# SUBCHRONIC TOXICITY ANALYSIS OF MIXTURES OF *Christia vespertilionis* (L.f.) Bakh. f. LEAF AND *Morinda citrifolia* L. FRUIT ETHANOLIC EXTRACTS IN MALE SPRAGUE DAWLEY RATS

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**Abstract.** *Christia vespertilionis*(L.f.) Bakh. f. (CV) leaves and *Morinda citrifoa* L.(MC) fruits have acquired much attention among Malaysians and are commercially available.Practitioners are concerned about the effectiveness and safety of the products. Thus, the study aims to evaluate the toxicity effects of mixtures of CV and MC in male Sprague Dawley rats in a 90-day subchronic oral toxicity study. A total of 36 rats were divided equally into six groups; control, 5% DMSO (vehicle), mixture of low dose CV and MC (75 mg/kg), mixture of low dose CV (75 mg/kg) and medium-dose MC (125 mg/kg), mixture of medium-dose CV (125 mg/kg) and low dose MC (75 mg/kg) and mixture of medium CV and MC (125 mg/kg). The extracts were orally gavaged daily for 90 days. On day 91, the rats were sacrificed, and the blood profiles and organs changes were evaluated. There was no mortality observed in groups of rats that received mixtures of CV and MC. Weekly body weights, haematology and serum biochemistry also showed no significant ($p>0.05$) differences compared to control. However, significant ($p<0.05$) differences were observed for hepatic necrosis and number of activated Kupffer cells in all herbal treated groups compared to control (from very mild to moderate scores). Kidneys showed a significant ($p<0.05$) score for the granular cast (very mild). In conclusion, mixtures of CV and MC induced hepatotoxicity and renal toxicity, and the no-observed-adverse-effect levels (NOAEL) of CV and MC mixture is lower than 150 mg/kg.

**Keywords:***Christia vespertilionis*(L.f.) Bakh. fleaves, *Morinda citrifolia* L.fruits, subchronic toxicity, hepatotoxicity.

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## Introduction

Toxicity studies are used in conjunction with animal model research to identify the safe dose level of a medicine used to treat diseases. This guideline allows for the characterisation of the adverse consequences of repeated oral gavage doses, which are discussed in accordance with the OECD recommendations. Medicinal herbs and fruits are effective in treating various diseases and ailments, including cancer.

Thousands of herbs and their preparations have lately been examined around the world to determine their pharmacologically active components, scientific validity, and uses, all of which are helping to spread and promote the herbs in treatments and medicines [1-5]. However, while herbal medicine is widely used, little has been done to ensure its safety.Nowdays, mostdrugs contained synthetic version of herbal compound [6-7].

*C. vespertilionis*(L.f.)Bakh. f. is also known as 'butterfly leaf .' It is a perennial herb with trifoliate leaves that is utilised as a decorative plant in Southeast Asian cultivated gardens. In the literature, there is relatively little information about this plant, collected from all possible scientific sources. . Researchers would have plenty of scope to research into the plant's pharmacological and phytochemical properties. This study provides current information on the plant's toxicity studies, useful to future researchers.From previous studies, the subacute toxicity study of the *C. vespertilionis*Bakh. f. extract showed hepatotoxicity (very mild, mild, and mild to moderate) in hepatic necrosis, hepatic degeneration, and hepatitis scores [8].

The Rubiaceae family includes *M. citrifolia*L., sometimes known as Noni. M. *citrifolia*L., an edible and tropical plant, has a long history as a medicinal plant in India and the Pacific Islands. The plant components contained bioactive chemicals, including leaves, fruit, roots, bark, flowers, and seeds [9].In recent years, an increasing number of pharmacological research on noni juice and isolated fruit components have been published. They are primarily linked to cancer, inflammation, and metabolic illnesses. On several websites, there are countless testimonials on Noni's positive health effects. Clinical evidence on Noni, on the other hand, is still hardly published in scholarly journals. Although the number of scientific papers on the noni fruit has expanded dramatically in recent years, the current knowledge is still inadequate.

In additional studies by West et al., (2006) and Wang et al., (2002), rats given *M. citrifolia* L. extracts orally for 13 weeksshowed no signs of toxicity on their haematology, serum biochemistry, or histopathology [10-11]. Noni extracts caused chronic toxicity effects of hepatocyte necrosis, increased liver injury marker AST (aspartate aminotransferase) and albumin reduction, injury symptoms (hypoactivity, excessive grooming, swollen eyes and hunched posture), and 40% mortality within 3 months, according to a study by Mohamad et al. published in 2017 [12].

The toxicology evaluations of *C. vesperlionis*Bakh. f.leaves and *M. citrifolia* L. fruits are essential as they reveal the side effects, especially on people who consume them as supplements and medicine. The health benefits of these herbal plants have been known and used in many countries for their medicinal properties. The increased use of these herbal plants has resulted in safety and efficacy concerns. Thus, the toxicity of these plants can be determined through *invivo* studies as a safeguard to public health and to increase public awareness of toxicology studies and provide a preclinical safety assessment before a human

safety dose or concentration used can be performed and evaluated. Therefore, the objective of this study is to investigate the subchronic oral toxicity effects of ethanolic mixture extracts of *C. vesperlionis* (L.F.) Bakh. f.leaves and *M. citrifolia* L. fruitsin male Sprague Dawley rats.

## Materials and methods

***Plant preparation.*** *C. vespertilionis (*L.f.) Bakh. f.and *M. citrifolia* L. were obtained from the MARDI research station located at Muadzam Shah, Pahang. The leaves and fruits were cut into small pieces and left to dry at room temperature. The dried leaves and fruits were ground into a fine powder and extracted using ethanol. Sample extraction was conducted bythe maceration process with a slight modification according to the method developed byNurul et al., [8]. The ratio between dried sample and ethanol was 0.02 g of the sample with 40 mL of ethanol. The mixture was placed in an orbital shaker (HeidophUnimax 1010, German) at 200 rpm at room temperature for 2 hours. The mixture was filtered through filter paper (Whatman No. 4). The filtrate was evaporated under vacuum reduced pressure of 60°C using a rotary evaporator (R-215, Buchirotavaporator, Switzerland) to produce a thick syrupy mass crude extract. The crude extract was kept at -4˚C in a Schott bottle. Different extract concentrations were prepared with 5% DMSO at 75 mg/kg, and125 mg/kg of body weight and given to rats via oral gavage. The extract solution was freshly prepared every week based on the body weight.The factor for choosing the extract concentration were based on the study by Nurul (2018) and Erhirhie et al. (2014)[13-14].

***Location of study.*** The rats were placed in the Animal Metabolism, Toxicology and Reproductive Centre (AMTREC), Malaysia Agricultural Research and Development Institute (MARDI) at Serdang, Selangor. The experiment was designed and conducted in accordance with the ethical approvalof the Animal Care Unit Committee (ACUC), Malaysia Agricultural Research and Development Institute (MARDI) approval reference number 20170717/R/MAEC0023.

***Animal management and routine.*** Male Sprague Dawley (SD) rats at 6 weeks of age with an average weight between 160-180 grams were used in this study. Rats were acclimatised to housing conditions in polycarbonate plastic cages with temperatures between 22-27˚C, humidity at the range 40-70% and balance of 12 hours light/ 12 hours dark cycle. The rats have been supplied by Alchemy Supplies Sdn. Bhd, Seri Kembangan, Malaysia.

***Experimental design.*** In the subchronic (90-days) oral toxicity studies, a total of 48 male SD rats were divided into six groups. The details for each group are shown in Table 1.

**Table 1. Experimental design of subchronic toxicity study**

| Group | Treatment |
|---|---|
| A | Low dose*Christiavespertilionis* (L.f.) Bakh. f. (75mg/kg) leaf and Low dose *Morindacitrifolia*L.(75mg/kg) fruit extract |
| B | Low dose*Christiavespertilionis* (L.f.) Bakh. f. (75mg/kg) leaf and Medium dose *Morindacitrifolia*L. (125mg/kg) fruit extract |
| C | Medium dose*Christiavespertilionis* (L.f.) Bakh. f. (125mg/kg) leaf and Low dose *Morindacitrifolia* L. (75mg/kg) fruit extract |
| D | Medium dose*Christiavespertilionis* (L.f.) Bakh. f. (125mg/kg) leaf and Medium dose *Morindacitrifolia* L. (125mg/kg) fruit extract |
| E | Vehicle 5% DMSO |
| F | Control |

An extract from *C. vespertilionis* (L.f.) Bakh. f. leaf and *M. citrifolia* L. fruit were dissolved in 5% DMSO prior to oral gavage in rats. In the control group, rats received normal saline and in the vehicle group, the rats received 5% DMSO by oral gavages. All rats had free access to water and commercial chow *ad libitum*. Rats were observed daily for any mortality or signs of toxicity and were weighed weekly. The rats were fasted the night before humanely euthanised. All rats were euthanised with inhalation of carbon dioxide ($CO_2$).

***Blood samplings.*** Blood samples were collected once at the end of the study using a 26 gauge needle and 5 mL syringe via cardiac puncture for haematology and clinical biochemistry analyses.

***Haematological and serum biochemical analysis.*** Total numbers of white blood cells (WBCs) and red blood cells (RBCs), and haemoglobin (Hb) concentrations were analysed using an automated haematology analyser (Cell Dyn® 3700, Abbott Diagnostics, USA). A blood smear was prepared and stained with Wright stain. The differential WBC count was performed manually by counting 100 WBCs on the blood smears. Packed cell volume (PCV), icterus index, and plasma protein concentration were determined manually using standard methods. Mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) were calculated manually by using standard formula, MCV= (PCV x 1000)/RBC and MCHC = Hb/PCV.

The blood samples analysed for creatinine, urea, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatine kinase (CK), total protein (TP), albumin (ALB) using an automated chemistry analyser (TRX 7010, Biorex Mannheim Germany). The total protein consists of albumin and globulin, and thus, the globulin results were calculated manually using the standard formula, Total protein−Albumin (TP-AL).

***Histopathological analysis.*** The general appearance of rats and all organs were examined for gross lesions. Toxicity effects were evaluated by the assessment of kidneys and liver histopathology. The tissues were fixed in a 10% buffered formalin solution for 24 hours and processed in an automated processor (Leica ASP300, Germany). The tissues were embedded with paraffin using a tissue embedding 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 a 45°C water bath prior to mounting on glass slides. All the glass slides were labelled with a diamond pen and the tissue sections were mounted on a hot plate overnight. The slides were then stained with haematoxylin and eosin stain (H&E) and examined under a light microscope at magnifications of 40x, 100x, 200x, 400x,600x and 1000x.

***Toxicity lesions characteristics.*** The tissues were scored as none=0 (no lesion observed), very mild=1 (less than 10% lesion were observed), mild= 1.5 (less than 30% lesion were observed), moderate=2 (less than 50% lesion were observed), moderate-severe=2.5 (less than 70% lesion were observed) and severe=3 (>70% lesion were observed) [8].

**Table 2. Lesion scored and percentage of area affected (%)**

| Score | Percentage lesion | |
|---|---|---|
| 0 | 0 | None |
| 0.5 | Less than 15% | Very mild |
| 1 | 15-30% | Mild |
| 1.5 | 30-45% | Mild to moderate |
| 2 | 45-60% | Moderate |
| 2.5 | 60-75% | Moderate to severe |
| 3 | More than 75% | Severe |

(Nurul *et al.*, 2018).

***Statistical analysis.*** Bodyweight, haematological, serum biochemical parameters and results of relative organ weight were expressed as mean±SD and mean±SEM. Data were analysed using one-way ANOVA and Tukey HSD tests using statistical analysis software, IBM SPSS Statistic 22.0. Histopathology results were expressed as mean±SEM and analysed using the Kruskal-Wallis test for global comparison of groups for all the parameters.

## Results and Discussion

***Bodyweight.*** The bodyweight results of all rats are shown in Table 3. No significant differences ($p>0.05$) were observed between the groups throughout the study.

**Table 3. Body weights (Mean ±SEM) of male Sprague Dawley rats that received repeated doses of ethanolic extracts of *Christiavespertilionis* (L.f.) Bakh. f. leaves and *Morindacitrifolia*L. fruits by oral gavage for a 90-day duration.**

| Group<br>Week | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| 1 | 203.67±10.34 [a] | 204.67±13.10 [a] | 206.33±15.92 [a] | 217.83±7.86 [a] | 189.40±9.22 [a] | 202.75±11.05 [a] |
| 2 | 216.00±6.57 [a] | 213.00±9.67 [a] | 224.83±13.93 [a] | 234.17±4.25 [a] | 213.40±7.00 [a] | 222.25±5.98 [a] |
| 3 | 241.67±8.74 [a] | 241.00±12.79 [a] | 253.00±15.60 [a] | 266.67±7.58 [a] | 234.00±6.77 [a] | 252.75±9.08 [a] |
| 4 | 248.67±8.65 [a] | 247.67±10.88 [a] | 267.00±14.09 [a] | 272.67±7.17 [a] | 249.40±7.00 [a] | 262.75±14.01 [a] |
| 5 | 271.00±8.91 [a] | 269.17±9.68 [a] | 283.50±13.73 [a] | 291.67±5.64 [a] | 252.20±7.51 [a] | 273.75±13.02 [a] |
| 6 | 289.50±13.16 [a] | 294.33±14.50 [a] | 299.00±19.12 [a] | 317.83±7.49 [a] | 271.80±8.82 [a] | 301.75±14.06 [a] |
| 7 | 302.50±10.29 [a] | 310.17±16.91 [a] | 316.67±18.11 [a] | 338.00±9.57 [a] | 289.00±7.79 [a] | 320.00±15.93 [a] |
| 8 | 319.50±11.54 [a] | 329.00±17.80 [a] | 331.83±18.16 [a] | 344.17±10.40 [a] | 302.00±7.25 [a] | 333.50±16.50 [a] |
| 9 | 344.00±13.98 [a] | 350.67±23.85 [a] | 365.50±28.91 [a] | 375.50±14.09 [a] | 310.20±9.86 [a] | 355.50±24.85 [a] |
| 10 | 339.00±17.02 [a] | 358.67±24.54 [a] | 375.00±28.38 [a] | 374.83±11.33 [a] | 320.00±10.48 [a] | 357.25±25.66 [a] |
| 11 | 343.67±21.43 [a] | 363.17±25.04 [a] | 379.00±29.20 [a] | 386.00±12.48 [a] | 324.80±8.86 [a] | 369.75±21.28 [a] |
| 12 | 342.83±20.04 [a] | 364.83±23.12 [a] | 383.67±27.71 [a] | 385.00±10.68 [a] | 326.80±7.96 [a] | 372.00±20.05 [a] |
| 13 | 345.50±27.92 [a] | 385.00±26.21 [a] | 396.17±30.38 [a] | 406.17±14.40 [a] | 334.00±12.71 [a] | 384.00±26.65 [a] |

Notes: Values in the same row with similar superscripts were not significantly different at $p>0.05$. Note: A: Mixture of low dose *C. vespertilionis* (L.f.) Bakh. f. leaves and *M. citrifolia* L. fruits, Control; B: Mixture of low dose *C. vespertilionis* (L.f.) Bakh. f. leaves and a medium-dose of *M. citrifolia* L. fruits ; C: Mixture of medium-dose *C. vespertilionis* (L.f.) Bakh. f. leaves and low dose *M. citrifolia* L. fruits; E: Mixture of medium-dose *C. vespertilionis* (L.f.) Bakh. f. leaves and *M. citrifolia* L. fruits; E: vehicle (5% DMSO) and F: control.

The bodyweight continued to increase by 20-30g per week in rats. Generally, the bodyweights wereincreased concomitantly throughout the experimental period.This mean the extract does not suppress the appetite of rats through out the study.

***Organs relative weight.*** The organs relative weight results of male Sprague Dawley rats in subchronic oral toxicity study are shown in Table 4. There were no significant differences ($p>0.05$) in relative weight of the liver, spleen, kidneys, testes, heart, lungs, and brain organs in this study.

**Table 4. The organs relative weights (mean±SEM) of male Sprague Dawley rats that received repeated doses of ethanolic extracts of *Christiavespertilionis* (L.f.) Bakh. f. leaves and *Morindacitrifolia* L. fruits by oral gavage for a 90-day duration.**

| Organ/Group | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| Spleen | 0.62 ± 0.02[a] | 0.70 ± 0.08[a] | 0.64 ± 0.07[a] | 0.64±0.06 [a] | 0.60±0.02 [a] | 0.65±0.12 [a] |
| Liver | 11.24 ± 0.89[a] | 12.21 ± 0.94[a] | 13.67 ± 1.61[a] | 13.38±0.68 [a] | 11.51±1.22 [a] | 13.56±1.74 [a] |
| Kidneys | 2.38 ± 0.06[a] | 2.61 ± 0.13[a] | 2.97 ± 0.27[a] | 2.69±0.14 [a] | 2.36±0.12 [a] | 2.65±0.24 [a] |
| Heart | 1.43±0.10 [a] | 1.41±0.11 [a] | 1.48±0.07 [a] | 1.45±0.04 [a] | 1.27±0.04 [a] | 1.68±0.12 [a] |
| Testes | 2.87 ± 0.17[a] | 3.01 ± 0.15[a] | 3.42 ± 0.30[a] | 3.25±0.15 [a] | 3.23±0.10 [a] | 3.24±0.06 [a] |
| Lungs | 2.65±0.13 [a] | 2.73±0.28 [a] | 2.73±0.26 [a] | 2.35±0.15 [a] | 2.67±0.16 [a] | 2.72±0.31 [a] |

Notes: Values in the same row with similar superscripts were not significantly different at $p>0.05$. Note: A: Mixture of low dose *C. vespertilionis* (L.F.) Bakh. F. leaves and *M. citrifolia* L. fruits, B: Mixture of low dose *C. vespertilionis* (L.F.) Bakh. F. leaves and a medium-dose of *M. citrifolia* L. fruits; C: Mixture of medium-dose *C. vespertilionis* (L.F.) Bakh. F. leaves and low dose *M. citrifolia* L. fruits; E: Mixture of medium-dose *C. vespertilionis* (L.F.) Bakh. F. leaves and *M. citrifolia* L. fruits; E: vehicle (5% DMSO) and F: control.

*C. vespertilionis* (L.F.) Bakh. f. leaf and *M. citrifolia* L. fruit extracts are widely consumed and commercialised in today's market.Animals such as rats are commonly used prior to clinical studies because of their ability to replicate human systems [14]. As a result, the current subchronic toxicity study in rodents intends to investigate the toxicity caused by repeated doses of oral administration over 90 days. This test offers information on the target organs and the potential for the test chemical to accumulate in the organism, which is then used to determine the no observed adverse effect level (NOAEL) dose following OECD Guideline 413 [15]. The weekly body weight gain did not differ significantly ($p>0.05$) between treated groups compared to controlThis suggests that the extracts did not affect the glucose, protein, or fat metabolism in rats, nor did they lower their appetite. Furthermore, there was no significant ($p>0.05$) difference in the weight of the organs (liver, kidney, spleen, heart, testes, and lungs) between the concurrent control and treated animals.Throughout this study, this indicating no cellular changes or injury happened such as hypertrophy and hyperplasia due to the direct exposure of toxicants on cells. Interestingly, the food and

water intakes were found to be unaltered during the 90-day treatment period when compared to a control group in this study.No gross lesions were linked with the extracts' harmful effects in the concurrent control group or the treated rats. However, histopathology was used to analyse the lesion score in the animals.An increase or decrease in the absolute or relative weight of an organ following the administration of a chemical or medicine indicates the chemical's deleterious effect and may be linked to changes in internal organ size [15].

***Haematological analyses.*** The haematology results are shown in Table 5. No significant differences were observed in the haemogram parameters. The PCV was not affected by the dose levels studied. There were no significant differences in haemoglobin concentration, RBC, MCV, icterus index and plasma protein at dose levels in all three studies. There were also no significant differences in leukogram parameters for WBC counts and differential leukocyte counts. All values were still within the reference range [16].

**Table 5.The haematology values (mean±SEM) of male Sprague Dawley rats that received repeated doses of ethanolic extracts of *Christiavespertilionis* (L.f.) Bakh. f. leaves and *Morindacitrifolia* L. fruits by oral gavage for a 90-day duration.**

| Parameter/Group | Reference range (13 weeks) | A | B | C | D | E | F |
|---|---|---|---|---|---|---|---|
| RBC ($10^{12}$/L) | 6.39-8.01 | 9.52 ± 0.27[a] | 10.08 ± 0.40 [a] | 9.76 ± 0.51 [a] | 9.98±0.56 [a] | 9.53±0.31 [a] | 9.27±0.09 [a] |
| Hb (g/L) | 135-159 | 183.00 ±5.42 [a] | 179.00 ±17.52 [a] | 189.33 ±12.25 [a] | 190.33±9.12 [a] | 184.80±3.84 [a] | 181.50±1.26 [a] |
| HCT | 42-49 | 0.63±0.01 [a] | 0.62±0.02 [a] | 0.64±0.01 [a] | 0.65±0.02 [a] | 0.64±0.01 [a] | 0.65±0.01 [a] |
| Platelet | 923-1580 | 1540.67±78.95 [a] | 1592.67±114.93 [a] | 1402.67±108.10 [a] | 1501±72.93 [a] | 1461.80±156.28 [a] | 1667.75±177.63 [a] |
| PCV (L/L) | 25.0-35.0 | 29.33 ± 1.58 [a] | 29.17 ±1.96 [a] | 32.50 ± 0.99 [a] | 33.50±1.45 [a] | 31.20±2.52 [a] | 35.35±3.42 [a] |
| MCV (fL) | 46.0-70.5 | 67.17 ± 0.54 [a] | 68.17 ±1.62 [a] | 69.33 ± 1.26 [a] | 68.50±0.76 [a] | 67.20±1.16 [a] | 70.00±1.08 [a] |
| MCHC (g/L) | 250.0-300.0 | 286.00 ± 2.68 [a] | 284.67 ± 2.84 [a] | 279.67 ±4.98 [a] | 279.83±2.09 [a] | 230.40±5.68 [a] | 279.75±5.79 [a] |
| WBC (× $10^9$/L) | 12.0-20.0 | 18.23±2.01 [a] | 18.00 ±3.34 [a] | 16.62 ± 0.99 [a] | 17.23±1.87 [a] | 18.22±4.41 [a] | 21.38±3.37 [a] |
| Neutrophils (× $10^9$/L) | 0.28-1.43 | 0.45 ± 0.01 [a] | 0.69 ± 0.01 [a] | 0.72 ± 0.01 [a] | 0.69 ± 0.01 [a] | 0.77 ± 0.07 [a] | 0.59 ± 0.04 [a] |
| Lymphocytes (× $10^9$/L) | 2.45-7.66 | 6.08 ± 0.01 [a] | 4.97 ± 0.01 [a] | 5.43 ± 0.01 [a] | 4.97 ± 0.01 [a] | 4.64 ± 0.01 [a] | 3.66 ± 0.0.01 [a] |
| Monocytes (× $10^9$/L) | 0.17-0.76 | 0.26 ± 0.04[a] | 0.17 ± 0.04 [a] | 0.13 ± 0.04[a] | 0.20 ± 0.04[a] | 0.16 ± 0.02 [a] | 0.12 ± 0.03[a] |
| Eosinophils (× $10^9$/L) | 0.03-0.21 | 0.16 ± 0.04[a] | 0.11 ± 0.04[a] | 0.11 ± 0.03[a] | 0.15± 0.04[a] | 0.07 ± 0.04 [a] | 0.09 ± 0.04[a] |
| Basophils (× $10^9$/L) | 0-0.02 | 0.10 ± 0.03[a] | 0.04 ± 0.03[a] | 0.08 ± 0.02[a] | 0.09 ± 0.03[a] | 0.07 ± 0.02 [a] | 0.05 ± 0.02[a] |
| Icterus Index | 2.00 | 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] | 2.00 ± 0.00 [a] |
| Plasma protein (g/L) | 65.0-75.0 | 71.00 ± 3.30 [a] | 72.33 ± 3.59 [a] | 72.67 ±0.84 [a] | 70.33±0.61 [a] | 73.20±1.50 [a] | 72.25±2.46 [a] |

Notes: Values in the same row with similar superscripts were not significantly different at p>0.05. Note: A: Mixture of low dose *C. vespertilionis* (L.f.) Bakh. f. leaves and *M. citrifolia* L. fruits, B: Mixture of low dose *C. vespertilionis* (L.f.) Bakh. f. leaves and a medium dose of *M. citrifolia* L. fruits ; C: Mixture of medium-dose *C. vespertilionis* (L.f.) Bakh. f. leaves and low dose *M. citrifolia* L. fruits; E: Mixture of medium-dose *C. vespertilionis* (L.f.) Bakh. f. leaves and *M. citrifolia* L. fruits; E: vehicle (5% DMSO) and F: control

***Serum biochemical parameters.*** Serum biochemical parameters were grouped as renal parameters (urea, creatinine) and liver parameters (total protein, albumin, globulin, creatinine kinase (CK), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP)) as presented in Table 6. Renal parameters showed no substantial difference between the control, vehicle and treated rats in all studies. Similarly, the liver function parameters showed no significant difference between the control, vehicle, and treated rats for all parameters measured in the subchronic toxicity study. All values still within the reference range [16].

**Table 6. The serum biochemical parameters (mean±SEM) of male Sprague Dawley rats that received repeated doses of ethanolic extracts of *Christiavespertilionis* (L.f.) Bakh. f. leaves and *Morindacitrifolia* L. fruits by oral gavage for a 90-day duration.**

| Parameter/Group | Reference range (13 weeks) | A | B | C | D | E | F |
|---|---|---|---|---|---|---|---|
| Urea (mmol/L) | 4.32-8.97 | 5.45 ± 0.38[a] | 5.57 ± 0.15[a] | 5.45 ± 0.37[a] | 5.18±0.32 [a] | 5.34±0.23 [a] | 5.75±0.44 [a] |
| Creatinine (µmol/L) | 35.4 – 79.6 | 43.33 ± 1.31[a] | 42.50 ± 2.05[a] | 39.00 ± 0.97[a] | 42.33±1.48 [a] | 46.60±3.23 [a] | 45.50±3.33 [a] |
| CK (U/L) | 300.0 – 520.0 | 209.00 ± 20.02[a] | 210.83 ± 41.07[a] | 177.33 ± 25.81[a] | 252.17±50.85 [a] | 339.60±67.93 [a] | 320.25±173.95 [a] |
| ALT (U/L) | 34.9 – 218.1 | 63.33± 10.83[a] | 70.50 ± 13.45[a] | 52.33 ± 9.43[a] | 73.00±16.23 [a] | 84.60±7.40 [a] | 83.25±1.89 [a] |
| AST (U/L) | 131.6 – 459.0 | 113.50 ± 7.68 [a] | 120.83± 9.25[a] | 120.67 ±11.57[a] | 129.67±10.10 [a] | 148.60±16.03 [a] | 140.50±30.52 [a] |
| Total protein (g/L) | 56.1 – 208.1 | 75.63 ± 1.35[a] | 76.33 ± 1.53[a] | 74.58 ± 1.88[a] | 75.42±2.11 [a] | 77.92±1.50 [a] | 76.45±0.83 [a] |
| Albumin (g/L) | 65.0 – 81.0 | 32.52± 0.65[a] | 35.67 ± 0.43[a] | 33.78 ±0.59[a] | 34.88±0.57 [a] | 35.24±0.58 [a] | 35.48±0.05 [a] |

Notes: Values in the same row with similar superscripts were not significantly different at p>0.05. Note: A: Mixture of low dose *C. vespertilionis* (L.F.) Bakh. F. leaves and *M. citrifolia* L. fruits, B: Mixture of low dose *C. vespertilionis* (L.F.) Bakh. F. leaves and a medium dose of *M. citrifolia* L. fruits; C: Mixture of medium-dose *C. Vespertilionis* (L.F.) Bakh. F. leaves and low dose *M. citrifolia* L. fruits; E: Mixture of medium-dose *C.vespertilionis* (L.F.) Bakh. F. leaves and *M. citrifolia* L. fruits; E: vehicle (5% DMSO) and F: control

Many enzymes are released or increased in the bloodstream due to organ or tissue damage, which can be determined by examining the serum biochemical analysis [16]. The importance of assessing liver and renal parameters in toxicity evaluations cannot be overstated. In this study, no significant differences in the activity of the tested enzymes were identified in any of the rat groups (p >0.05). Table 6 summarises the serum enzymes' activity, including AST, ALT, CK, total protein, and albumin concentrations. These variables are

frequently used to evaluate the state of liver function [19]. Because the liver is the central organ for chemical detoxification, a liver function test is essential. Changes in ALT and AST levels are a sensitive indicator of the extent of liver cell injury [20]. ALT and AST levels rise and are released into the bloodstream when hepatocytes are damaged.Table 6 shows that no significant changes in urea and creatinine concentrations were reported in any rat group. This means that both extracts do not affect normal urea and creatinine concentrations. The concentrations of plasma urea and creatinine are frequently employed as indicators of renal glomerular function, and they will rise as a result of renal damage [21]. Previous studies by Hadija et al. (2003) and Nurul et al. (2018), both of which found no significance in blood and serum biochemistry analysis despite repeated dosages ofsigle extract of *C. vespertilionis* (L.f.) Bakh. f. leaf and single extract of *M. citrifolia*L.fruitgiven for 28 days [8, 22], both of which found no relevance in blood and serum biochemistry analysis.

**Lesions score.** The Kruskal Wallis test was used to analyse the global comparison for organ toxicity among all groups. The results of histopathological changes in liver and kidney tissues are summarised in Table 7. Liver histological examination of treated rats with the mixture of *C. vespertilionis* (L.f.) Bakh. f. leaf and *M. citrifolia*L.fruit extract have revealed some abnormalities. Significant ($p<0.05$) differences in hepatic necrosis, regeneration and number of activated Kupffer cells were observed in all herbal treatment groups compared to control (from very mild to moderate scores). The histological changes in the kidney tissues showed very mild significant changes ($p<0.05$) in the granular cast.

**Table 7.Lesion scores of liver and kidney of male Sprague Dawley rats that received repeated doses of ethanolic extracts of *Christiavespertilionis* (L.f.) Bakh. f. leaves and *Morindacitrifolia* L. fruits by oral gavage for a 90-day duration.**

| Organ | Mean score of lesions | A | B | C | D | E | F | Kruskal Wallis Test for global comparison of organ lesions among groups. Asymptotic significant (p<0.05) |
|---|---|---|---|---|---|---|---|---|
| Liver | **Activated Kupffer cell** | 0.83±0.17 [a] | 1.25±0.11 [a] | 1.25±0.11 [a] | 1.25±0.11 [a] | 0.00±0.00 [b] | 0.00±0.00 [b] | **0.01*** |
| | Sinusoid dilatation | 0.00±0.00 [a] | 0.00±0.00 [a] | 0.17±0.17 [a] | 0.00±0.00 [a] | 0.00±0.00 [a] | 0.00±0.00 [a] | 0.48 |
| | **Eosinophilic cytoplasm** | 0.42±0.27 [ab] | 1.00±0.00 [bc] | 1.10±0.24 [bc] | 1.33±0.11 [c] | 0.00±0.00 [a] | 0.00±0.00 [a] | **0.01*** |
| | Cytoplasmic vacoulation | 0.33±0.22 [a] | 0.17±0.17 [a] | 0.42±0.27 [a] | 0.00±0.00 [a] | 0.00±0.00 [a] | 0.00±0.00 [a] | 0.34 |
| | **Pyknotic cells** | 0.00±0.00 [a] | 0.00±0.00 [a] | 0.33±0.22 [ab] | 0.83±0.17 [b] | 0.00±0.00 [a] | 0.00±0.00 [a] | **0.02*** |
| | Karyolysis | 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] | 0.00±0.00 [a] | 1.00 |
| | Karyohexxis | 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] | 0.00±0.00 [a] | 1.00 |
| | **Regeneration** | 1.00±0.00 [b] | 1.03±0.08 [b] | 1.08±0.24 [b] | 1.33±0.11 [b] | 0.00±0.00 [a] | 0.00±0.00 [a] | **0.01*** |
| | Inflammatory cells | 0.17±0.17 [a] | 0.00±0.00 [a] | 0.00±0.00 [a] | 0.17±0.17 [a] | 0.00±0.00 [a] | 0.00±0.00 [a] | 0.61 |
| | Mean score | 0.31±0.09 | 0.38±0.04 | 0.48±0.14 | 0.55±0.07 | 0.00±0.00 [a] | 0.00±0.00 [a] | 0.38 |
| Kidney | **Granular cast** | 0.17±0.17 [a] | 0.00±0.00 [a] | 0.33±0.21 [a] | 0.67±0.21 [a] | 0.00±0.00 [a] | 0.00±0.00 [a] | **0.04*** |
| | Cellular cast | 0.00±0.00 [a] | 0.50±0.22 [a] | 0.17±0.17 [a] | 0.50±0.22 [a] | 0.00±0.00 [a] | 0.00±0.00 [a] | 0.08 |
| | Protein cast | 0.17±0.17 [a] | 0.17±0.17 [a] | 0.00±0.00 [a] | 0.00±0.00 [a] | 0.00±0.00 [a] | 0.00±0.00 [a] | 0.61 |
| | 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] | 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] | 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] | 0.00±0.00 [a] | 1.00 |
| | Mean score | 0.06±0.06 [a] | 0.11±0.07 [a] | 0.08±0.06 [a] | 0.20±0.07 [a] | 0.00±0.00 [a] | 0.00±0.00 [a] | 0.62 |

Notes: A: Mixture of low dose *C. vespertilionis* (L.F.) Bakh. F. leaves and *M. citrifolia* L. fruits, B: Mixture of low dose *C. vespertilionis* (L.F.) Bakh. F. leaves and a medium dose of *M. citrifolia* L. fruits ;C: Mixture of medium-dose *C. vespertilionis* (L.F.) Bakh. F. leaves and low dose *Morindacitrifolia* L. fruits; E: Mixture of medium-dose *C. vespertilionis* (L.F.) Bakh. F. leaves and *M. citrifolia* L. fruits; E: vehicle (5% DMSO) and F: control. * indicate a significant difference (p<0.05).

The liver and kidneys, which are involved in the metabolism, detoxification, storage, and excretion of xenobiotics and their metabolites, are vulnerable to exogenous chemicals [23]. Histopathological evaluation is necessary to support serum biochemistry study and to determine toxicity levels in organs and tissues. Figure 1 is the normal histology of liver in control group as showed the normal color of hepatocyte and also the normal size of hepatocytes.When all herbal medication treated groups were compared to control groups, the histology data demonstrated significance ($p<0.05$) on lesion hepatic necrosis (eosinophilic cytoplasm) and several activated Kupffer cells (scored from very mild to moderate scores). This investigation discovered hepatic necrosis at the periportal and midzonal levels as showed in Figure 2 to Figure 5. The histopathological findings in this subchronic study were in contrast to those of Rosly et al., (2011), who found no toxicity despite using large amounts of *M. citrifolia* L. fruits in their studies [24]. Rosly and colleagues (2011) results are primarily attributable to the rats being fed mixed ground pellets ad libitum, which is a rudimentary manner of feeding. As a result, the rats only received a modest amount of fruits throughout the day compared to the oral gavage administration.Meanwhile, Figure 4 showed the dilated of sinusoid due to the atrophied size of hepatocytes at periportal towards midzonal area. Atrophied of hepatocytes is one of the criteria for the response of liver towards toxic substances.

Furthermore, the treated groups had significantly higher regeneration (as showed in Figure 6) ratings accompanied by the lesion of activated Kupffer cells (Figure 7) and inflammatory cells (Figure 8) than the control groups in this study. This means that the extract tests are carried out by inflicting liver injury on experimental animals and calculating the treatment's favourable effects on the rate of hepatocyte regeneration. Both Karthik et al., (2017) and Nurul et al., (2018) found that both extracts could be hepatoprotective following the onset of acute liver damage [8, 27]. The Kupffer cells are the resident of macrophage in liver. Kupffer cells will be activated (presence as round shape at the sinusoid of hepatocyte) and the present of inflammatory cells (lymphocytes) as the response of hepatocytes toward the toxic substances as liver is the site for the metobilsm and process of the foreign substance that circulate in blood.To offer appropriate qualitative data on assessing the quality of the chemical composition of *C. vespertilionis* (L.f.) Bakh. f. leaves and *M. citrifolia* L. fruits, comprehensive phytochemical profiling is required. As a result, valid analytical methods are required for a comparative assessment of the leaves and fruits for the quantitative determination of a significant marker, as the putative bioactive compounds on the market are mainly uncontrolled, and a consumer survey of the quality of available products is desirable.

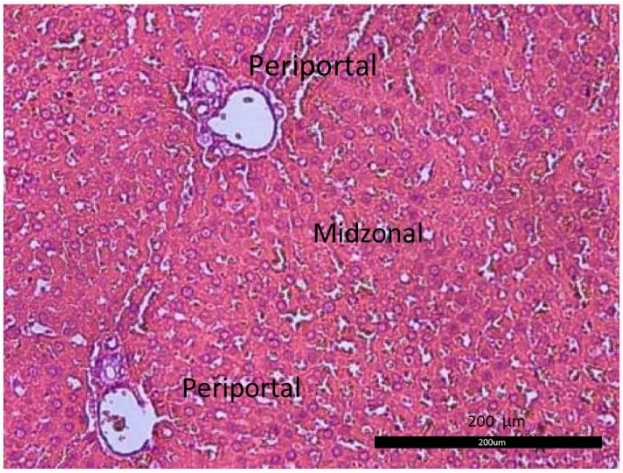


**Figure 1.Pictomicrograph of rat's normal histology of liver in the control group at 13th week of the study period (H&E, x200 magnification). This showed thenormal size of hepatocyte and at periporal and midzonal areas.**

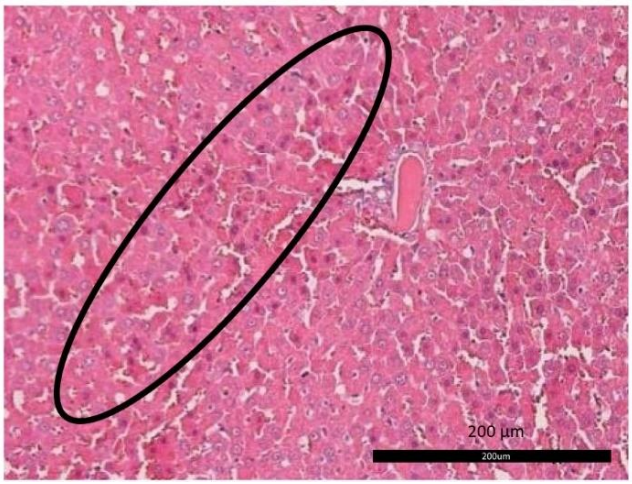


**Figure 2.Pictomicrograph of the liver section of a rat treated with LD *Christiavespertilionis* (L.f.) Bakh. f. + LD *Morindacitrifolia*L., showing eosinophilic cytoplasm (score 0.5) at the periportal towards midzonal area (H&E, x200 magnification).**

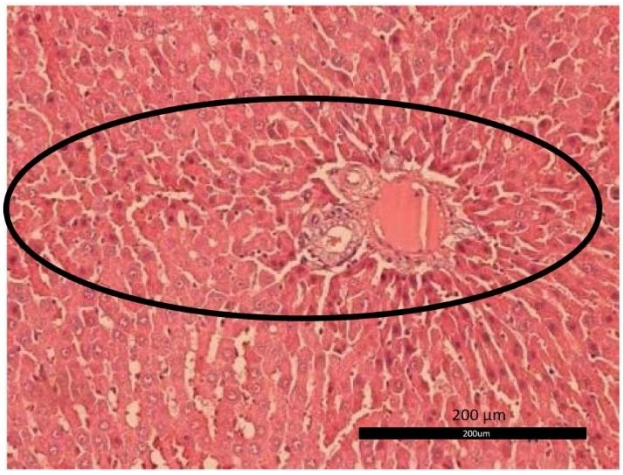


**Figure 3.Pictomicrograph of the liver section of a rat treated with LD *Christiavespertilionis* (L.f.) Bakh. f.+ MD *Morindacitrifolia*L., showing eosinophilic cytoplasm (score 0.5) at the periportal area (H&E, x200 magnification).**

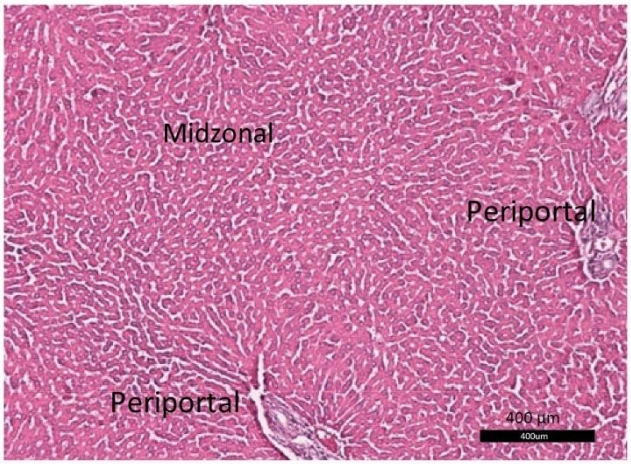


**Figure 4.Pictomicrograph of a liver section of a rat treated with MD *Christiavespertilionis* (L.f.) Bakh. f. + LD *Morindacitrifolia*L., showing diffused dilated sinusoid (score 2) as a result of atrophied hepatocytes at the periportal towards the centrilobular area (H&E, x100 magnification).**

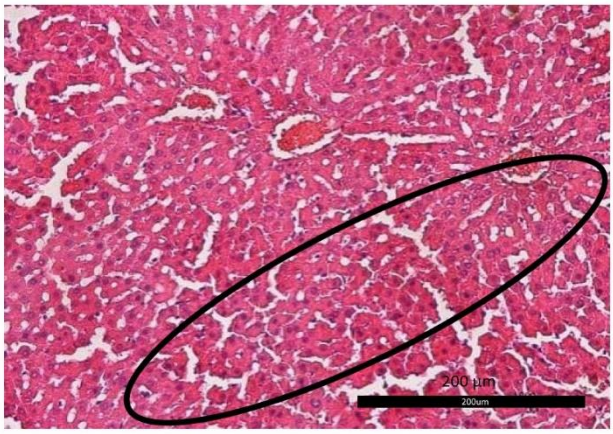


**Figure 5.Pictomicrograph of liver in a rat treated with MD *Christiavespertilionis* (L.f.) Bakh. f. + MD *Morindacitrifolia*L., showing eosinophilic cytoplasm (score 1.5) at the periportal towards the centrilobular area (H&E, x200 magnification).**

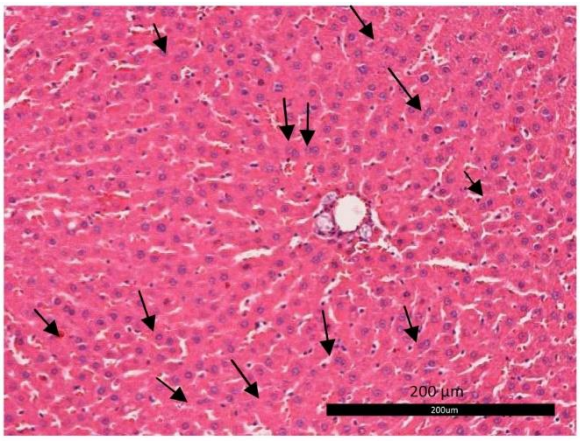


**Figure 6.Pictomicrograph of a liver section of a rat treated with extracts showing regeneration (score 1) (arrows) at the affected periportal area (H&E, x200 magnification).**

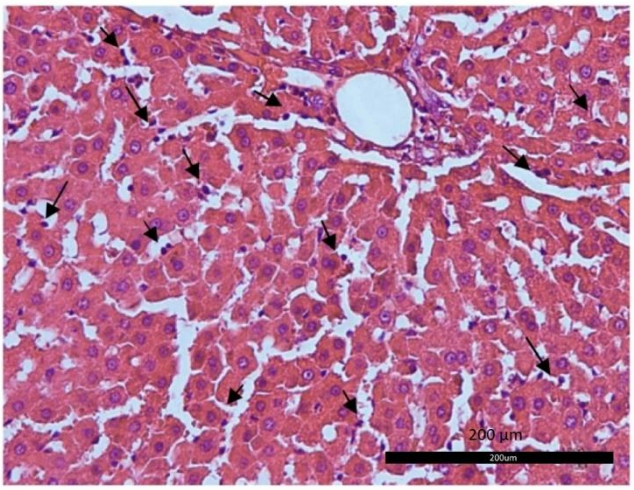


**Figure 7.Pictomicrograph of a liver section of a rat treated with both extracts showing activated Kupffer cells (arrows) (score 1) at the periportal area (H&E, x200 magnification).**

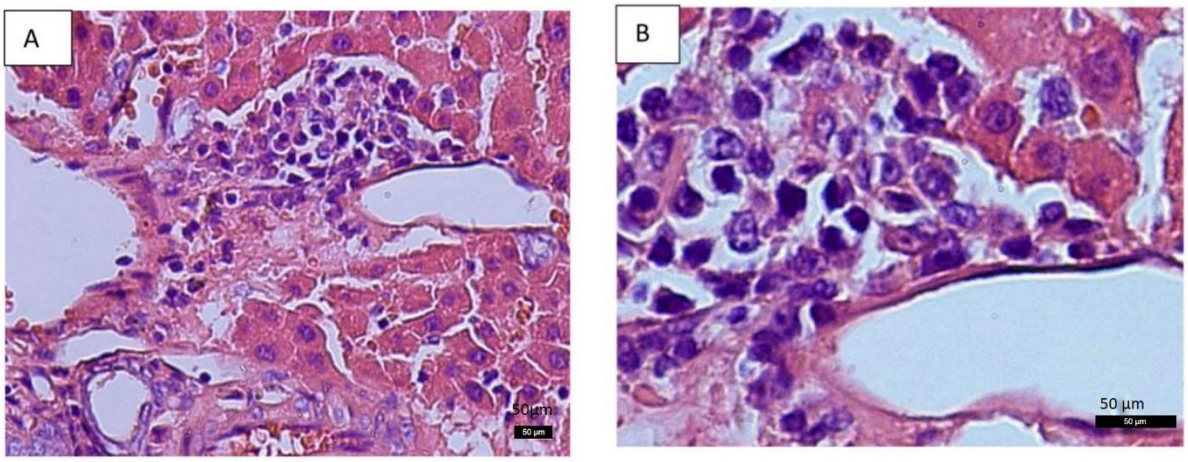


**Figure 8.Pictomicrograps of the liver section showing inflammatory cells (score 1) at the periportal area (H&E, x600 magnification (A), x1000 magnification (B))**

Probably, the alterations in renal histology did not correspond to changes in renal function, as Tamara and colleagues discovered.(2019). The extract may cause structural damage if used for a more extended period, according to the results of histopathological analysis for a more extended period [25]. The kidneys in this investigation had a significant ($p<0.05$) score for the granular cast (very mild) as showed in Figure 9 and Figure 10. Granular cast are more often associated with necrotic luminal cellular debris and are potentially a minor inflammatory infiltrate, indicating initial tubular injury [26]. They are also more often associated with necrotic luminal cellular debris and are potentially a moderate inflammatory infiltrate.

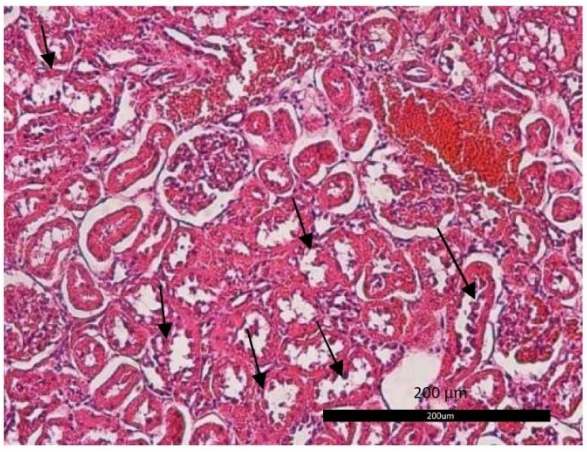


**Figure 9.Pictomicrograph of kidney section of a rat treated with MD *Christiavespertilionis* (L.f.) Bakh. f.+ MD*Morindacitrifolia* L. showing cellular casts (arrows) (score 0.5) (H&E, x200 magnification)**

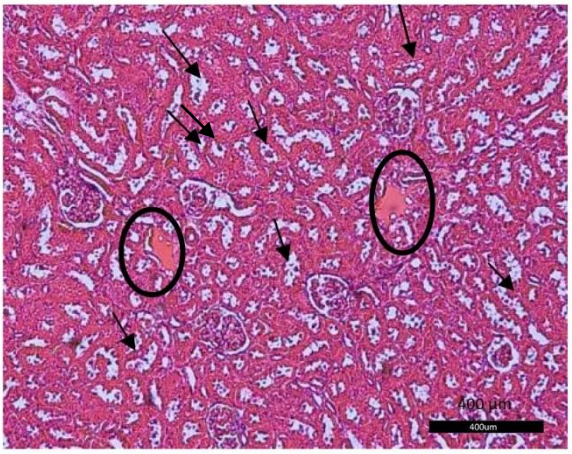


**Figure 10.Pictomicrograph of a kidney section of a rat treated with MD *Christiavespertilionis* (L.f.) Bakh. f. + MD *Morindacitrifolia* L. showing protein cast (encircle)(score 0.5) and granular casts (arrows) (score 0.5) (H&E, x100 magnification**

High-quality pharmacological research is necessary to shed light on the potential mechanisms of action. . Alkaloids, flavonoids, terpenoids, phytosterols, phenols, triterpenes, fatty acids, alkanes, and long-chained alcohols have been found in both leaves and fruit extracts[28-33].Based on this present study, this can be prove that the potential bioactive compounds may result on the toxicity toward liver and kidney from very mild to moderate toxicity as showed in the lesion of histopathological analysis. However, the inclusion of several phytochemicals as bioactive substances in both extracts as added value to the medical efficacy and application potentials because the listed phytochemical have been study as the anticancer also. To our knowledge, this is the first study to compare the toxicity extracts of *C. vespertilionis* (L.f.) Bakh. f. leaves and *M. citrifolia* L. fruits to proceed later with cancer investigations, as both have been claimed to have anti-cancer potential. Later on, the appropriate concentration of extracts may cause minimal toxicity to the rats in a way to see the potential of extracts toward the cancer study.

## Conclusion

The findings of this investigation indicate that ethanolic compounds generated from *C. vespertilionis*(L.f.) Bakh. f. leaf and *M. citrifolia* L. do not appear to cause any invivo harm. . They neither caused any lethality nor produced any remarkable physiological, behavioural, haematological and serum biochemical adverse effects in subchronic toxicity studies in rats. However, they may cause adverse effects on the histopathology analyses at specific doses. These results are in agreement with Nurul, et al. (2018) and Nurul (2018), who demonstrated that both liver and kidney toxicities were induced by the ethanolic extract of *C. vespertilionis* (L.f.) Bakh. f.leaves in subacute and subchronic toxicity studies. Therefore, based on the findings of this study, it is suggested that excessive consumption of herbal mixtures, especially over an extended period of time, is capable of causing liver and kidney damage and should be avoided

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## Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

## Disclosure of conflict of interest

The authors have no disclosures to declare.

## Compliance with ethical standards

The work is compliant with ethical standards. The experiment was designed and conducted in accordance with the ethical approval of the Animal Care Unit Committee (ACUC), Malaysia Agricultural Research and Development Institute (MARDI) approval reference number 20170717/R/MAEC0023.

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