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Acute and subacute oral toxicity of *Litsea elliptica* Blume essential oil in rats*

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Abstract: *Litsea elliptica* Blume has been traditionally used to treat headache, fever, and stomach ulcer, and has also been used as an insect repellent. The acute and subacute toxicities of *L. elliptica* essential oil were evaluated orally by gavage in female Sprague-Dawley rats. For the acute toxicity study, *L. elliptica* essential oil was administered in doses from 500 to 4 000 mg/kg (single dose), and in the subacute toxicity test, the following doses were used: 125, 250, and 500 mg/kg, for 28 consecutive days. In the acute toxicity study, *L. elliptica* essential oil caused dose-dependent adverse behaviours and mortality. The median lethal dose value was 3 488.86 mg/kg and the acute non-observed-adversed-effect level value was found to be 500 mg/kg. The subacute toxicity study of *L. elliptica* essential oil did not reveal alterations in body weight, and food and water consumptions. The haematological and biochemical analyses did not show significant differences between control and treated groups in most of the parameters examined, except for the hemoglobin, mean cell hemoglobin concentration, mean cell volume, mean cell hemoglobin, serum albumin, and serum sodium. However, these differences were still within the normal range. No abnormalities or histopathological changes were observed in the liver, pancreatic islet of Langerhans, and renal glomerulous and tubular cells of all treated groups. In conclusion, *L. elliptica* essential oil can be classified in the U group, which is defined as a group unlikely to present an acute hazard according to World Health Organization (WHO) classification.

Key words: *Litsea elliptica*, Acute toxicity, Subacute toxicity, Median lethal dose (LD_{50}), Natural insecticide, Non-observed-adversed-effect level (NOAEL)
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1 Introduction

The widespread application of insecticides in household products and public health programs creates a major concern due to their effects on human and environmental health. Certain synthetic pesticides provide a broad range of toxic effects (Isman and Machial, 2006). Insecticides have caused significant negative effects on the non-target organisms; therefore, a toxicity evaluation is important (Celik and Suzek, 2008). Proliferations of research were carried out to produce insecticides from natural products as alternatives to synthetic insecticides in order to reduce their negative health impacts. The use of natural products as insecticides is environmentally desirable and economically profitable (Mittal and Subbarao, 2003). As such, the use of plants for insecticidal purpose is becoming more popular due to virtually non-existent adverse effects.

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Litsea elliptica (Family: Lauraceae) Blume is a well-known tropical tree used in herbal or traditional medicine in the South East Asia region (Jiwajinda *et al*., 2002). Crushed leaves of *L. elliptica* are applied around the forehead for treatment of headache (Grosvenor *et al*., 1995), and also can be used as an herbal medicine to treat stomach ulcers and fever (Jiwajinda *et al*., 2002). The extract of its leaves has also been proven to have chemopreventative activities, reducing the occurrence of stomach cancer in Thailand (Bhamarapravati *et al*., 2003) and Nakahara *et al*. (2002) also reported that its leaves have a significant antimutagenic activity.

L. elliptica was reported to have potential insecticidal activities (Rohani *et al*., 1997). An aqueous cream with 1/3 active compound from *L. elliptica* has a protective role up to 96.6% against mosquito bites (Ibrahim and Zaridah, 1998). The methanol extract of *L. elliptica* leaves has been shown to control the vector of dengue fever (Hidayatulfathi *et al*., 2003). Significant insecticidal activities have been found against larvae of *Aedes aegypti* and *Aedes albopictus* (Hidayatulfathi *et al*., 2003) and adults of *A. aegypti* (Hidayatulfathi *et al*., 2004).

Based on previous studies, the *L. elliptica* essential oil could be useful as an insecticide, thereby reducing the use of synthetic pesticides. Prior to effective formulation of *L. elliptica* essential oil as mosquito control, evaluation of its toxic effects is required. The toxicity evaluation is based on the duration of exposure and, according to Environmental Protection Agency (EPA), 14 d and 28 d are recommended for acute and subacute toxicity studies, respectively (EPA, 2000; 2002). According to EPA (2002), the main purpose of the acute toxicity study is to determine the median lethal dose (50% death) LD_{50} and non-observed-adversed-effect level (NOAEL) value by evaluating the mortality rate and signs of toxicity. According to EPA (2000), the suggested dose for subacute toxicity study is based on the NOAEL. Therefore, the chosen dose in subacute toxicity study should be NOAEL, 1/2NOAEL, and 1/4NOAEL. The present work evaluated the acute and subacute oral toxicities of *L. elliptica* essential oil in female Sprague-Dawley (SD) rats and the results obtained from this study will provide the safety information of this extract before its commercialization as a natural product pesticide.

2 Materials and methods

2.1 Plant materials and extraction

The leaves of *L. elliptica* were obtained from Bangi Forest Reserve, Selangor, Malaysia. The voucher specimen (FRI41999) was deposited at the Herbarium of the Forest Research Institute Malaysia, Kepong. Then, the leaves were dried at room temperature (25–28 °C), and ground to produce fine particle. The extraction of essential oil was done using Clevenger apparatus (Duran and Favorit, Germany) by a water steam distillation method. The extraction was done for at least 8 h and the temperature was adjusted to maintain the boiling conditions. Sodium sulphate dehydrates (Na_2SO_4) was added to remove the remaining water in the essential oil to obtain 100% purity with the density of 860 mg/ml.

2.2 Animals

Female SD rats weighing 180–220 g were obtained from the Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia. The animals were kept in plastic cages in environmental conditions (22–24 °C, 12 h:12 h dark/light cycle), fed a mouse pellet diet (mouse pellet 702 P, Gold Coin Feedmills (M) Sdn. Bhd., Pelabuhaan Utara, Malaysia), and allowed to drink water ad libitum without distraction. All the animal handling protocols were approved by the Animal Ethics Committee of Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia.

2.3 Acute toxicity

The assessment of acute toxicity was performed according to the EPA (2002) test guidelines. Healthy rats were fasted overnight, but allowed access to water ad libitum, and were randomly divided into seven groups (n=10). The first group (control group) received distilled water only. The other six groups were orally treated with a single dose of *L. elliptica* essential oil at 500, 1000, 2000, 2500, 3000, or 4000 mg/kg, respectively. The doses in this acute toxicity study were based on the results from a range-finding study, where the observations on mortality and toxicity signs were made. All the treatments were administered by force-feeding. Animals were observed for signs of toxicity, body weight, food consumption, and water intake as well as mortality for

a period of 14 d after treatment. The toxicity signs and symptoms were observed in individual cages during the first 3 h after the essential oil administration, and subsequently monitored daily throughout the duration of the study (Konan *et al*., 2007). According to the mortality of rats observed within 14 d, the LD_{50} value was calculated. At Day 15, all surviving animals were sacrificed, internal organs were excised, and organ weights were measured.

2.4 Subacute toxicity

The subacute toxicity study was performed according to the EPA (2000) test guidelines. The animals were divided randomly into four groups with 10 rats per group. *L. elliptica* essential oil was administered orally by gavage with doses 125, 250, or 500 mg/kg for 28 consecutive days, while the control rats received distilled water only. The chosen doses were based on the dose of NOAEL that was obtained from the acute toxicity study, which dose being 500 mg/kg. Toxicity signs and mortality were monitored daily, whereas body weight changes, and food and water consumptions were monitored weekly. At the end of the study, animals were fasted overnight, anesthetized with diethyl ether, and cardiac puncture was done to obtain blood samples. The heparinised blood samples were used for determining haematological parameters. Meanwhile, the non-heparinized tube was used for blood chemistry analysis and a fluoride tube was used for blood glucose determination. Following dissection, the liver, the pancreas, the heart, the kidneys, and the spleen were removed and weighed immediately.

2.5 Hematological and biochemical analyses

An automatic hematology analyzer (ABC vet. Isolab Sdn. Bhd., France) was used to analyze haematological parameters, including red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin (Hb), haematocrit (Hct), mean cell hemoglobin (MCH), mean cell volume (MCV), mean cell hemoglobin concentration (MCHC), and platelet (Plt) count. For biochemical analysis, blood was centrifuged at 1500×*g* for 10 min to obtain serum, and stored at −40 °C. Blood urea nitrogen (BUN), creatinine, potassium, sodium, chloride, calcium, phosphorus, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, and albumin were assayed using automated chemical analysis: Hitachi 902 (Roche Diagnostic Sdn. Bhd., Japan). Enzymatic glucose-oxidase kits (Catalogue No. TR 15104, Trace Scientific, Melbourne, Australia) were used to analyze plasma glucose levels.

2.6 Morphological analysis

The macroscopic external features of the selected organs were performed to detect any abnormal signs. All the organs were perfused with 0.9% (9 g/L) saline solution, and fixed in 10% buffered formalin solution at room temperature. The organs were processed according to Ochei and Kolhatkar (2000), enclosed with paraffin and subjected to haematoxylin-eosin (H&E) staining for microscopic histological examination under 40× magnification by histopathological experts.

2.7 Statistical analysis

All studies mentioned above were done in triplicate except for the LD_{50} study. The LD_{50} was calculated using probit analysis (SPSS 11.5). All values were expressed as mean±standard error of the mean (SEM) and were analyzed by one-way analysis of variance (ANOVA) followed by Scheffe post hoc test, and statistically significant findings were considered those in which $P<0.05$.

3 Results

3.1 Acute toxicity study

The acute toxicity of *L. elliptica* essential oil given as single doses orally is presented in Table 1.

Table 1 Effects of different oral single doses of *L. elliptica* essential oil in rats for acute toxicity

L. elliptica essential oil (mg/kg)	D/T	Symptom
500	0/10	None
1000	0/10	Hypoactivity
2000	0/10	Hypoactivity
2500	1/10	Hypoactivity
3000	5/10	Hypoactivity, lacrimation, piloerection
4000	6/10	Hypoactivity, lacrimation, piloerection

All treated rats were carefully examined for up to 14 d after the dose for toxicity signs and lethality. D/T: number of dead rats/number of treated rats. None: no toxicity symptoms observed during the observation period

The NOAEL of *L. elliptica* essential oil was 500 mg/kg. The rats showed the signs of toxicity such as hypoactivity, lacrimation, and piloerection, in a dose-dependent manner, and these signs remained until death. Mortality was observed in the groups receiving 2500, 3000, and 4000 mg/kg with one, five, and six deaths, respectively (Table 1). From the acute toxicity data, the calculated LD_{50} obtained was 3488.86 mg/kg.

The body weight of surviving animals is shown in Table 2. On Day 7, there was a significant body weight decrease in group 3000 mg/kg compared to the control ($P<0.05$). On Day 14, there were significant decreases in body weights in groups 2000, 2500, and 3000 mg/kg compared to the control ($P<0.05$), and in group 3000 mg/kg compared to the 500 and 1000 mg/kg groups ($P<0.05$). Group 4000 mg/kg was not included, since there were too few surviving animals in this group. As for selected organs weights, there were significant changes only in the renal and the heart for absolute and relative weights in all *L. elliptica* treated groups, except the 500 mg/kg group (Table 2).

3.2 Subacute toxicity study

Neither toxicity signs nor death was observed throughout the study following administration of *L. elliptica* essential oil for 28 consecutive days. *L. elliptica* essential oil did not cause any significant changes in body and organ weights, as shown in Table 3. The food and water consumptions also revealed no differences among treated and untreated groups (statistical analysis was not done since two animals were kept per cage).

3.3 Hematological and biochemical parameters

The hematological and biochemical profiles of the control and treated groups are shown in Tables 4 and 5, respectively. The RBCs, WBCs, Hct, and Plt values did not show any differences among treated and control groups. *L. elliptica* essential oil significantly increased the Hb concentration in all doses, though decreased the MCH and MCHC levels in 125 and 250 mg/kg of *L. elliptica* essential oil groups ($P<0.05$). *L. elliptica* essential oil at a dose of 250 mg/kg significantly decreased the level of MCV, in contrast with control group ($P<0.05$). However, all the haematological values in all treated groups were still within the normal range. For the biochemical profile, all the data showed no statistical differences among treated and control groups.

3.4 Morphological analysis

Gross pathological examination revealed no detectable abnormalities in the selected organs. In addition, histopathological examination found no detectable alteration neither in the control nor the treated groups (Fig. 1).

Table 2 Body and organ weights of rats in acute toxicity study in control and groups treated with different doses of *L. elliptica* essential oil

Parameter	Control	Treatment of *Litsea elliptica* essential oil				
		500 mg/kg	1000 mg/kg	2000 mg/kg	2500 mg/kg	3000 mg/kg
Body weight loss (%)						
Day 7	5.98±0.08	5.03±0.07	4.80±0.02	3.63±0.05	2.44±0.04	0.98±0.02
Day 14	10.29±0.13	7.91±0.09	7.43±0.05	5.32±0.06[a]	3.90±0.04[a]	2.46±0.04[c]
Absolute weight (g)						
Liver	7.81±1.04	7.84±1.29	8.67±0.90	8.41±1.29	8.35±0.77	8.52±0.78
Renal	1.34±0.06	1.34±0.07	1.03±0.05[b]	1.01±0.08[b]	1.28±0.10[d]	1.51±0.11[bde]
Spleen	0.44±0.10	0.48±0.06	0.45±0.08	0.48±0.16	0.50±0.11	0.49±0.18
Heart	0.32±0.03	0.33±0.03	0.35±0.03	0.36±0.03	0.39±0.05[b]	0.39±0.07[b]
Relative weight (%)						
Liver	3.40±0.48	3.49±0.58	3.87±0.38	3.86±0.56	3.92±0.40	4.09±0.26
Renal	0.58±0.04	0.60±0.04	0.46±0.02[b]	0.46±0.03[b]	0.60±0.05[d]	0.73±0.06[bde]
Spleen	0.19±0.04	0.27±0.03	0.20±0.03	0.22±0.07	0.23±0.05	0.24±0.10
Heart	0.14±0.01	0.15±0.02	0.15±0.01	0.16±0.02	0.18±0.02[a]	0.19±0.03[a]

Data are expressed as mean±SEM, $n=10$ for each group. [a] Significant at $P<0.05$ as compared with control only; [b] Significant at $P<0.05$ as compared with the control and 500 mg/kg; [c] Significant at $P<0.05$ as compared with the control, 500 and 1000 mg/kg; [d] Significant at $P<0.05$ as compared with 1000 and 2000 mg/kg; [e] Significant at $P<0.05$ as compared with 2500 mg/kg

Table 3 Body and organ weights of rats in subacute toxicity study in control and groups treated with different doses of *L. elliptica* essential oil

Parameter	Control	Treatment of *Litsea elliptica* essential oil		
		125 mg/kg	250 mg/kg	500 mg/kg
Body weight (g)				
Day 0	200.00±2.89	197.00±2.13	198.00±1.87	197.00±2.00
Day 7	209.00±4.33	208.00±2.50	207.00±2.90	201.50±3.42
Day 14	212.50±4.30	212.00±2.71	211.50±3.66	204.50±2.93
Day 21	214.50±4.68	213.00±3.67	213.00±3.51	210.50±4.68
Day 28	217.50±4.90	217.00±3.35	214.00±4.58	212.50±3.75
Absolute weight (g)				
Liver	5.53±0.86	5.98±0.93	5.69±0.80	6.09±0.38
Renal	1.29±0.15	1.37±0.13	1.34±0.21	1.35±0.12
Spleen	0.38±0.07	0.44±0.07	0.45±0.07	0.45±0.10
Heart	0.75±0.09	0.72±0.07	0.75±0.08	0.71±0.11
Pancreas	1.16±0.32	1.01±0.23	1.02±0.17	1.02±0.34
Relative weight (%)				
Liver	2.54±0.34	2.76±0.38	2.65±0.29	2.87±0.18
Renal	0.60±0.06	0.63±0.06	0.63±0.07	0.64±0.04
Spleen	0.18±0.03	0.21±0.04	0.21±0.03	0.21±0.03
Heart	0.35±0.05	0.34±0.02	0.35±0.04	0.33±0.04
Pancreas	0.54±0.13	0.47±0.10	0.48±0.07	0.48±0.15

Data are expressed as mean±SEM, n=10 for each group. No statistical difference was found between the control and *L. elliptica* essential oil treated groups (P>0.05)

Table 4 Hematological values of rats in subacute toxicity study in control and groups treated with different doses of *L. elliptica* essential oil (Taib *et al*., 2009)

Parameter	Control	Treatment of *L. elliptica* essential oil		
		125 mg/kg	250 mg/kg	500 mg/kg
RBC ($\times10^{12}$ L^{-1})	7.82±0.15	7.92±0.11	8.27±0.18	8.16±0.16
WBC ($\times10^{9}$ L^{-1})	10.23±0.74	12.09±1.20	11.53±0.72	12.64±1.39
Hb (g/dl)	13.45±0.32	14.62±0.22*	14.71±0.21*	14.82±0.27*
HCT (l/l)	0.40±0.01	0.39±0.01	0.40±0.01	0.41±0.01
MCV (fl)	51.60±0.65	49.80±0.39	48.80±0.63*	50.30±0.47
MCH (pg)	17.18±0.18	18.48±0.13*	17.80±0.25	18.19±0.16*
MCHC (g/dl)	33.26±0.41	37.14±0.15*	36.46±0.13*	36.14±0.27*
PLT ($\times10^{9}$ L^{-1})	655.90±63.72	432.50±141.44	491.90±119.00	424.40±70.82

Data are expressed as mean±SEM, n=10 for each group. * Significant difference compared with the control group (P<0.05). RBC: red blood cell; WBC: white blood cell; Hb: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelets

Table 5 Blood chemistry values of rats in subacute toxicity study in control and groups treated with different doses of *L. elliptica* essential oil

Parameter	Control	Treatment of *L. elliptica* essential oil		
		125 mg/kg	250 mg/kg	500 mg/kg
Urea (mmol/L)	7.09±1.17	6.48±0.62	7.01±1.68	7.68±2.01
Creatinin (mmol/L)	0.07±0.01	0.07±0.00	0.07±0.01	0.07±0.01
Sodium (mmol/L)	142.79±1.74	141.66±2.54	139.84±2.34*	139.07±1.49*
Potassium (mmol/L)	5.74±0.66	5.53±0.33	5.51±0.70	5.38±0.65
Calcium (mmol/L)	3.03±0.12	2.91±0.09	2.94±0.17	2.87±0.09
Cloride (mmol/L)	100.76±1.68	99.58±1.84	99.92±1.69	99.69±4.76
Phosphate (mmol/L)	1.72±0.20	1.97±0.21	1.94±0.29	1.98±0.29
ALT (U/L)	38.98±10.98	38.34±8.38	37.33±7.86	39.79±11.77
AST (U/L)	102.08±18.83	114.29±20.63	114.63±24.03	115.41±29.57
Albumin (g/L)	44.49±3.97	40.87±2.75	40.18±3.39	39.66±4.21*
Total protein (g/L)	75.16±6.91	72.33±3.07	72.85±6.06	74.44±5.98
Glucose (mmol/L)	6.90±0.25	6.82±0.26	7.55±0.22	7.84±0.41

Data are expressed as mean±SEM, n=10 for each group. * Significant difference compared with the control group (P<0.05)

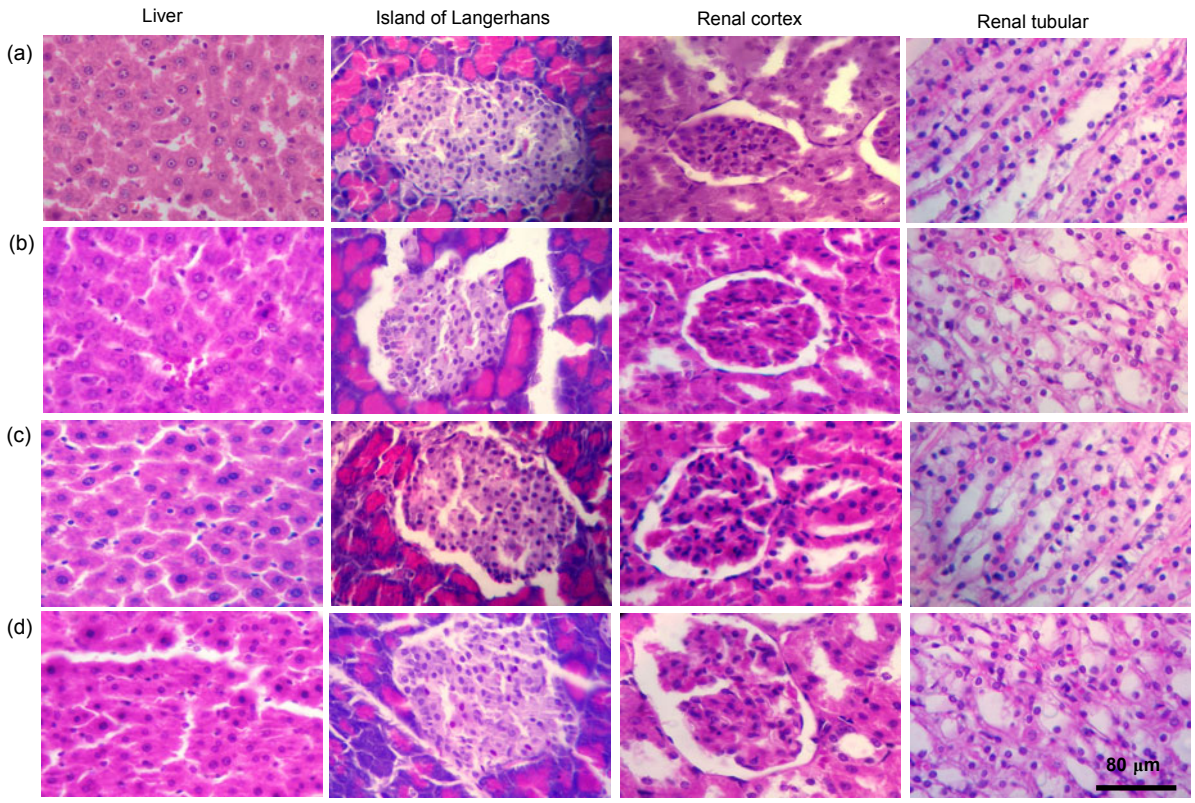

Fig. 1 Effects of *L. elliptica* essential oil on the liver, island of Langerhans, renal cortex, and renal tubular
H&E staining of control (a) and treated groups of 125 (b), 250 (c), and 500 mg/kg (d). No abnormality was found in all treated or control groups

4 Discussion

In this toxicity study, using probit analysis, LD_{50} of *L. elliptica* essential oil was estimated to be 3488.86 mg/kg, thus the essential oil can be classified in the U group as unlikely to present an acute hazard in normal use by World Health Organization (WHO, 2005) recommending classification guidelines of pesticides. Administration of a single oral dose of the *L. elliptica* essential oil had produced some toxicity symptoms, which probably were the result of disturbances on the function of autonomic nervous system (ANS) and central nerves system (CNS). Lacrimation is one of the toxic symptoms shown to be a result of the muscarinic effect of cholinergic poisoning (Liang, 1996) and exposure to organophosphate insecticides (Costa, 2006). According to Gotoh *et al*., (2006), hypoactivity was suggested to be due to a decrease in locomotor activity controlled by the CNS. Acute oral toxicity study showed that *L. elliptica* essential oil possessed a similarity in some toxicity symptoms produced by organophosphate insecticides such as malathion, diazinon, and chlorpyrifos, which also have some neurotoxic symptoms (Kwong, 2002).

The acute oral toxicity study of *L. elliptica* essential oil also caused a significant decrease in body weight at higher doses, and might be associated with the toxic symptoms that occurred, which lead the rats to become anorectic. According to Lansdown (1993), changes in body and organ weights are probably due to the toxic effects of the xenobiotic. Furthermore, the changes in organ-to-weight ratio or the relative weight could also be due to organ injury as a result of exposure to the toxic material (Wang *et al*., 2007). In the present study, an increase in absolute and relative weights of the kidneys and the heart is most probably due to oedema (Costa-Silva *et al*., 2008). However, the acute oral administration of *L. elliptica* essential oil did not induce any significant toxicity symptoms at the dose concentration of 500 mg/kg and this dose

has been suggested to be the NOAEL for this study.

Based on the acute toxicity study of the *L. elliptica* essential oil, the doses to be evaluated in subacute toxicity study for 28 d repeated dose given were found to be 125 (1/4NOAEL), 250 (1/2NOAEL), and 500 mg/kg (NOAEL). The 28-d toxicity test has been accepted in practice for a subacute oral toxicity study. Subacute oral toxicity study has been applied in safety assessment studies to provide safety information prior to the commercialization of a certain product (Arts *et al*., 2004; Bautista *et al*., 2004). In the subacute toxicity study, no toxicity signs were detected throughout the experiments; therefore, the present results suggest that *L. elliptica* essential oil administered orally is non-toxic to rats.

The haematological system is sensitive to toxic chemicals and can be used as an important index to monitor the physiological changes in human and animal (Li *et al*., 2010). Therefore, toxic chemicals risk evaluation involved the analysis of blood parameters. The synthetic insecticides such as α-cypermethrin, carbendazim, and chlorpyrifos at various doses given are reported to cause anemia in rats (WHO, 1995). However, the hematological parameters obtained from this study showed that the *L. elliptica* essential oil did not influence hematological parameter values (Sanderson and Philips, 1981); therefore, there is potential for future utilization of *L. elliptica* essential oil.

Generally, all biochemical parameters observed in the subacute toxicity study did not show any significant changes compared with the control group and all the values were still within the normal ranges (Petterino and Argentino-Storino, 2006). *L. elliptica* essential oil did not cause alterations in the values of transaminase enzymes, which are good biomarkers predicting possible toxicity of the liver (EI Hilaly *et al*., 2004). Similarly, no alteration was observed in the glucose or creatinine level, which reflects normal pancreas or renal function, respectively. All the biochemical data were consistent with histological evaluation of the liver, the pancreas, and the kidneys which did not reveal any significant changes due to administration of *L. elliptica* essential oil for a 28-d duration.

In conclusion, *L. elliptica* essential oil could be categorized as NOAEL crude drug, as it acts harmlessly under the current normal usage, and this phenomenon is considered to be of no toxicological concern. However, this is the first study to investigate the toxicity of *L. elliptica* essential oil in rats, and a subchronic toxicity test should also be conducted to establish the adverse effects of a repeated response to *L. elliptica* essential oil.

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