxicity is an expression of being poisonous, indicating the state of adverse effects led by the interaction between toxicants and cells. This interaction may vary depending on the chemical properties of the toxicants and the cell membrane, as it may occur on the cell surface, within the cell body, or in the tissues beneath as well as at the extracellular matrix. The toxic effects may take place prior to the binding of the toxicants to the vital organs such as liver and kidneys. Hence, evaluation of toxic properties of a substance is crucial when considering for public health protection because exposure to chemicals can be hazardous and results to adverse effects on human being. In practice, the evaluation typically includes acute, sub acute, chronic, carcinogenic and reproductive effects [18]. A toxic substance might elicit interesting pharmacological effects at a lower non-toxic dose. Toxicity results from animals will be crucial in definitively judging the safety of medicinal plants if they are found to have sufficient pharmacological products. Acute toxicity is mainly to obtain an appropriate dose for long-term toxicity tests [19].

Natural products from medicinal plants have formed the basic foundation for the treatment of various ailments for decades [20]. In order to achieve discovery of new pharmacological active compound, screening natural products for pharmacological activity, evaluation and assessment of the toxic characteristic of isolated lead compound (LD_{50}) are the beginning steps. Hence the current study was performed to evaluate the sub acute toxicity of isolated compound in animal models (rats). Data from the sub acute toxicity study may (1) provide initial ground information on the mode of toxic action of a drug, (2) help in dose determination in animal studies and (3) help determine LD_{50} values that provide many benefits to arrive at an effective dose of a new lead drug compound [21].

The rats were treated with different doses of PF orally from the range of 250 – 1000 mg/kg body weight which did not produce significant signs of toxicity. Hence, if a high dose (e.g., 1000 mg/kg) is found to be nontoxic and survivable, no further acute testing is required and will not be conducted. In this study, PF at a dose of 1000mg/kg had no adverse effect on the tested rats up to 28 days of observation supported by previous reports [22]. There was no significant toxicity and no mortality was observed in the PF treated test groups when compared to the control groups during the entire study period which may indicate that the drug is safe and nontoxic [23]. Therefore, this study indicates that does not cause sub acute toxicity effects at the dose tested and with an LD50 value greater than 200 mg/kg.

In this study the food and water were well-accepted by the rats treated with PF suggesting that the drug did not cause any alterations in carbohydrate, protein or fat metabolism in these experimental animals. Therefore, PF can be considered as non-toxic up to the tested experimental dose.

The body weight and organ weight changes serve as a sensitive and authenticated indicator of the general health status of animals [24]. However, the observed changes in body and organ weight could be attributed to the nutritive components in the drug PF [25].

The clinical biochemistry analyses were performed to assess the possible alterations in both hepatic and renal functions influenced by PF. Liver and kidney function analysis is very important in the toxicity evaluation of the drug PF as they are both necessary for the healthy survival of any living organism [26]. Liver and Renal dysfunction can be monitored by concurrent analysis of glucose protein, urea, creatinine and total protein and their normal levels reflect the normal liver and renal function [27]. In the current study, changes in serum urea and creatinine levels in PF treated groups showed non-significant differences indicating a normal and healthy renal function.

Evaluation of haematological parameters can be used to determine the degree of the deleterious effect of PF on the blood of an animal [28]. These analyses are relevant to risk evaluation as changes in the haematological system have higher predictive value for human toxicity when the data are translated from the animal studies [29]. A haemogram was undertaken for all the PF treated and control groups and the results show no significant deleterious effects. The nonsignificant effect of the PF on red blood cells, Hb, and platelets indicates that the PF does not affect the erythropoiesis, morphology, or osmotic fragility of the red blood cells [30]. A normal haematological profile of PF treated groups which further justified the non-toxic nature of PF.

Histopathology helps in diagnosing the tissue damages of an animal subjected to toxic stress [31]. Even though bio-chemical studies give an idea of the pathological state of the animal, a clear picture of cytoarchitectural changes produced during chemical intoxication can be traced by histopathological studies [32]. Heart increases the rate of metabolism in an organism, by increase the rate of food and oxygen delivery to the cells (or body) and the rate of removal of waste products from the cells (or body). Liver and kidneys play significant roles in metabolic activities of body. Liver is the major organ involved in drug metabolism and kidneys are the site for drug reabsorption and excretion [33]. Histopathological sections of heart tissues of the treated groups showed

normal myocardial fibres and pulmonary vessel even at the higher dose level of chronic treatment groups of PF. Similarly, sections of livers showed normal structure of the central vein and surrounding hepatocytes, and sections of kidneys showed normal structural features suggesting the preserved renal integrity of the sub acute treatment groups. The glomeruli and renal tubules namely the proximal and distal, convoluted tubules exhibit the normal architecture indicating the absence of renal toxicity.

CONCLUSIONS

In conclusions, sub acute administration of isolated compound PF from the methanol flower extract of *Punica granatum L.* shows no significant adverse effects and did not produce any significant dose related changes on the experimental parameters such as hematological, blood biochemistry and also body weight, behavioural, and mortality. Histopathological studies also showed appearance of normal architecture of the vital organs of the rats, in both control and treated groups and did not induce any toxic effects at different doses. Therefore, the study concluded that the PF of *Punica granatum* L. at the given doses did not produce any significant toxic effect in rats during 28days period of the treatment and the tested compound is safe and non-toxic and could be well used for pharmacological and therapeutic purposes,

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