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Sub-Acute Oral Toxicity Study of Ethanol Extract of Oroxylum Indicum Leaf in C57BL/6 Mice

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Oroxylum indicumalso known as 'pucukbeko' in Malaysia is often consumed as raw salad (ulam) due to the belief that the plant has numerous therapeutics activities that could improve health. Despite its medicinal potential,however, there has been very limited data on the plant's safety and toxicity profile particularly for long term consumption. More depth insight and evidence-based studies are needed to verify its safety as a potential herb. Therefore, this study aims to investigate sub acute oral toxicity of ethanol extract of O. indicum in C57BL/6 male mice. Twenty-five mice (n=5) were orally administered at single dose of normal saline (control), vehicle (5% DMSO), extracts (100, 200 and 500 mg/kg bw), respectively in accordance with OECD Guideline 420 for 28 days. Liver, kidneys, heart, lungs, testes, spleen,and blood samples were collected to determine the effects of the extract on the relative organ weight, tissue changes, and blood profile alterations in the end of the study. The sub-acute toxicity results demonstrated no lethal effects and abnormal behavioural changes in mice treated with an expansion dose up to a maximum of 500 mg/kg. No significant ($p>0.05$) changes in body weights, relative organ weight and haematological evaluation. Nevertheless, there were significant differences ($p<0.05$) in the urea, mean corpuscular volume (MCV), and alanine transaminase (ALT) values but the levels were still within the acceptable range. Histopathological analysis of the liver and kidney tissues also revealed no striking lesions. This study displays that mice treated with an increasing dose ofO. indicum leaf ethanolic extract up to a maximum 500mg/kg bw did not cause any toxicological effects and considered safe to be consumed and used for therapeutic purposes

Keywords: C57BL/6 mice; Ethanol Extract; Haemato-Biochemistry; Oroxylum Indicum; Subacute oral toxicity study.

The uses of herbal medicines are rapidly increasing across the world, and people are opting for these products as an alternative treatment of many health conditions and diseases[1,2]. Several factors that contribute to the demand of public in using herbal remedies are due to various claims about the efficacy or effectiveness of the use of medicinal plant[3] and because high incidence of side effects derived from commercial drug[4]. The improvements in the quality, efficacy, and safety of

herbal medicines with the development of science and technology towards the use of herbal remedies gives another option to people on the use of this product for many therapeutic purposes[5]. To the date, up to 70,000 plant species have been screened for biological activities[6] and about 70% end up as a commercial drug[7]. With the increasing population and health conscious minded among people, the demands and pharmaceutical investment on herbs as a healthcare option is believed to have increased tremendously.

Oroxylum indicum is widely distributed throughout India subcontinent and South East Asia countries, including Malaysia[8]. In Malaysia, it is commonly known as "pucuk beko, bonglai or bolai kayu" and eaten raw as a salad or cook with coconut milk. The leaves from the plant have previously been used among the older generation as herbal remedies and passed down to the new generation to be applied. Various scientific studies reported that *O. indicum* possesses anticancer[9], antioxidant and hepatoprotective[10], immunomodulaory properties[11] and gastro-protective properties[12]. Despite the various claimed benefits of *O. indicum* leaf extract, the toxicity profiling of this plant is still lacking and have not been scientifically proven. Acute toxicity of ethanolic extract of *Oroxylum Indicum* are proven to be safe up to 500 mg/kg bw. This study was a continuation of a previous acute toxicity study of *O. indicum* leaf extract where the results suggests that the herbs considered safe in acute toxicity level[13]. Hence, this study aims to investigate the subacute oral toxicity of *O. indicum* leaf ethanolic extract in mice and to determine the prolonged toxic effect of the plant extract. Subacute toxicity study may support initial acute toxicity study of this plant where the duration of treatment for 28 days provides facts about the plausible adverse effects likely to arise for longer exposure.

MATERIALS AND METHODS

Preparation of ethanolic extract

The plant leaves were purchased from a local market in Pengkalan Chepa, Kelantan in January 2021. The leaflets have 2-4 pairs, 7.0 x 3.5-8.0 cm, ovate-elliptic, acuminate, base obliquely rounded-obtuse. The leaves of *O. indicum* were then washed with distilled water and oven-dried at 40°C for 4 days. The dried leaves of *O. indicum* were ground into fine particles form and 150g of the pulverized leaves were soaked in 1.5 L of ethanol for 72 hours at room temperature. Then, the extract was filtered using Whatman filter paper (No. 1001-240, Grade 1) and was dried and evaporated by using a rotary evaporator controlled in a water bath at 60°C. The final yield products were stored under -20°C. The extract was tested for its sterility by inoculating 0.1g of the extract in 1ml of sterile nutrient broth. The extract was considered sterile with the absence of turbidity after incubated at 37ÚC for 24 hours. The plant part was identified by the botanist from Herbarium, Universiti Kebangsaan Malaysia, Selangor, Malaysia. A voucher specimen (ID025/2020) was deposited at the herbarium for reference.

Experimental animals and husbandry

A total of 25 males C57BL/6 mice aged between 6 to 8 weeks with a bodyweight of 25 to 30 g were obtained from the Animal Research and Service Centre, Universiti Sains Malaysia, Health Campus, Kubang Kerian, Kelantan. The mice were placed in polycarbonate cages with optimum environmental conditions of temperature (24 ± 2°C), relative humidity (45 ± 5%), and lightning duration (12 hours daylight and 12 hours dark hours) were provided. Filtered tap water and commercial mice pellets were provided *ad libitum*. Wood shaving was provided as the bedding which was cleaned and changed daily. All mice underwent an acclimatization period for 5 days prior to the toxicity experiment. The research was conducted according to the guidelines and approval by the Institutional Animal Care and Use Committee (IACUC), Universiti Malaysia Kelantan (IACUC Ref No: UMK/FPV/ACUC/FYP/16/2020).

Subacute oral toxicity study

The study was designed according to Organisation for Economic Co-operation and Development Guideline Test No. 420 (OECD, 2001)[14]. The mice were randomly assigned into 5 experimental groups consisted of 5 mice per group. All the mice were fasted overnight from the feed but had free access to water before experimentation. The experimental groups were orally gavage with a single dose of 100 mg/kg bw, 200 mg/kg bw, and 500 mg/kg bw of extracts accordingly, whereas the control and vehicle groups were treated with normal saline and 5% dimethyl sulfoxide (DMSO) respectively. No

food was provided for the first 2 hours after *O. indicum* leaf extract administration. The extract was administered once daily, in the morning for 28 days. The conditions of all experimental mice were closely observed for the first 30 minutes to 6 hours, intermittently at 7 to 24 hours, and twice daily for 28 days for any abnormalities or signs of toxicity and mortality. The conditions which were accessed include changes in body weight, food and water intake, coat colour, mucous membrane, respiratory rate, neurological signs, and behavioural changes.

Necropsy, gross organ examination and sample collection

All surviving mice were sacrificed with inhalation of carbon dioxide and decapitation using a guillotine. The internal organs mainly liver, kidneys, lungs, heart, brain, and spleen were isolated, cleaned, and weighed. The organs were examined for the abnormalities and changes in the sizes, colour, consistency, and texture. After that, all organs were preserved in 10% formalin for further histopathology evaluation. The relative organ weights were determined as follows:

Relative organ weight = weight of organ/ bodyweight of the mice on the sacrifice day x 100% [13].

Haematology and serum biochemistry analyses

Blood samples were also collected in heparin tubes using 25G needles via cardiac puncture. The serum samples were centrifuged at 5000 rpm for 30 seconds prior to analysis using microcentrifuge. The complete blood count tested were total white blood cell count and differentials count, total red blood cell count, haemoglobin concentration, packed cell volume, mean corpuscular volume, mean corpuscular haemoglobin concentration, and platelet count. The serum samples were submitted for biochemical analysis for creatinine and blood urea nitrogen (BUN), alanine aminotransferase (ALT), and total protein, including albumin and globulin. All haematological procedures were conducted using IDEXX VetTest Chemistry analyser at Faculty of Veterinary Medicine, Universiti Malaysia Kelantan.

Histopathological evaluation

After 48 hours of formalin preservation, a small block of tissue was placed on the cassette and processed by using an automated tissue processor. Then, the tissues were sectioned into a thickness of 5 ìM using a rotary microtome and dried overnight in an oven at 37°C. Haematoxylin and Eosin (H&E) were used to stain the sectioned tissues for the examination of abnormalities and lesions. All the tissue sections were scored according to the established scoring method by [15].

Statistical analysis

Data were analysed statistically using Statistical Package for Social Science (SPSS) software version 23. Test of analysis of variance (ANOVA) was conducted to compare the data variations between and within groups. Post hoc analysis using Duncan test was further carried out. The results were considered statistical significance when $p<0.05$.

RESULTS

Behaviour, physical observations, body weight changes, relative organs weight and mortality rate if mice upon treatment.

All the toxicity signs including pain, stress, abnormal behavioural, physical changes and mortality were absent in all groups of mice. Body weights of the mice taken at initial of experimentation (day 0), day 7, and day 14, day

Table 1. The body weight (mean ± SEM) in subacute oral toxicity study of *Oroxylum indicum* leaf ethanolic extract in mice

Day/Week	Control	Vehicle	Low dose (100 mg)	Medium dose (200 mg)	High dose (500 mg)
Day 0-7 (Week 1)	25.4 ± 0.20[a]	26.10 ± 0.10[a]	25.53 ± 0.32[a]	25.78 ± 0.37[a]	25.73 ± 0.33[a]
Day 14 (Week 2)	25.43 ± 0.07[a]	26.17 ± 0.33[a]	25.68 ± 0.28[a]	25.72 ± 0.29[a]	26.02 ± 0.35[a]
Day 21 (Week 3)	25.90 ± 0.20[a]	26.77 ± 0.32[a]	26.02 ± 0.22[a]	26.08 ± 0.36[a]	26.20 ± 0.27[a]
Day 28 (Week 4)	26.30 ± 0.06[a]	26.80 ± 0.41[a]	26.40 ± 0.24[a]	26.28 ± 0.42[a]	26.68 ± 0.25[a]

*Values in the same row with similar superscript letter were not significantly different ($p > 0.05$)

28 of post extract administration did not show any significant ($p>0.05$) changes compared to control groups (Table 1). There were no significant differences ($p>0.05$) observed in the organ's weights of liver, kidneys, heart, lungs, testes, and spleen (Table 2). Administration of the extract at different doses (100, 200, and 500 mg/kg bw) resulted in no death of the mice within 28 days period of the study and observation (Table 2). This finding indicates that the inoculation of *O. indicum* leaf ethanolic extract up to 500 mg/kg bw for 28 days is safe.

Haematological and serum biochemical analyses

There were no significant differences ($p>0.05$) in the hemogram parameters in all groups in both treatment and control groups (Table 3). For the serum biochemistry analysis of kidney, liver, and protein, there were no significant differences ($p>0.05$) for both treatment and control groups (Table 4).

Table 2. The relative organs weights (mean ± SEM) in subacute oral toxicity study of *Oroxylum indicum* leaf ethanolic extract in mice

Organs	Control	Vehicle	Low dose (100 mg)	Medium dose (200 mg)	High dose (500 mg)
Liver	1.23 ± 0.12[a]	1.36 ± 0.09[a]	1.43 ± 0.13[a]	1.50 ± 0.09[a]	1.40 ± 0.15[a]
Kidneys	0.57 ± 0.20[a]	0.20 ± 0.01[a]	0.38 ± 0.10[a]	0.38 ± 0.08[a]	0.40 ± 0.12[a]
Lungs	0.17 ± 0.03[a]	0.16 ± 0.06[a]	0.28 ± 0.02[a]	0.15 ± 0.05[a]	0.23 ± 0.13[a]
Heart	0.30 ± 0.10[a]	0.10 ± 0.01[a]	0.18 ± 0.05[a]	0.25 ± 0.09[a]	0.20 ± 0.07[a]
Spleen	0.26 ± 0.03[a]	0.25 ± 0.02[a]	0.30 ± 0.05[a]	0.24 ± 0.01[a]	0.27 ± 0.04[a]

*Values in the same row with similar superscript letter were not significantly different ($p > 0.05$).

Table 3. The haematological evaluation (mean ± SEM) in subacute oral toxicity study of *Oroxylum indicum* leaf ethanolic extract in mice

Parameters	Control	Vehicle	Low dose (100 mg)	Medium dose (200 mg)	High dose (500 mg)
RBC ($x10^{12}$/L)	6.56 ± 1.00[a]	6.13 ± 0.23[a]	4.69 ± 1.14[a]	5.31 ± 0.96[a]	5.04 ± 0.69[a]
Hb (g/L)	12.23 ± 1.37[a]	10.37 ± 0.12[a]	12.42 ± 2.37[a]	10.03 ± 1.79[a]	9.08 ± 1.06[a]
HCT(L/L)	23.60 ± 1.02[a]	27.75 ± 1.95[a]	24.43 ± 5.46[a]	9.98 ± 5.76[a]	6.70 ± 3.86[a]
MCV (Fl)	44.40 ± 0.20[a]	44.9 ± 0.30[a]	45.23 ± 0.74[a]	46.17 ± 0.22[a]	45.30 ± 0.66[a]
MCHC(g/L)	38.70 ± 3.0[a]	37.90 ± 3.20[a]	50.73 ± 9.60[a]	42.73 ± 2.67[a]	40.90 ± 1.67[a]
Platelets (10^9/L)	185.67 ± 25.43[a]	208.00 ± 54.15[a]	170.75 ± 29.05[a]	175.75 ± 30.04[a]	233.20 ± 56.18[a]
WBC($x10^9$/L)	1.87 ± 0.28[a]	3.13 ± 0.81[a]	4.10 ± 0.92[a]	2.00 ± 0.35[a]	2.00 ± 0.21[a]
Neutrophils ($x10^9$/L)	0.26 ± 0.08[a]	0.17 ± 0.10[a]	0.12 ± 0.05[a]	0.11 ± 0.03[a]	0.16 ± 0.01[a]
Lymphocytes (x109/L)	1.63 ± 0.46[a]	2.53 ± 0.83[a]	3.35 ± 0.84[a]	1.73 ± 0.33[a]	1.60 ± 0.26[a]
Monocytes (x109/L)	0.02 ± 0.002[a]	0.02 ± 0.01[a]	0.01 ± 0.05[a]	0.03 ± 0.02[a]	0.01 ± 0.05[a]

*Values in the same row with similar superscript letter were not significantly different ($p > 0.05$)

Table 4. The serum biochemistry evaluation (mean ± SEM) in subacute oral toxicity study of *Oroxylum indicum* leaf ethanolic extract in mice

Parameters	Control	Vehicle	Low dose (100 mg)	Medium dose (200 mg)	High dose (500 mg)
Urea (mmol/L)	26.40 ± 1.00[a]	26.00 ± 3.30[a]	30.20 ± 2.01[a]	28.50 ± 1.50[a]	25.00 ± 4.16[a]
Creatinine (µmol/L)	0.12 ± 0.04[a]	0.26 ± 0.12[a]	0.15 ± 0.01[a]	0.22 ± 0.06[a]	0.28 ± 0.05[a]
ALT (U/L)	104.20 ± 1.02[a]	90.70 ± 0.95[a]	115.80 ± 1.80[a]	122.50 ± 2.01[a]	102.30 ± 2.16[a]
Total protein (g/L)	6.56 ± 0.50[a]	6.20 ± 0.23[a]	6.88 ± 0.60[a]	7.07 ± 0.74[a]	6.01 ± 0.22[a]
Albumin (g/L)	3.40 ± 0.12[a]	3.01 ± 0.10[a]	2.60 ± 0.28[a]	2.69 ± 0.50[a]	2.10 ± 0.16[a]
Globulin (g/L)	4.60 ± 0.30[a]	3.80 ± 0.29[a]	3.58 ± 0.60[a]	3.20 ± 0.43[a]	4.20 ± 0.15[a]

*Values in the same row with similar superscript letter were not significantly different ($p > 0.05$)

Organ gross examination and histopathological evaluation of the organs

No gross lesions were observed related to toxicity in both treatment and control groups. The histopathological scoring revealed no abnormalities and lesions were noticed from the tissue sections of the liver and kidney of mice in all treatments and control groups as shown in Figures 1-10. Figure 1: Liver section of the control group mice; Figure 2: Kidney section of the control group mice; Figure 3: Liver section of the vehicle group mice; Figure 4: Kidney section of the vehicle group mice; Figure 5: Liver section of the low dose group mice; Figure 6: Kidney section of the low dose group mice; Figure 7: Liver section of the medium dose group mice; Figure 8: Kidney section of the medium dose group mice; Figure 9: Liver section of the high dose group mice; Figure 10: Kidney section of the high dose group mice. (H&E, 40x magnification)

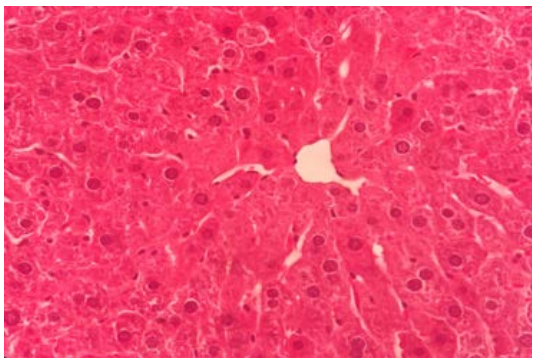

Fig. 1. Liver section of the control group mice

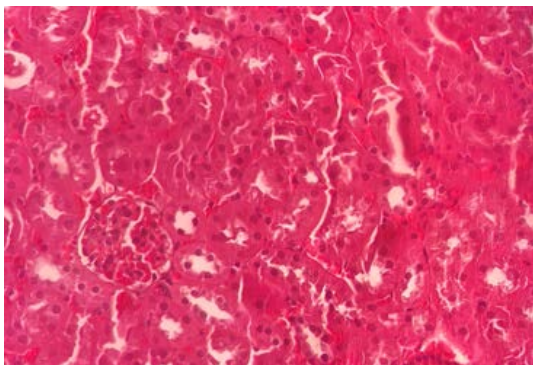

Fig. 2. Kidney section of the control group mice

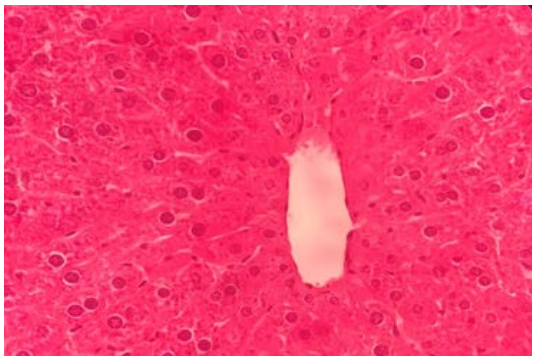

Fig. 3. Liver section of the vehicle group mice

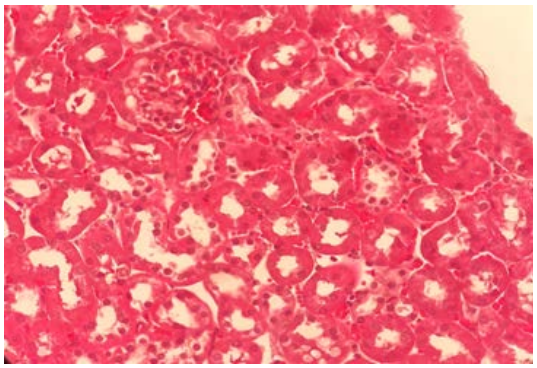

Fig. 4. Kidney section of the vehicle group mice

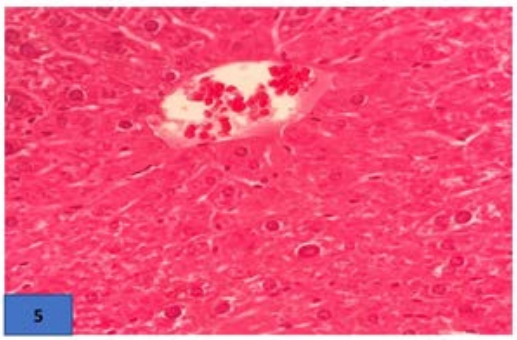

Fig. 5. Liver section of the low dose group mice

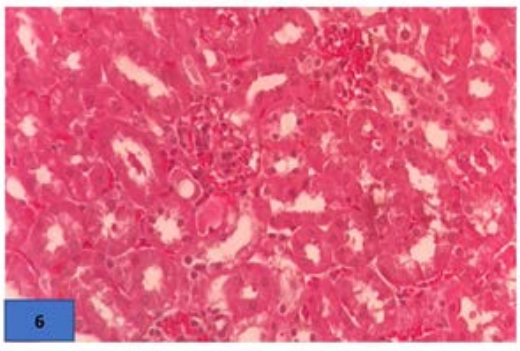

Fig. 6. Kidney section of the low dose group mice

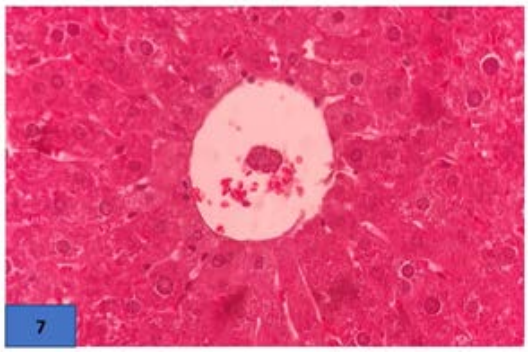

Fig. 7. Liver section of the medium dose group mice

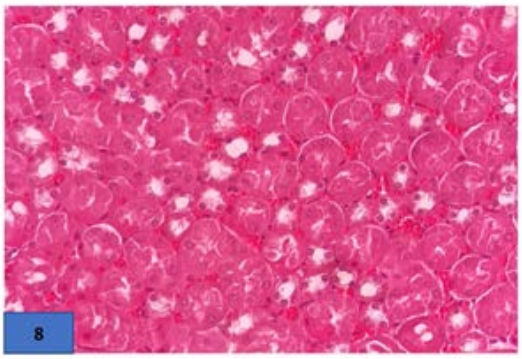

Fig. 8. Kidney section of the medium dose group mice

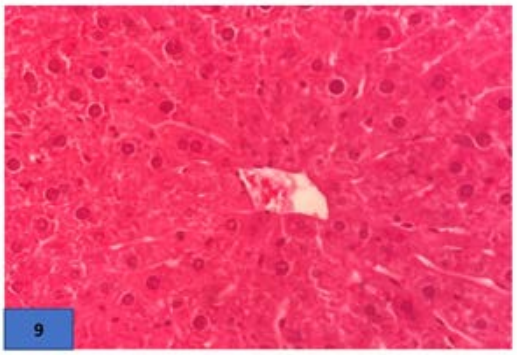

Fig. 9. Liver section of the high dose group mice

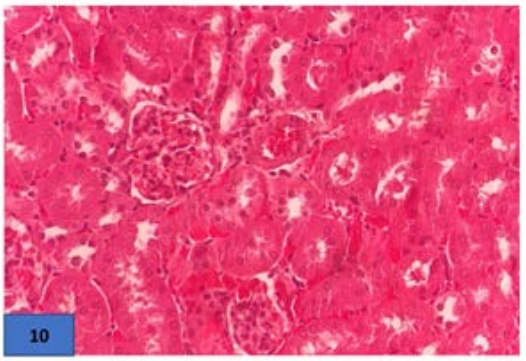

Fig. 10. Kidney section of the high dose group mice

DISCUSSION

To the best of our knowledge, to date, there is no subacute toxicity study of *O. indicum* to support its medicinal potential even though the plant is extensively used by local people as raw salad. In this 28-day oral subacute toxicity study revealed that no death was found in mice indicating that the LD_{50} of *O. indicum* ethanolic lead extract could be more than 500 mg/kg. Therefore, the long-term consumption of the plant extract below 500 mg/kg may be considered relatively safe.

Several parameters were assessed in this study. Administration of *O. indicum* extract up to maximum dose of 500 mg/kg did not cause any mortality among mice and did not showed any abnormal behavioural changes that indicates signs of toxicity such as lethargy, inactive, isolated itself and no fur-grooming. Mortality rates and behaviour changes are important parameters in toxicity study[16,17]. In addition, the LD_{50} of the *O. indicum* extract was up to 500 mg/kg bw which suggests a wide safety margin for therapeutic doses.

Body weight change is a good indicator of animal health. Body weight changes not only influenced by weight gain after food intake, but also will be affected by the internal organ weight. Body weight changes can be caused by adverse effects of chemicals or drugs consumption. Changes in body weight due to exposure to potentially toxic substances could indicate an effect of toxicity[13]. If more than 10% of body weight loss from initial body weight after consuming crude extract, it may indicate to the toxic effects of the administration. The increasing body weight of mice for all group with no significant different ($P>0.05$) in the end suggests the herbs is safe to be consumed. This result agrees with [17,18], who also recorded a significant increase in the body weight of mice and rats respectively when administered with ethanolic plant extracts**.** It can be stated that leaves did not interfere energy metabolism of animal. Similarly,

no significant different of relative organ weight of control and treated group (P> 0.05), indicating no cellular changes or injury happened such as hypertrophy and hyperplasia due to the direct exposure of toxicants on cells.

The blood can be used as parameters analyses to the systemic toxicity of any chemical or compound as the blood cells are first exposed to toxicants and most susceptible target. The toxicants may directly harm the cells in the bloodstream or have an effect on the hematopoietic tissue in the bone marrow. This can lead to a decrease of blood cells count and osmotic fragility and affects the health status of the animal[19,20]. Furthermore, the severity of toxicity can be predicted from the significant alteration of the blood parameters[15]. In this study, there were no significant differences among all groups for haemogram analyses except in mean corpuscular volume. The readings, however, remained within the acceptable value.

Substances such as compound, medication and xenobiotics entering the body must undergo metabolism and excretion processes majorly in the liver and kidneys. Any severe organ dysfunction may result in compound accumulation or toxic-concentrated metabolites[21]. The serum level of creatinine and BUN were used to estimate kidney status in this study. Creatinine and BUN are by-products of protein metabolism in the kidney glomerulus and circulated in the blood[22]. Moreover, Alanine aminotransferase (ALT) and nitrogenous wastes marker of the kidney such as blood urea nitrogen (BUN) and creatinine are typically used as markers for the liver and kidney toxicities as they directly involve in detoxification of metabolites[23,24]. In this study, there were no significant differences (p>0.05) for both treatment and control groups indicating that the extracts were not toxic at the dosage up to 500 mg/kg. The histopathological results supported evidence related to the body weight, haematology and biochemical findings as histopathology is an essential parameter for assessing pathological changes in the organs due to systemic toxicity.

CONCLUSION

In conclusion, the oral sub-acute toxicity of *Oroxylum indicum* leaf extract has shown to be more than 500 mg/kg and this study suggest the plant is generally safe for long-term consumption. However, for clearer and more comprehensive picture of the toxicity effect of *O. indicum,* a well-designed of chronic toxicity study is encouraging to be carry out.

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