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Original Article

CHRONIC ORAL TOXICITY STUDIES OF CRUDE ETHANOLIC EXTRACT AND ETHANOLIC FRACTION OF *PELLIONIA HEYNEANA* WEDD. LEAF IN WISTAR RATS

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ABSTRACT

Objective: *Pellionia heyneana* Wedd. leaf has been used by the Cholanaikan tribe as a traditional medicine to enhance immunity and also to treat various liver ailments. However, no scientific reports are available regarding its long term toxicity studies. The objective of the present study was to investigate the chronic oral toxicity study of *P. heyneana* leaf.

Methods: In the present study, scientific evaluation of oral toxicity of *P. heyneana* crude ethanolic leaf extract (PHLE) and leaf ethanolic fraction (PHEF) were carried out in Wistar rats. Animals were fed with three varying concentrations (500 mg/kg, 1000 mg/kg and 1500 mg/kg) of PHLE and PHEF for 90 d. During the study period, all the animals were closely observed for any morbidity or mortality, food and water intake, body weight *etc*. The effect of PHLE and PHEF on animal behaviour, metabolism, liver function, kidney function, blood glucose level, *in vivo* antioxidant status, haematological parameters, histopathology of internal organs etc. were evaluated after 90 d chronic toxicity study.

Results: All the animals administered with PHLE/PHEF up to 1500 mg/kg dose did not show any deleterious changes in normal metabolism, histopathology of internal organs, haematological and biochemical indices.

Conclusion: All these results revealed that PHLE and PHEF of *P. heyneana* are non toxic in long term oral administration in Wistar rats.

Keywords: *Pellionia heyneana,* Chronic oral toxicity, Cholanaikan tribe, Histopathology

INTRODUCTION

The pivotal role played by medicinal plants in ensuring the health care needs of humans have seen an upsurge in the last few decades. As has been pointed out by the WHO, about 80% of the world's population rely predominantly on plants and plant extracts for health care [1]. In the present scenario, a large number of ethnomedicinal plants are used in the pharmaceutical industries for finding new therapeutic agents for the various fields of biomedicine [2]. This is because of the widespread misconception that the plants used in traditional medicine are harmless and carry no risk for its use due to their natural origin. But as with all other medicines, the long term administration of certain herbal medicines may cause adverse effects both in humans and animals [3]. Hence a comprehensive toxicity study is imperative in order to establish its safety, efficacy and quality and also to ascertain the nontoxic nature of the medicinal plants used in traditional medicine.

P. heyneana Wedd. is an herb belonging to Urticaceae family, frequently growing in moist forests. Cholanaikans, the most primitive and one of the remaining hunter-gatherer tribes of South India call this plant as 'Elaven', and believe that the plant juice makes them young and energetic. They use the leaf juice of *P. heyneana* in their traditional medicine for treating immunodeficiency in children [4] and various liver diseases [5]. Even though the plant has significant medicinal property, no detailed toxicity study has been conducted so far, as regards the safe administration of the plant. Hence, the present studies have been carried out to evaluate the chronic oral toxicity of the plant.

MATERIALS AND METHODS

Chemicals and instruments

All the chemicals for the study were purchased from Sigma Aldrich, USA. The biochemical kits were purchased from Coral Clinical System, Goa, India. Rotary evaporator (Buchi R-215, Switzerland) and auto hematology analyser-vet (Mindray China) were used.

Collection and authentication of plant material

P. heyneana Wedd. plants were collected from Kallar, Thiruvananthapuram district of Kerala, India, and authenticated by the plant taxonomist of the Institute. Voucher specimens were deposited at the Jawaharlal Nehru Tropical Botanic Garden and Research Institute Herbarium (TBGT 57060 dated 10/12/2010).

Preparation of solvent extract/fractions

The leaf powder of *P. heyneana* was extracted with 95% ethanol for 48 h, using a soxhlet apparatus. The extract was then filtered and the filtrate was concentrated under reduced pressure using rotary evaporator, to get the crude extract (PHLE).

For preparing serial fractions, *P. heyneana* leaf powder was first extracted with hexane using soxhlet apparatus, powder was then dried and again extracted with chloroform and finally with ethanol to get; hexane fraction (PHHF), chloroform fraction (PHCF) and ethanolic fraction (PHEF) respectively.

Based on the antioxidant studies (data not shown), the most promising fractions namely PHLE and PHEF were selected for toxicological evaluation.

Animals

Wistar ♂ and ♀rats (200-235 g) were obtained from the Institute's Animal House. All the animals were housed in polypropylene cages under standard conditions with temperature 25±2 °C, relative humidity 60±10%, room air changes 15±3 times/h and a 12 h light-dark cycles, fed commercial rat feed (Lipton India Ltd; Mumbai, India) and boiled water *ad libitum*. Animals were acclimatized for 1 w before the initiation of the experiment. The study was carried out according to NIH guidelines after getting the approval of the Institute's Animal Ethics Committee (No: B-19/11/2013/05C).

Chronic toxicity study

Wistar albino rats were divided into seven groups of 6 animals (3♂

and 3♀/group) each. Group 1, the control group was treated with 0.5% Tween-80 (vehicle) for 90 d. Groups II, III and IV were treated with PHLE extract 500, 1000 and 1500 mg/kg/d, p. o. and Groups V, VI and VII were treated with PHEF extract 500, 1000 and 1500 mg/kg/d, p. o. respectively [6]. Toxic manifestations such as signs of toxicity, mortality and body weight changes were monitored daily. At the end of the study, all animals were fasted for 24 h and sacrificed by carbon dioxide inhalation on 91st day. Blood samples for haematological and biochemical analysis (estimation of plasma markers of hepatic injury, evaluation of kidney function, blood glucose level) were collected by cardiac puncture. Detailed gross necropsy, including careful examination of the body external surface, orifices and cranial, thoracic and abdominal cavities and their contents were performed in all the groups. Liver samples were subjected to antioxidant assays (estimation of malondialdehyde (MDA), assay of catalase (CAT), determination of reduced glutathione (GSH)). The internal organs (lungs, heart, liver, spleen and kidney) were collected, weighed and preserved in 10% formaldehyde solution for histopathological examination.

Estimation of serum biochemical parameters of liver and kidney

The collected blood was allowed to coagulate for 1h at room temperature. It was centrifuged at 1500 rpm for 15 min at 37 ºC to separate the serum. The serum was then subjected to the assay of hepatic marker enzymes, namely aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), γ-glutamyl transferase (GGT) and other serum parameters namely, serum bilirubin (SB), total cholesterol (TC), triglycerides (TGL) and for evaluation of kidney function, estimation of urea and creatinine were carried out using the commercial kits by Coral clinical system, Goa, India.

Estimation of blood glucose

The blood glucose level is directly measured by placing one drop of blood on the test strip of blood glucometer and reading the values displayed.

Estimation of liver malondialdehyde

Malondialdehyde in the rat liver was estimated by the modified procedure [7]. Liver homogenate (10% w/v) from each group (1 ml) mixed with 100 µl of 8.1% SDS and 600 µl of 20% acetic acid solution was kept for 2 min at room temperature. Then 600 µl of 0.8% solution of TBA was added, heated at 95 ºC for 60 min in a water bath and cooled with ice cold water at 4 ºC. A mixture of n-butanol and pyridine (15:1 v/v) were added, shaken vigorously and centrifuged at 1 000 0 rpm for 5 min. The absorbance of the organic layer was measured at 532 nm. Lipid peroxidation was expressed as nmol/g wet liver.

Assay of liver catalase

Catalase in the rat liver was assayed according to the standard method [8]. To 0.9 ml of phosphate buffer (0.01M, pH-7.0) 0.1 ml of liver homogenate (10% w/v) and 0.4 ml of H_2O_2 (0.2 M) were added. After 60 sec, 2 ml of dichromate-acetic acid reagent (5%) was added. The tubes were kept in boiling water bath for 10 min and the colour developed was read at 620 nm. Standard H_2O_2 in the range of 2-10 µl was taken with blank containing reagent alone. CAT activity was measured proportionately to the rate of H_2O_2 reduction. Dichromate in acetic acid was converted to perchromic acid and then chromic acetate, when heated in the presence of H_2O_2. Chromic acetate formed was measured at 620 nm. Absorbance values were compared with a standard curve generated from known catalase and the activities were expressed as U/mg protein. The amount of protein/mg of tissue was determined by the standard method [9].

Determination of liver reduced glutathione

Homogenized rat liver samples (10% w/v) from the entire group (0.2 ml) were mixed with 1.8 ml of 1 mM EDTA solution. To this, 3.0 ml precipitating reagent (1.67 g of met phosphoric acid, 0.2 g of EDTA disodium salt, 30 g sodium chloride, 1000 ml distilled water) was added, mixed thoroughly and kept for 5 min before centrifugation. To 2 ml of the supernatant, 4.0 ml of 0.3 M disodium hydrogen phosphate solution and 1.0 ml of DTNB (5, 5'dithio-bis (2-nitro benzoic acid)) reagent were added and absorbance was read at 412 nm. Absorbance values were compared with a standard curve generated from the known GSH [10].

Haematological analysis

Blood samples were collected in EDTA coated vials, mixed well and the haematological parameters namely, white blood cell (WBC) count, lymphocyte (LYM) count, monocyte (MONO) count, granulocyte (GRAN) count, LYM%, MONO%, GRAN%, red blood cell (RBC) count, hemoglobin (HGB) concentration, hematocrit (HCT) %, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), red blood cell distribution width (RDW), platelet (PLT) count, mean platelet volume (MPV), platelet distribution width (PDW) and platelet crit (PCT) % were estimated using veterinary auto hematology analyzer.

Histopathological investigations

A portion of the internal organ (liver, kidney, heart, lungs, spleen) obtained from all the groups were sliced into two pieces of approximately 6 mm^3 size and preserved in 10% formalin solution for 24 h. They were subjected to dehydration with acetone of strength 70, 80 and 100 % respectively, each for 1 h. Infiltration and impregnation were done by treatment with paraffin wax, twice for 1 h. Paraffin was used to prepare paraffin 'L' moulds. Specimens were cut into sections of 3-7 µm thickness and stained with haematoxylin and eosin. The thin sections of the liver were made into permanent slides and examined under high resolution microscope with photographic facility and photomicrographs were taken.

Statistical analysis

All the data were expressed as mean±standard deviation (SD). The significance of difference among the group was assessed using one way analysis of variance (ANOVA) followed by Dunnett's post-test using SPSS software version-20. The $p \leq 0.05$ was considered statistically significant.

RESULTS

Chronic toxicity study

In chronic toxicity studies, PHEF and PHLE administration up to 1500 mg/kg dose did not result in mortality or any change in behaviour of the animals.

The drug treated animals did not show any decrease in body weight and they showed only a general increase in body weight (fig. 1). The mean increase in body weight within the group from the first day to the 90th day of the experiment was; 135.45±8.75 g (NC), 126.00±7.46 g (PHLE 500 mg/kg), 118.63±6.53 g (PHLE 1000 mg/kg), 148.82±7.53 g (PHLE 1500 mg/kg), 170.43±5.34 g (PHEF 500 mg/kg), 165.62±5.74g (PHEF 1000 mg/kg) and 164.47±6.41 g (PHEF 1500 mg/kg). The growth rate of each group was found to be 1.51±0.08 g (NC), 1.40±0.08 g (PHLE 500 mg/kg), 1.32±0.07 g (PHLE 1000 mg/kg), 1.65±0.08 g (PHLE 1500 mg/kg), 1.89±0. 06 g (PHEF 500 mg/kg), 1.84±0.06 g (PHEF 1000 mg/kg) and 1.83±0.07 g (PHEF 1500 mg/kg). The mean organ body weight of internal organs like lungs, heart, liver, spleen and kidneys of all the experimental Wistar rats after 90 d toxicity study is represented in fig. 2. Internal organ weight relative to 100 g body weight of all the drugs treated animals, showed no significant ($p \leq 0.05$) difference from the normal control values. No significant change in food and water intake of the test animals were observed at all the test doses compared to the normal control.

The detailed postmortem examination of the physical appearance of internal organs of all the doses of PHLE and PHEF treated animals did not show any macroscopic differences in size, color or texture compared to the normal control animals.

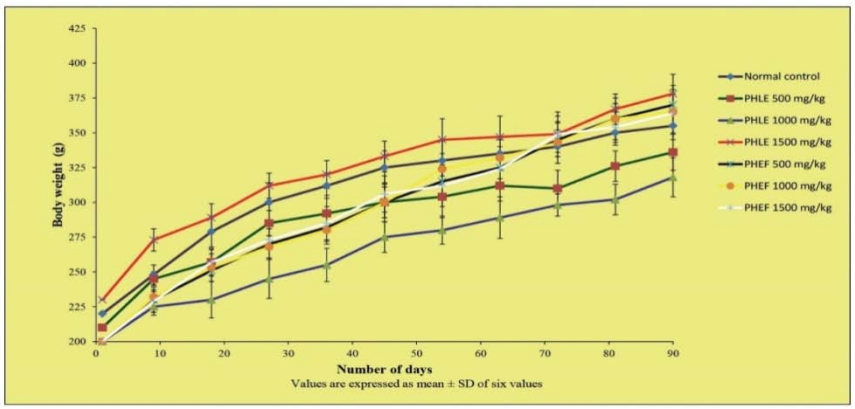

Fig. 1: Effect of PHLE and PHEF in body weight of Wistar rats in chronic toxicity study

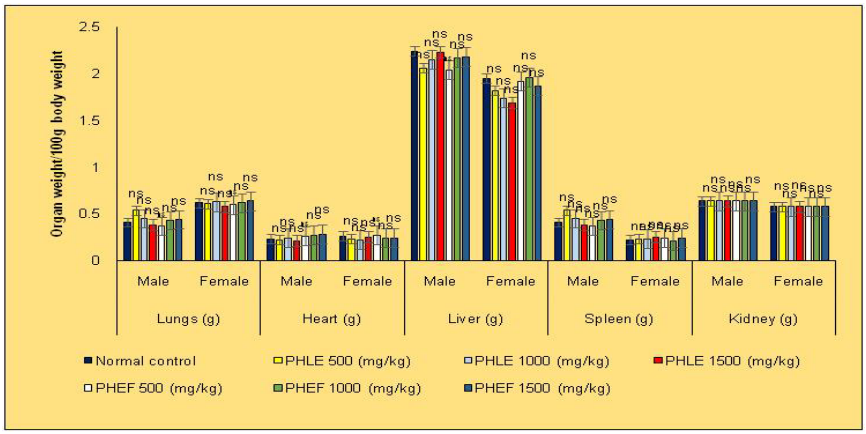

Fig. 2: Effect of PHLE and PHEF on internal organ weight of Wistar rats after chronic toxicity study

Values are expressed as mean±SD of six values, one way ANOVA followed by Dunnett's multiple comparison test, ^{ns}-no significant difference when compared to normal control

Estimation of serum biochemical parameters of liver and kidney

Effect of PHLE and PHEF on serum hepatic marker enzymes, namely AST, ALT, ALP, GGT and other serum parameters namely, SB, TC, TP and TGL after the 90 d toxicity study are represented in table 1. All the parameters showed normal values and there was no significant ($p \leq 0.05$) difference in the drug treated groups when compared to the normal control group.

Renal function was evaluated by estimating the urea and creatinine values in the serum. Urea and creatinine values of all doses of PHLE and PHEF treated animals together with the normal control values were shown in table 1. There was no significant ($p \leq 0.05$) difference observed in the serum urea and creatinine values of drug treated animals when compared to normal control animals. The maximum value of urea found in the drug treated animals were 41.32±1.65 mg/dl (PHLE 1500 mg/kg) and minimal value was 36.56±1.45 mg/dl (PHLE 1000 mg/kg), whereas the normal control value was found to be 37.82±1.34 mg/dl. All the creatinine values were found to be within the range of 0.42±0.02 to 0.44±0.06 mg/dl.

Estimation of blood glucose

The blood glucose level of all the animals was found to be within the normal range. There was no significant change in the glucose levels of animals treated with all the doses of PHLE and PHEF when compared with normal control animals (table 1).

Table 1: Effect of PHLE and PHEF on serum biochemical parameters in chronic toxicity study

Parameters	Treatment groups						
	Normal control	PHLE 500 (mg/kg)	PHLE 1000 (mg/kg)	PHLE 1500 (mg/kg)	PHEF 500 (mg/kg)	PHEF 1000 (mg/kg)	PHEF 1500 (mg/kg)
AST (IU/l)	65.36±3.47	68.12±2.87^{ns}	65.47±3.82^{ns}	73.65±3.47^{ns}	65.87±2.47^{ns}	71.83±3.61^{ns}	71.65±3.21^{ns}
ALT (IU/l)	40.39±1.45	43.78±1.67^{ns}	46.65±1.28^{ns}	49.85±1.84^{ns}	46.57±1.87^{ns}	48.69±1.86^{ns}	49.63±1.35^{ns}
ALP (IU/l)	91.36±3.19	94.54±1.34^{ns}	96.87±1.43^{ns}	94.54±1.96^{ns}	90.36±2.69^{ns}	98.12±1.58^{ns}	100.79±1.47^{ns}
GGT (IU/l)	7.13±1.34	6.45±1.40^{ns}	7.82±1.34^{ns}	7.72±1.86^{ns}	6.11±1.25^{ns}	6.86±1.57^{ns}	7.13±1.65^{ns}
SB (mg/dl)	0.38±0.04	0.39±0.04^{ns}	0.36±0.05^{ns}	0.42±0.01^{ns}	0.34±0.04^{ns}	0.39±0.05^{ns}	0.46±0.06^{ns}
TC (mg/dl)	99.65±3.32	96.62±2.54^{ns}	98.62±2.37^{ns}	97.88±2.54^{ns}	93.61±2.67^{ns}	99.88±2.54^{ns}	103.57±3.48^{ns}
TP (g/dl)	5.70±0.25	5.96±0.31^{ns}	5.86±0.34^{ns}	5.13±0.36^{ns}	5.12±0.31^{ns}	4.93±0.31^{ns}	4.68±0.36^{ns}
TGL (mg/dl)	95.33±2.67	98.13±3.88^{ns}	97.43±2.64^{ns}	99.65±1.34^{ns}	91.37±3.45^{ns}	102.68±3.43^{ns}	123.69±3.6^{ns}
Glucose (mg/dl)	121.23±4.23	112.45±4.61^{ns}	118.65±4.51^{ns}	111.56±4.54^{ns}	118.46±4.61^{ns}	119.68±4.62^{ns}	121.43±5.2^{ns}
Urea (mg/dl)	37.82±1.34	40.84±1.32^{ns}	36.56±1.45^{ns}	41.32±1.65^{ns}	36.56±1.75^{ns}	37.35±1.63^{ns}	40.56±1.42^{ns}
Creatinine (mg/dl)	0.42±0.06	0.43±0.05^{ns}	0.42±0.03^{ns}	0.44±0.06^{ns}	0.43±0.04^{ns}	0.43±0.02^{ns}	0.42±0.02^{ns}

Values are expressed as mean±SD, n = 6, one way ANOVA followed by Dunnett's multiple comparison test, ^{ns}-no significant difference when compared to normal control.

Evaluation of *in vivo* antioxidant status of liver

MDA, CAT and GSH of the liver in the entire plant drug treated groups showed no significant difference from the normal control group at 90 d chronic toxicity study (fig. 3-5). PHLE and PHEF up to 1500 mg/kg did not show any negative impact on the *in vivo* antioxidant status of the liver.

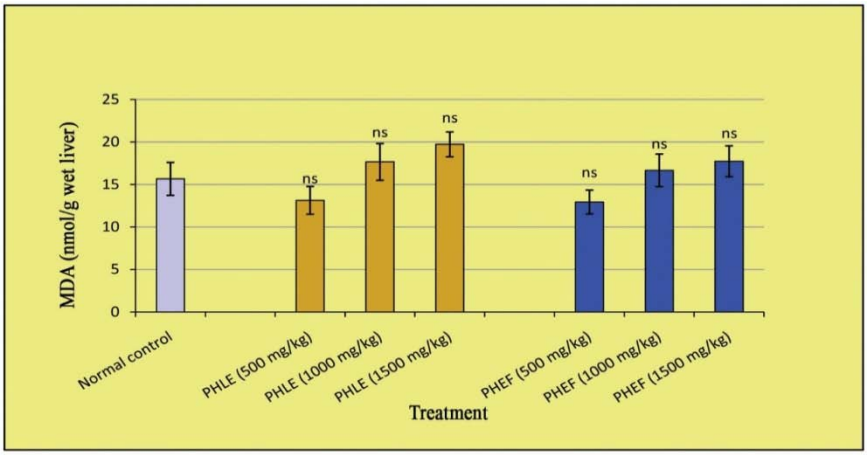

Fig. 3: Effect of PHLE and PHEF on hepatic MDA after chronic toxicity study

Values are expressed as mean±SD of six values, one way ANOVA followed by Dunnett's multiple comparison test, [ns]- no significant difference when compared to normal control

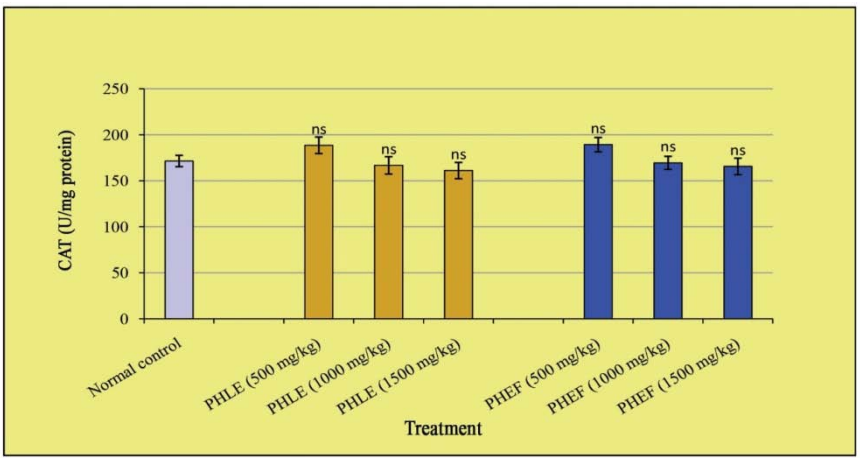

Fig. 4: Effect of PHLE and PHEF on hepatic CAT after chronic toxicity study

Values are expressed as mean±SD of six values, one way ANOVA followed by Dunnett's multiple comparison test, [ns]- no significant difference when compared to normal control

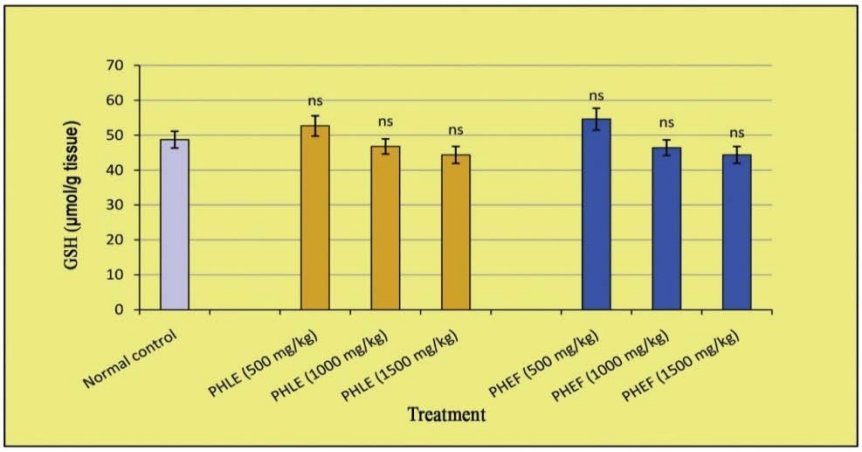

Fig. 5: Effect of PHLE and PHEF on hepatic GSH after chronic toxicity study

Values are expressed as mean±SD of six values, one way ANOVA followed by Dunnett's multiple comparison test, [ns]- no significant difference when compared to normal control

Haematological analysis

The haematological analysis report of blood samples of drug treated animals after 90 d of PHLE and PHEF administration is represented in table 2 and table 3 respectively. The eighteen haematological parameters of all the plant drug treated groups showed no significant difference ($p \leq 0.05$) from the normal control values. There were no significant changes in RBC, WBC, granulocytes, lymphocytes, monocyte, platelet count *etc*. up to 1500 mg/kg of PHLE and PHEF treated animals.

Table 2: Effect of *P. heyneana* leaf ethanolic extract on haematological parameters in chronic toxicity study

Hematological parameters	Groups			
	Normal control	PHLE 500 (mg/kg)	PHLE 1000 (mg/kg)	PHLE 1500 (mg/kg)
WBC (×10³/µl)	11.00±1.20	13.40±2.34^{ns}	14.00±2.00^{ns}	16.10±1.20^{ns}
LYM (×10³/µl)	7.40±0.45	10.30±1.45^{ns}	10.20±1.75^{ns}	6.70±0.24^{ns}
MONO (×10³/µl)	0.30±0.04	0.30±0.06^{ns}	0.30±0.01^{ns}	2.50±0.12^{ns}
GRAN (×10³/µl)	3.30±0.54	2.80±0.30^{ns}	3.50±0.09^{ns}	6.90±0.45^{ns}
LYM%	67.40±2.30	76.50±2.10^{ns}	72.80±2.50^{ns}	61.40±3.22^{ns}
MONO%	3.00±0.45	2.50±0.45^{ns}	2.50±0.14^{ns}	5.70±1.54^{ns}
GRAN%	29.60±1.28	21.00±1.50^{ns}	24.70±2.15^{ns}	32.90±2.46^{ns}
RBC (×10⁶/µl)	9.00±0.34	9.07±0.34^{ns}	8.29±0.43^{ns}	9.06±0.34^{ns}
HGB (g/dl)	15.20±1.20	15.90±1.24^{ns}	15.70±0.98^{ns}	16.70±1.23^{ns}
HCT (%)	47.50±2.40	48.40±1.78^{ns}	47.90±1.50^{ns}	51.00±1.94^{ns}
MCV (fl)	52.80±2.75	53.40±1.29^{ns}	57.90±2.30^{ns}	56.30±2.45^{ns}
MCHC (g/dl)	16.80±1.73	17.50±1.20^{ns}	18.90±2.56^{ns}	18.40±1.2^{ns}
MCH (pg)	32.00±2.64	32.80±1.56^{ns}	32.70±3.45^{ns}	32.70±3.59^{ns}
RDW (%)	15.30±1.63	15.00±1.46^{ns}	17.80±1.74^{ns}	9.80±1.40^{ns}
PLT (×10³/µl)	1078.00±28.53	1112.00±12.50^{ns}	999.00±23.57^{ns}	1030.00±42.34^{ns}
MPV (fl)	5.30±0.10	5.300±0.20^{ns}	6.10±0.63^{ns}	5.80±0.10^{ns}
PDW	16.20±2.59	16.20±1.67^{ns}	17.00±2.64^{ns}	16.70±2.84^{ns}
PCT (%)	0.57±0.04	0.59±0.03^{ns}	0.61±0.03^{ns}	0.59±0.04^{ns}

Values are expressed as mean±SD, n = 6, one way ANOVA followed by Dunnett's multiple comparison test, ^{ns}-no significant difference when compared to normal control.

Table 3: Effect of *P. heyneana* leaf ethanolic fraction on haematological parameters in chronic toxicity study

Hematological parameters	Groups			
	Normal control	PHEF 500 (mg/kg)	PHEF 1000 (mg/kg)	PHEF 1500 (mg/kg)
WBC (×10³/µl)	11.00±1.20	12.38±2.13^{ns}	13.89±2.28^{ns}	15.91±1.20^{ns}
LYM (×10³/µl)	7.40±0.45	9.13±1.5^{ns}	10.14±1.64^{ns}	10.93±0.32^{ns}
MONO (×10³/µl)	0.30±0.04	0.34±0.05^{ns}	0.31±0.03^{ns}	0.50±0.12^{ns}
GRAN (×10³/µl)	3.30±0.54	2.91±0.2^{ns}	3.44±0.06^{ns}	3.48±0.41^{ns}
LYM%	67.40±2.30	73.75±2.14^{ns}	73.00±2.43^{ns}	56.12±3.21^{ns}
MONO%	3.00±0.45	2.75±0.38^{ns}	2.23±0.16^{ns}	3.14±1.62^{ns}
GRAN%	29.60±1.28	23.51±1.47^{ns}	24.77±2.47^{ns}	21.87±2.51^{ns}
RBC (×10⁶/µl)	9.00±0.34	9.42±0.43^{ns}	8.68±0.52^{ns}	9.25±0.43^{ns}
HGB (g/dl)	15.20±1.20	16.82±1.32^{ns}	15.43±0.86^{ns}	16.55±1.28^{ns}
HCT (%)	47.50±2.4	49.34±1.65^{ns}	47.67±1.48^{ns}	50.36±1.83^{ns}
MCV (fl)	52.80±2.75	53.65±1.34^{ns}	55.37±2.54^{ns}	56.37±2.17^{ns}
MCHC (g/dl)	16.80±1.73	16.98±1.23^{ns}	18.65±2.23^{ns}	18.17±1.59^{ns}
MCH (pg)	32.00±2.64	32.54±1.43^{ns}	33.63±3.12^{ns}	32.74±3.43^{ns}
RDW (%)	15.30±1.63	15.81±1.27^{ns}	17.15±1.15^{ns}	14.61±1.63^{ns}
PLT (×10³/µl)	1078.00±28.53	1169.58±14.69^{ns}	995.98±21.86^{ns}	1189.45±47.32^{ns}
MPV (fl)	5.30±0.10	5.88±1.28^{ns}	6.46±1.54^{ns}	5.72±2.83^{ns}
PDW	16.20±2.59	15.65±1.45^{ns}	16.76±2.45^{ns}	16.43±2.13^{ns}
PCT (%)	0.57±0.04	0.58±0.02^{ns}	0.62±0.04^{ns}	0.58±.03^{ns}

Values are expressed as mean±SD, n = 6, one way ANOVA followed by Dunnett's multiple comparison test, ^{ns}-no significant difference when compared to normal control.

Histopathological investigations

Histopathological examination of liver, heart, kidney, lungs and spleen of all the drugs treated animals showed normal architecture similar to the normal control group (fig. 6). Liver sections were normal with hepatic lobules consisting of branching and anastomosing plates of hepatic cells with clear sinusoidal space (fig. 6A). Abnormalities like necrotic or fatty changes were completely absent and Kupffer cells were less in number in all the liver sections examined. The interlobular septa with the portal area consisting of branches of portal veins and hepatic arteries are also visible and clearly demarcated.

Microphotographs of the heart showed the normal architecture with normal muscle fibers. Abnormalities such as enlargement or disaggregation of connective tissues, interfibrillar hemorrhages, congestion and focal areas of disrupted cardiac muscle fibers were absent in all the drug treated groups. Focal areas of infiltration and significant lesions of pathological importance were completely absent (fig. 6B).

Microscopic examination of the kidney showed normal renal cytoarchitecture with normal renal corpuscles and renal tubules which are surrounded by basement membrane. The glomeruli and Bowman's capsule appeared normal. The glomerular epithelium and the capsular space also appeared normal in the control and the treated groups (fig. 6C). Section of the medullary areas also exhibited normal collection of papillary ducts and ducts of Loops of Henle.

Lung sections showed the cells lining the alveoli and the surrounding capillaries are normal, one cell thick, and are in very close contact with

each other to facilitate gas diffusion and no prominent thickening of the alveolar wall has been observed (fig. 6D).

Splenic sections of all the drug treated animals showed normal cytoarchitecture comparable to the normal spleen. Areas of red and white pulp along with central arteries were observed. The germinal centers were also visible along with the connective tissue trabeculae and all appeared normal for both control group as well as for the treated groups (fig. 6E)

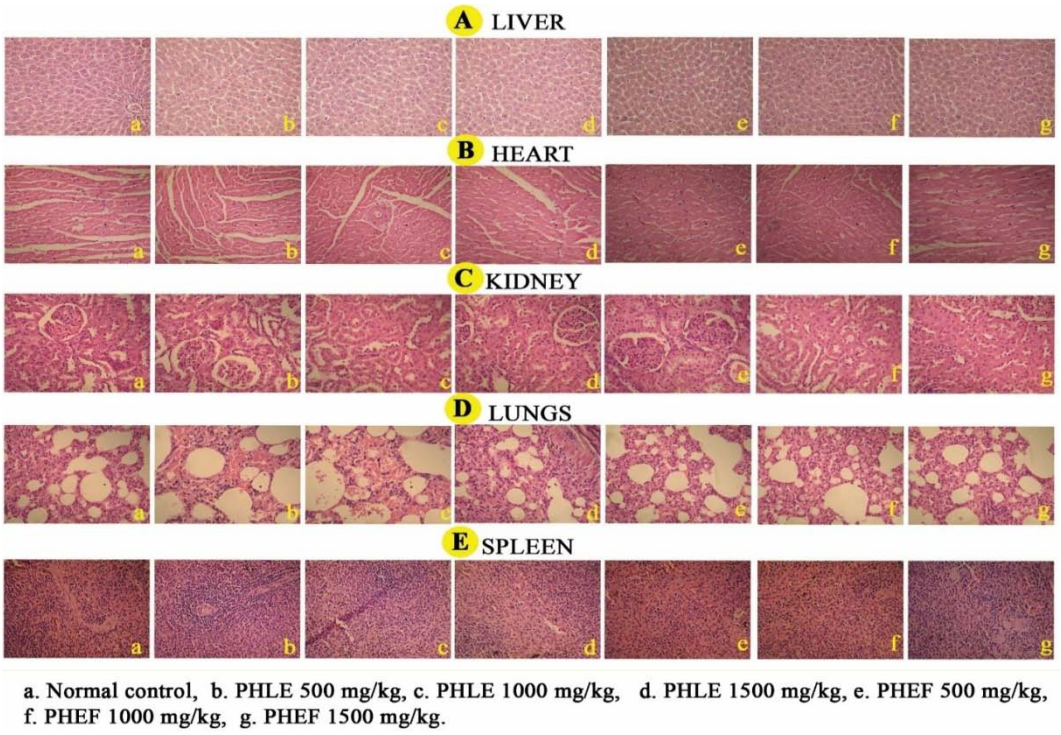

Fig. 6: Effects of PHLE and PHEF on various rat organ histomorphologies in chronic oral toxicity study

DISCUSSION

Chronic toxicity studies will help to assess the undesirable effects of continuous or repeated exposure of plant extracts or compounds over a portion of the average life span of experimental animals, such as rodents. Specifically, they provide information on target organ toxicity and are designed to identify unobservable adverse effect levels [11]. In the present study, administration of PHLE and PHEF up to 1500 mg/kg for 90 d did not show any clinical signs of toxicity or mortality in either sex. Changes in body weight serve as a sensitive indicator of the general health status of animals [12]. The gradual increment in body weight shown by the entire drug treated animals has been found to be statistically insignificant from the normal control values. All these evaluations confirmed that the PHLE and PHEF or their secondary metabolites had played no adverse role in the normal metabolism of the Wistar rats. Furthermore, the diet and water were well-accepted by the rats treated with PHLE and PHEF and this points out the fact that the drugs did not possibly cause any alterations in carbohydrate, protein or fat metabolism in these experimental animals as has been stressed by Klaassen [13].

The liver is the target site for most of the toxic metabolism, which may lead to the damage of membrane permeability of this organ and the leakage of liver enzymes into the blood stream [14]. Hence quantification of liver marker enzymes in animal system is one of the most frequent approaches adopted to evaluate the toxicity of a plant extract [15]. The serum biochemical parameters were evaluated to understand the possible alterations in hepatic function influenced by the extracts. The nonsignificant changes in AST, ALT, ALP, GGT, TC, TP and SB values in the drug treated rats suggested that chronic administration of PHLE and PHEF is non-hepatotoxic even at higher doses (up to 1500 mg/kg). The non-significant change in the levels of AST and ALT also points out that the drugs have no toxic effect on heart tissue [16].

Estimation of biochemical parameters pertaining to kidney functions *viz*., urea and creatinine supports the nontoxic effects of the extracts on kidney functions. Since, almost all drugs, chemicals and xenobiotics are eliminated through renal excretion, a comprehensive study on the effect of the plant extract on kidney function is essential in the toxicity studies [17]. An increase in the levels of urea and creatinine are significant indicators of the damaged functional nephrons [18]. The results of serum biochemical parameters related to kidney functions *viz*. urea and creatinine had demonstrated no significant differences with respect to control group animals and the same shows nontoxic effect of *P. heyneana* on kidney function.

Normal glucose level in the blood is maintained by the combined action of two hormones, namely glucagon and insulin, produced by hypothalamus and pancreas respectively. Any type of toxicity in these organs will interfere with the normal secretion of this hormone and alter the normal glucose level in the blood. Normal glucose level shown by the entire drug treated groups in the present study confirms that PHLE and PHEF had no adverse influence in maintaining normal glucose level in blood.

MDA is one of the end products in lipid peroxidation and its increased level in liver indicates the hepatic damage. Reduced glutathione is an intracellular non-enzymatic antioxidant, which protects cells against free radicals, peroxides and other toxic compounds [19]. Catalase is an antioxidant enzyme present in all major organs, especially in the liver, which promotes the conversion of hydrogen peroxide into molecular oxygen and water without the production of free radicals [20]. Estimation of MDA, GSH and CAT in the liver of drug treated animals reflected no changes and all the antioxidant parameters were found to be remain within the normal range. All these findings imply the maintenance of normal hepatic non-enzymatic and enzymatic antioxidant mechanisms during PHLE and PHEF treatment.

The haematopoietic system is one of the most sensitive targets of toxic compounds and is an important index of physiological and pathological status in man and animals [21]. Furthermore, haematological analysis is relevant in risk evaluation as changes in the haematological system have higher predictive value for human toxicity when the data are translated from animal studies [22]. After 90 d of treatment, there were no significant treatment-related changes in all the eighteen haematological parameters between control and treated groups. The non-significant effect of the extract on RBC count, HGB concentration, HCT %, MCV, MCHC, MCH and RDW indicates that PHLE and PHEF do not affect the erythropoiesis, morphology or osmotic fragility of the red blood cells [23]. White blood cells are the first line of cellular defense that respond to infectious agents, tissue injury, or inflammatory process. No significant changes were observed in the lymphocyte, monocyte and granulocyte count in drug treated groups, which further confirmed the above findings. All these normal haematological profiles of PHLE and PHEF treated groups justified the non-toxic nature of *P. heyneana* extract and fraction.

The internal organs are highly sensitive to toxic drugs and the resultant hypertrophy of these organs provides the firsthand indication of toxicity which may be useful in predicting toxicity level, enzyme induction, physiologic perturbations and acute injury [24]. The lungs, heart, liver, spleen and kidney weight of the Wistar rats in the drug treated groups have compared favorably with those of the controls at all the doses of PHLE and PHEF investigated. The autopsy also revealed that there were no treatment related changes in both control and treated groups. No hypertrophy of organs was observed in this study amongst all the groups studied. In addition, the microscopic examination revealed that none of the organs from the extract treated rats showed any alteration in cell structure or any unfavorable effects. No pathologies were recorded in the histological sections of the vital organs (heart, liver, spleen, kidney and lungs) of the control group. These results revealed that the extract did not affect the secretory ability of the organs and is supported by the previous observations [25]. It is also possible that the extract did not cause any cellular constriction or inflammation of the organs which would have resulted in swelling and increase in weight. This finding is well corroborated by the histological findings which did not show any pathological changes in the lungs, heart, liver, spleen and kidney of the treated animals.

CONCLUSION

Based on above findings, it is clear that the experimental animals did not show any deleterious changes in organ, haematological and biochemical indices during the course of repeated administration of PHLE and PHEF and no mortality was observed during the period. On the basis of findings that emerged from the present investigation, it can be established that *P. heyneana* has no toxic effect on chronic oral administration in rodents and supports the tribal use of the plant in the traditional system of Kerala. Taking into consideration all the above facts, it can be very well judged that both extracts did not affect the major biochemical functions in the body of Wistar rats during the 90 d toxicity study. Therefore, it is confirmed that the prolonged oral administration of PHLE and PHEF is safe without any toxic effect.

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CONFLICT OF INTERESTS

Declared none

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