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Research Article

ACUTE AND SUBACUTE TOXICITY STUDY OF THE ETHANOL EXTRACTS OF *PUNICA GRANATUM* (LINN). WHOLE FRUIT AND SEEDS AND SYNTHETIC ELLAGIC ACID IN SWISS ALBINO MICE

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ABSTRACT

Objective: The objective of the present study was to evaluate the acute and sub-acute toxicity profile of ethanolic extracts of *Punica granatum* (L).whole fruit (EPWF) and seeds (EPS) and synthetic ellagic acid (EA) in Swiss albino mice following OECD guidelines.

Methods: In acute toxicity study, mice were orally administered 2000 mg/kg body weight of extracts and synthetic compound. In subacute toxicity study, the animals were orally administered with the extracts and synthetic compound (2000 mg/kg body weight) daily for 28 days whereas, control group received normal saline.

Results: There were no behavioral alterations or mortality recorded in the treated groups. The LD50 value was more than 2000 mg/kg body weight. Test groups did not record any significant alterations ($p>0.05$) in body weight gain, food and water intake. The hematological and biochemical parameters and organ weights did not record any significant alterations ($p>0.05$) in the treated groups when compared to control. A detailed examination of histoarchitecture of the liver and kidney did not reveal any observable cellular damage in the treated groups compared to control.

Conclusion: The overall finding of this study suggests that *Punica granatum* (L) whole fruit (EPWF) and seeds (EPS) ethanolic extract and synthetic ellagic acid (EA) is safe up to 2000 mg/kg body weight oral administration and can be considered as non toxic.

Keywords: Punica granatum (L), Ellagic acid, ethanolic extract, acute toxicity, subacute toxicity.

INTRODUCTION

Epidemiological studies consistently show that increased consumption of plant-based, antioxidant-rich foods, i.e., fruits, vegetables, whole grains, and nuts, is associated with the reduced risk for several chronic diseases. Present estimates indicate that about eighty percent of the world's population relies on traditional medicine for health care delivery [1, 2].

The exclusive use of herbal drugs for the management of variety of ailments continues due to easy access, better compatibility and for economic reasons. Studies of medicinal plants using scientific approaches showed that various biological components of medicinal plants exhibit a variety of properties and can be used to treat various ailments. However, a number of studies have reported the toxic effects of herbal medicines [3, 4].

Adverse effects of herbs have been reported including allergic reactions, hepatotoxicity [5] nephrotoxicity [6-9], cardiac toxicity [10-12], neurotoxicity [13] and even death [14]. Therefore, a pre-clinical toxicity study is indispensable to validate their safe medicinal use.

The pomegranate, *Punica granatum* L., an ancient, mystical, and highly distinctive fruit, is the predominant member of the Punicaceae family. The pomegranate tree, which is said to have flourished in Eden, has been used extensively in the folk medicine for a number of therapeutic purposes [15].

In addition, it has recently been reported that pomegranate contains some species of flavonoids and anthocyanidins in their seed oil and juice, and shows an antioxidant activity three times more potently than red wine and green tea extract [16-22].

Furthermore, the chemo preventive and adjuvant therapeutic applications of pomegranate to human breast cancer have been warranted recently [23]. Owing to these significant biological activities, pomegranate juice is being increasingly popularized.Ellagic acid has antiproliferative and antioxidant properties in a number of in vitro and small-animal models [24-26].

These properties of ellagic acid have spurred preliminary research into the potential health benefits of ellagic acid consumption [27]. As with other polyphenolic antioxidants, ellagic acid has a chemo protective effect in cellular models by reducing oxidative stress [28].

Despite of the popular use, exploring various medicinal importances of the various parts of the plant, there is no report on the toxicity study of whole fruit (peel+ seeds) and seed extracts in comparison to synthetic ellagic acid.

The present investigation was therefore carried out to evaluate the safety profile of the ethanol extract of *P.granatum* whole fruit (peel+ seeds) and seeds and synthetic ellagic acid in Swiss albino mice.

MATERIALS AND METHODS

Collection of Plant Material and Preparation of Extract

The fruits of *Punica granatum* were collected from the local market, Mangalore and the specimens were identified.

The whole fruit (peel+ seeds) and seeds of *P.granatum* were dried in hot air oven at 40°-50°C for a period of one week. The dried plant material was powdered using mixer grinder, and subjected to soxhlet extraction with 99% ethanol for 24 hours. The mixture was evaporated to dryness in a rotary flash evaporator (Rotavap Model No.PBU-6) and stored in refrigerator. The condensed extracts were used for invivo acute and subacute toxicity studies.

Synthetic compound, Ellagic acid was purchased from Sigma Aldrich.

Experimental Animals

Adult female Swiss Albino mice (6-8 weeks old/20-25g) were procured from the Institutional Animal House, K.S Hegde Medical Academy, Nitte University, Mangalore. Animal care and handling was carried out according to the guidelines set by WHO (World Health Organization, Geneva, Switzerland).

They were housed under standard animal house conditions and fed with standard laboratory pellets and water *ad libitum.* The

experimental protocol was approved by the Institutional animal ethical committee.

Acute oral toxicity

Acute oral toxicity study was conducted according to the guidelines of Organization for Economic Co-operation and Development (OECD, 425) [29].

Twenty four animals were randomly allocated into four groups of six animals each.

Group I (Control): animals were administered orally with vehicle (normal saline).

Group II, III and IV - administered with 2000 mg/ kg body weight of standard compound Ellagic acid (E.A), ethanolic extract of *P.granatum* whole fruit (EPWF) and seeds (EPS) respectively via oral gavage.

Doses were prepared using distilled water and dose volume was not more than 1 ml/100g body weight. The animals were observed continuously for the first 4 h to record any changes in general behavior and other physiological activities [30, 31]. Mortality rate was recorded after 24 hours.

Sub-acute oral toxicity

The sub-acute oral toxicity study was conducted according to the guidelines of the Organization for Economic Co-operation and Development (OECD, 407) [32]. Twenty four animals were randomly divided into four groups of six animals each. Group I (C) - control, received normal saline (vehicle) for 28 days. Group II-E.A, Group III-EPWF and Group IV-EPS were orally administered 2000 mg/kg body weight of the standard compound and *P.granatum* ethanolic extracts once daily for 28 consecutive days.

Food and water intake was recorded daily; body weight was recorded once in a week throughout the study period.

Serum Isolation and Hematology

At the end of experimental period, the animals were euthanized, blood samples collected through cardiac puncture and taken into heparinized tube for hematological studies and non-heparinized tube from which serum was isolated by centrifugation at 3000 rpm for 10 minutes and used for biochemical estimations.

Hematological parameters namely, White blood cell (WBC) count, red blood cell (RBC) count, haemoglobin (Hb) levels, percentage of lymphocytes (LY), Monocytes (MO),Granulocytes(GR) and Platelet Count (PLT) was recorded using Hematology Analyser (VET –ERMA PCE 210 VET).

Serum Biochemical Parameters

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), serum albumin, bilirubin, total protein (liver damage) and urea, uric acid and creatinine (kidney damage) were analysed using commercially available kits in a semi auto analyser (Star 21 plus (E114923).

Also serum lipid profile [total cholesterol (TC), triglyceride (TG) and high density lipoprotein (HDL-C)] were assessed and low density lipoprotein (LDL-C) and very low density lipoprotein (VLDL-C) were calculated by Friedewald's formula [33].

Organ Weight and Histopathology

Animals were sacrificed under mild ether anesthesia. After sacrifice, organ weights (liver, kidney, spleen and thymus) were recorded.

Vital organs like Liver and kidneys were excised from the anesthetized animal, rinsed in 0.9% saline. Tissue pieces were fixed in 10 % paraformaldehyde for paraffin histology and processed in paraffin embedding as per the standard protocol. Sections of each tissue were stained with hematoxylin and eosin, and observed for possible histopathological damages.

Statistical Analysis

The data are expressed as Mean±SD. Statistical significance between the groups was analysed by means of analysis of variance followed by Tukey's multiple comparison tests. The criterion for statistical significance was set at $P \leq 0.05$.

RESULTS

Acute Oral Toxicity

Cage side observations did not record any behavioral changes during the first four hours of EA, EPWF and EPS extract at 2000 mg/kg body weight administration. After 24 hours there was no mortality recorded in treated groups.

Sub-acute oral toxicity

Body weight gain, food and water intake

EA, EPWF and EPS groups did not record any significant alterations ($p>0.05$) in body weight gain (Table 1). Further there was no alteration in food and water intake in all the treated groups as compared to control.

Table 1: Effect of Ellagic Acid and *P.granatum* ethanolic extracts subacute oral administration on Body Weight in Swiss Albino Mice.

Groups	Initial Body Weight(g)	Final Body Weight(g)
Control	23.75±1.58	25.5±1.35
EA	23.6±1.87	25.2±1.77
EPWF	22.83±1.11	25.2±1.28
EPS	23.96±1.46	24.4±1.67

Data are expressed as Mean±SD. (n=6). P>0.05, Non significant.

Hematology

The hematological parameters (RBC, WBC, Hb, LY%, MO%, GR% and Platelet Count) did not record any significant alterations ($p>0.05$) in any of the treated groups when compared to control (Table 2).

Table2. Effect of Ellagic Acid and *P.granatum* ethanolic extracts subacute oral administration on Hematological Parameters in Swiss Albino Mice.

Groups	RBC($X10^6$/µl)	WBC($X10^3$/µl)	Hb(g/dl)	LY (%)	MO (%)	GR (%)	PLT($X10^3$/µl)
Control	6.0675±0.97	4.45±0.22	10.55±0.82	74.08±0.09	7.68±0.09	18.25±0.08	314±0.19
EA	6.55±0.76	4.58±0.61	10.86±0.94	72.08±0.02	7.80±0.01	17.92±0.07	387±0.63
EPWF	6.42±0.46	4.65±0.34	11.2±0.63	72.32±0.09	7.08±0.07	18.82±0.02	356±0.52
EPS	6.694±0.52	4.9±0.51	10.26±0.47	76.10±0.03	7.92±0.05	17.76±0.06	301±0.35

RBC: Red blood corpuscle, WBC: White blood corpuscle, Hb: Hemoglobin; LY: Lymphocyte, MO: Monocyte, GR: Granulocyte, PLT: Platelet. Data are expressed as Mean±SD, (n=6). P>0.05, Non significant.

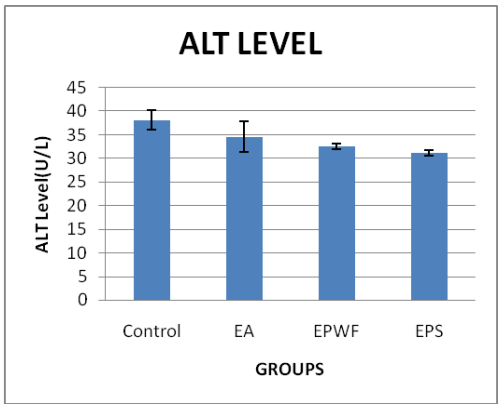


Figure 1: Control, EA (Ellagic acid), EPWF (Ethanolic Pomegranate Whole Fruit), EPS (Ethanolic pomegranate seeds)

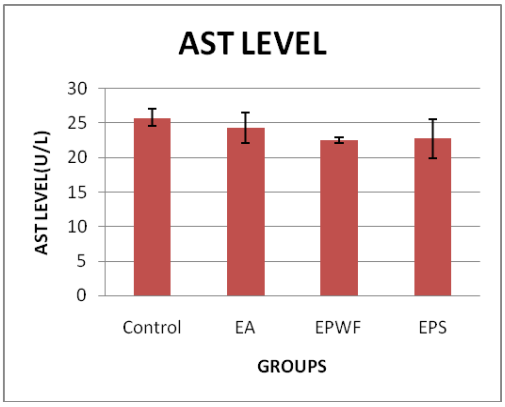


Figure 2: Control, EA (Ellagic acid), EPWF (Ethanolic Pomegranate Whole Fruit), EPS (Ethanolic pomegranate seeds

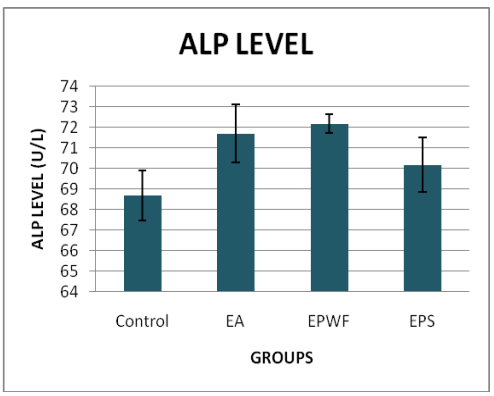


Figure 3: Control, EA (Ellagic acid), EPWF (Ethanolic Pomegranate Whole Fruit), EPS (Ethanolic pomegranate seeds)

Total Protein (TP), Albumin and Total Bilirubin levels did not record any significant (p>0.05) alterations in all the treated groups when compared to control (Figure 4, 5&6).

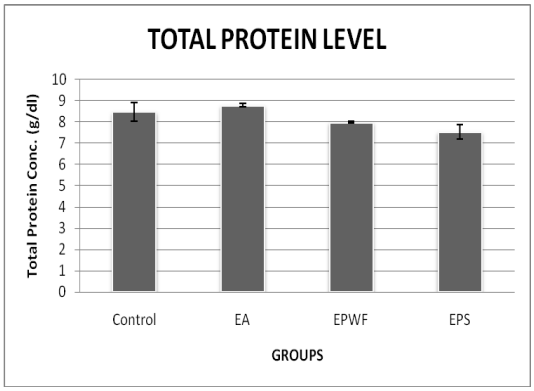


Figure 4: Control, EA (Ellagic acid), EPWF (Ethanolic Pomegranate Whole Fruit), EPS (Ethanolic pomegranate seeds)

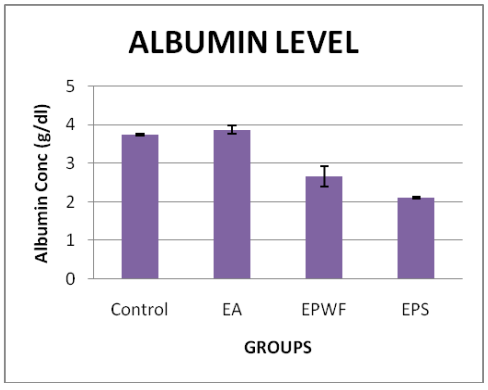


Figure 5: Control, EA (Ellagic acid), EPWF (Ethanolic Pomegranate Whole Fruit), EPS (Ethanolic pomegranate seeds)

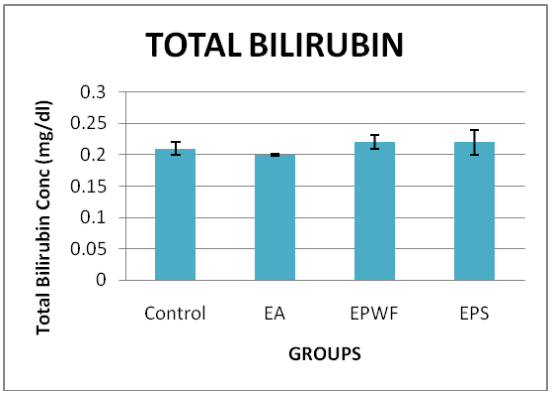


Figure 6: Control, EA (Ellagic acid), EPWF (Ethanolic Pomegranate Whole Fruit), EPS (Ethanolic pomegranate seeds)

Serum biomarkers of kidney damage – Creatinine, Uric Acid and Urea levels recorded non significant (p>0.05) alterations in all the test groups when compared to control (Figure 7, 8&9).

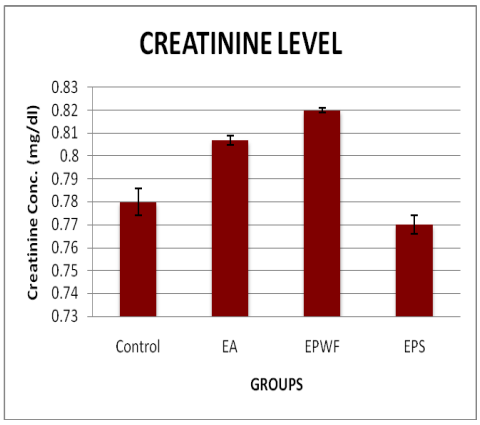


Figure 7: Control, EA (Ellagic acid), EPWF (Ethanolic Pomegranate Whole Fruit), EPS (Ethanolic pomegranate seeds)

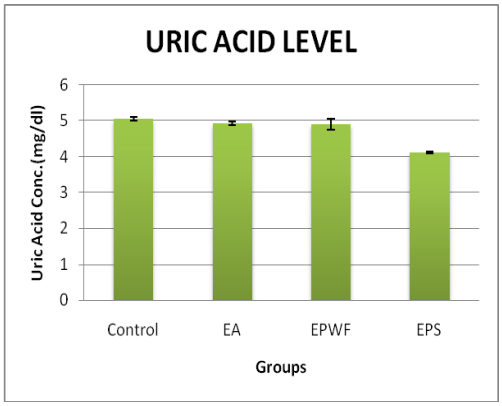


Figure 8: Control, EA (Ellagic acid), EPWF (Ethanolic Pomegranate Whole Fruit), EPS (Ethanolic pomegranate seeds)

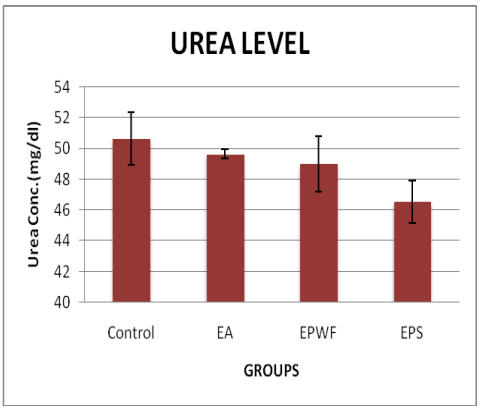


Figure 9: Control, EA (Ellagic acid), EPWF (Ethanolic Pomegranate Whole Fruit), EPS (Ethanolic pomegranate seeds)

Total cholesterol, LDL and HDL levels recorded moderate non-significant increase (p>0.05) in all the treated groups compared to control whereas Triglyceride and VLDL levels recorded moderate non-significant reduction (p>0.05) in all the treated groups compared to control (Table 3).

Table 3: Effect of Ellagic Acid and *P.granatum* ethanolic extracts subacute oral administration on plasma lipid and lipoprotein level in Swiss Albino Mice

	GROUPS			
	Control	**Ellagic Acid**	**EPWF**	**EPS**
Total Cholesterol(mg/dl)	112.66±4.57	122.25±2.51	128.4±2.26	119.85±2.75
Triglyceride(mg/dl)	57.35±2.28	54.7±1.27	47.85±2.19	43.72±0.67
VLDL-C(mg/dl)	11.47±0.45	10±0.25	9.57±0.43	8.74±0.13
LDL-C(mg/dl)	44.16±2.15	48.48±0.10	45.80±1.20	45.14±1.21
HDL-C(mg/dl)	70.55±0.57	70.775±0.40	75.93±1.80	64.7±0.76

Data are expressed as Mean±SD, (n=6). P>0.05,Non significant.

Organ Weight and Histopathology

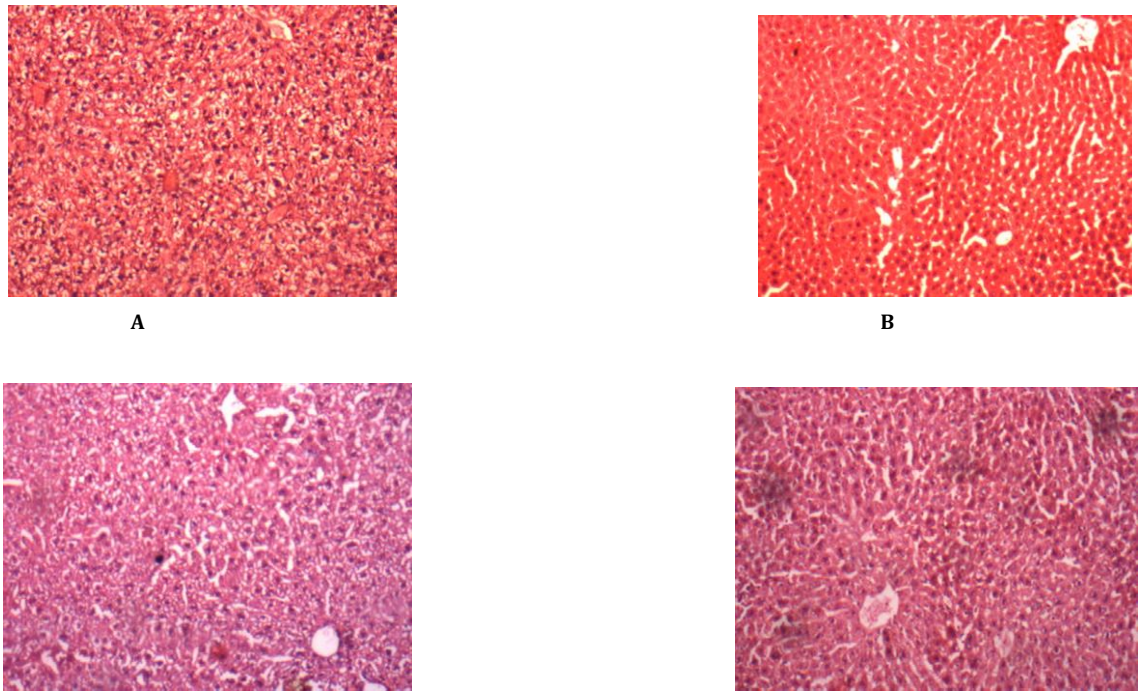
Organ weights of liver, kidney, spleen and thymus did not record any significant alterations (P>0.05) in all the treated groups when compared to control (Table 4).

Table 4. Effect of Ellagic Acid and *P.granatum* ethanolic extracts subacute oral administration on Organ weights in Swiss Albino Mice.

	Groups			
Organs	**Control**	**EA**	**EPWF**	**EPS**
Liver	1.36±0.15	1.33±0.12	1.18±0.10	1.15±0.03
Kidneys	0.67±0.095	0.6±0.08	0.55±0.1	0.675±0.09
Spleen	0.07±0.02	0.09±0.005	0.097±0.009	0.092±0.005
Thymus	0.14±0.03	0.15±0.03	0.16±0.02	0.17±0.021

Data are expressed as Mean±SD, (n=6). P>0.05,Non significant.

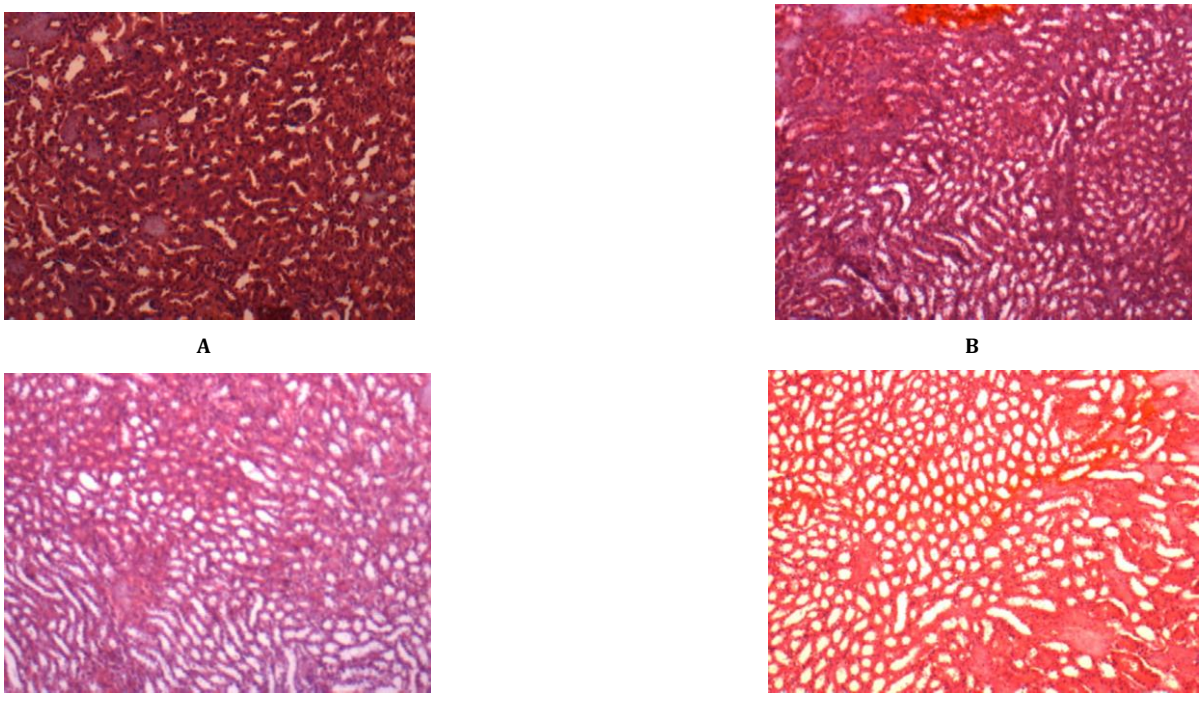
A detailed examination of histoarchitecture of the liver and kidney did not reveal any observable cellular damage. The cellular morphology, nuclear characteristics and tissue integrity of organs of treated groups were similar to the control group (Figure 10 &11).



A **B**

C **D**

Figure 10(A-D): Photomicrograph showing normal architecture of liver in control (A), EA(B), EPWF(C) and EPS(D) administered mice for 28 days



A **B**

C **D**

Figure 11(A-D): Photomicrograph showing normal architecture of kidney in control (A), EA(B), EPWF(C) and EPS(D) administered mice for 28 days

DISCUSSION

Natural products from including plants, animals and minerals have been used for basic treatment of human diseases[34].Herbal medicines have received a great attention as alternatives to synthetic pharmaceutical products in recent times, leading to the increase in their demand [35].

In recent years, herbal drugs are exclusively used for the treatment of various diseases and are still practiced in rural communities. Therefore it is important to ascertain the safety and efficacy of herbal products by experimental screening method, as well as to establish the active components of these herbal remedies [36].

The primary aim of toxicological assessment of any herbal medicine is to identify adverse effects and to determine limits of exposure level at which such effects occur. Two important factors which are taken into consideration in evaluating the safety of any herbal drug are the nature and significance of the adverse effect and in addition, the exposure level where the effect is observed. Toxicity testing can reveal some of the risks that may be associated with use of herbs especially in sensitive populations.

The present study was aimed to investigate the possible toxic effects of ethanolic extracts of *P.granatum* whole fruit (EPWF) and seeds (EPS) and synthetic ellagic acid (EA) in Swiss albino mice.

Patel C et al. [37] carried out acute toxicity study of standardised pomegranate fruit extract in rats and mice and found the oral LD50 to be greater than 5g/kg body weight.

This is similar to the present study which showed that the ethanol extract of *P.granatum* whole fruit and seeds and synthetic ellagic acid is practically non toxic at single dose oral administration in mice. In the acute oral toxicity study of the extracts and synthetic compound, no changes in the behaviour of mice were observed. There was no mortality observed at the end of 24 h period. Hence, the LD50 of EA, EPWF and EPS is thought to be greater than 2000 mg.

Subacute toxicity is repeat-dose study performed to expose any deleterious changes in organ, hematological and biochemical indices that may arise in the course of repeated administration of a test substance, usually ranges from weeks to a few months.

Tasaki M et al, assessed the subchronic toxicity of Ellagic acid in F344 rats and estimated the no-observed-effect level (NOEL) to be 5% (3011 mg/kg b.w./day) for males and the no-observed-adverse-effect level (NOAEL) and NOEL in females to be 5% (3254 mg/kg b w/day) and <1.25% (778 mg/kg b.w./day), respectively [38].

This is similar to the present subacute toxicity study where various parameters were thoroughly studied. The body weight, food and water intake were found to be unaltered during the 28 days treatment period when compared to control group. Also there were no significant changes in different vital organ weights. No mortality was observed during this period.

The effect of EA, EPWF and EPS on some hematologic parameters in mice is as shown in Table 2. The general lack of significant changes in blood indices is an indication of safety of the extracts and synthetic compound. The observed non-significant increase in WBC count could emphasize the beneficial effect of EA, EPWF and EPS in improving the immunity and general well-being of the animals [39]. Also, the observed non-significant difference in hemoglobin concentration in the treated groups justifies the fact that the extracts at the said doses does not induce anaemia, making it safe. All the other hematological parameters in all treated group remained normal without any significant alterations.

The release of Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) from the liver and heart and an elevation in the levels of these enzymes are good indices of damage to these organs [40].

The non-significant changes in ALT and AST in the treated groups indicate that the extracts and the synthetic compound had no deleterious effect on liver and cardiac functions. Alkaline Phosphatase (ALP) which has both hepatic and bone sources, showed a non significant increase in the treated groups. The general lack of significant changes in the aminotransferase and ALP together with normal liver weight is an indication that EA,EPWF and EPS is safe and offers no deleterious effect on heart and liver.

Similarly, no significant alterations were observed in Total Protein, Albumin, Total Bilirubin, Creatinine, Uric Acid and Urea level which is a good indicator of liver and kidney functions.

The observed non significant increase in total cholesterol, LDL and HDL levels may have occurred due to increased secretion of total cholesterol, LDL and HDL by the liver. However serum lipid profile was not significantly altered by treatment with EA, EPWF and EPS and this suggests that the extracts and synthetic compound does not impair lipid metabolism.

The biochemical analysis was further supported by the histopathology findings which revealed no pathological changes in the liver and kidney of treated mice.

Thus, the present work evaluated the acute and subacute toxicity of the ethanolic extract of *P.granatum* and synthetic ellagic acid. The results of this study demonstrated that the extracts and the synthetic compound may be considered relatively safe without any toxicity. Due to its non-toxic effects on the organ systems, there is a clear potential for the utilization of *P. granatum* extracts and synthetic ellagic acid for therapeutic use.

CONCLUSION

In conclusion, the present study provides valuable data on the acute and subacute toxicity profile of the ethanol extracts of *Punica granatum* (Linn) whole fruit and seeds and synthetic ellagic acid in Swiss albino mice. The present investigation demonstrated that the extracts and the synthetic compound at level up to 2000mg/kg body weight did not cause any adverse effects and considered as non toxic and safe.

ACKNOWLEDGEMENT

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