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Original Research Article

Toxicity evaluation of standardized and nanoliposomal extracts of *Labisia pumila* whole plant (Blume, Myrsinaceae) in Sprague Dawley rats

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Abstract

Purpose: *To investigate the toxicity of Labisia pumila standardized extract (LPE) and its liposomal extract (LLP).*
Methods: *For acute toxicity study, LPE or LLP was orally administered (2000 mg/kg) in single doses to Sprague Dawley rats and the routine activity of the rats was continuously monitored for a total of 14 days. After 14 days of treatment, all rats were sacrificed and their vital organs were excised, weighed and macroscopically examined, while for a repeated dose toxicity study, the rats were orally administered with LPE or LLP at the selected doses (250, 500 and 1000 mg/kg) for a period of 28 days. The animals were sacrificed (anaesthetized by sodium pentobarbitone and blood was collected by cardiac puncture), followed by examination of their body organs and blood serum.*
Results: *LPE and LLP at 2000 mg/kg did not produce mortality or significant changes in the general behaviour, body weight and organ gross appearance of the rats. In repeated dose toxicity study no significant changes in, growth, organ weights, haematological parameters, biochemical values and histological features of vital organs of the treated groups, compared to the control group.*
Conclusion: *The no-adverse-effect-level for LPE and LLP is (1000 mg/kg/day) when administered orally for 28 days.*

Keywords: *Labisia pumila, Acute oral toxicity, Repeated dose toxicity*

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INTRODUCTION

Labisia pumila Blume, var. alata (Family: Myrsinaceae), known as Kacip Fatimah in Malaysia. Traditionally, *L. pumila* has been used due to its medicinal values in health products and female tonics. Furthermore, *L. pumila* is used in other ailments such as dysmenorrhoea, gonorrhoea, dysentery and flatulence [1]. Recent pharmacological studies have reported that *L.*

pumila has phytoestrogenic, anti-agiogenesis, anticancer, antioxidant, antibacterial, antifungal, anti-aging and anti-inflammatoryactivities. The benefits of the traditional use of *L. pumila* have also been supported by identification and isolation of many active compounds including phenolics, flavonoids and resorcinol compounds [2]. Gallic acid (as one of the isolated compounds from this plant) possessed many pharmacological activities such as antioxidant, antiangiogenesis, antiobesity, and anticancer effects. Rutin is also reported for different pharmacological effects, for example hepatic protective, antidiabetic, antihypertensive, anti-inflammatory, anticancer and antioxidant activities. Gallic acid and rutin both found in *L. pumila* extract [3-8]. Sub-acute, teratogenic and reproductive toxicity studies of *L. pumila* water extract at (50, 1000 and 800 mg/kg), respectively without any side effects were reported [9-11]. The present study has been designed to perform the acute oral toxicity study at (2000 mg/kg) and repeated dose oral toxicity study of LPE and LLP at (250, 500 and 1000 mg/kg), respectively. Furthermore, the present study also describe authenticity of plant species, parts of plant, type of extract and method of extraction. So, this study was aimed to evaluate the acute and repeated dose oral toxicity studies on standardized *L. pumila* extract (LPE) and liposome of *L. pumila* (LLP).

EXPERIMENTAL

Chemical and reagents

Soybean lecithin was procured from Hong Aun kimia Sdn Bhd., Malaysia. Acetone, chloroform and ethanol were obtained from Quality Reagent Chemical Malaysia.

Preparation of LPE and LLP

The whole plant of *L. pumila* var. alata was extracted with ethanol (50%) using soxhlet apparatus. The crude extract was filtered, concentrated under reduced pressure using a rotary evaporator, and further dried in oven. The dried extract was then kept in the fridge at -20^{o}C prior to use. Liposome formulation of ethanol (50%) extract of *L. pumila* was prepared as described by [12].

Acute oral toxicity study

Male and female Sprague Dawley rats at 8-10 weeks of age were used. The animals were acclimatized to laboratory conditions for a week prior to the experiment. The rats were housed with free access to feed (normal laboratory chow, Gold Coin) and tap water *ad libitum*. The rats were maintained at 28±2°C under a light/dark cycle of 12 h. All the procedures were done according to the OECD guidelines (2008) [13 and 15] and approved by the Animal Ethics Committee of Universiti Sains Malaysia (USM/Animal Ethics approval no. 2014/597).

LPE and LLP were administered orally (2000 mg/kg) in single doses to the rats (n=5). Another group of 5 rats without treatment was used as control group. The general behaviour of the rats were continuously monitored for 1 h after dosing, periodically during the first 24 h and daily thereafter for a total of 14 days. After 14 days of treatment, all animals were euthanized in CO_2 chamber, and selected vital organs were excised, weighed, and macroscopically examined [14].

Repeated dose toxicity study

Animals (70 rats) were divided into 7 groups (5 rats/sex/group) and received the test materials orally. Group 1 served as control receiving vehicle only. Animals in group 2 to group 7 received 250, 500 and 1000 mg/kg of LPE or LLP, respectively. Animals were treated once daily consecutively for 28 days. The animals were observed daily for clinical signs and mortality. At the end of the experiment, animals were anaesthetized by sodium pentobarbitone and blood was collected by cardiac puncture from overnight fasted animals. Vital organs such as brain, heart, lungs, liver, spleen, kidneys, and testes/ovaries were removed and weighed. Haematological, biochemical and histological analysis were done [15]. Measurements were made by (Gribbles Lab., Penang, Malaysia).

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM) and were analysed using Statistical Package for the Social Sciences (SPSS 20.0, IBM). The data were considered significant at $p < 0.05$.

RESULTS

Toxicity of LPE and LLP

For acute oral toxicity study, lethal effects were not observed after administration of LPE or LLP at dose (2000 mg/kg). No behavioural changes were observed during the observation period. There were no significant changes in the organ-to-body weight ratios (Table 1) of the treated rats compared to normal control group. Therefore, the

LD_{50} value for oral administration of LPE and LLP was greater than 2000 mg/kg.

In repeated dose oral toxicity study, there was no mortality of animals at any dose. Gross observations did not show any changes and all animals of both groups remain healthy. There were no abnormalities founded with respect to eye colour, touch response, salivation, grip strength and tail pinch. No locomotor dysfunction, convulsions or tremors were observed in all animals.

There were no significant differences observed for the weekly body weight and organ-to-body weight ratios of rats treated with LPE or LLP for 28 days (Table 2 and Table 3) except for LLP 250 mg/kg, LPE 500 mg/kg, and LLP 500 mg/kg for female groups during 14 days as presented in Table 2. The haematology results (Table 4) showed no statistically significant differences in the values for treated groups compared to normal control group except for haemoglobin (Hb), packed cell volume (PCV), mean cell volume (MCV), and neutrophiles in female groups only but still within the normal range as previously reported [16]. A biochemical examination results are summarized in Table 5, the levels of biochemical parameters were not statistically significant compared to normal control groups except for urea and uric acid in female treated groups, and for total cholesterol (TC), triglycerides (TG), and high density lipoprotein (HDL) in female and male treated groups but remained within the normal range except for urea level in female treated groups (LPE 500 mg/kg and LLP at 250, 500, 1000 mg/kg). Among the indicators of liver function, alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were not affected by LPE or LLP in both male and female treated groups.

The histological examination of liver and kidney tissues of normal control group and treated groups did not show any pathological abnormalities. Both control and treated groups showed normal hepatic architecture. Mild periportal inflammation was seen in female and male normal control groups (only 1 out of 5 rats), female LPE 500 mg/kg group (only 1 out of 5 rats) and male LPE 250 and 1000 mg/kg groups (only 1 out of 5 rats), while it didn't show any periportal chronic inflammation in liver of LLP treated groups in both male and female rats.

Table 1: Organ-to-body weight ratios of rats treated with LPE and LLP for acute toxicity study

Organ	Normal control water (10 mL/kg)	Treated group with LPE (2000 mg/kg)	Treated group with LLP (2000 mg/kg)
Heart	0.35±0.01	0.35±0.01	0.32±0.01
Liver	3.12±0.09	2.85±0.08	3.11±0.09
kidney (R)	0.32±0.01	0.33±0.01	0.31±0.01
kidney (L)	0.32±0.01	0.32±0.01	0.32±0.01
Lung	0.63±0.04	0.62±0.04	0.64±0.02
adrenal (R)	0.02±0.00	0.02±0.00	0.02±0.00
adrenal (L)	0.02±0.00	0.02±0.00	0.02±0.00
Spleen	0.26±0.01	0.28±0.01	0.27±0.02
Uterus	0.22±0.04	0.26±0.06	0.23±0.01
Ovaries	0.18±0.03	0.19±0.02	0.19±0.01
Thymus	0.19±0.01	0.21±0.02	0.18±0.01
Brain	0.75±0.03	0.72±0.01	0.74±0.01

Values are expressed as mean ± SEM. No significant difference between normal control group vs. treated groups, where R = right and L=left

Table 2: Weekly body weight (g) of rats treated with LPE and LLP for 28 days

Group	0 day	7 days	14 days	21 days	28 days
Normal control	218.15±8.16	233.5±9.47	249.1±11.10	233.4±9.95	239.66±11.07
LPE 250 mg/kg	217.96±7.38	226.77±6.73	233.9±8.85	224.5±9.57	235.74±9.27
LPE 500 mg/kg	219.1±4.78	223.85±3.91	232.4±7.05	220.1±5.50	235.69±6.53
LPE 1000 mg/kg	209.44±3.81	218.49±6.99	230.6±7.35	218±7.48	227.51±8.97
LLP 250 mg/kg	217.49±7.63	226.84±5.82	226.53±6.16	235.94±7.02	228.24±6.82
LLP 500 mg/kg	217.86±5.34	232.66±6.47	233.97±7.30	243.06±7.54	234.77±8.90
LLP 1000 mg/kg	230.81±5.28	229.54±6.41	227.32±6.02	235.21±5.85	224.91±8.04

Values are expressed as mean ±SEM. (n=10); * = $p< 0.05$, ** = $p< 0.01$, *** = $p< 0.001$ for treated groups vs. normal control

Table 3: Organ-to-body weight ratios of rats treated with LPE and LLP for 28 days

% Organ weight/ Bodyweight	Normal control	LPE 250 mg/kg	LPE 500 mg/kg	LPE 1000 mg/kg	LLP 250 mg/kg	LLP 500 mg/kg	LLP 1000 mg/kg
Brain	0.74±0.025	0.75±0.025	0.75±0.16	0.75±0.035	0.74±0.02	0.73±0.025	0.72±0.05
Heart	0.34±0.015	0.35±0.015	0.36±0.01	0.35±0.015	0.33±0.01	0.34±0.025	0.32±0.015
Lung	0.60±0.025	0.61±0.04	0.63±0.03	0.62±0.065	0.57±0.025	0.57±0.025	0.58±0.045
Liver	2.68±0.075	2.61±0.085	2.65±0.09	2.61±0.095	2.74±0.1	2.73±0.17	2.52±0.105
Spleen	0.21±0.01	0.22±0.015	0.21±0.01	0.21±0.015	0.20±0.01	0.20±0.01	0.19±0.01
Kidney (L)	0.32±0.015	0.32±0.01	0.30±0.02	0.32±0.015	0.30±0.015	0.30±0.015	0.29±0.01
Kidney (R)	0.32±0.015	0.33±0.015	0.32±0.015	0.32±0.01	0.31±0.015	0.30±0.015	0.30±0.015
Ovaries	0.34±0.025	0.34±0.025	0.32±0.02	0.32±0.02	0.35±0.025	0.33±0.02	0.32±0.02
Uterus	0.42±0.04	0.41±0.035	0.40±0.03	0.39±0.035	0.40±0.03	0.42±0.025	0.40±0.03
Thymus	0.20±0.02	0.17±0.025	0.19±0.025	0.21±0.035	0.19±0.025	0.19±0.025	0.19±0.03

Values are expressed as mean ±SEM. (n=10). No statistical significant difference for treated groups vs. normal control.

Table 4: Haematological values of rats treated with LPE and LLP for 28 days

Hematological parameters	Normal control	LPE 250 mg/kg	LPE 500 mg/kg	LPE 1000 mg/kg	LLP 250 mg/kg	LLP 500 mg/kg	LLP 1000 mg/kg
Hb (g/dL)	169.3±2.58	167.20±2.49	162.60±13.91	170.10±4.23	154.60±2.36	160.30±3.40	154.90±1.19
RBC ($\times10^{12}$/L)	9.23±0.18	9.18±0.14	8.17±0.93	9.04±0.20	8.25±0.16	8.71±0.14	8.46±0.13
PCV (L/L)	0.52±0.01	0.53±0.01	0.49±0.06	0.53±0.02	0.48±0.02	0.49±0.01	0.46±0.01*
MCV (gl)	56.90±0.97	58.20±0.91	59.80±1.52	58.50±1.38	58.30±1.31	55.50±1.03	54.30±0.44
White cell count ($x10^9$/L)	5.32±0.96	4.44±0.40	4.69±0.96	6.32±1.06	4.08±0.68	3.70±0.63	3.50±0.84
Neutrophil ($\times10^9$/L)	1.73±0.38	1.63±0.17	1.89±0.59	1.83±0.38	1.19±0.19	0.83±0.07***	0.76±0.17***
Lymphocytes ($\times10^9$/L)	3.35±0.66	2.65±0.33	2.57±0.48	4.15±0.66	2.68±0.48	2.68±0.51	2.53±0.74
Monocytes ($\times10^9$/L)	0.16±0.05	0.11±0.03	0.14±0.07	0.17±0.05	0.13±0.03	0.17±0.04	0.14±0.27
Eosinophils ($\times10^9$/L)	0.10±0.02	0.12±0.02	0.16±0.07	0.115±0.03	0.13±0.03	0.13±0.02	0.12±0.01
Platelets ($\times10^9$/L)	1065.10±87.45	778±112.16	676.60±155.56	604±77.98	726.90±97.43	962.40±60.66	874.50±50.37

Values are expressed as mean ±SEM. (n=10);* = $p< 0.05$, ** = $p< 0.01$, *** = $p< 0.001$ for treated groups vs. normal control. Hb = haemoglobin, RBC = red blood cells, PCV = packed cell volume and MCV = mean cell volume.

Table 5: Biochemical parameters of rats treated with LPE and LLP for 28 days

Biochemical parameters	Normal control	LPE 250 mg/kg	LPE 500 mg/kg	LPE 1000 mg/kg	LLP 250 mg/kg	LLP 500 mg/kg	LLP 1000 mg/kg
Urea (mmol/L)	8.00±0.27	8.00±0.37	8.69±0.34	8.06±0.36	6.96±0.31*	6.73±0.34**	6.06±0.34**
Creatinine (umol/L)	49.20±2.14	54.20±2.19	54.10±2.47	47.90±3.51	45.20±2.14	43.00±1.95	41.10±3.48
Uric acid (mmol/L)	0.13±0.01	0.14±0.01	0.13±0.01	0.12±0.01	0.15±0.03	0.13±0.02	0.13±0.02
TP (g/L)	80.60±1.44	82.20±1.61	80.60±0.82	79.30±1.65	76.50±1.59	77.40±1.25	74.50±1.56
ALP (U/L)	117.10±13.67	118.10±15.57	138.90±22.40	136.00±22.31	136.70±14.73	130.00±22.47	111.70±12.90
AST (U/L)	274.20±12.59	299.20±17.50	272.90±16.60	310.80±20.74	233.60±20.84	244.90±24.20	227.90±24.87
ALT (U/L)	50.80±3.17	56.00±3.08	54.40±4.55	50.50±3.14	59.20±9.17	53.80±10.52	43.80±4.74
Glu. (mmol/L)	3.78±0.51	3.83±0.29	4.21±0.28	4.04±0.38	5.57±0.70	5.33±0.86	5.67±0.27
TC (mmol/L)	3.02±0.18	3.30±0.27	3.13±0.16	3.02±0.23	1.68±0.10	1.80±0.16	1.68±0.13
TG (mmol/L)	0.35±0.02	0.45±0.03*	0.43±0.02	0.42±0.03	0.71±0.06***	0.73±0.12**	0.54±0.07**
LDL (mmol/L)	1.09±0.21	1.41±0.25	1.15±0.19	1.13±0.43	0.95±0.07	1.02±0.13	0.98±0.12
HDL (mmol/L)	1.77±0.17	1.69±0.17	1.82±0.12	1.70±0.22	0.41±0.03***	0.45±0.04***	0.46±0.05***

Values are expressed as mean ±SEM. (n= 10);* $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$ for treated groups vs. normal control. TP = total protein, ALP = alkaline phosphate, AST = Aspartate aminotransferase, ALT = alanine aminotransferase, Glu = glucose, TC = cholesterol, TG = triglycerides, LDL = low density lipoprotein and HDL = high density lipoprotein.

No ballooning degeneration, inflammation or other pathological abnormalities were observed in liver tissue samples of male and female rats (Plates 1 and 2). Kidney exhibited normal renal cortex containing normal glomeruli. Renal tubules were lined with typical cuboidal epithelium and had distinct lumen. Chronic pyelitis was observed in 1 female rat in normal control and LPE 1000 mg/kg groups (Plates 3 and 4). Thus these changes were not considered to be treatment related in this study, because these microscopic changes were observed in normal control group.

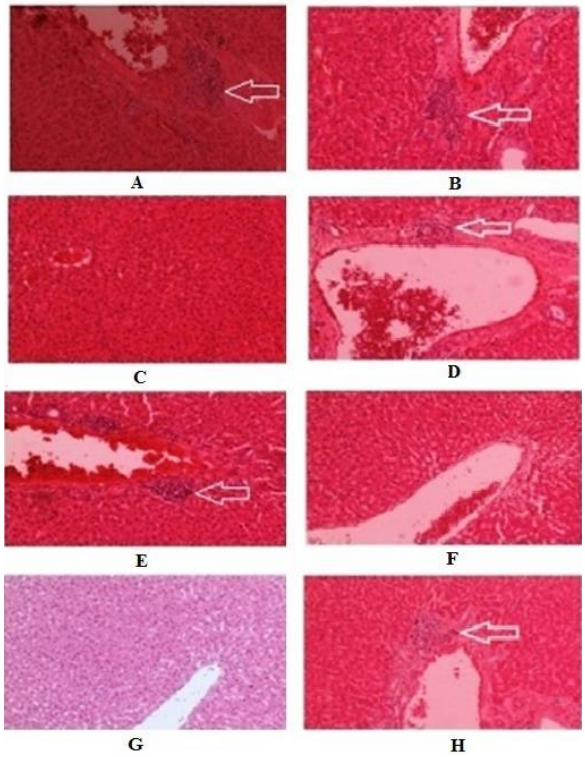

Plate 1: Effects of different doses of LPE on liver histology of female and male rats in repeated dose toxicity study for 28 days as assessed by H & E staining. (A): female normal control with arrow showing mild periportal chronic inflammation (1 out of 5 rats) (B): male normal control with arrow showing mild periportal chronic inflammation (only 1 out of 5 rats) (C) female treated group with 250 mg/kg of LPE (D) male treated group with 250 mg/kg of LPE with arrow showing mild periportal chronic inflammation (only 1 out of 5 rats) (E) female treated group with 500 mg/kg of LPE with arrow showing mild periportal chronic inflammation (only 1 out of 5 rats) (F) male treated group with 500 mg/kg of LPE (G) female treated group with 1000 mg/kg of LPE (H) male treated group with 1000 mg/kg of LPE with arrow showing mild periportal chronic inflammation (only 1 out of 5 rats) (Magnification × 10).

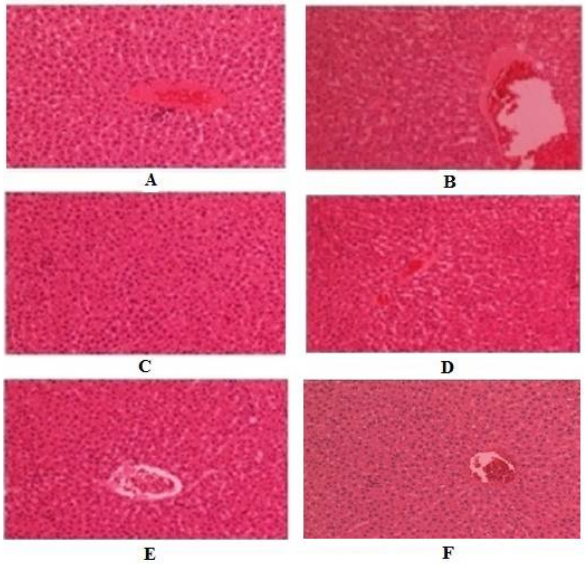

Plate 2: Effects of different doses of LLP on liver histology of female and male rats in repeated dose toxicity study for 28 days as assessed by H & E staining. (A) female treated group with 250 mg/kg of LLP (B) male treated group with 250 mg/kg of LLP (C) female treated group with 500 mg/kg of LLP (D) male treated group with 500 mg/kg of LLP (E) female treated group with 1000 mg/kg of LLP (F) male treated group with 1000 mg/kg of LLP (Magnification × 10)

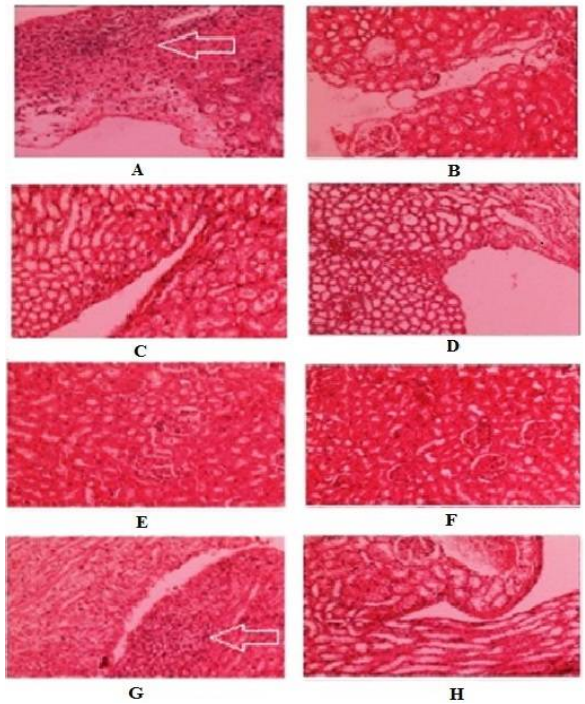

Plate 3: Effects of different doses of LPE on kidney histology of female and male rats in repeated dose toxicity study for 28 days as assessed by H & E staining (A): female normal control showing normal renal tubules with chronic pyelitis (only 1 out of 5 rats) (B): male normal control group (C): female treated group with 250 mg/kg of LPE (D): male treated group with 250 mg/kg of LPE (E): female treated group with 500 mg/kg of LPE (F): male treated group with 500 mg/kg of LPE (G): female treated group with 1000

mg/kg of LPE showing normal tubules with chronic pyelitis (only 1 out of 5 rats) (H): male treated group with 1000 mg/kg of LPE
(Magnification × 10)

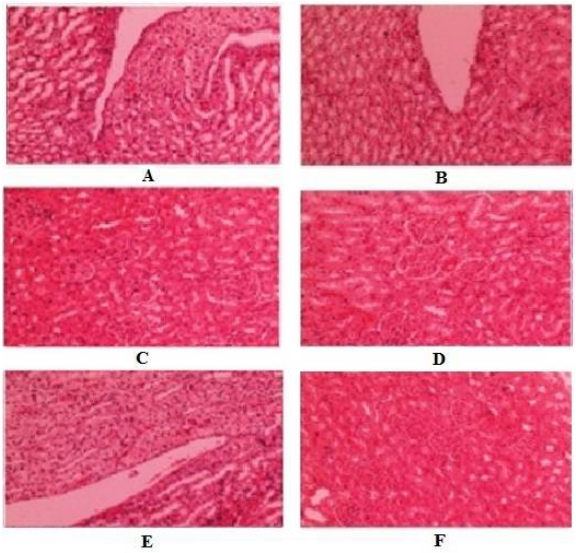

Plate 4: Effects of different doses of LLP on kidney histology of female and male rats in repeated dose toxicity study for 28 days as assessed by H & E staining (A): female treated group with 250 mg/kg of LLP (B): male treated group with 250 mg/kg of LLP (C): female treated group with 500 mg/kg of LLP (D): male treated group with 500 mg/kg of LLP (E): female treated group with 1000 mg/kg of LLP (F): male treated group with 1000 mg/kg of LLP. All groups in this plate showing normal glomeruli and tubules (Magnification × 10)

DISCUSSION

Herbal medicines are gaining popularity in developing countries because of their less side effects. Such remedies are often believed to be harmless, since these treatments are “natural” and commonly used for self-medication without supervision. Although medicinal plants may cause several biological activities in humans, a little is known regarding the potential toxicity in many of these bioactive substances.

The results of acute toxicity study indicated that LPE and LLP didn't cause visible signs of toxicity or mortality. Generally, a reduction in body weight and internal organ weights is a simple and sensitive index of toxicity after exposure to potentially toxic substances [17]. In the present study, LPE and LLP (2000 mg/kg, per oral) did not significantly affect body or organ weight as compared to the normal control groups, which suggests that the extract did not disturb rat growth; this is with the agreement to the toxicity classification reported by (Loomis and Hayes 1996) [18].

However a 28-days sub-chronic oral toxicity testing using repeated doses is widely considered satisfactory to assess any possible health hazard, and is important even for herbal supplements. The present results revealed that all parameters in term of organ-to-body weight ratios, haematological biochemical values were found normal between LPE and LLP treated groups compared to normal control group [16]. Histological examination of the vital organs including the liver and kidney from the treated and normal rats showed no apparent histological changes, chronic pyelitis (inflammation of renal pelvis and calyces), was only focally seen at pelvis of the kidney. It is often caused by ascending bacterial infection. The renal parenchyma was normal in this study. Overall the histological examination indicated evidence of safety at the tested doses when administered orally. The safety of treatment was also confirmed by the absence of behavioural changes and absence of difference in feed consumption between the treated and normal control groups. Hence, the results clearly showed that oral administration of LPE and LLP are safe at the evaluated doses (250, 500 and 1000 mg/Kg) in Sprague Dawley rats.

CONCLUSION

The acute and repeated dose oral toxicity assay of LPE and LLP could be very useful in its future clinical studies. LPE and LLP seemed to be non-toxic as was seen after its acute and repeated dose oral administrations. The oral lethal dose is in excess of 1000 mg/kg. The no-adverse-effect-level from the present study was determined to be 1000 mg/kg per day for rats under the condition tested.

DECLARATIONS

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Conflict of interest

No conflict of interest associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Guarantors of

integrity of entire study, Zhari Ismail, Amirin Sadikun, Mohammed Ali Ahmed Saeed and Abdul Hakeem Memon; study concepts/study design or data acquisition or data analysis/interpretation, all authors; manuscript drafting or manuscript revision for important intellectual content, all authors; manuscript final version approval, all authors; literature research, Mohammed Ali Ahmed Saeed and Sultan Ayesh Mohammed Saghir; experimental studies, Mohammed Ali Ahmed Saeed, Abdul Hakeem Memon, Mohd Shahrul Ridzuan Hamil and Sultan Ayesh Mohammed Saghir; histology assay, Gurjeet Kaur; statistical analysis, Mohammed Ali Ahmed Saeed and Hooi Kheng Beh; and manuscript editing, Zhari Ismail, Amirin Sadikun and Abdul Hakeem Memon.

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