

## SUB-ACUTE TOXICITY STUDY OF DIABECINE RECONSTITUTE IN SPRAGUE-DAWLEY RATS: A PRELIMINARY STUDY IN THE EVALUATION AS AN ANTIHYPERGLYCEMIA AGENT

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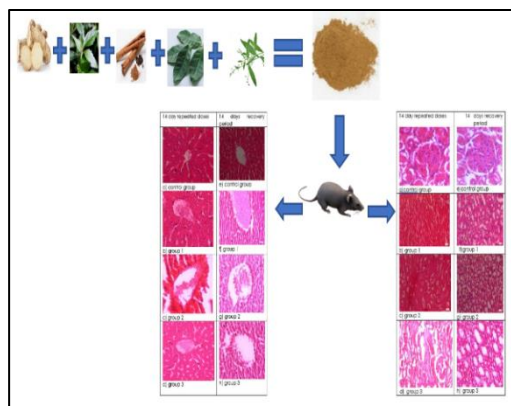
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### Graphical abstract



### Abstract

Diabecine reconstitute is a combination of well-known herbs such as *Cinnamomum zeylanicum*, *Eugenia polyantha*, *Curcuma xanthorrhiza*, *Orthosiphon stamineus* and *Andrographis paniculata* which are believed to give promising finding especially in the treatment of diabetes mellitus. However, no scientific studies have been performed to investigate its toxicological effect. Therefore, this study aimed to evaluate its safety profile using sub-acute toxicity (14 days repeated doses) in rat model. The sub-acute toxicity study was evaluated by daily administration of different doses (250, 600 and 2000 mg/kg) of diabecine reconstitute for 14 days. Clinical signs, body weight changes, food and water intake, white blood count and relative organ weight were recorded. While the liver and kidney were removed for histological analysis. The result indicated that administration of diabecine reconstitute did not show significant differences in food consumption, water intake and white blood count ( $p > 0.05$ ) as compared to the control. However, there is significant difference observed in body weight and relative organ weight ( $p < 0.05$ ). Diabecine reconstitute at 600 and 2000 mg/kg proved to be toxic to kidney and liver in histopathology studies. However, the diabecine reconstitute at concentration of 250 mg/kg is safe to be used and showed no toxicity effect. Therefore, diabecine reconstitute at dose of 250 mg/kg was found to be toxicologically safe and it may be a good potential polyherbal formula to be incorporated in the diabetes mellitus treatment.

**Keywords:** Diabecine reconstitute, Sprague Dawley rats, 14 days sub-acute toxicity, histopathology, polyherbal

### Abstrak

Diabecine reconstitute is a combination of well-known herbs such as *Cinnamomum zeylanicum*, *Eugenia polyantha*, *Curcuma xanthorrhiza*, *Orthosiphon stamineus* and *Andrographis paniculata* which are believed to give promising finding especially in the treatment of diabetes mellitus.

However, no scientific studies have been performed to investigate its toxicological effect. Therefore, this study aimed to evaluate its safety profile using sub-acute toxicity (14 days repeated doses) in rat model. The sub-acute toxicity study was evaluated by daily administration of different doses (250, 600 and 2000 mg/kg) of diabecine reconstitute for 14 days. Clinical signs, body weight changes, food and water intake, white blood count and relative organ weight were recorded. While the liver and kidney were removed for histological analysis. The result indicated that administration of diabecine reconstitute did not show significant differences in food consumption, water intake and white blood count ( $p>0.05$ ) as compared to the control. However, there is significant difference observed in body weight and relative organ weight ( $p<0.05$ ). Diabecine reconstitute at 600 and 2000 mg/kg proved to be toxic to kidney and liver in histopathology studies. However, the diabecine reconstitute at concentration of 250 mg/kg is safe to be used and showed no toxicity effect. Therefore, diabecine reconstitute at dose of 250 mg/kg was found to be toxicologically safe and it may be a good potential polyherbal formula to be incorporated in the diabetes mellitus treatment.

**Kata kunci:** Diabecine reconstitute, tikus jenis Sprague-Dawley, 14 hari sub-akut toksisiti, histopatologi, polihierba

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## 1.0 INTRODUCTION

Natural remedies and herbal preparations are widely used in developing countries to treat various diseases. This practice provides an alternative way to compensate for any deficiencies in current synthetic medicines. Plant-based products have been used for centuries in medical purposes and approximately 80% of the world population depends on this alternative way to fulfil their health needs (1).

Numerous herbs have been reported to possess antihyperglycemia activity. Some of them have demonstrated hypoglycemic effects via animal studies or clinical trials (2; 3) for example *Morus alba*, *Panax quinquefolius* and *Cinnamomum cassia* and their formulations have been approved by Korean authorities to be used as antidiabetic drugs (4). However, it is believed that herbal medicine formulations with multiple herbs will give superior effects when compared to the similar herbs taken separately (5; 6; 7; 8).

Diabecine reconstitute is herbal supplement for diabetes mellitus using combination of standardized extract of herbs which is *Cinnamomum zeylanicum*, *Eugenia polyantha*, *Andrographis paniculata*, *Curcuma xanthorizza* and *Orthosiphon stamineus*. All these medical plants are reported to have several bioactive compounds which are believed to possess antidiabetic activity. *Cinnamomum zeylanicum* is claimed to have catechin which could regenerate the  $\beta$ -cells in streptozotocin-induced diabetic rats (9). Recently, the antihyperglycemic effect of *C. zeylanicum* has entered human trials as it could lower the elevated levels of glucose and improved insulin sensitivity in people with diabetes (10). *E. polyantha* and *A. paniculata* have gallic acid and

andrographolide that can reduce blood glucose level in alloxan- induced diabetic rats (11; 12). Curcumin in *C. xanthorizza* is reported to possess an important role in protecting advanced glycation, reduces blood glucose and the levels of glycosylated hemoglobin in diabetic animal (13). Meanwhile *O. stamineus* is documented to have rosmarinic acid which has a potential to regulate the key enzymes of carbohydrate metabolism (14).

Natural product supplements can be considered safe to use but some of them are known to give toxicity effect at high dose or have adverse effect after prolonged use (15). The toxicity data of the single plant in diabecine reconstitute is widely reported. However, the safety and toxicity data on the combination of these five herbs have not been reported elsewhere until now. Therefore, this current study aimed to evaluate the toxicological effect of diabecine reconstitute in female Sprague-dawley rats and to identify its safe dose for the development a novel antihyperglycemia agent.

## 2.0 METHODOLOGY

### Materials

Diethyl ether, hydrochloric acid, xylene, acetic acid, glycerol and ethanol were purchased from Merck Sdn. Bhd. Potassium acetate, Wright's stain and paraffin plasticized pellet were obtained from BDH (Leicestershire, England). Formaldehyde was purchased from HmbG chemicals. Harris hematoxylin solution and eosin Y were purchased from Sigma-Aldrich Sdn. Bhd. Streptozotocin (STZ) (98% HPLC) was purchased from Sigma. Tri-sodium Citrate-2-hydrate

and Citric acid-1-hydrate were obtained from HmbG Chemicals. Glibenclamide was purchased from Pharmaniaga.

### Plant Material & Experimental Design

Diabecine reconstitute was supplied by Proliv Life Sciences Sdn. Bhd. All extracts were diluted in distilled water according to the body weight of each rats.

### Sub-Acute Toxicity Study

#### Sub- Acute Oral Toxicity Study (Repeated Dose 14 Days)

The experiment was conducted following the protocols described in OECD Guideline 402. The procedures and experimental protocols used in this study were approved by the Ethics Committee of Universiti Malaysia Terengganu with registration number UMT/JKEPHT/2017/3.

Twenty-four healthy female Sprague-Dawley rats at the age of 8-10 weeks and with body weight of 180-200g were randomly divided into four groups of six rats per group and received the following treatments:

|         |   |
|---------|---|
| Control | Normal rats received distilled water            |
| Group 1 | Normal rats received 250 mg/kg BW of diabecine  |
| Group 2 | Normal rats received 600 mg/kg BW of diabecine  |
| Group 3 | Normal rats received 2000 mg/kg BW of diabecine |

These doses were selected based on the previous *in vitro* cytotoxicity study (16) of diabecine towards diabetic models of 3T3-L1, WRL-68 and 1.1B4 Cell Lines. All groups were treated by oral gavage once daily for 14 days. Food consumption, water intake and body weight gained were recorded at day 0, 3, 7 and 14 during treatment. Blood was withdrawn from tail vein by sterile needle on day 0, 7 and 14. All blood samples were subjected to thin blood smear for white blood cells differential counting. Half of the survived rats (n=3) from each group was sacrificed on day 14 of sub-acute toxicity study to obtain organs such as liver and kidney for their relative weight. The organs were analysed via histological examination for any abnormalities and toxicological signs.

### Fourteen Days Recovery Period Study

Half of the survived rats (n=3) were returned back to their own cage and were kept for another 14 days observation period. During the period, all groups of rats were not treated with any diabecine reconstitute and they had free access to food pellets (10% of body weight) and water. The occurrence of toxicity was observed twice daily.

### Histological Analysis

Upon dissection, livers and kidneys were removed from the carcasses. Samples were fixed with 10% buffered formalin for 24 hours before the dehydration process using a tissue processing machine. Then, samples were embedded into molds with paraffin wax and sectioned at 5  $\mu$ m. Leica™ DM LB2 light microscope (Germany) that was equipped with the Leica™ Image Analyzer System and LAS 4.0 system software and a x20 objective lens were used in the histological analysis.

## 3.0 RESULTS AND DISCUSSION

### Sub-acute Toxicity Study

#### Food Consumption and Water Intake

Following the OECD guidelines 420, any adverse effect of the test substances can be determined using several parameters such as food consumption, water intake, body weight, relative organ weight gain, clinical biochemistry (White blood cell count) macroscopic pathology of internal organ. All these parameters were analysed as it can provide a first signs of toxicity and give an insight information on low observed adverse effect level (LOAEL) and no observed adverse effect level (NOAEL) of the tested substances (17).

The oral administration of diabecine reconstitute (250, 600 and 2000 mg/kg bw) showed no significant differences in food consumption and water intake in the treated rats as compared to the control (Table 1 & 2) during the sub-acute and recovery period. The determination of food consumption and water intake in the toxicity study of herbal medicine for therapeutic purpose is an important parameter as an adequate intake of nutrients is crucial to maintain the physiological state of the animals and to achieve the accurate response to the herbal tested (18). In this current study, the food and water intake of the treated rats were not statistically different as compared to control which suggest that diabecine reconstitute does not suppress or induce appetite.

**Table 1** Effect of the diabecine reconstitute on food consumption. Data are expressed as mean  $\pm$  SEM and analyzed by one-way ANOVA followed by Tukey's test. (\* p< 0.05 as compared to control)

| Group   | Day 3 (g/day)    | Day 7 (g/day)   | Day 14 (g/day)  | Day 21 (g/day)  | Day 28 (g/day)  |
|---------|------------------|-----------------|-----------------|-----------------|-----------------|
| Control | 35.0 $\pm$ 0.01  | 41.0 $\pm$ 0.00 | 30.0 $\pm$ 0.00 | 20.0 $\pm$ 0.02 | 28.0 $\pm$ 0.00 |
| Group 1 | 40.0 $\pm$ 00.00 | 40.0 $\pm$ 0.00 | 40.0 $\pm$ 0.01 | 23.0 $\pm$ 0.00 | 30.0 $\pm$ 0.00 |
| Group 2 | 40.0 $\pm$ 00.00 | 40.0 $\pm$ 0.00 | 32.0 $\pm$ 0.02 | 25.0 $\pm$ 0.00 | 31.0 $\pm$ 0.00 |
| Group 3 | 42.0 $\pm$ 0.003 | 30.0 $\pm$ 0.02 | 32.0 $\pm$ 0.02 | 24.0 $\pm$ 0.04 | 35.0 $\pm$ 0.05 |

**Table 2** Effect of the diabecine reconstitute on water intake. Data are expressed as mean  $\pm$  SEM and analyzed by one-way ANOVA followed by Tukey's test. (\*  $p < 0.05$  as compared to control)

| Group   | Day 3<br>(ml/day)     | Day 7<br>(ml/day)   | Day14<br>(ml/day)    | Day21<br>(ml/day)     | Day28<br>(ml/day)     |
|---------|-----------------------|---------------------|----------------------|-----------------------|-----------------------|
| Control | 138.0 $\pm$<br>24.00  | 75.0 $\pm$<br>50.00 | 175.0 $\pm$<br>50.00 | 175.00 $\pm$<br>50.00 | 185.00 $\pm$<br>30.00 |
| Group 1 | 150.0 $\pm$<br>100.00 | 100.0 $\pm$<br>0.00 | 175.0 $\pm$<br>20.00 | 190.00 $\pm$<br>20.00 | 180.00 $\pm$<br>40.00 |
| Group 2 | 150.0 $\pm$<br>100.00 | 100.0 $\pm$<br>0.00 | 125.0 $\pm$<br>50.00 | 180.00 $\pm$<br>40.00 | 175.00 $\pm$<br>50.00 |
| Group 3 | 200.0 $\pm$<br>00.00  | 100.0 $\pm$<br>0.00 | 125.0 $\pm$<br>50.00 | 200.00 $\pm$<br>0.00  | 190.00 $\pm$<br>20.00 |

### Body Weight

Table 3 shows the changes in the body weight of rats fed at different doses (250, 600 and 2000 mg/kg) of diabecine reconstitute. The weight loss is a simple and sensitive index to determine any toxicity effect after the exposure of the test substances (19). Increased in body weight has been observed in all groups from day 0 until day 7 and slightly decreased until day 28. The decrease might be attributed by numbers of rats which are only three remaining rats in the recovery period (day 15-28), as half of the rats are sacrificed on day 14. The significant differences have been observed during day 14 (group 2) and day 21 (group 2 and 3) as compared to control. This significantly higher body weight suggests that the rats were in good health even though received high concentration of diabecine reconstitute.

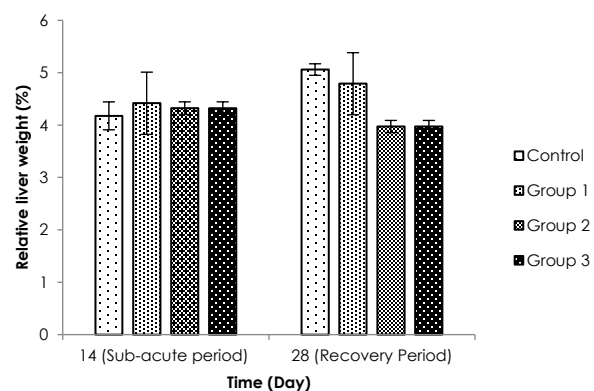
**Table 3** Effect of the diabecine reconstitute on body weight. Data are expressed as mean  $\pm$  SEM and analyzed by independent t-test (\*  $p < 0.05$  as compared to control)

| Group   | Day 0<br>(g)       | Day 3<br>(g)      | Day 7<br>(g)      | Day 14<br>(g)      | Day 21<br>(g)      | Day 28<br>(g)      |
|---------|--------------------|-------------------|-------------------|--------------------|--------------------|--------------------|
| Control | 197 $\pm$<br>0.01  | 203 $\pm$<br>0.00 | 205 $\pm$<br>0.00 | 163 $\pm$<br>0.01  | 120 $\pm$<br>0.01  | 130 $\pm$<br>0.015 |
| Group 1 | 200 $\pm$<br>0.01  | 197 $\pm$<br>0.01 | 218 $\pm$<br>0.01 | 177 $\pm$<br>0.01  | 173 $\pm$<br>0.02* | 170 $\pm$<br>0.02  |
| Group 2 | 202 $\pm$<br>0.01  | 202 $\pm$<br>0.01 | 208 $\pm$<br>0.01 | 198 $\pm$<br>0.01* | 180 $\pm$<br>0.02* | 187 $\pm$<br>0.014 |
| Group 3 | 205 $\pm$ 0<br>.01 | 200 $\pm$<br>0.01 | 205 $\pm$<br>0.01 | 187 $\pm$<br>0.01  | 130 $\pm$<br>0.01  | 163 $\pm$<br>0.03  |

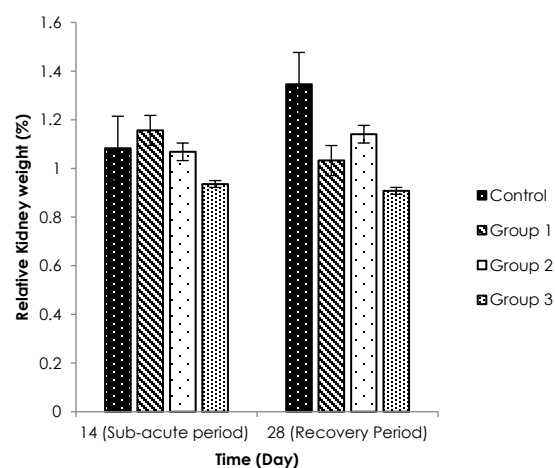
### Relative Organ Weight (ROW)

Figure 1 and 2 display the relative weight of the liver and kidney in the sub-acute and recovery period. There was a significant difference in the ROW of liver in group 2 and 3 during recovery period when compared to the control. However, there was no significant difference observed in the kidney in both periods of study.

The significantly decreased liver weight of the treated rats in group 2 and 3 in the recovery period may indicate a potential toxic effect after exposure to the diabecine reconstitute at high dose. Generally, any significant changes of the organ can be correlated to the toxic effect of the test substances (20). Therefore, liver might be susceptible to the toxic effects of the diabecine reconstitute at high doses. This finding is supported by the report of Ogunlala et al. 2013 (20) who found the significant increment in the ROW of the liver and kidney after the treatment of *Caesalpinia bonduc*.



**Figure 1** Relative organ weight on sub-acute and recovery period. Data are expressed as mean  $\pm$  SEM and analyzed by independent t-test (\*  $p < 0.05$  as compared to control)

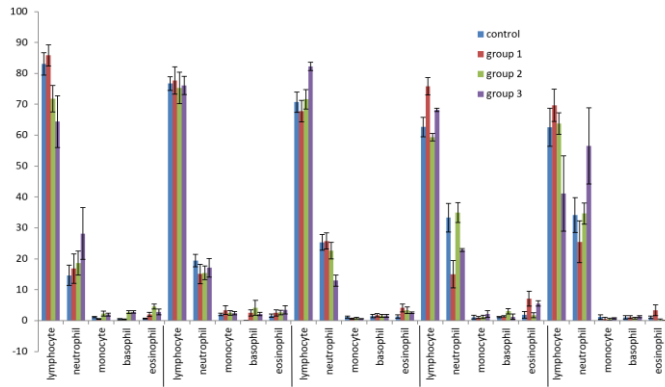


**Figure 2** Relative organ weight on sub-acute and recovery period. Data are expressed as mean  $\pm$  SEM and analyzed by independent t-test (\*  $p < 0.05$  as compared to control)

### White Blood Cell Count (WBC)

The evaluation of haematological parameters can be used to examine any deleterious effects of foreign substances including herbal extracts on the blood profile of the animal. Hematopoietic system serves as a crucial index in pathological and physiological status and very sensitive to toxic compounds (18).

Figure 3 shows the exposure of Sprague-dawley rats to the diabcine reconstitute (250, 600, 2000 mg/kg) produced transient and small changes in lymphocytes, neutrophils, monocytes, basophil and eosinophil but the values were not significantly difference when compared to the control group. This finding suggests that diabcine reconstitute may have no or less toxicological effects on the hematopoietic system (1).



(\* p < 0.05 as compared to control)

**Figure 3** Relative organ weight on sub-acute and recovery period. Data are expressed as mean ± SEM and analyzed by independent t-test

**Histopathology**

**Histopathology of Liver**

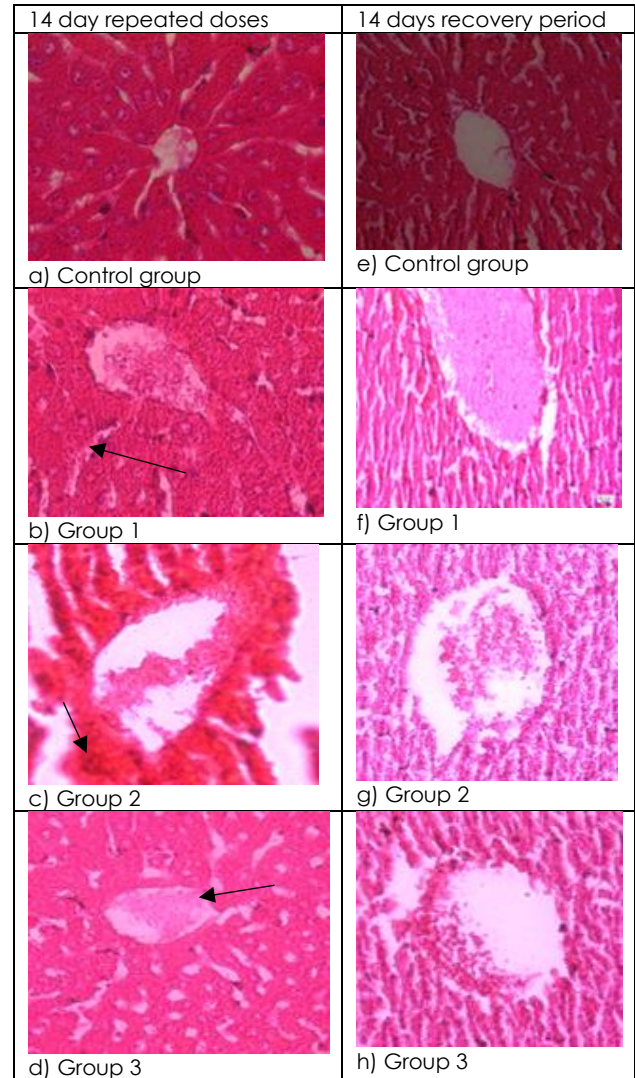
The liver of control untreated animal in did not exhibit any gross lesions during macroscopic examination. Microscopic examination showed that hepatocytes are in normal cell arrangement (Figure 4a and 4e). No significant lesion such as fatty liver or hepatocytes degeneration observed in animals in control.

The liver of white rats in Group 1 showed erythrocytes aggregation with mild centralized thrombi during sub-acute study (Figure 4b). The vein also showed early formation of fibrin indication certain degrees of injury of the endothelium (Figure 4b). During the recovery period the thrombus detached from endothelium wall (Figure 4f) while hyperemia subsided. Hyperemia conditions refer to an increase in blood flow to a particular tissue and organ which leads to increased vascularization and redness. In the context of liver, hyperemia occurs due to several factors such as infections, inflammation, and toxins. The subsided hyperemia conditions during recovery period indicated that the toxicity effect of diabcine subsided during the recovery period.

The vein of animal in Group 2 showed mild hemolysis of erythrocytes and thrombus from the endothelial wall with moderate hyperemia of surrounding hepatocytes during sub-acute study (Figure 4c). However, the hyperemic condition subsides during the recovery period with moderate thrombus. However, few hepatocytes progress into

necrosis state where several cells start to loss their cell structure (Figure 4g).

Animal in Group 3 showed moderate thrombosis with fibrinous materials. The erythrocytes showed degree of hemolysis while more generalize hyperemia was observed on surrounding tissue (Figure 4d). The conditions showed improvement with lateral thrombi on the endothelium wall and mild hyperemia (Figure 4h) (21).



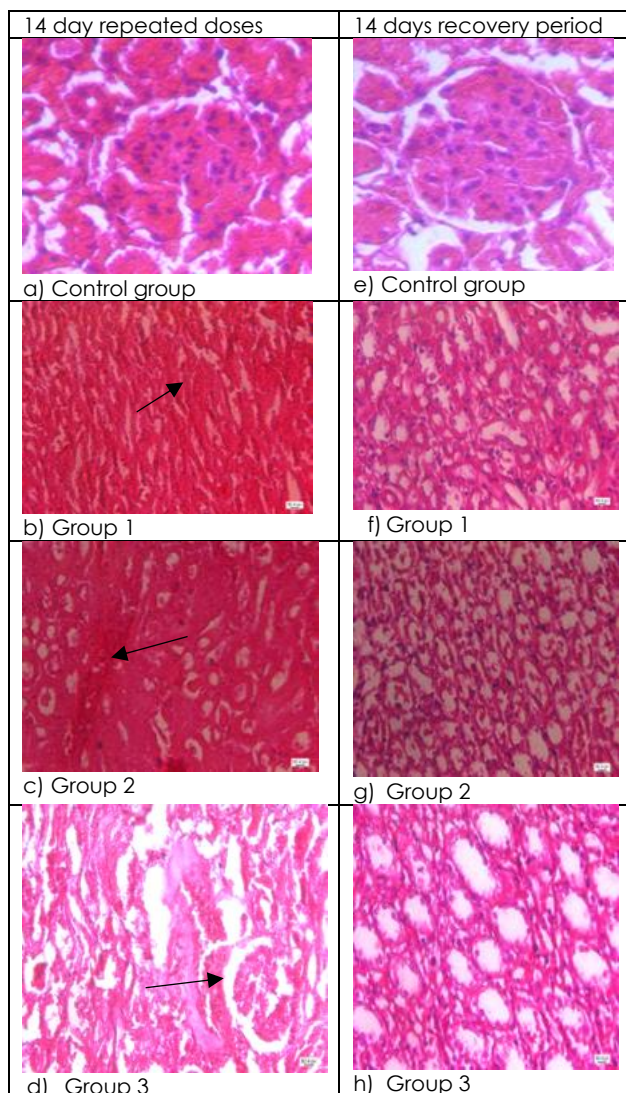
**Figure 4** Photomicrographs (X40) of liver histopathology in sub-acute (a-d) and recovery period (e-h).

**Histopathology of Kidney**

Kidney of control groups for both studies showed normal histology appearance without any lesions (Figure 5a & 5e). Both figures 5a and 5e showed glomerulus surrounded by Bowman capsules with normal proximal and distal tubules. However, kidney from animal in Group 1 showed generalizes hyperemic condition with swollen tubules (Figure 5b). During the recovery period, the kidney tissue exhibited moderate degeneration, which is evidence for intoxication (Figure 5f).

Swollen tubules were evidenced in kidney of white rats in Group 2 with severe hyperemia and fibrinous formation (Figure 5c). The condition progresses into fatty degeneration during recovery period but with less hyperemic condition. (Figure 5g). Necrosis and fibrinous formation were evidenced in kidney of white rat of Group 3 with degrees of glomerulo-nephritis (Figure 5d). Moderate fatty degeneration was also observed during the recovery period (Figure 5h) with degree of necrotic cells.

Other than liver, kidney is the second target organ which plays important role to the excretion of the metabolism and foreign substances (Effendy *et al.*, 2006). The toxic compounds in the plant extract will cause disruption of glomerular functions and pathological changes. Finally, these disorders will affect the renal tubular functions. This study highly suggested the presence of toxic compounds in diabecine during 14 days of sub-acute toxicity study which led to the disruption of renal tubular functions. However, this toxic compound has been detoxification during recovery periods.



**Figure 5** Photomicrographs (X40) of kidney histopathology in sub-acute (a-d) and recovery period (e-h)

## 4.0 CONCLUSION

In conclusion, diabecine reconstitute at concentration of 250 mg/kg BW is safe to use and showed no or less toxicity effect. However, diabecine reconstitute at 600 mg/kg and 2000 mg/kg BW evidenced toxic to kidney and liver. Therefore, diabecin at 250 mg/kg BW is potent to be used as an alternative antidiabetic remedy. However, further and depth evaluation such as chronic and sub-chronic toxicity study need to be done in the future to ensure its safety for human consumption.

## Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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