Original Article

Hepatotoxicity in acute and subchronic oral gavage of ethanolic extract of Christia vespertilionis (L.f.) Bakh. f. leaf in male sprague dawley rats

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Abstract

Increased interest in *Christia vespertilionis* (L.f.) Bakh. f. leaves is due to their real potential of their health-enhancing properties. This study aims to evaluate the possible *in vivo* toxicity in 14-day acute and 90-day subchronic oral toxicity of ethanolic extract *Christia vespertilionis* (L.f.) Bakh. f. in male Sprague Dawley rats. In the acute study, the rats were divided into control, 5% DMSO (vehicle) and 2000 mg/kg groups. For the subchronic toxicity study, a total of 30 rats were divided into five groups consisting of a control, 5% DMSO (vehicle), low dose (75 mg/kg), medium dose (125 mg/kg) and high dose (250 mg/kg) groups. Rats were analysed for body weight, relative organ weight, haematologically, serum biochemically and histopathologically. The results showed that the single dose and daily dose induced no mortality and haematological changes in male Sprague Dawley rats meanwhile the serum biochemistry results of the acute study showed changes in creatinine kinase (CK) and AST. The histopathology results showed there was significant (p<0.05) hepatic necrosis (mild to moderate) and degeneration (very mild) of liver tissue in the treated group (2000 mg/kg) affecting the periportal area. The same observation was noted in the subchronic study where there were significant histopathological changes in the liver parameters but no significant change on the kidney tissue assessment when comparing the control and treated groups. This study showed significant differences (p<0.05) in hepatic necrosis and activated kupffer cells. The lethal oral dose 50 (LD50) of *Christia vespertilionis* (L.f.) Bakh. f. is greater than 2000 mg/kg and NOAEL is less than 75 mg/kg.

Keywords: *Christia vespertilionis* (L.f.) Bakh. f., LD_{50}, NOAEL, hepatotoxicity, acute, subchronic

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Introduction

Medicinal plants have gained a high level of interest among researchers due to the belief that these sources of medicine are safe and harmless since they originate from nature and have traditionally been used. It has been reported by the World Health Organization (WHO) that many traditional and complementary practitioners often reject medicinal products derived from medicinal plants (WHO, 2015). In both developed and developing countries herbal medicines are considered to provide safer treatment than allopathic or modern medicines because of cultural desirability, accessibility and little expense, besides having originated from natural sources. However, their biological source does not ensure that there are no risks associated with the use of natural products as medicinal products.

Several studies on using plants as alternative sources for curative agents have been established and revised, including the isolation of phytochemical compounds and the structural elucidation of bioactive compounds for the production of medicine that can function as pharmacological component either by using a whole plant or part of it as a herbal remedy. However, some medicinal plants have been used based solely on claims made by practitioners without a clinical trial or demonstration that they were safe to use. There is mounting evidence that many herbal medicines cause substantial toxicity in their consumers (Martin, 2013). Consequently, much greater scientific focus than before is now being given to determine the possible toxicity of herbal medicines. It is important to conduct clinical research on the health of these plants until they can be transformed into a modern alternative medicine. Toxicology evaluation of *Christia vespertilionis* (L.f.) Bakh's f . leaves is very important to get to know and educate the public on their adverse effects particularly on those of us are who are using them as a complement and as a medicine in daily use.

There have been many *in vitro* studies of *Christia vespertilionis* (L.f.) Bakh. f. rather than *in vivo* studies. All *in vitro* studies of this plant have been involved with the cell lines such as MCF-7 (breast cancer), HepG2 (hepatocellular carcinoma), WRL-68 (normal human liver cells), CRL 2522 (normal human skin fibroblast) and HaCat (human epidermal keratinocyte) (Nurliana *et al*., 2019). This plant is also scientifically documented as *Christia vespertilionis* (L.f.) Bakh's f . or the commercially recognized Rerama leaf. This leaf originates from the Papilionaceae family, a genus of Christia. This leaf grows as a perennial in shady tropical forests. Southeastern Asia including Cambodia, Thailand, Vietnam, China, Indonesia, Myanmar and Malaysia are known to be native home of this herb. In Malaysia's markets, this leaf has been commercialized and consumed in tea bag form by healthy people as well as cancer patients. In addition, this leaf is also claimed to have anti-tumour and anti cancer properties (Brach and Song, 2006; Ravindran *et al*., 2019). However, there is still lack of clinical trial on the safe dose and use in the human body other than the clinical trial of 28-days reported by Nurul *et al*. (2018). Various sections of this plant (mainly the leaves) have been reported to be effective in many aspects of *in vitro* treatment study including as anti-plasmodial and for high cytotoxicity to the human cervical cancer cell line (HeLa) and the embryonic lung cell line (MRC5) (Nguyen-Pouplin *et al*., 2007), inhibiting neuroendocrine and hepatic sacroma tumour development (Wu *et al*., 2012; Hofer *et al*., 2013). Although, several pharmacological studies have been performed on this plant (as anti-plasmodial, anti-tumour), there is no experimental evidence of its toxicity. Despite all these studies of the effectiveness on *Christia vespertilionis* (L.f.)Bakh, f., these leaves have been subject to a limited safety test.

Therefore, standardization of protection and quality control measurement has become important to ensure the supply of high-quality medicinal plant materials. Toxicity analysis refers to a substance or a chemical's potential to induce adverse effects in the host (Merlin *et al*., 2019). Certain degrees of toxicity by a chemical substance may harm and affect the whole organism as well as sub-structural aspects of an organism, such as the cells (cytotoxicity) or organ (organotoxicity). Any substance given orally can be harmful or harmless at certain doses or lower doses.Thus, there is a range of possible effects between these two limits, due to subtle long-term subchronic toxicity to immediate lethality (Lee *et al*., 2014;Saganuwan *et al*., 2016; Thomas *et al*., 2017). Toxicity in medicinal plants may be caused by phytotoxins. Phytotoxins are generally referred to as secondary plant compounds or bioactive phytochemical compounds and are often considered to have evolved in mammalian systems. Many of the chemicals that have been shown to be toxic are components of plants that are part of the human diet. The purpose of this research was therefore to evaluate the oral lethal dose (LD_{50}) and the non-observed-adverse-effect level (NOAEL) of ethanolic extract *Christia vespertilionis* (L.f.) Bakh. f. leaves in both acute and subchronic oral toxicity in male Sprague Dawley rats.

Materials and Methods

Materials in the present study are the same as those indicated in the previous subacute toxicity study of leaf extract reported by Nurul *et al*. This study was conducted at the Toxicology Laboratory Animal Metabolism, Toxicology and Reproductive Centre (AMTREC), Malaysia Agricultural Research and Development Institute (MARDI) at Serdang, Selangor. The study was planned and carried out in conjunction with the ethical approval of the Animal Care Unit Committee (ACUC), Malaysia Agricultural Research and Development Institute (MARDI) (Approval Number: 20170717/R/MAEC24).

Collection of Plant Materials and Preparation of ethanolic extract of Christia vespertilionis (L.f.) Bakh. f. leaves: The plant *Christia vespertilionis* (L.f.) Bakh. f. was acquired from the MARDI research center situated in Muadzam Shah, Pahang, Malaysia. The plant was identified and authenticated by the Department of Biodiversity, Institute of Bioscience, Universiti Putra Malaysia and deposited in the herbarium (SK3167/17).

Plant material and leaves part were separated and washed thoroughly with water for the first time to remove dirt and soil deposits. Using soxhlet apparatus by the method of continous hot percolation, 250g of fine ground dry powder of *Christia vespertilionis* (L.f.) Bakh. f. leaves were extracted with absolute 100% ethanol and passed through filter sheet No. 80. The filtered solution resulting from the dark greenish-brown extract was then concentrated at a higher pressure to prepare up to 100 mL as a final crude extract in the Rota evaporator for 1 hour. Each week, the extract used in rats was freshly prepared with a weekly body weight by diluting the concentrated raw extracts with 5% DMSO to form 75 mg / kg, 125 mg / kg, 250 mg / kg and 2000 mg / kg doses. The output yield of the extract was found to be 10%.

Experiemental Design: The average male rats used in this study (Alchemy Supplies Sdn. Bhd.) weighed 170 grams and were 6 weeks old. Male rats are certainly more accurate than female rats to assess chemical toxicological properties, making male rats more likely to be picked. The rats were acclimatized for 5 days and randomized to different groups with similar average body weights. The rats were housed in plastic resin cages with a range of temperatures of 22-27 °C, a humidity range of 40% and a 24-hour consistency of balanceed light and dark. The rats were fed with a standard feed pellets diet (Alchemy Supplies Sdn. Bhd, Seri Kembangan, Malaysia) ad libitum. Acute and subchronic toxicity studies were conducted *in vivo* using the upper and lower dose limit test procedure in accordance with the OECD Test Guidelines. Normal saline was given to rats in the control group with 5 percent Dimethyl Sulfoxide (DMSO) for rats in the vehicle group. The 5 percent DMSO to vehicle group was used as a standard to normalize the results later on since all extract groups were mixed with 5 percent DMSO for extract preparation weekly.

Acute toxicity study: A total of 18 male Sprague Dawley (SD) rats were used for this study. Rats were segregated (n=6) into the control group of vehicles with 5 percent DMSO and 2000 mg / kg extract group. Extracts were administered orally and the subjects were observed for the next 14 days. Animals were closely observed for 2,4,8 and 12 hours after administration of the extract for behavioral changes and signs of toxicity. There was also observation for signs of mortality in each group within 24 hours. Surviving animals were additionally monitored for 14 days for any signs of delayed toxicity. The LD_{50} (medium lethal dose) was assessed in this study. At day 15 of the experiment, rats were humanely euthanized with the inhalation of carbon dioxide (CO_2). Blood and serum were collected via cardiac puncture with 23 gauge needles and 3 mL syringe for further analysis.

Subchronic toxicity study: A total of six rats were systematically selected from a population of 30 rats. The rats were segregated into five groups: control, vehicle (5 percent DMS0), 75 mg / kg, 125 mg / kg and 250 mg / kg extract. The extracts were administered orally continuously for 90 days and given daily (in the morning). Rats were monitored for mortality over a period of 3 months. In addition, surviving animals were observed for 90 days for additional toxicity assessments. The NOAEL (non-observed-adverse-effect level) was estimated in this study. At day 91, rats were humanely euthanized with carbon dioxide (CO2) inhalation. Blood and serum were collected via cardiac puncture with 23 gauge needles and 3 mL syringe for further analysis.

Haematology analysis: Blood samples were collected using the EDTA tube. Later, the blood was processed within 12 hours of collection. An automated haematology analyzer (Cell Dyn ® 3700, Abbott Diagnostics, USA) was used to analyze the total number of white blood cells (WBCs), red blood cells (RBCs) and haemoglobin (HB) concentrations. The WBC difference count was conducted manually by numbering a total of 100 WBCs on the peripheral blood stained with Wright stain. In addition, packed cell volume (PCV), icterus index and plasma protein concentration were manually prepared and calculated using standard methods In this study, the icterus index was the measurement of the plasma's yellow colour.This colour is normally and almost exclusively due to the presence of bilirubin, a hemoglobin waste product from the red blood cells. In addition, there was manual calculation of mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) using the standard formula, MCV= (PCV x 1000)/RBC and MCHC = Hb / PCV.

Serum biochemistry analysis: Blood samples were collected and dispensed into a plain tube. To obtain the serum, blood tubes were centrifuged (Centrifuge S417R, Eppendorf, CA) immediately to prevent blood lysis for 15 minutes at 3000 rpm. The serum obtained was further analyzed within 24 hours of collection using an automated clinical chemistry analyser (TRX 7010, Biorex Mannheim, Germany) for both liver and kidney parameters. Kidney parameters include creatinine and urea, while liver parameters include the total bilirubin, total protein (TP), albumin (ALB), globulin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and creatine kinase (CK).

Histopathological analysis: For histopathological analysis, the selected organs of each rat included liver, kidneys, spleen and testicles in chilly [pH 4] standard salt water for a while (a few seconds) just to be washed from blood. Later, organs were trimmed of fat, weighed and checked for gross lesions. Organs were then fixed in a 10% buffered solution of formalin. The tissue fixation process lasted 24 hours in a clean 10% formalin solution. After 24 hours, 10% of the formalin solution was changed in each sample collection tube. Organ samples were processed at the Histopathology Laboratory, Faculty of Veterinary Medicine, UPM, Serdang. All specimens were cut to about 0.5 cm thick. The size of each specimen was the same in diameter and thickness. Samples were then placed in labeled cassettes. Afterward, the cassettes were inserted into a 10% formalin solution overnight, before being positioned in an automated processor (Leica ASP300,

Germany) and undergoing a series of tissue processing steps for approximately 16 hours. By using the processor machine (Leica EG1160, Germany), samples were then embedded with paraffin to form a block. Samples were trimmed using rotary microtome cutters (Leica RM2155, Germany) of approximately 3-5 µm thickness The cut tissues were directly placed in a 45 ° C water bath prior to slide mounting. All glass slides were labeled with a diamond pen and mounted on a hot plate (54 ° C) overnight to remove the paraffin from the tissue sections. Later on, all the slides underwent a series of steps for the Haematoxylin and Eosin (H&E) staining protocol. After staining, the slides were mounted with DPX Mountant (Sigma-Aldrich, United States) and covered with glass slip. The samples were finally evaluated using a standard light microscope under 10X, 20X, 40X, 60X and 100X magnification.

Lesion characteristics: In both present studies, basic five histological lesions in liver tissues were evaluated and scored including, inflammation, activated kupffer cells, hydropic degeneration, regeneration and necrosis. Meanwhile, in the renal tissue, histological lesions such as cellular cast, granular cast, protein cast, inflammation, hydropic degeneration and necrosis were evaluated and were scored. Lesions scored in both liver and kidney tissues were reported and scored as 0 (normal), 0.5 (very mild), 1.0 (mild), 1.5 (mild to moderate), 2 (moderate), 2.5 (moderate to severe) and 3.0 (severe) as referred to in Nurul *et al*., (2018). Lesions were scored individually for each sample on the basis of the percentage of lesions present in the tissues. Detailed descriptions of the method of scoring lesions are presented in Table 1. In addition, the percentage of lesions was calculated and converted to the area concerned as shown in the tabulated results.

Table 1 Lesion scores and percentage of area affected (mm^2)

Score	Percentage lesion	Actual size of lesion (mm^2)
0	0	0
0.5	Less than 15%	33.75
1	15-30%	33.76-67.4
1.5	30-45%	67.5-101.24
2	45-60%	101.25-134.99
2.5	60-75%	135-168.74
3	More than 75%	168.75-225

Statistical Analysis: Weekly body weight, haematological , serum biochemical parameters and results of relative organ weight are expressed as mean±SEM. The data was analysed using one-way ANOVA and post-hoc analysis with Turkey HSD test using statistical analysis software, IBM SPSS Statistic 22.0. Meanwhile, the semi-quantitative results of histopathology, were analysed using Kruskal-Wallis test for global comparison all groups for all parameters and expressed as mean±SEM. The non-parametric Mann-Whitney-U test was used to compare the two lesion scores between the control and the vehicle groups and the extract (significant P-value was presented as superscript in the table).

Results

Body Weight: The average body weight of rats in the oral toxicity study of *Christia vespertilionis* (L.f.) Bakh. f. extract that was taken for 2 weeks are presented in Table 2. Rats in groups A, B and C continued to gain weight from week 1 to week 2. Based on daily oral gavage, there were also no significant changes in body weight of all the rats in subchronic study (Table 3) All animals displayed a normal increase in average weekly body weight without significant difference between both the control and the treated groups although, after the oral administration of ethanol leaf extracts of *Christia vespertilionis* (L.f.) Bakh. f. insignificant increases in body weight of rats indicated that the administration of the extract did not affect the development of the animals.

Table 2 Effect of ethanol leaf extract of *Christia vespertilionis* (L.f.) Bakh. f. on the body weight (Mean ±SEM) of rats at 14 days

	Group		
Week	**Control**	**Vehicle**	**2000 mg/kg of *Christia vespertilionis* (L.f.) Bakh. f.**
1	160±2.20 [a]	165±3.15 [a]	167±1.28 [a]
2	199±1.45 [a]	193±5.7 [a]	195±2.19 [a]

Notes: Values in the same row with similar superscripts were not significantly different at $p>0.05$. The superscript represents the post-hoc test from ANOVA comparing 3 groups.

Table 3 Effect of ethanol leaf extract of *Christia vespertilionis* (L.f.) Bakh. f. on the body weight (Mean ±SEM) of rats at 90 days

Week	Group				
	Control	Vehicle	Low Dose	Medium Dose	High Dose
1	170±4.98 [a]	166±6.82 [a]	164±3.05 [a]	171±7.54 [a]	168±4.69 [a]
2	174±19.21 [a]	190±4.69 [a]	186±6.47 [a]	200±6.20 [a]	206±6.33 [a]
3	210± 22.49 [a]	216±3.46 [a]	205±6.98 [a]	221±7.93 [a]	234±6.34 [a]
4	234±23.24 [a]	237±4.18 [a]	229±6.65 [a]	241±9.07 [a]	255±6.89 [a]
5	252±26.68 [a]	255±5.61 [a]	244±5.72 [a]	254±11.46 [a]	272±8.84 [a]
6	265±27.97 [a]	264±5.64 [a]	254±5.40 [a]	266±12.94 [a]	288±10.85 [a]
7	273±18.34 [a]	277±6.09 [a]	272±6.73 [a]	273±12.90 [a]	299±11.92 [a]
8	304±31.07 [a]	301±5.94 [a]	286±6.95 [a]	300±11.81 [a]	317±7.02 [a]
9	329±34.16 [a]	325±14.46 [a]	303±7.20 [a]	305±13.32 [a]	336±13.65 [a]
10	320±30.07 [a]	320±11.07 [a]	305±9.30 [a]	315±11.23 [a]	343±13.03 [a]
11	342±35.53 [a]	334±10.69 [a]	320±6.38 [a]	329±13.43 [a]	358±12.67 [a]
12	344±32.67 [a]	336±9.38 [a]	319±6.25 [a]	332±13.92 [a]	357±10.35 [a]
13	346±29.76 [a]	337±12.96 [a]	322±6.93 [a]	335±14.77 [a]	356±8.11 [a]

Values in the same row with similar superscripts were not significantly different at $p>0.05$. The superscript represents the post-hoc test from ANOVA comparing all groups.

Mortality: No mortality was observed at 2000 mg/kg body of ethanolic leaf extract of *Christia vespertilionis* (L.f.) Bakh. f. in male Sprague Dawley rats. Furthermore, no mortality was observed in groups which were daily administrated with extracts at 75mg/kg, 125 mg/kg and 250 mg/kg extract body weight of *Christia vespertilionis* (L.f.) Bakh. f. in male Sprague Dawley rats.

LD_{50} Value: The LD_{50} value of ethanol leaf extract of *Christia vespertilionis* (L.f.) Bakh. f., calculated from Acute Oral Toxicity (Guideline 425) was found to be more than 2000 mg/kg body weight.

Haematology analysis: Based on Tables 4 and 5, there was no significant difference in the haemogram parameters. Not all dose levels studies affected PCV values. There was also no significant difference in concentrations of haemoglobin , RBC, MCV, icterus index and plasma protein at all dose levels in both studies. There was also no significant difference in WBC counts and leukocytes parameters All values were still within the reference ranges.

Table 4 The haematology values (mean±SEM) of Sprague Dawley rats in acute oral toxicity study of ethanolic extract of *Christia vespertilionis* (L.f.) Bakh. f.

Parameter/Group	Control	Vehicle	2000 mg/kg of *Christia vespertilionis* (L.f.) Bakh. f.
RBC (10^{12} /L)	8.29 ± 0.47[a]	8.25 ± 0.27 [a]	8.43 ± 0.17 [a]
Hb (g/L)	149.5 ± 1.78 [a]	151.79 ± 2.60 [a]	149.83 ± 3.66 [a]
PCV (L/L)	0.48 ± 0.02 [a]	0.42 ± 0.02 [a]	0.36 ± 0.02 [a]
MCV (fL)	63.33 ± 1.23 [a]	62.67 ± 0.42 [a]	61.83 ± 1.14 [a]
MCHC (g/L)	283.83 ± 1.92 [a]	290.33 ± 1.36 [a]	287.50 ± 2.63 [a]
WBC (× 10^9/L)	6.10±0.72 [a]	6.17 ±0.73 [a]	6.92 ± 0.90 [a]
Neutrophils (× 10^9/L)	0.93 ± 0.01 [a]	1.02 ± 0.01 [a]	1.12 ± 0.01 [a]
Lymphocytes (× 10^9/L)	4.51 ± 0.06 [a]	4.62 ± 0.07 [a]	5.11 ± 0.09 [a]
Monocytes (× 10^9/L)	0.25 ±0.03 [a]	0.15 ± 0.02 [a]	0.10 ±0.06 [a]
Eosinophils (× 10^9/L)	0.22 ± 0.02 [a]	0.12 ± 0.03 [a]	0.10 ±0.05 [a]
Basophils (× 10^9/L)	0.16 ± 0.03 [a]	0.19 ± 0.02[a]	0.22± 0.04 [a]
Icterus Index	2.00 ± 0.00 [a]	2.00 ± 0.00 [a]	2.00 ± 0.00 [a]
Plasma protein (g/L)	77.5 ± 0.50 [a]	76.83 ± 0.75 [a]	76.33 ± 0.76 [a]

Notes: Values in the same row with similar superscripts were not significantly different at $p>0.05$. The superscript represents the post-hoc test from ANOVA comparing 3 groups.

Table 5 The haematology values (mean±SEM) of Sprague Dawley rats in subchronic oral toxicity study of ethanolic extract of *Christia vespertilionis* (L.f.) Bakh. f.

Parameter/Group	Control	Vehicle	Low Dose	Medium Dose	High Dose
RBC (10^{12} /L	7.01 ± 1.19[a]	7.03 ± 1.30 [a]	9.08 ± 0.57 [a]	7.92 ± 1.25 [a]	9.00 ± 0.19[a]
Hb (g/L)	133.33 ± 22.29[a]	136.67 ± 23.20 [a]	171.50 ± 9.61 [a]	152.83 ± 24.76 [a]	166.33 ± 3.49[a]
PCV (L/L)	0.31 ± 0.03 [a]	0.34 ± 0.02[a]	0.38 ± 0.03 [a]	0.41 ± 0.02 [a]	0.39 ± 0.01 [a]
MCV (fL)	59.16 ± 0.60 [a]	58.33±0.71 [a]	59.33±0.67 [a]	59.00±1.03 [a]	58.17±0.48 [a]
MCHC (g/L)	323.33 ± 3.37 [a]	335.67±8.54[a]	319.50±2.31[a]	324.50±3.06[a]	317.83±3.65 [a]
WBC (× 10^9/L)	5.82 ± 1.41 [a]	4.60 ± 1.44 [a]	7.60 ± 1.41 [a]	6.07 ± 1.45 [a]	6.55 ± 1.57 [a]
Neutrophils (× 10^9/L)	0.77 ± 0.07 [a]	0.59 ± 0.04 [a]	0.45 ± 0.01 [a]	0.69 ± 0.01 [a]	0.72 ± 0.01 [a]
Lymphocytes (× 10^9/L)	4.64 ± 0.01 [a]	3.66 ± 0.0.01 [a]	6.08 ± 0.01 [a]	4.97 ± 0.01 [a]	5.43 ± 0.01 [a]
Monocytes (× 10^9/L)	0.16 ± 0.02 [a]	0.12 ± 0.03[a]	0.26 ± 0.04[a]	0.17 ± 0.04 [a]	0.13 ± 0.04[a]
Eosinophils (× 10^9/L)	0.07 ± 0.04 [a]	0.09 ± 0.04[a]	0.16 ± 0.04[a]	0.11 ± 0.04[a]	0.11 ± 0.03[a]
Basophils (× 10^9/L)	0.07 ± 0.02 [a]	0.05 ± 0.02[a]	0.10 ± 0.03[a]	0.04 ± 0.03[a]	0.08 ± 0.02[a]
Icterus Index	2.00 ± 0.00 [a]	2.00 ± 0.00 [a]	2.00 ± 0.00 [a]	2.00 ± 0.00 [a]	2.00 ± 0.00 [a]
Plasma protein (g/L)	80.50 ± 0.86 [a]	75.67 ± 1.310 [a]	75.17 ± 1.83 [a]	78.33 ± 1.20 [a]	76.67 ± 2.04 [a]

Values in the same row with similar superscripts were not significantly different at $p>0.05$. The superscript represents the post-hoc test from ANOVA comparing all groups.

Serum biochemistry analysis: Serum biochemical parameters were grouped as kidney parameters (urea, creatinine) and liver parameter (total protein, albumin, globulin, creatinine kinase (CK), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) are presented in Tables 6 and 7. Regarding the renal parameters, there was no significant difference between the control, vehicle and treated rats in this study. There was no significant difference in kidney parameters measured except for the CK and AST parameters in the acute study, which showed significant differences between the control group in the vehicle and the treated groups. In the meantime, there was no significant difference in serum biochemistry analysis of both liver and kidney parameters in the subchronic study

Table 6 The serum biochemical parameters (mean±SEM) of Sprague Dawley rats in acute oral toxicity study of ethanolic extract of *Christia vespertilionis* (L.f.) Bakh. f..

Parameter/Group	Control	Vehicle	2000 mg/kg of *Christia vespertilionis* (L.f.) Bakh. f.
Urea (mmol/L)	5.87 ± 0.38[a]	5.53 ± 0.19[a]	6.37 ± 0.48[a]
Creatinine (µmol/L)	60.33 ± 1.98[a]	65.33 ± 1.26[a]	62.50 ± 0.99[a]
CK (U/L)	400.33 ± 76.91[b]	266.67 ± 25.36[a]	258.50 ± 27.87[a]
ALT (U/L)	61.98 ± 5.35[a]	50.02 ± 3.00[a]	54.57 ± 3.66[a]
ALP(U/L)	276.83 ± 17.61[a]	258.33 ± 17.34[a]	227.83 ± 9.22[a]
AST (U/L)	154.13 ± 15.54[b]	99.88 ± 7.25[a]	104.83 ± 4.33[a]
Total protein (g/L)	75.12 ± 0.82[a]	75.07 ± 2.14[a]	74.90 ± 1.95[a]
Albumin (g/L)	35.18 ± 0.87[a]	36.43 ± 0.71[a]	35.38 ± 1.84[a]
Globulin (g/L)	39.94 ± 0.05 [a]	38.64 ± 1.40 [a]	39.52 ± 0.11[a]

Notes: Values in the same row with similar superscripts were not significantly different at *p>0.05.* . The superscript represents the post-hoc test from ANOVA comparing 3 groups.

Table 7 The serum biochemical parameters (mean±SEM) of Sprague Dawley rats in subchronic oral toxicity study of ethanolic extract of *Christia vespertilionis* (L.f.) Bakh. f .

Parameter/Group	Control	Vehicle	Low Dose	Medium Dose	High Dose
Urea (mmol/L)	6.03 ± 0.58[a]	6.33 ± 0.12[a]	6.77 ± 0.39[a]	6.18 ± 0.16[a]	6.78 ± 0.31[a]
Creatinine (µmol/L)	68.00 ± 2.13[a]	66.00 ± 1.88[a]	65.33 ± 0.80[a]	65.00 ± 1.34[a]	71.67 ± 3.21[a]
CK (U/L)	426.17 ± 91.77[a]	318.33 ± 29.07[a]	521.83 ± 86.89[a]	387.67 ± 48.79[a]	496.00 ± 93.81[a]
ALT (U/L)	89.35 ± 17.52[a]	111.15 ± 30.23[a]	96.40 ± 12.01[a]	65.75 ± 7.16[a]	69.97 ± 6.14[a]
ALP(U/L)	147.83 ± 8.91[a]	124.17 ± 5.97[a]	134.83 ± 6.56[a]	124.50 ± 4.55[a]	127.83 ± 6.94[a]
AST (U/L)	151.07 ± 16.57[a]	176.28 ± 30.15[a]	280.83 ± 56.93[a]	221.17 ± 40.45[a]	180.52 ± 18.38[a]
Total protein (g/L)	64.47 ± 0.87[a]	69.80 ± 1.15[a]	73.75 ± 1.41[a]	71.15 ± 1.15[a]	74.97 ± 2.03[a]
Albumin (g/L)	30.57 ± 0.67[a]	34.45 ± 1.17[a]	33.20 ± 1.28[a]	30.30 ± 0.88[a]	31.72 ± 0.75[a]
Globulin (g/L)	33.90 ± 0.20[a]	35.35 ± 0.02[a]	40.55 ± 0.13[a]	40.85 ±0.27[a]	43.25 ± 1.28[a]

Values in the same row with similar superscripts were not significantly different at *p>0.05.* The superscript represents the post-hoc test from ANOVA comparing all groups.

Organ Weight: The relative weight of specific organs in the acute oral toxicity study are shown in Tables 8 and 9, respectively. There was no significant difference (p>0.05) in the relative weight of the liver, spleen, kidneys and testicular organs either acute or subchronic studies.

Table 8 The organs relative weights (mean±SEM) of Sprague Dawley rats in acute oral toxicity study of ethanol extract of *Christia vespertilionis* (L.f.) Bakh. f.

Organ/Group	Control	Vehicle	2000 mg/kg of *Christia vespertilionis* (L.f.) Bakh. f.
Spleen	0.57 ± 0.03[a]	0.50 ± 0.05[a]	0.51 ± 0.06[a]
Liver	7.41 ± 0.61[a]	7.85 ± 0.62[a]	6.37 ± 0.22[a]
Kidneys	1.65 ± 0.13[a]	1.62 ± 0.08[a]	1.39 ± 0.04[a]
Testes	2.29 ± 0.11[a]	2.88 ± 0.09[a]	2.54 ± 0.08[a]

Notes: Values in the same row with similar superscripts were not significantly different at *p>0.05.* The superscript represents the post-hoc test from ANOVA comparing 3 groups.

Table 9 The organs relative weight (mean±SEM) of SD rats in subchronic oral toxicity study of ethanol extract of*Christia vespertilionis* (L.f.) Bakh. f..

Organ/Group	Control	Vehicle	Low Dose	Medium Dose	High Dose
Liver	10.69 ± 1.16[a]	11.32 ± 0.45[a]	9.60 ± 0.50[a]	10.55 ± 0.73[a]	11.01 ± 0.57[a]
Kidneys	2.28 ± 0.28[a]	2.20 ± 0.04[a]	2.16 ± 0.11[a]	2.62 ± 0.13[a]	2.97 ± 0.12[a]
Lungs	2.25 ± 0.31[a]	2.77 ± 0.26[a]	2.17 ± 0.11[a]	2.61 ± 0.16[a]	2.66 ± 0.14[a]
Heart	1.03 ± 0.11[a]	1.00 ± 0.05[a]	0.91 ± 0.05[a]	1.07 ± 0.08[a]	1.24 ± 0.03[a]
Brain	1.52 ± 0.04[a]	1.56 ± 0.03[a]	1.51 ± 0.06[a]	1.63 ± 0.02[a]	1.59 ± 0.02[a]
Testes	3.20 ± 0.17[a]	3.18 ± 0.26[a]	3.28 ± 0.13[a]	3.07 ± 0.07[a]	3.60 ± 0.21[a]
Spleen	0.65 ± 0.06[a]	0.52 ± 0.03 [a]	0.61 ± 0.04[a]	0.68 ± 0.02[a]	0.85 ± 0.04[a]

Value in the same column with similar superscripts were not significantly different at *p>0.05.* The superscript represents the post-hoc test from ANOVA comparing all groups.

Histopathology: In the acute study, the histological evaluation of livers of treated rats with the *Christia vespertilionis* (L.f.) Bakh. f. leaves extract revealed some abnormalities. There were significant differences (p<0.05), tested by Kruskal Wallis in lesions observed for necrosis and regeneration liver tissue in the treated group (2000 mg/kg)**.** Similar results were observed when the same data was tested using Mann Whitney U test for comparison between the two groups (treated 2000mg/kg group to control group) presented by superscripts (Table 10). Due to the broad generalized pattern of necrosis and lesions scores in liver tissues in these studies, the liver was scored based on the area affected. The acute study shows that the affected area in the liver tissues was significant in the periportal area (Table 11).

Table 10 Lesion scores of liver and kidneys of Sprague Dawley rats in acute oral toxicity

Organ	Mean score of lesions	Control	Vehicle	2000 mg/kg of *Christia vespertilionis* (L.f.) Bakh. f.	Kruskal Wallis Test for global comparison of organ lesions among groups. Asymptotic significant (p<0.05)
	Scored and Percentage of area affected (mm2)				
Liver	**Degeneration (vacuolated cytoplasm)**	0[a]	0[a]	45.22±14.17[b]	0.08
	Necrosis (karyolysis and eosinophilic cytoplasm)	0 [a]	0 [a]	101.24±18.22 [b]	**0.01***
	Pyknotic cell	0 [a]	0 [a]	5.74±5.74 [a]	0.37
	Regeneration	0 [a]	0 [a]	33.75±7.43 [a]	**0.03***
	Inflammation	0 [a]	0 [a]	33.75±7.43 [a]	0.37
	Activated Kupffer cell	0 [a]	0 [a]	11.14±7.09 [a]	0.12
	Mean score	0	0	48.59±13.50 [b]	0.16
Kidney	Granular cast	0 [a]	0 [a]	0 [a]	1.00
	Cellular cast	0 [a]	0 [a]	0 [a]	1.00
	Protein cast	0 [a]	0 [a]	0 [a]	1.00
	Pyknotic cell	0 [a]	0 [a]	0 [a]	1.00
	Inflammation	0 [a]	0 [a]	0 [a]	1.00
	Hydropic degeneration	0 [a]	0 [a]	0 [a]	1.00
	Mean score	0	0	0	1.00

Notes: Values in the same row with similar superscripts were not significantly different at $p>0.05$. The superscript represents the post-hoc test from ANOVA comparing 3 groups. Note: 0 mm^2: none, < 33.75 mm^2: less than 15%, < 67.49 mm2: 15~30%, < 101.24 mm^2: 30~45%, < 134.99 mm^2: 45~60%, < 168.74 mm^2: 60~75% and > 168.74 mm^2: more than 75%.

Table 11 Lesion scores based on area affected in liver of Sprague Dawley rats in acute oral toxicity study of ethanolic extract of *Christia vespertilionis* (L.f.) Bakh. f.

Organ	Area	Control	Vehicle	2000 mg/kg of *Christia vespertilionis* (L.f.) Bakh. f.	Kruskal Wallis Test for global comparison of organ lesions among groups Asymptotic significant (p<0.05)
	Scored and Percentage of area affected (mm2)				
Liver	Centrilobular	0.00±0.00[a]	0.00±0.00 [a]	11.14±7.43 [a]	0.12
	Midzonal	0.00±0.00 [a]	0.00±0.00 [a]	11.14±7.43 [a]	0.12
	Periportal	0.00±0.00 [a]	0.25±0.25 [a]	126.55±25.31[b]	**0.01***
	Mean	0.00±0.00	0.00±0.00	43.20±15.52	0.08

Notes: Values in the same row with similar superscripts were not significantly different at $p>0.05$. The superscript represents the post-hoc test from ANOVA comparing 3 groups.

Liver histological examination of treated rats with the *Christia vespertilionis* (L.f.) Bakh. f. leaf extract also revealed some abnormalities. Significant differences (p<0.05) were observed for necrosis and regeneration of liver tissue in the treated group (75mg / kg, 125mg / kg and 250mg / kg) in the subchronic oral toxicity study. Subchronic toxicity study results showed significant differences (p<0.05) in hepatic necrosis and activated kupffer cells. Mild to moderate hepatic necrosis was observed in both high and medium dose groups (Table 12), while only mild lesions were observed in the low dose group. On the other hand, the number of activated kuffer cells was significantly higher (p<0.05) in the low and the medium dose groups compared to the high dose group (Table 12). Also, the same as cases in acute study, due to the generalized pattern of necrosis and lesions score in liver tissues, the livers were scored based on the area affected. For percentage of affected area, it was revealed that high dose groups had significant (p<0.05) extension coverage from the periportal towards the centrilobular area. Comparing low dose and medium dose groups (Table 13) both had lesion coverage from the periportal towards the midzonal areas. The results suggested that *Christia vespertilionis* (L.f.) Bakh. f. extract induces dose-dependent subchronic oral hepatotoxicity in rats. As hepatic necrosis was predominantly seen compared to hepatic necrosis and hepatic degeneration in subacute toxicity studies of this leaf extract, it is suggested that subchronic toxicity study of *Christia vespertilionis* (L.f.) Bakh. f. extract induces more permanent damage to the hepatocytes compared to previous subacute (28days) studies.

Table 12 Lesion scores in mm^2 of liver and kidneys of Sprague Dawley rats in subchronic oral toxicity study of ethanolic extract of *Christia vespertilionis* (L.f.) Bakh. f.

Organ	Mean score of lesions	Control	Vehicle	Low Dose	Medium Dose	High Dose	Kruskal Wallis Test for global comparison of organ lesions among groups Asymptotic significant (p<0.05)
Liver	Degeneration (vacuolated cytoplasm)	0.00±0.00 [a]	0.00±0.00 [a]	33.75±0.32 [a]	33.75±0.32 [a]	28.35±0.20 [a]	0.14
	Necrosis (karyolysis and eosinophilic cytoplasm)	0.00±0.00 [a]	0.00±0.00 [a]	72.22±0.25[b]	95.84±0.38[b]	112.72±0.29[b]	**0.02***
	Pyknotic cells	0.00±0.00 [a]	0.00±0.00 [a]	28.35±0.66 [a]	33.75±0.77 [a]	50.55±0.82 [a]	0.17
	Regeneration	0.00±0.00 [a]	0.00±0.00 [a]	67.4±0.00 [a]	78.97±0.61 [a]	67.4±0.00 [a]	0.06
	Inflammation	0.00±0.00 [a]	0.00±0.00 [a]	22.28±0.52 [a]	22.28±0.52 [a]	11.48±0.41 [a]	0.33
	Activated Kupffer cells	0.00±0.00 [a]	0.00±0.00 [a]	84.37±0.61[b]	67.40±0.77[b]	50.55±0.82[a]	**0.01***
	Mean score	0	0	49.20±0.34	49.88±0.56	53.25±0.42	0.
Kidney	Granular cast	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	1.00
	Cellular cast	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	1.00
	Protein cast	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	1.00
	Pyknotic cell	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	1.00
	Inflammation	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	1.00
	Hydropic degeneration	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	1.00
	Mean score	0	0	0	0	0	1.00

Value in the same column with similar superscripts were not significantly different at $p>0.05$. The superscript represents the post-hoc test from ANOVA comparing all groups.
Note: 0 mm^2: none, < 33.75 mm^2: less than 15%, < 67.49 mm2:
15~30%, < 101.24 mm^2: 30~45%, < 134.99 mm^2: 45~60%, < 168.74 mm^2: 60~75% and > 168.74 mm^2: more than 75%.

Table 13 Lesion scores based on the area affected in the liver of Sprague Dawley rats in subchronic oral toxicity study of ethanolic extract of *Christia vespertilionis* (L.f.) Bakh. f.

Organ	Area	Control	Vehicle	Low Dose	Medium Dose	High Dose	Kruskal Wallis Test for global comparison of organ lesions among groups Asymptotic significant (p<0.05)
Liver	**Centrilobular**	0.00±0.00 [a]	0.00±0.00 [a]	16.88±0.27 [a]	22.28±0.41 [a]	33.75±0.32[b]	**0.01***
	Midzonal	0.00±0.00 [a]	0.00±0.00 [a]	33.75±0.22 [a]	33.75±0.22[b]	45.16±0.52[b]	**0.04***
	Periportal	0.00±0.00 [a]	0.00±0.00 [a]	67.40±0.77[b]	95.84±0.20[b]	101.24±0.32 [b]	**0.01***
	Mean	0.00±0.00	0.00±0.00	39.09±0.42	37.07±0.30	57.96±0.21	0.02*

Value in the same column with similar superscripts were not significantly different at $p>0.05$. The superscript represents the post-hoc test from ANOVA comparing all groups.
Note: 0 mm^2: none, < 33.75 mm^2: less than 15%, < 67.49 mm2:
15~30%, < 101.24 mm^2: 30~45%, < 134.99 mm^2: 45~60%, < 168.74 mm^2: 60~75% and > 168.74 mm^2: more than 75%.

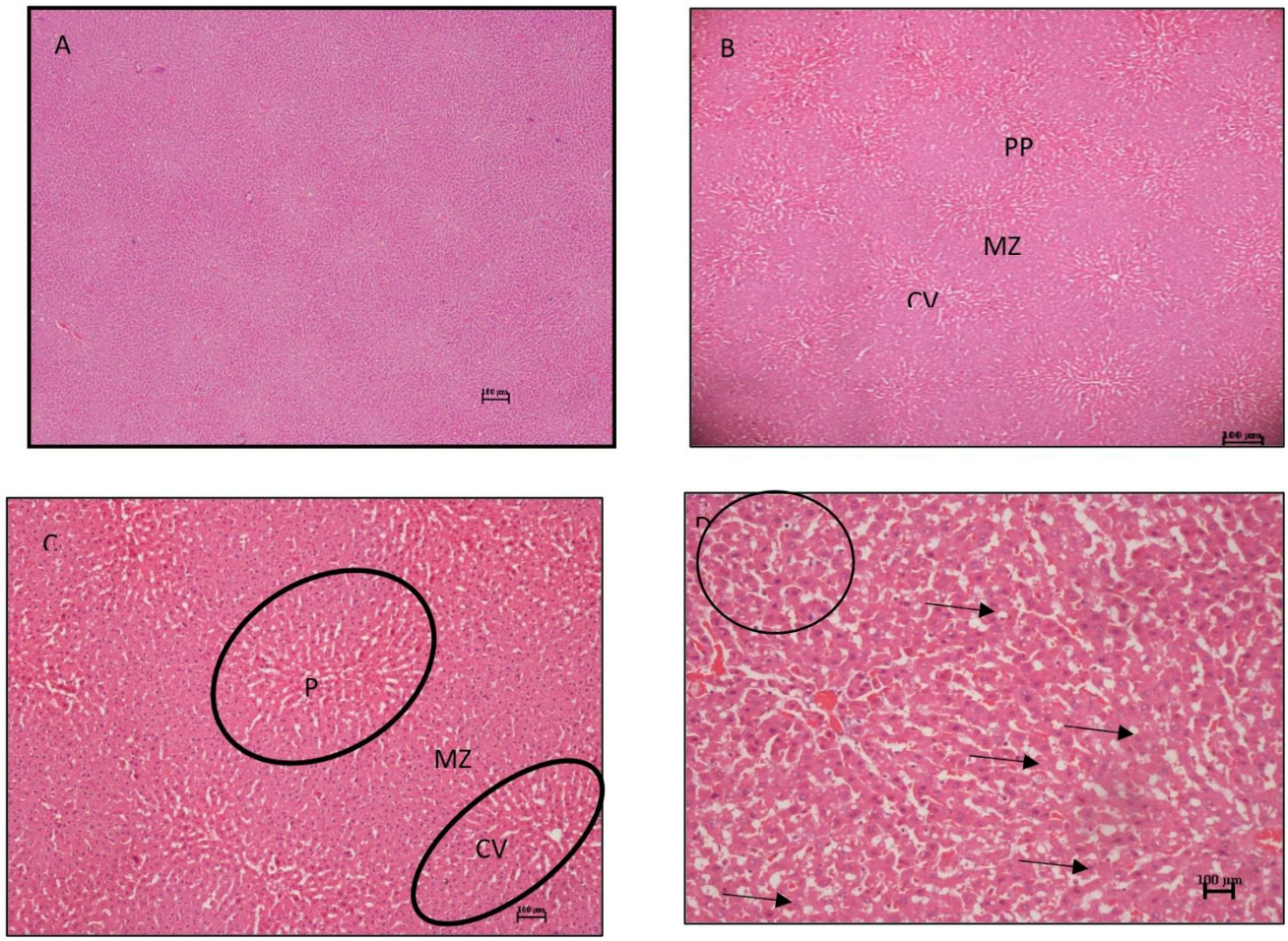

Figure 1 A: Photomicrograph of liver section of a control in acute toxicity study of ethanolic extract of *Christia vespertilionis* (L.f.) Bakh. f. sacrificed at the end of study period (x40).Figure B: Photomicrograph of liver section of a rat in acute toxicity admistrated with 2000mg/kg body weight sacrified at the end of study period showing necrosis at both periportal and centrilobular areas (encircled)(x40). Figure C: Photomicrograph of liver section of a rat in acute toxicity study with extract at 2000 mg/kg body weight, showing necrosis at the periportal and centrilobular area but midzonal area was not affected (x100).Figure D: Photomicrograph of liver section of a rat in acute toxicity study administrated with 2000 mg/kg body weight showing sinusoid dilatation with atrophied hepatocytes and degeneration (cytoplasmic vacuolation) (arrow) (x200)(H&E stain).

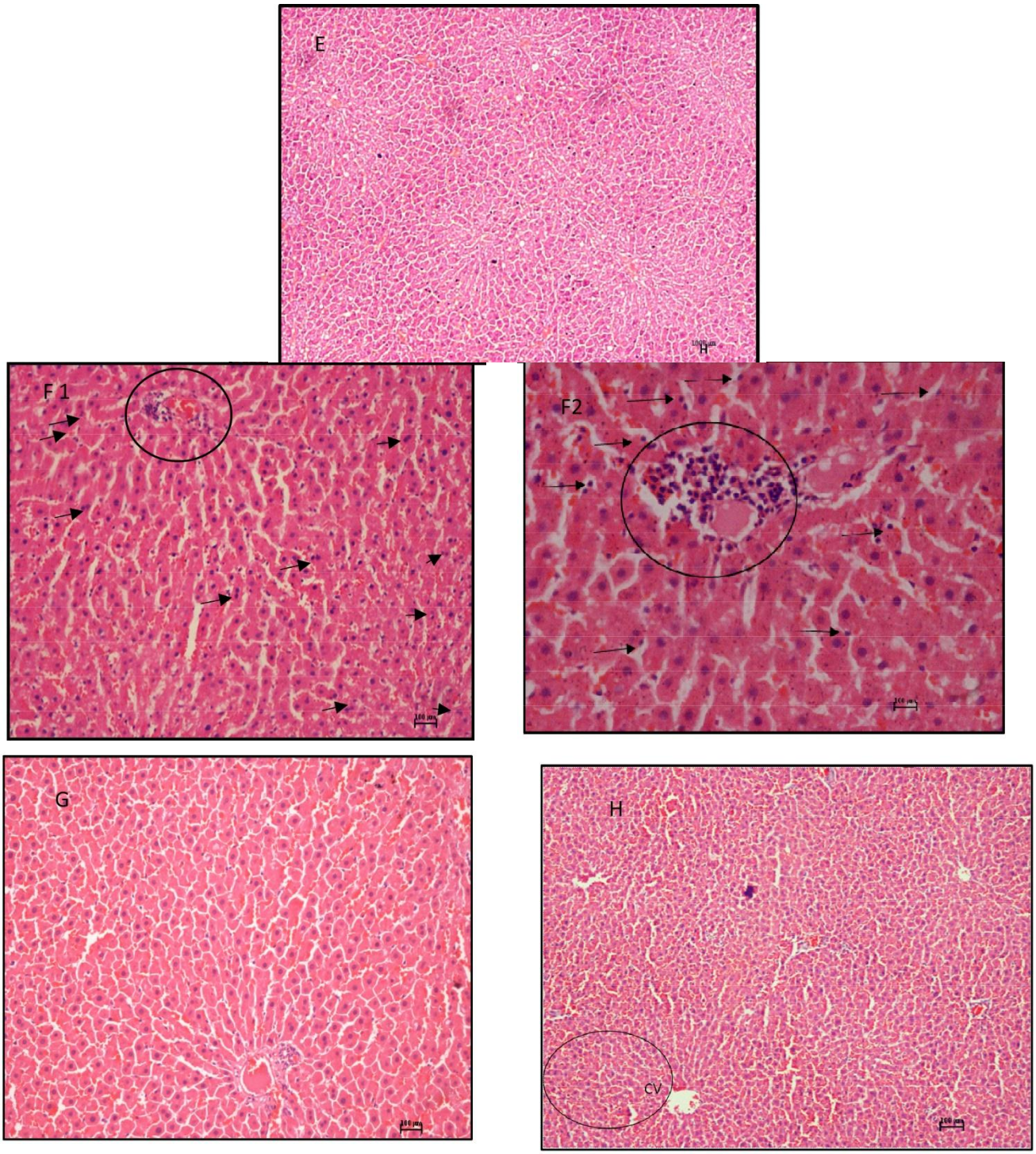

Figure 2 E: Photomicrograph of liver section of a rat in control group of subchronic toxicity study of ethanolic extract of *Christia vespertilionis* (L.f.) Bakh. f. (x40).Figure F : Photomicrograph of liver section of a rat in low dose group of subchronic toxicity study showing inflammatory cells (encircled) at periportal and regeneration (arrow) at periportal-midzonal area, picture 2 is the focus (encircled) of picture 1(x200, x400).Figure G: Photomicrograph of liver section of a rat in high dose group in subchronic toxicity study showing necrosis at periportal area to midzonal area, showing atrophied of hepatocytes (encircle) and karyolysis (arrow) (x400).Figure H: Photomicrograph of liver section of a rat in high dose group in subchronic toxicity study showing necrosis in all three areas, showing atrophied of hepatocytes (encircle) and karyolysis (arrow)(x200) (H&E stain).

The results of histology changes in the kidney tissues for acute and subchronic studies showed no significant change (p>0.05). This histology of the kidneys was the same as in subacute study of *Christia vespertilionis* (L.f.) Bakh. f. leaf extract in previous studies.

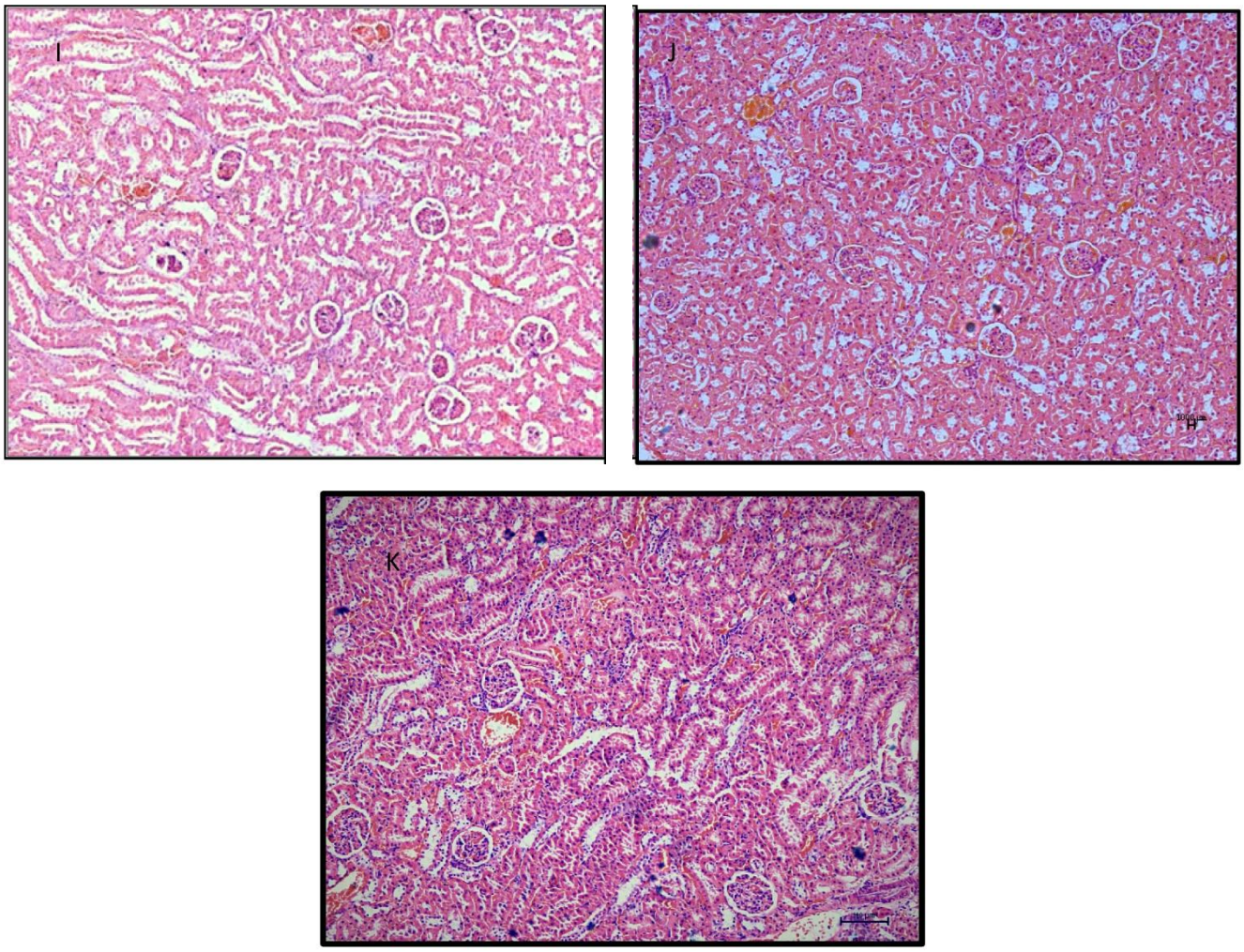

Figure 3 I: Photomicrograph of normal kidney section of a rat in acute study toxicity *Christia vespertilionis* (L.f.) Bakh. f. (H&E stain, x100).Figure J: Photomicrograph of normal kidney section of a rat in medium dose subchronic study toxicity (x200). Figure K: Photomicrograph of normal kidney section of a rat in high dose subchronic study toxicity (x100)(H&E stain)

Discussion

Phytomedicines as an alternative medicine derived from herbs are used in many countries nowadays. Despite the use of herbal medicine as an alternative source of medicine being wide- spread worldwide, there have been very few studies to provide knowledge about their efficacy and safety as well as the effectiveness of herbals used by consumers(Jaspreet *et al.,* 2017). Toxicological assessment, therefore, through toxicity studies, plays a vital role in the testing of herbal products as a way of confirming their safety and efficacy. To date, *in vivo* animal testing in experimental design is considered to be the gold standard in herbal toxicity testing to be applied to human physiology, because of time and ethical and experimental costs. Specific guidelines for toxicological assessment include the dose and time in the animal design to be considered for the human dose. In the present study, acute with single-dose herbal administration and repeated daily herbal dose in subchronic studies were intended to test the possible toxicity of *Christia vespertilionis* (L.f.) Bakh. f. leaf extract in male Sprague Dawley rats.

The acute toxicity study mainly tests for LD_{50}, primarily as an initial step to be taken in the toxicity study to determine the median lethal dose of toxin required to kill half of the population tested after a specified test period (OECD, 2001). In addition, the acute toxicity study also assesses adverse reactions that occur immediately or shortly after a single oral short time frame of exposure to a chemical or as adverse reactions within a short period of time when a single dose of a chemical multiple doses are administered within 24 hours. In the present toxicity evaluations, a dose of 2000 mg/kg did not cause any obvious therapeutic abnormalities or death after 14-day of observation as reported, The subchronic study aimed to evaluate the no-observed-adverse-effect-level (NOAEL) of extract given orally to rats daily and continuously for 90-day as stated in the guidelines (OECD, 2013). Along with the acute study, throughout the 90-day subchronic study, also reported no mortality in the control and the rats administrated with extract. As a result, even at a high dose, the administration of extract rats showed a marginal level of toxicity to the subsistence of the animals.

In addition to mortality, clinical and histopathological analysis are also major crucial

observations to signify the toxicity effects on organs in the treated groups especially in the group of animals given a high dose once and daily (Wang *et al.*, 2017). During the 14- day and 90-day study period, it was observed that the intake of food and water was normal with non- significant body weight variations in both the control and treated groups. Weekly body weight is one of the criteria used to assess the health condition of a tested animal (Tanya *et al.*, 2013). It suggests a pharmacological intervention of lipids, carbohydrates and protein metabolism within the body of the animal because these nutrients play a major role in demonstrating differences in the body's physiological functions. Body weight is also related to the weight of the organs. Toxins from the extract may result in an enlargement in size due to the hyperthrophy of cells or a decrease in size due to the atrophy of cells of the vital organs in this study (Awotunde *et al.*, 2017). Liver, kidneys, heart, lungs and spleen are the vital organs of our body that are the main target areas of any toxic substance metabolically (National Research Council , 2011). Supported results of weekly body weights based on the statistics stated that the organs weight reported in both studies also showed no significant change in the control and the treated groups. At the end of the study period, the animals were humanely sacrificed and there were no lesions found in macroscopic examinations of kidneys and livers of the control, vehicle and treated groups. Therefore, it can be proposed that *Christia vespertilionis* (L.f.) Bakh. f. extract is practically non-toxic.

The haematology results in both present studies showed that there was no significant difference between the control and the treated groups. Acute study with administration of 2000 mg/kg concentration and daily 90-days orally gavaged extract at 75 mg/kg, 125 mg/kg and even at 250 mg/kg did not show any effect on the haematopoietic system. The assessment of haematological parameters can also be used to determine the extent of the damaging effect of foreign compounds, including plant extracts, on the blood constituents of the animal (Muriithi *et al.*, 2015). As an example, the use of oral herbals that showed significant difference on haematology parameter, as reported, was the test of methanolic extracts of *V. lasiopus* extract that had shown an increase in erythrocytes and hematocrit counts. This result may reflect the phytochemical content that stimulates the formation or secretion of erythropoietin in the normal mice's stem cells (Muriithi *et al.*, 2015). Based on the white blood cell (wbc) differential count in both studies, it was also found that there was no significant difference in the control, vehicle and treatment groups. Significant increases in white blood cells and differential leukocytes had been reported in animals tested with garlic extract, Allum sativum (Linn) (Iranloye, 2002) and seed extracts of from *Citrus paradise Macfad* (Adeneye, 2008). Increase in wbc differential counts may have resulted in an extract that had stimulated the granulocyte-macrophage colony factor and macrophage colony stimulating factor including the interleukins IL -2 IL-4 and IL-5. This response will lead to increased regulation, proliferation, differentiation and maturation of the committed stem cells responsible for the production of white blood cells. Based on the present results, they can be related to the results reported by Nurul *et al*. (2018), even for 28-day orally gavage of *Christia vespertilionis* (L.f.) Bakh. f. that also showed no significant difference in haematological parameters. Therefore, based on the acute, subacute and subchronic studies, *Christia vespertilionis* (L.f.) Bakh. f. does not affect the haematological paramaters in rats.

Serum biochemical analysis to help evaluate organ damage, cell damage, enzyme induction, activation or inhibition of enzymes is becoming very common in toxicology studies. Kidneys and the liver are two main organs that play a significant role in detoxifying the body. The estimation of some biochemical parameters, such as the activity of enzymes in tissues and blood, plays a key role in disease and toxicity (Stockham and Scott, 2008). Single administration of 2000 mg/kg of the ethanolic extract of *Christia vespertilionis* (L.f.) Bakh. f. showed significant changes in the liver parameters but not significant in kidney parameters. Daily administration of extract in 90-days for low, medium even high concentration groups showed no significant change on serum biochemistry analysis of liver and kidney parameters. This result can be related also to the repeated 28-day oral gavage of extract that had already been reported by Nurul *et al*. (2018). By giving the 2000 mg/kg once, it was reported that both CK and AST in acute study had significantly decreased compared to the control group. In addition, the acute study took only 14 days of the study period, so the circulating half-life of the enzyme also affected the measured serum activity. The serum half-life of total CK is approximately 0.5 to 1 hour and the AST is 17 hours in rats (Dana, 2006). The half-life factor in serum enzymes parameters might be related to the duration of the study being conducted. Administration of ethanolic extract of *Christia vespertilionis* (L.f.) Bakh. f. did not show any change in 90-day study, whereas it showed changes in 14-day study. There are possibilities of the enzymes returning to normal ranges if the study period is prolonged. This result was in accordance with the study by Jasdanwala ,2015. Both haematological and serum biochemistry analyses in both studies followed reference as a result in study by Delwata *et al*., (2018). Besides, studies by Oladuton *et al*., (2019), also showed the same result where the AST level was significant in acute study while in repeated doses 28-days insiginificant in all biochemistry parameters.

Histopatological analysis is a must to support the clinical findings from this present study. Further analysis on histopathological assessment aims to prove the extract adverse-effect on the rats specifically on liver and kidney through a microscopic view. Many plant derived medicines have the ability to cause liver injury. Herbal medicines related to hepatotoxicity is the second most common cause of drug-induced liver injury nowadays. Histological examination is the benchmark for assessing treatment associated to pathological changes in tissues and organs (Haouas *et al.*, 2014; Musumeci, 2014; Shubin *et al.*, 2016). Histology of kidneys in both studies showed no significant change of lesion scores. However, there were significant changes in lesions of the liver of the treated samples evaluation on degeneration (very

mild) and hepatic necrosis (mild to moderate). There is a possibility that this change may reflect a cellular adaptation beneficial to the host tissue. Based on the present study, rats administrated with 2000 mg/kg of extract once, resulted in degeneration of hepatocytes showed by lesion scores of cytoplasmic vacuolation. Cytoplasmic vacoulation occurred as a result of excess concentration of glycogen, primarily the monoparticulate form from natural compounds in herbal medicine (Nadia *et al*., 2014). Besides, the lesion scores of cytoplasmic vacoulation often accompanies cell death or necrosis. In addition, cytoplasmic vacoulation of hepatocyte was reported as a result of disturbance of oxidative phosphorylation in mitochondria with suppression of production of ATP and failure of the ATP dependent sodium pump in the cell membrane, resulting in an accumulation of sodium intracellularly and consequent inflow of water into the different cellular compartments which results in cellular swelling (Asyura *et al*., 2016).These lesion scores were also supported by the use of extract in hepatocyte of rats in oral gavage of extract of pomegranate (Mansouri *et al*., 2016). It was reported in a previous study that 90-day rats administration with extract of ethanolic *C. nutans* showed cytoplasmic vacoulation (Asyura *et al*., 2016). In contrast to this present subchronic study, extract of *Christia vespertilionis* (L.f.) Bakh. f. did not show the cytoplasmic vocuolation of hepatocytes.

Necrosis scores in both studies showed significant differences between control, vehicle and treated groups. In this present study, lesion scores of necrosis was mainly representing the single cell necrosis in the form of intercellular rounded eosinophilic bodies (apoptotic bodies) surrounded by clear holes. Apoptotic or necrotic alteration may be followed by organelles swelling, in particular mitochondria, endoplasmic reticulum and lysosome rupture, which may lead to amorphous eosinophilic cytoplasm as an initial sign of hepatocyte necrosis sequencing prior to the shrinkage and dissolution of the nucleus (Paula *et al*., 2013). In 90-day subchronic study, lesion scores for necrosis were observed to be mild to moderate in both high dose and medium dose groups (Table 13), while low dose group only had mild lesions, which means the score was associated with the increase in concentration administrated daily on rats. Previous studies, with ingestion of herbal medicine, showed necrosis of hepatocytes followed by elevated levels of liver parameters including the ALT and AST activities (Paula *et al*., 2013). Besides, the scores on the activated Kupffer cells in acute and subchronic study had resulted in significant differences between the control, the vehicle and the treated groups. In 90-day study, the number of activated kuffer cells was significantly (p<0.05) higher in low and medium dose groups compared to the high dose group. In liver tissue, Kupffer cells is the resident liver macrophages and change to the activate Kupffer cell due to related on inflammation induced by toxin or xenobiotic. Other than infiltration of inflammatory cells in tissues, inflammation is triggered by various signals, including pro-inflammatory cytokines and chemokines, which are released from injured hepatocytes and activated Kupffer cells (Liu *et al*., 2016). Both studies showed significance in the regeneration score when the treated group was compared with the control group. The regeneration ability of liver tissue work was due to the reaction of the hepatocytes towards toxicant (Miyaoka *et al*., 2012). During the regeneration process, hepatocytes become hypertrophy in the first response, followed by the proliferation of the non-epithelial compartment (hyperplasia). The ability of the extract to induce hepatotoxicity followed by regeneration as reported in previous study with repeated ingestion orally basil leaf or *Ocimum sanctum* alcoholic extract (Lahon and Das, 2011). Regeneration of hepatocyte after being exposed to toxin will make the extract experience hepatoprotective activity. Mainly, lesion scores on regeneration of the hepatocytes took place at the periportal towards the midzonal area in both studies.

Further evaluation on the percentage of the affected area showed that a dose of 2000 mg/kg of *Christia vespertilionis* (L.f.) Bakh. f. extract had caused significant lesions most prominently on the periportal area. Zonal affected subchronic study was most pronounced in the generalise area, but most prominent on the periportal (low dose), the periportal to the midzonal (medium dose) and periportal towards centrilubular area (high dose) often accompanied by hepatocyte atrophy. Localized zonal effects are related to the duration and the dose involved (Rolf and Madlen, 2014). Different zonals in hepatocytes are biochemically different from toxicants in the physiology of the liver. Metabolic reaction flows from the periportal zone as Zone 1 to the midzonal zone as Zone 2 to the centrilobular zone as Zone 3. In general, hepatocytes of Zone 1 (hepatic parenchymal cells) are effective for oxidative metabolism, fatty acid oxidation, gluconeogenesis, bile acid extraction, ammonia detoxification and urea and glutathione conjugation. Zone 3 hepatocytes are effective for the biotransformation of glycolysis, liponeogenesis and CYP450 (El Kasmi *et al*., 2013; Amin *et al*., 2014). Of particular significance for its role in the understanding of drug toxicity, xenobiotic (drug) metabolism occurs primarily in Zone 3 hepatocytes, which are particularly undergoing phase I as a phase of metabolic activity. On the other hand, elevated levels of some phase II as phase detoxification activity can also be observed in zone 1. Based on all the gavaged results of *Christia vespertilionis* (L.f.) Bakh. f. extract in the present study, it can be concluded that there is a possibility that the bioactive compounds activity in the extract responded to the host cell and body. Furthermore, in a previous phytochemical study by Smitha and Jain (2019), the leaves showed presence of alkaloids, flavonoids (quercetin), proteins, glycoside, phytosterols, tannin, diterpene, coumarin and quinine in the preliminary phytochemical tests (Smitha and Jain, 2019). These bioactive compounds can lead to acute hepatocellular injury. As an example, a study has shown that the alkaloid content in medicinal herbs cause an imbalance in oxidative stress enzyme especially in hepatocellular (Amin *et al*., 2014). The imbalance of reactive oxygen and antioxidative enzyme such as gluthatione peroxidise occur as regulating alkaloid induced hepatotoxicity. Phytosterol as the possible compound in extract *Christia vespertilionis* (L.f.) Bakh. f. involved

in the present study will promote liver injury and activate the Kupffer cells (William *et al.*, 2000; Teschke *et al.*, 2015). Quercetin, as part of the flavonoid family, found in this leaf extract, has been reported to create hepatoprotective activity by reducing liver oxidative damage, ductular proliferation and fibrosis in studies of rats with biliary-obstructed (Teschke *et al*., 2015). In order to confirm and relate the findings of the present studies, it is recommended that further research should be carried out to study the possible bioactive compounds content in the extract.

In conclusion, *Christia vespertilionis* (L.f.) Bach. f. ethanolic leaves extract did not induce mortality in the acute toxicity study (2000 mg / kg). This extract in the acute study showed no significant change in weekly body weight, haematology and organ weight but was significant in the CK and AST values compared to the control group. Histopathological assessment showed very mild to moderate hepatocellular injury in treated groups compared to the control. The same result also showed in the subchronic toxicity study, leaf extract did not cause mortality. In addition, this extract in the subchronic study showed no significant effect on weekly body weights, haematology, serum biochemistry and body weight. Meanwhile, histopathological assessment in the subchronic study has shown that there have been significant effects on liver parameters for histopathological changes but no significant effects on the assessment of renal tissue when the control and treatment groups have been compared. This subchronic study showed significant differences (p<0.05) in hepatic necrosis and activated copper cells. Mild to moderate hepatic necrosis was observed in both high and medium dose groups, while only mild lesions were observed in the low dose group. On the other hand, the number of activated kuffer cells was significantly higher (p<0.05) in the low and medium dose groups compared to the high dose group. They were dose-dependent based on low-dose, medium-and high-dose levels of necrosis, degeneration, inflammatory cells and activated kupfer cells. Several of these observations support the conclusion that the liver is a plausible target organ for this extract, although the mechanism of toxic effect on the liver is unclear. According to these results, NOAEL levels were lower than 75 mg / kg for rats in the 90-day toxicity study, which provides the basis for the clinical use of Christia vespertilionis (L.f.) Bakh. f. and for the determination of a reasonable safe dose. The NOAEL is less than 75 mg / kg with equivalent to 1774 mg of total gavage extract for 90 days.

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