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Blood Profiles and Histopathological Changes of Liver and Kidney Tissues from Male Sprague Dawley Rats Treated with Ethanol Extracts of *Clinacanthus nutans* Leaf

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Abstract

Background: *Clinacanthus nutans* (*C. nutans*) or commonly known as 'Sabah snake grass' or 'Belalai Gajah' is a widely known herb used to treat Herpes simplex virus (HPV), skin rashes and snake bite.

Objective: This study aim is to evaluate the toxicity of ethanol extract of *C. nutans* leaf extract on 90-day sub chronic toxicity study in male Sprague Dawley rats.

Method: A total of 40, 6-week male Sprague Dawley rats were divided into 5 groups (n=8) namely control, vehicle (10% DMSO), and 3 treatment groups which received a daily oral dose of *C. nutans* leaf extract at 75 (low dose), 125 (medium dose), and 250 (high dose) mg/kg for 90 days via oral gavage. Blood sample were collected at the end of the experiment for evaluation of haematology and serum biochemistry. Selected organs including liver and kidneys were collected for histopathological examination. The toxicity were evaluated by observing and evaluating the changes of body weight, haematology and serum biochemistry parameters and histopathology changes of liver and kidney tissues.

Results: There was no mortality sign of sub chronic toxicity observed during the observation period. Body weight, haematology parameters and organ relative weight showed no significant difference in control and treatment groups meanwhile in serum biochemistry parameters, observed a significant difference ($P<0.05$) in level of LDH and creatinine kinase in high dose group showed significant lower($P<0.05$) compared to control. Nevertheless the levels of LDH and creatinine kinase were still in normal range. Significant abnormal histopathological changes ($P<0.05$) such as centrilobular sinusoid dilatation/centrilobular necrosis, hydropic degeneration/cytoplasmic vacuolation and inflammation were observed in liver tissues in medium and high dose groups. Kidney tissue showed a significant abnormal histopathology changes ($P<0.05$) such as granular cast and cellular cast were observed in medium and high dose group.

Conclusion: It is concluded that 125-250 mg/kg ethanol extract of *C. nutans* leaves induce hepatotoxicity and renal toxicity.

Keywords: Sub chronic oral toxicity study; *Clinacanthus nutans*; Haematology; Serum biochemistry; Histopathology changes

Introduction

Clincanthus nutans (*C. nutans*) is a popular local herb belongs to family Acanthaceae that grows in tropical Asian countries [1]. It is locally known as 'Sabah snake grass' or 'rumput belalai gajah' (Malaysia). Meanwhile in Thailand and Indonesia, *C. nutans* is known as 'Phayo yo' and 'Dandang Gendis', respectively. In Thailand, *C. nutans* are traditionally used for skin rashes, snake and insects bites, diabetes mellitus and diarrhoea treatment [1,2]. Apart from that, it is also used as anti-viral against herpes simplex virus (HSV) and varicella-zoster virus (VZV) [3]. The leaves of *C. nutans* are boiled and traditionally used as medicine to treat dysentery in Sukajadi village in West Java, Indonesia [4].

Nowadays, the leaves of *C. nutans* have gained its popularity as supplement and medicine to treat various types of illness especially cancer. It is believed the consumption of *C. nutans* leaves as tea can cure the illness without knowing its side effects. The therapeutic effect of *C. nutans* against cancer has not been clinically tested in laboratory animals although the consumption of *C. nutans* is increasing without knowing its toxicology effects. Only a few toxicity studies of *C. nutans* leaves have been carried out in the laboratory rats to determine its toxicity. Previous studies carried out by P'ng et al. [5] and Peng et al. [6] has shown extract of *C. nutans* leaves did not cause toxicity in laboratory mice. However a study by Chin et al. [7] reported extract of *C. nutans* leaves cause a significant increase in liver weight and serum biochemistry parameters (serum total proteins and albumin/globulin ratios) which suggest hepatotoxicity.

The toxicology evaluations of *C. nutans* leaves are very important in order to know the side effects especially the people who consume it as a supplement and medicine. The health promoting benefits of both

herbal plants have been known and being used in many countries especially for its medicinal properties. The increasing use of these herbal plants has resulted in concerns of the safety and effectiveness. Hence, through the toxicity studies in laboratory animal, the toxicology of these plants can be determined as a safeguard to the public health and to raise public awareness on the toxicity of *M. citrifolia* fruits and *C. nutans* leaves. The toxicology studies also provide a pre-clinical safety evaluation before it can be performed and evaluated in human. Thus the objective of this study is to investigate sub chronic oral toxicity of ethanol extract of *C. nutans* leaves in male Sprague Dawley rats.

Materials and Methods

Plant preparation and extraction

Clinacanthus nutans leaves were obtained from Malaysian Agriculture Research and Development Institute (MARDI) research station located at Muadzam Shah, Pahang. The *C.nutans* leaves were washed and dried under the sun for 48 h. Lastly, the dried samples were ground into powder and were kept in a container at 4°C. The ground samples were extracted with 70% ethanol (Merck, German) at a ratio 1 g of sample to 40 mL of ethanol. Each mixture was placed in an orbital shaker (Heidoph Unimax 1010, German) at 200 rpm at room temperature approximately for 2 h [8]. The mixture was filtered twice through a filter paper (Whatman No. 4). Later, the ethanol was removed by a rotary evaporator (BUCHI Rotavapor R-200, Switzerland). The semisolid extracts were obtained after was freeze-dried in a freeze-dryer (The VIRTIS Company, USA). The semisolid extracts was considered as *C. nutans* leaf extracts and used in this study. Each extracts were dissolved with 10% DMSO and were given to the rats via oral gavage. The dissolved extracts were freshly prepared every week based on the body weight.

Animal management, routine and experimental design

All animals used in the experiments were subjected to approval by the Animal Care and Use Committee (ACUC) MARDI, Serdang and were conducted at the Animal Metabolism, Toxicology and Reproductive Centre (AMTREC), MARDI, Serdang. A total 40 male Sprague Dawley (SD) rats at age of 5-6 weeks old with an average weight between 160-180 g were used in the studies. The rats were acclimated to the housing conditions in polycarbonate plastic cage with temperature within the range of 22-27°C, humidity at the range 40%-70% and balance of 12 h light/12 h dark cycle. The bedding and water were replaced every day and the cages were cleaned when it is necessary. The rats were divided into five groups (n=8) namely control (group A), vehicle (10% DMSO) (group B), and 3 treatment groups; 75 (low dose) (group C), 125 (medium dose) (group D) and 250 (high dose) (group E) mg/kg of body weight. The extracts of *C. nutans* leaves were dissolved in 10% DMSO before orally given to the rats. In control group, the rats received no treatment, while in vehicle group the rats were given 10% DMSO by oral gavage. All rats had free access to water and commercial chow ad libitum. The rats were observed daily for any mortality and signs of toxicity, and were weighed weekly throughout the study period.

Haematology Analysis

Blood sampling

At the end of the experimental period all rats were humanely sacrificed with an overdose of sodium pentobarbital euthanasia solution via intramuscular injection. Blood samples were collected by using 21 gauge needle and 3 mL syringe via posterior vena cava. 1 mL of blood samples were collected into anticoagulant blood collection tube (EDTA tube). The blood was mixed well to prevent from clotting stored on ice.

Complete blood count

The blood samples were analysed for complete blood count using an automated haematology analyser (Cell Dyn® 3700, Abbott Diagnostics, USA) for the total RBC, WBC, platelet count, haemoglobin (Hb) concentration, mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) were calculated. Blood smear was stained using Wright stain and examined under a light microscope at 10X, 20X, 40X, 60X and 100X magnifications. Differential WBC count was determined manually by counting 100 WBC on the blood smears. The absolute values of each type of WBC (lymphocytes neutrophils, eosinophils, monocytes and basophils were calculated by multiply the percentage of each WBC type to the total WBC count from the automated analyser.

Capillary blood preparation

Microhaematocrits were filled up about third quarter of the capillary with blood from the EDTA tubes. The capillary tubes were wiped with tissue until it is cleaned and dried. The end of the capillary tubes was sealed by flame from Bunsen burner with the heated up technique. The capillary tubes were placed in a microhaematocrit centrifuge (Hettich Haematocrit 210, Germany) and centrifuged at 10,000 rpm for 5 minutes. This procedure was run to separate blood and plasma. RBC will be at the bottom of the capillary tubes and the plasma will be at the top of the capillary.

Packed cell volume

The centrifuged capillary tubes were further analysed for packed cell volume (PCV) using microhaematocrit reader (Hawksley). The base of RBC was intersected with the base line of the reader. Meanwhile the top of plasma was intersected with the top line of the reader by moving the holder left or right, before the middle line of the reader was adjusted to intersect with the top of RBC and the measuring ruler. The PCV percentage was obtained from the middle line and the measuring reader intercept. The values were converted into percentage (L/L).

Icterus index

The centrifuged capillary tubes were used to determine the icterus index. The plasma separated in the capillary tube was compared with an icteric index standard board colour degree to determine the icteric standard index of the samples.

Plasma protein determination

The centrifuge capillary tubes were later analysed for plasma protein concentration. The top of the capillary tubes were broken and the plasma liquid was dropped onto a prism refractometer (Atago T2-NE,

Japan). The plasma protein concentration was determined by observing the refractometer and the concentration value were read according to the scale.

Serum biochemistry analysis

Blood samples were collected into plain tube and were centrifuged (Centrifuge S417R, Eppendorf, CA) for 15 minutes at 3000 rpm to obtain the serum. The serum was further analysed for cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-cholesterol), creatinine, urea, total bilirubin, total protein (TP), albumin (ALB), globulin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), inorganic phosphorus (IP), alkaline phosphatase (ALP), uric acid (UA), lactate dehydrogenase (LDH), creatine kinase (CK), glucose using an automated clinical chemistry analyser (TRX 7010, Biorex Mannheim, Germany).

Histopathological analysis

At the end of the experimental period, all rats were sacrificed with overdose of xylazine and ketamine. Selected organs of each rat such as were collected in cold [pH 4] normal saline. The organs were cleaned from blood, weighed and examine for gross lesions for gross examination. The organs were then fixed in 10% buffered formalin solution. The process of tissue fixation was in 48 h duration in clean 10% formalin solution. The organs samples were processed at the Serology laboratory, Faculty Veterinary Medicine, UPM, Serdang. All of the samples were trimmed about 0.5 cm thickness and were placed in cassettes. Later the cassettes were placed into a 10% formalin solution overnight, before they were placed and undergone series of dehydrated process for about 16 h in an automated processor (Leica ASP300, Germany). The samples then were embedded with paraffin to form a block and left cool by a processor machine (Leica EG1160, Germany). The samples were trimmed about 3-5 µm thickness using a sectioning rotary microtome (Leica RM2155, Germany), and directly placed the tissue sectioning in 45°C water bath before mounting on slides. All the glass slides were labeled with a diamond pen and continue mounted on a hot plate (54°C) overnight. Later all the slides were undergone a series of steps for Haematoxylin and Eosin (H&E) staining protocol. Lastly, the samples were examined under a light microscope at 10X, 20X, 40X, 60X and 100X magnifications.

Lesion characteristics

Toxicological lesions such as inflammation, activated kupffer cells, hydropic degeneration, periportal necrosis, midzonal mecrosis, and centrilobular necrosis in the liver were examined and were scored. Meanwhile in kidney tissue, toxicological lesions such as cellular cast, granular cast, protein cast, inflammation, hydropic degeneration/ cytoplasmic vacuoaltion and necrosis were examined and were scored. The lesions scoring in both liver and kidney tissues were scored as 0=normal, 1=mild (1%-30%), 2=moderate (31%-70%) and 3=severe (>70%), based on the percentages of tissues affected.

Statistical analysis

The average body weight, haematology and serum biochemistry data were expressed as Mean ± SEM and analysed by using one-way ANOVA and Tukey test. Meanwhile for histopathology examination, data was expressed as Mean ± SEM and analysed using by using Kruskal Wallis for global comparison of groups of all parameters and on-parametric and Mann-Whitney-U test for comparison between two groups. These data's were analysed using statistical software, IBM SPSS Statistic 21.0.

Results

Average body weight

The average body weight of rats in oral toxicity study of *C. nutans* extract that were taken weekly and are presented in Figure 1. Rats in groups A, B, C, D and E continuously gained weight from week 1 to week 12. The body weight (mean ± SEM) of the rats at week 12 were arranged in an ascending order are 390.09 ± 13.38 g (group B), 390.89 ± 16.24 g (group C), 434.56 ± 23.7 g (group E), 445.90 ± 11.09 g (group A) and 464.89 ± 15.59 g (group D). There were no significant differences (P>0.05) in the body weight between all groups throughout the study period.

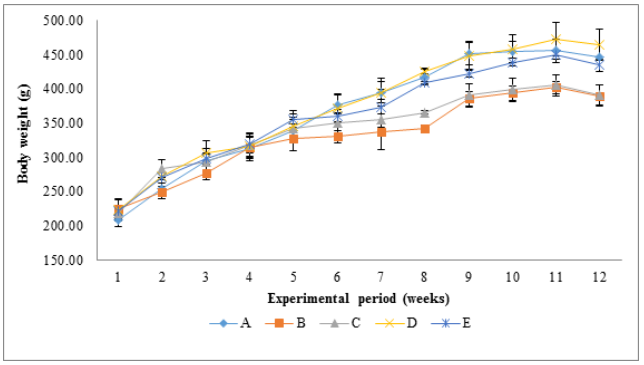

Figure 1: Mean (± SEM) body weights of male SD rats received repeated doses of ethanol extract of *C. nutans* leaves by oral gavage in 90-day duration. (**Note:** A: Control; B: Vehicle (10% DMSO); C: Low Dose; D: Medium Dose; E: High Dose).

Haematology, serum biochemical analyses and organ to body weight ratio

The haematology results of rats in sub chronic oral toxicity study of *C. nutans* leaf extract at week 12 are shown in Table 1 and Table 2. There were no significant differences (P>0.05) observed in the results between all groups compared to control. The serum biochemical results of rats in sub chronic oral toxicity study of *C. nutans* extract at week 12 are shown in Table 3. The results showed significant differences (P<0.05) in some of the results. The LDH level of rats in high dose group (1595.00 ± 164.39 U/L) was significantly (P<0.05) lower compared to control (2659.30 ± 82.13 U/L). The similar trend was seen in the CK value of rats in high dose group (441.71 ± 58.41 U/L) compared to control (941.83 ± 89.78 U/L). However, the values were still within the reference ranges (Patterino and Argentino-Stroino, 2006). The results of liver, kidneys, lungs, heart, thymus, thyroids, brain, adrenals, testes and spleen ratio to the body weight (%) of rats in in sub chronic oral toxicity study of *C. nutans* extract are presented in Table 4. There were no significant differences (P>0.05) in the ratio at week 12 of the study between the groups.

Histopathological scoring of mean lesion scores

Kruskal Wallis test for the global comparison of organ toxicity among groups showed significant results (P<0.05) for the sinusoidal dilatation, cytoplasmic vacuolation and inflammation in the liver, and granular cast in the kidney (Table 5). Mann-Whitney U test showed significant differences (P<0.05) for the sinusoidal dilatation, cytoplasmic vacuolation and inflammation in the liver of medium and high dose groups compared to control (Table 6). Meanwhile in the kidney, granular cast was significantly different (P<0.05) in medium and high dose groups compared to control (Table 6). Results of sub chronic oral toxicity of *C. nutans* leaf extract showed significant lesions in the liver and kidney of medium and high dose groups compared to low dose group. Lesions observed in the liver and kidney is shown in Figures 2 and 3. It is concluded that administration of *C. nutans* leaves ethanol extract, daily for 90 days at high (250 mg/kg of body weight) and medium (125 mg.kg of body weight) doses induce mild hepatic and renal toxicity in rats. Administration of the extract daily for 90 days at low dose (75 mg/kg of body weight) induces no hepatic and renal toxicity.

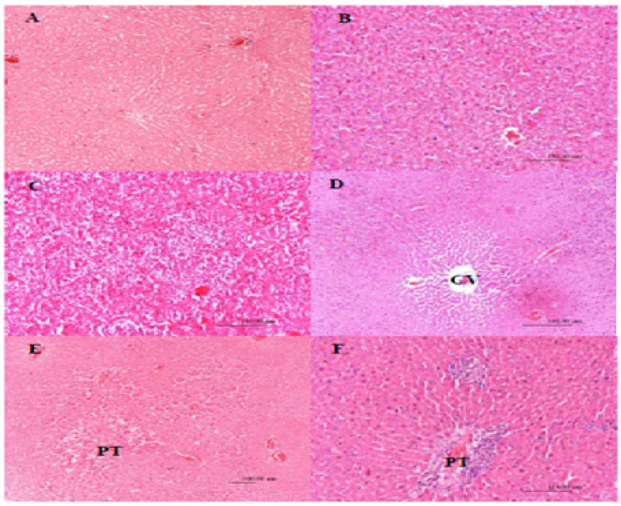

Figure 2: Photomicrographs of rat's liver sections at the end of experimental period (day 90). (A) Normal liver section from a rat in control group (H&E stain, X200). (B) Mild cytoplasmic vacuolation from a rat in high dose *C. nutans* group (H&E stain, X200). (C) Marked cytoplasmic vacuolation (scored as 3) from a rat in high dose *C. nutans* group (H&E stain, X200). (D) Sinusoidal dilatations were observed around the central vein (CV) (centrilobular necrosis). Midzonal necrosis was observed in the areas. The lesions were characterised by presence of necrotic cells with eosinophilic cytoplasm and pyknotic nuclei (scored as 1) from a rat in high dose *C. nutans* group (H&E stain, X200). (E) Periportal and midzonal necrosis characterised by with pyknotic nuclei, eosinophilic cytoplasm and atrophied hepatocytes was observed (scored as 1) from a rat in high dose *C. nutans* group (H&E, X200). (F) Mild hepatitis was observed around the portal triad (PT) and in liver parenchyma (scored as 1) from a rat in high dose *C. nutans* group (H&E, X2000).

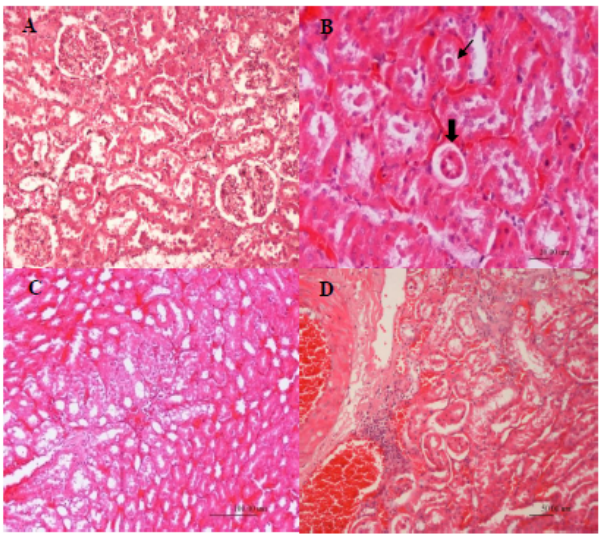

Figure 3: Photomicrographs of rat's kidney sections at the end of experimental period (day 90). (A) Normal renal section from a rat in control group (H&E stain, X200). (B) Granular cast (thin arrow) and cellular cast (thick arrow) were present in the renal tubules (scored as 1) from a rat in high dose *C. nutans* group (H&E stain, X400). (C) Prominent cytoplasmic vacuolation of renal tubular cells indicating or known as hydropic degeneration (scored as 1) from a rat in high dose *C. nutans* group (H&E stain, X200). (D) Infiltration of lymphocytes in the interstitial tissues (scored as 1) from a rat in high dose *C. nutans* group (H&E stain, X200).

Discussion

The herbal industry is a fast growing industry worldwide. The increment demand in herbal supplements, health functional food, and skin care and herbs functional drinks has led to an increment of growing of herbal industry [9]. High demand in alternative medicine is also a contributing factor to the use of herbal products such as Traditional Chinese Medicine, Ayurveda and Jamu [9]. *Clinacanthus nutans* (rumput belalai gajah) is popular Malaysian herbs that are currently being studied for pharmaceuticals, medicinal and market potential.

Clinacanthus nutans either in crude, extract or also its biochemical compound is used in dose-dependent method for multiple purposes. For example, treatment 100 µg/mL of *C. nutans* leaf extract is a good anti-oxidant in several of cancer cell lines [10]. Basically, selected doses of ethanol extract of *C. nutans* administration via oral gavage to the rats were based on the OECD guidelines for testing chemicals version 408 (repeated dose 90-day oral toxicity study). In sub chronic oral toxicity study (90-day) (OECD versions 407 and 408), at least three dose levels are used and the dose level is selected from four fixed levels, 5, 50, 300 and 2000 mg/kg of body weight. Thus dose levels at 75 mg/kg, 125 mg/kg and 125 mg/kg of extract was used in the studies.

Sprague Dawley rats were used as animal model in the toxicity studies of ethanol extract of *Morinda citrifolia* and *Clinacanthus nutans*. The Sprague Dawley rat is an outbred multipurpose breed of albino rat. It was developed by R. Dawley, 1975 with the collaboration

of Sprague Dawley Company, Madison, Wisconsin. Sprague Dawley rat extensively used in the medical research and laboratory lab due to its advantages such as easy to breed, short life spans, calmness and easy to handle. The criteria of the Sprague Dawley rats make it an ideal model for this research. The Sprague Dawley rat also has been used to study the toxicity of Malaysia herbal plant such as Mitragyna speciose Korth (MS) (ketum), Carica papaya Linn (betik), and Momordica charantia (peria katak) [11-13].

Body weight is one of parameters used for evaluating health status of experimental animals [14]. Herbal extracts can contribute suppression of animal's appetite which leads to reduction in body weight of animals [15]. Decrease in body weight could also be associated with normal physiological adaptation responses of the body towards plant extracts or compounds [16]. A study conducted by Harizal et al. [17] reported an increased in animal body weight is related to the accumulation of the body fats rather than the toxicity effects after administration of single dose of methanol extract of Mitragyna speciosa Korth (ketum). The weekly body weight of sub chronic toxicity studies of *C. nutans* is shown in the Figure 1. In the current study, repeated administration of the extract for 90 day showed increases in body weight throughout the study period. Apart from that, no significant differences (P>0.05) were observed in the weekly average body weight between the groups of all rats throughout the study period. No physical changes were observed in this study.

Haematology is the study of the morphology and physiology of blood. It is an important toxicology evaluation of any compounds including herbal plant, chemicals and drugs [18]. Blood is a bodily fluid that delivers necessary substance such as oxygen and nutrients (example: glucose, amino acids and fatty acids) to the tissues and cells. It also transports metabolic waste products to the kidneys and liver to be filtered and excreted. The red blood cells (RBCs) or also called erythrocytes is one types of blood cells that delivering oxygen (O_2) to the body tissues and cells. Low RBCs is associated with anaemia and sometimes toxicity [19,20]. A study by Waldron [21] and Flora et al. [22] found the number of RBCs in cases of lead poisoning was lower compared to the normal. Meanwhile in plant toxicity, Adedapo et al. [23] reported low RBCs (anaemia) in rodents after crude extract of Euphorbia was orally administrated to the rats. Differ with a study by Okokon et al., an increase of RBCs values were reported after administration of ethanol extract of Croton Zambesicus in rats. This is due to the presence of alkaloids in the root extract that stimulate erythropoiesis. In this present study, the sub chronic oral toxicity study of *C. nutans* extract (Table 1) did not showed any significant differences (P>0.05) in the values of RBCs, PCV, haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), icterus index and plasma protein when compared to the control. The normal values of haematology parameters in this study indicate the plant extract did not induce anaemia and jaundice within the 90 days of animal trials.

Group/Parameter	RBC (10^{12}/L)	Hb (g/L)	PCV (L/L)	MCV (fL)	MCHC (g/L)	Icterus index	Plasma protein (g/L)
A	9.42 ± 0.17	173.2 ± 0.32	0.56 ± 0.02	56.56 ± 1.03	307.11 ± 7.47	2.00 ± 0.00	80.00 ± 0.00
B	10.00 ± 0.44	176.1 ± 0.73	0.54 ± 0.03	54.44 ± 3.66	328.00 ± 21.56	2.00 ± 0.00	78.33 ± 1.67
C	9.84 ± 0.90	172.4 ± 0.44	0.53 ± 1.09	54.84 ± 0.94	317.77 ± 2.58	2.00 ± 0.00	81.63 ± 2.27
D	9.55 ± 0.90	166.1 ± 0.47	0.52 ± 1.03	54.42 ± 0.95	319.77 ± 2.58	2.00 ± 0.00	76.25 ± 1.83
E	9.77 ± 0.14	172.1 ± 0.48	0.52 ± 0.01	53.14 ± 1.16	295.73 ± 30.91	2.00 ± 0.00	77.50 ± 2.50

Table 1: The erythron parameters, icterus index and plasma protein values (mean ± SEM) of Sprague Dawley (SD) rats in subchronic oral toxicity study of ethanol extract of *C. nutans* leaves at the end of experimental period (Values in the same column with similar superscripts were not significantly different at P>0.05. Note: A: Control; B: Vehicle (10% DMSO); C: Low Dose; D: Medium Dose; E: High Dose.).

The white blood cells (WBCs) or also called leukocytes are the cells in the immune systems which play a role in body defence from foreign materials and infectious diseases. The leukocytes are produced in the haematopoietic stem cells or also known as bone marrow. Abnormal low count of normal WBCs or neutropenia with low level of RBCs (anaemia) can be characterised as leukaemia. Meanwhile an increase in the number of white cells (leucocytosis) in the blood is a sign of infection [24-26]. A study by Okokon et al. reported leucocytosis in rats after orally administration of ethanol extract of Croton Zambesicus and might be a sign of infection. The present study showed administration of *C. nutans* leaf extracts for 90-day (Table 2 and Table 3) showed no significant differences (P>0.05) in the leukocytes count. The differential WBCs count such as neutrophil, lymphocytes, monocytes, eosinophils and basophils also showed no significant differences (P>0.05) in these study.

Parameter/Group	A	B	C	D	E
WBC (x 10^9/L)	5.74 ± 0.68	7.54 ± 0.51	7.05 ± 0.85	7.15 ± 0.68	6.78 ± 0.63
Neutrophils (x 10^9/L)	0.96 ± 0.87	2.15 ± 0.49	1.53 ± 0.38	1.77 ± 0.45	0.96 ± 0.20
Lymphocytes (x 10^9/L)	4.43 ± 0.36	5.00 ± 0.29	4.88 ± 0.50	4.85 ± 0.31	5.27 ± 0.89
Monocytes (x 10^9/L)	0.34 ± 0.07	0.28 ± 0.04	0.44 ± 0.09	0.36 ± 0.04	0.39 ± 0.04

Eosinophils (x 10^9/L)	0.00 ± 0.00	0.12 ± 0.04	0.17 ± 0.03	0.15 ± 0.01	0.18 ± 0.03
Basophils (x 10^9/L)	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01

Table 2: The total white blood cell (WBC) and WBC differential count (mean ± SEM) of Sprague Dawley (SD) rats in subchronic oral toxicity study of ethanol extract of *C. nutans* leaves at the end of experimental period (Values in the same row with similar superscripts were not significantly different at P>0.05. Note: A: Control; B: Vehicle (10% DMSO); C: Low Dose; D: Medium Dose; E: High Dose).

Serum biochemical analysis provides a valuable tool in evaluating the effects of herbal extracts in tissue [18]. Increased serum enzymes for examples ALP, ALT and GGT are due to cell membrane and tissue damage [27]. Previous study revealed administration of 4000 mg/kg of body weight of Herniaria glabra extract in rodents showed sign of hepatotoxicity with evidence of marked elevation in ALT and AST levels [16]. Increased levels of serum AST and CK together with LDH are an indication of muscle injury or increased muscle activity or myocardial infarction [27,28]. Previous study has shown injection of doxorubicin produces cardio toxicity in rats as indicated by the increment of LDH and CK levels in the serum [29]. Meanwhile decrease in the muscle enzymes are related to decrease in muscle activity [28,30].

Parameter/Group	A	B	C	D	E
IP (mmol/L)	4.24 ± 0.32	4.09 ± 0.28	3.90 ± 0.31	3.89 ± 0.19	3.96 ± 0.15
Urea (mmol/L)	7.93 ± 0.60	6.76 ± 0.50	7.95 ± 0.53	7.51 ± 0.41	7.68 ± 0.70
Creatinine (µmol/L)	85.66 ± 2.88	78.50 ± 5.19	82.50 ± 2.81	92.00 ± 4.31	87.50 ± 3.58
Glucose (mmol/L)	5.68 ± 0.81	6.40 ± 2.07	7.58 ± 1.87	5.32 ± 1.40	6.05 ± 1.25
Cholesterol (mmol/L)	2.47 ± 0.34	2.12 ± 0.26	2.07 ± 0.16	2.38 ± 0.18	1.96 ± 0.16
T.Bil (µmol/L)	1.53 ± 0.10	1.35 ± 0.13	1.511 ± 0.13	1.11 ± 0.09	1.08 ± 0.14
ALT (U/L)	57.91 ± 4.64	51.90 ± 4.25	45.73 ± 2.76	55.06 ± 3.76	48.22 ± 4.71
ALP (U/L)	121.17 ± 10.85	111.83 ± 4.93	98.12 ± 10.22	119.00 ± 6.33	110.50 ± 13.73
GGT (U/L)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
AST (U/L)	180.45 ± 10.49	160.52 ± 12.55	127.79 ± 8.66	153.33 ± 16.09	142.05 ± 11.10
CK (U/L)	941.83 ± 89.78	807.00 ± 155.37	567.12 ± 100.75	644.62 ± 127.66	441.71 ± 58.41
LDH (U/L)	2659.30 ± 82.13	2220.00 ± 312.81	1688.10 ± 310.42	1932.90 ± 343.63	1595.00 ± 164.39
TP (g/L)	91.96 ± 3.76	84.50 ± 6.36	87.21 ± 3.78	92.07 ± 4.25	88.35 ± 2.87
ALB (U/L)	46.58 ± 2.20	39.13 ± 3.60	41.32 ± 1.55	45.30 ± 2.11	43.24 ± 1.48
Globulin (g/L)	45.53 ± 1.56	45.37 ± 2.76	40.92 ± 1.90	47.77 ± 2.14	45.11 ± 1.39
TG (µmol/L)	0.65 ± 0.05	0.60 ± 0.07	0.46 ± 0.02	0.66 ± 0.07	0.58 ± 0.05
UA (µmol/L)	354.93 ± 48.34	343.60 ± 45.27	229.62 ± 23.18	313.05 ± 34.39	286.34 ± 15.92
HDL-C (mmol/L)	0.99 ± 0.04	0.84 ± 0.09	1.00 ± 0.05	1.09 ± 0.07	1.02 ± 0.05
LDL-C (mmol/L)	1.35 ± 0.29	1.16 ± 0.15	0.97 ± 0.10	1.15 ± 0.09	0.82 ± 0.10

Table 3: The serum biochemical parameter (mean ± SEM) of Sprague Dawley (SD) rats in subchronic oral toxicity study of ethanol extract of *C. nutans* leaves at the end of experimental period (Values in the same row with different superscripts were significantly different at P<0.05. Note: A: Control; B: 10% DMSO; C: Low Dose; D: Medium Dose; E: High Dose).

The current study of sub chronic (Table 4) toxicity study of *C. nutans* leaf extract showed decreases in AST, LDH and CK levels in the serum, which suggested decreased muscle activity rather than toxicity. These findings were different from previous study P'ng et al. [5] and Chin et al. [7] who observed no significant differences (P>0.01) in the serum biochemical parameters of rats treated with methanol extract of *C. nutans* leaves up to 900 mg/kg of body weight in both 14-day and 28-day toxicity studies. Decreased serum biochemical values were also reported by [31] that showed a significant decrease in creatinine after ethanol extract of *C. nutans* leaves at 1000 mg/kg of body weight for 90-day was administrated to rats. Although a few serum biochemical values in this study and also study done by Chavalittumrong et al. were

decrease, the values were still within normal ranges. However, decrease in those serum biochemistry values might also be related to the body compensatory mechanisms, body utilisation, alterations, or maintaining of the normal conditions. Decrease in LDH level is mainly due to the inhibition or reduction of synthesis of aspartate and alpha-ketoglutarate in the Krebs cycle. The inhibition of the synthesis of aspartate and alpha-ketoglutarate enzymes is due to reduce amount of ADP which causes accumulation of NADH and in turn inhibit synthesis of a number of enzymes [32]. Decrease or normal level of serum biochemical parameters could also be related to the half-life of the enzymes in the blood. The concentration of previously increased enzymes, for example due to acute injury, will reduce one-half of the increased concentration over a period of time. Another reason for decrease in serum biochemical parameters is the cytoprotective effect of the extracts towards muscle or liver. Histopathological examination of liver tissue is required to support the cytoprotective effect of the herbs.

Organ/Group	A	B	C	D	E
Liver	3.31 ± 0.03	3.15 ± 0.23	2.99 ± 0.22	2.86 ± 0.17	2.87 ± 0.10
Kidneys	0.68 ± 0.02	0.68 ± 0.03	0.74 ± 0.06	0.64 ± 0.04	0.67 ± 0.04
Lungs	0.51 ± 0.03	0.61 ± 0.06	0.50 ± 0.05	0.46 ± 0.03	0.47 ± 0.02
Heart	0.31 ± 0.01	0.32 ± 0.02	0.37 ± 0.04	0.29 ± 0.16	0.28 ± 0.01
Thymus	0.07 ± 0.00	0.08 ± 0.01	0.10 ± 0.01	0.08 ± 0.00	0.09 ± 0.01
Thyroids	0.16 ± 0.01	0.19 ± 0.02	0.22 ± 0.02	0.18 ± 0.01	0.18 ± 0.01
Brain	0.42 ± 0.02	0.46 ± 0.01	0.53 ± 0.05	0.46 ± 0.02	0.44 ± 0.03
Adrenals	0.05 ± 0.01	0.05 ± 0.02	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
Testes	0.73 ± 0.03	0.81 ± 0.05	0.94 ± 0.13	0.78 ± 0.03	0.82 ± 0.03
Spleen	0.17 ± 0.01	0.14 ± 0.01	0.18 ± 0.01	0.14 ± 0.03	0.21 ± 0.06

Table 4: The ratio of organ to relative body weight (%) (mean ± SEM) of Sprague Dawley (SD) rats in subchronic oral toxicity study of ethanol extract of *C. nutans* leaves at the end of experimental period.

The lesions scoring for liver and kidneys in the toxicity study of *C. nutans* were described with none when no toxicity was observed, one for 1%-30% toxicity was observed, two for 30%-70% toxicity was observed ad three more than 70% toxicity was observed. In the present study, histopathological examination revealed that daily administration of *C. nutans* extracts for 90 days induced toxicity in the liver and kidney tissues for medium (125 mg/kg) and high doses (250 mg/kg). Lesion scoring results of the organs were significantly different ($P<0.05$) from control. Histopathological lesions of sinusoidal dilatation, cytoplasmic vacuolation, inflammation, cellular cast and granular cast were scored mildly in the liver and kidneys of herbal treated groups. However the findings were not related to the results of serum biochemical parameters of liver and kidneys where all the values were within the normal ranges. Histopathological findings in this study was comparable to Saganuwan et al. [30] where they reported that aqueous leaf extract of Morinda Lucida at dose of 1626.5 mg/kg administrated to rats causes tubular degeneration of kidney and mild mononuclear cellular infiltration in liver. Similar to our findings, they found serum biochemical parameters particularly AST was not increased, but indeed significantly decreased ($P<0.05$). Glucose, creatinine, total protein, and bilirubin were also significantly decreased ($P<0.05$) compared to control. All the values, however, were still within the normal ranges.

		Group					
Organ	Mean scores of lesions	A	B	C	D	E	Kruskal Wallis test for global comparisons of organs lesions among groups. (Asymptotic Significant $P<0.05$)
	Inflammation	0	0	0	1.00 ± 0.00	1.12 ± 0.29	0.00*
	Activated Kupffer cells	0	0	0	0	0	1.00
Liver	Hydropic degeneration	0	0	0.80 ± 0.20	1.00 ± 0.00	1.50 ± 0.32	0.01*
	Periportal necrosis	0	0	0	0.14 ± 0.14	0.75 ± 0.36	0.07**
	Midzonal necrosis	0	0	0	0.14 ± 0.14	0.75 ± 0.36	0.07**

	Centrilobular necrosis	0	0	0	0.57 ± 0.20	1.12 ± 0.29	0.03*
	Mean score	0	0	0.13 ± 0.03	0.47 ± 0.08	0.87 ± 0.27	0.19
Kidney	Inflammation	0	0	0.40 ± 0.24	0.42 ± 0.20	0.50 ± 0.18	0.26
	Hydropic degeneration/cytoplasmic vacuolation	0	0	0.14 ± 0.14	0.14 ± 0.14	0.40 ± 0.24	0.44
	Necrosis	0	0	0	0	0	1.00
	Cellular cast	0	0	0	0	0.12 ± 0.12	0.64
	Granular cast	0	0	0.40 ± 0.24	0.71 ± 0.18	0.75 ± 0.16	0.02*
	Protein cast	0	0	0	0	0	1.00
	Mean score	0	0	0.15 ± 0.10	0.21 ± 0.08	0.29 ± 0.07	0.56

Table 5: Result of scoring of lesion of scores in the liver and kidneys of Sprague Dawley (SD) rats in all groups of sub chronic oral toxicity study of ethanol extract of *C. nutans* leaves at the end of the experimental period (Note: A: Control; B: 10% DMSO; C: Low Dose; D: Medium Dose; E: High Dose (n=8 rats in each groups). The symbol * denotes the values were significantly different (P<0.05) between treated groups and control. The symbol ** denotes the values were almost significantly different (P>0.05) between treated groups and control.).

A previous study by Deschpande et al. [33] on the effects of ethanol extract of turmeric in rats and mice for 90 days revealed some abnormalities in the liver tissues. The administration of high dose of turmeric (5%) for 90 days showed a significant difference for focal necrosis with regeneration in the hepatocytes of both mice and rats. A study by Abalaka et al. [34] on the effects of extract of *Adenuim obesum* stem bark in rats revealed vacuolation of hepatocytes and congestion of blood vessels after being administrated with 5000 mg/kg of body weight of ethanol extract of *Adenuim obesum* stem bark. Serum biochemical parameters of the rats and mice particularly AST, ALP and ALT were all normal. Arsad et al. [35] also reported similar lesions in the kidney and liver tissues as observed in our study, although their results of the toxicity of *Rhaphidophora decursiva* (Roxb.) Schott extract were not significantly different (P>0.05) compared to control in both serum and histopathological findings. Chin et al. reported administration of methanol extract of *C. nutans* leaf up to 900 mg/kg of body weight showed significant (P<0.01) increase in total serum protein, albumin to globulin ratio and relative liver weight in rats. Increase in the total serum protein and albumin could be due to dehydration as a result of in appetence. While an increase in liver weight might related to liver injury. However histopathology examination was not conducted in the study. sub chronic toxicity studies conducted by Rosly et al. [36] showed administration of *M. citrifolia* crude fruits up to 5000 mg/kg of body weight and 2000 mg/kg of body weight showed significant (P<0.05) decrease in total protein, and decreased in total white blood cells and spleen weight in rats, respectively. Nevertheless the values were still within the normal ranges. The histopathology examination of liver and kidneys revealed no significant differences (P>0.05) related to the treatments. There was no toxicity observed by Rosly et al. [36] although they used high concentrations of *M. citrifolia* fruits in their studies. The main reasons for that are the fruits were given to the rats in a crude form, and the crude fruit powder mixed with the ground rat pellet was fed to the rat ad libitum; the rats received the fruits in a small quantity throughout the day compared to oral gavage administration. Hepatic necrosis at the periportal and midzonal was observed in sub chronic oral toxicity of *C. nutans* leaf extract although it was not significant different (P>0.05) compared to control. Meanwhile centrilobular necrosis/centrilobular sinusoidal dilatation was observed in the study and significantly different (P<0.05) in sub chronic toxicity study of *C. nutans* for medium and high doses. Renal granular cast was observed and significant (P<0.05) in oral sub chronic toxicity study of *C. nutans* leaf extract for medium and high doses, respectively. Meanwhile renal cellular cast was observed in sub chronic oral toxicity study of *C. nutans* leaf extracts although it was significant (P<0.05) only in high dose. Hydropic degeneration/cytoplasmic vacuolation was observed in sub chronic study of *C. nutans* leaf extracts although it was not significantly different (P>0.05) compared to control.

Organ	Groups (Asymptotic significant ([P<0.05])									
		A *vs* B	A *vs* C	A *vs* D	A *vs* E	B *vs* C	B *vs* D	B *vs* E	C *vs* D	C *vs* E	D *vs* E
Liver	Inflammation	1.00	1.00	0.01*	0.01*	0.04	0.00	0.02	0.23	0.32	0.56
	Activated Kupffer cells	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	Hydropic degeneration	1.00	0.10	0.00*	0.01*	0.04	0.00	0.02	0.23	0.32	0.56
	Periportal necrosis	1.00	1.00	0.39	0.13	1.00	0.51	0.51	0.39	0.07	0.14
	Midzonal necrosis	1.00	1.00	0.39	0.13	1.00	0.51	0.51	0.39	0.07	0.14

	Centrilobular necrosis	1.00	1.00	0.04*	0.05*	1.00	0.10	0.10	0.07	0.00	0.14
	Mean score	1.00	0.85	0.30	0.23	0.68	0.35	0.36	0.38	0.29	0.42
Kidney	Inflammation	1.00	0.13	0.31	0.10	0.23	0.19	0.14	0.92	0.73	0.78
	Hydropic degeneration/cytoplasmic vacuolation	1.00	0.39	1.00	0.39	0.51	0.51	0.23	1.00	0.33	0.33
	Necrosis	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	Cellular cast	1.00	1.00	0.01*	0.01*	1.00	1.00	0.54	1.00	0.42	0.35
	Granular cast	1.00	0.13	0.01*	0.01*	0.23	0.05	0.03	0.29	0.22	0.88
	Protein cast	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	Mean score	1.00	0.60	0.55	0.55	0.66	0.62	0.49	0.86	0.61	0.72

Table 6: Results of Mann-Whitney U test for comparison between groups of the toxicity lesions in liver and kidney of Sprague Dawley (SD) rats in sub chronic oral toxicity study of ethanol extract of *C. nutans* leaves. (Note: A: Control; B: 10% DMSO; C: Low Dose; D: Medium Dose; E: High Dose (n=8 rats in each groups). The symbol * denotes the values were significantly different (P<0.05) between treated groups and control.)

Conclusion

90 days daily administration of ethanol extract of *C. nutans* leaf induced mild degree of toxicity in liver and kidneys of the rats. High dose (250 mg/kg of body weight) of the extract induced more prominent histopathological lesions. Prolong daily administration (more than 90 days) of the extract will probably induced moderate to severe toxicity in liver and kidney.

Conflict of Interest

The authors declare that thy have no competing interest.

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