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Histopathological changes in placenta and liver of pregnant rats administered with aqueous extract of *Dioscorea hispida* var. *daemona* (Roxb) Prain & Burkill

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ABSTRACT

Dioscorea hispida var. *daemona* (Roxb) Prain & Burkill (DH), also known a tropical yam or intoxicating yam is a bitter wild tuber which is consumed as a staple food and traditionally used as a remedy in Malaysia. However, DH is also notorious for its intoxicating effects and there is currently a dearth of study of possible effects of DH on liver and placental tissues and hence its safe consumption warrants in-depth investigation. This study was therefore designed to investigate into the effect of DH on liver and placenta of pregnant rat *via* histopathological examination. Thirty pregnant Sprague-Dawley rats were randomly divided into five groups consisting of a control (distilled water) and four DH aqueous extract groups (250, 500, 1000 and 2000 mg/kg body weight). The extracts were administered *via* oral gavage daily throughout the study and animals were sacrificed on day 21. Paraffin-embedded, hematoxylin and eosin stained sections of placenta and liver were examined. Significant changes ($p < 0.05$) were observed on relative liver and placental weights of animals treated with 2000 mg/kg body weight DH extract. The placental numbers were decreased with the increased of DH extract concentration. Liver histological examination in all treated groups showed that tissues underwent degeneration characterized by hepatocyte swelling, cytoplasmic vacuolation, cytolysis, margination and clumping of nucleus chromatin. Changes of the basal and labyrinth zone were observed in placental tissues in all treated groups. Glycogen cells were reduced with fibrin deposition in the basal zone, while irregular vessel formation was demonstrated in the labyrinth zone. UHPLC-ESI-MS analysis showed the presence four steroidal saponins DH. In conclusion, DH aqueous extract exert hepatotoxicity and adverse effects on the placenta of rats. However, the underlying mechanism and phytochemicals inducing the observed toxicity require further investigation.

1. Introduction

Natural products with therapeutic properties have been used for thousands of years by several cultures. Since the dawn of human kind, natural products such as minerals, plant and animal products have been the main source of medicines to treat and manage a plethora of human diseases. Interestingly, Malaysia is a country with a long-standing tradition in the use of such natural products to assuage human suffering. Endowed by a rich floral biodiversity and natural forests, Malaysia has the inherent potential to provide a very large panoply of plants for medicinal purposes (Burkill, 1935; Jamia, 2006).

One plant that is commonly used both as a food and for its medicinal properties in Malaysia is *Dioscorea hispida* (Dioscoreaceae) (Nashriyah et al., 2012; Hudzari et al., 2011). *D. hispida* is commonly known as tropical yam, wild tuber, *Ubi Gadong,* or intoxicating yam. It can be widely found in tropical regions like Indonesia, Thailand and Malaysia. The tuber of this plant is traditionally consumed as staple food due to the high carbohydrate content (Tajuddin et al., 2013). Studies have reported the biological activities of *D. hispida* such as antioxidant, analgesic, anti-inflammatory, antioxidant and anti-tumor activity

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(Muhammad Azhar et al., 2014, Panduraga et al., 2011).

Various studies have reported the toxicity effects of *D. hispida* chemical compounds like alkaloid, hydrogen cyanide, steroidal saponin and tannin (Adedayo et al., 2011; Chanida et al., 2014). Dioscorine isolated from *D. hispida* has demonstrated its neurotoxicity, which overly exert a stimulation of the central nervous system, causing hyperthermia and mydiatric (Sasiwatpaisit et al., 2014). Furthermore, hydrogen cyanide is able to cause unfavorable health complications like nausea, vomiting, stomach discomfort, and diarrhea (Ashri et al., 2014). In fact, saponin extracted from plants of same genus, *D. villosa* has been reported to cause impairment in pregnancy (Atsukwei et al., 2015). In addition, diosgenin from *D. villosa* induced embryonic development arrest in mouse at about the 16-cell stage (Ruyani et al., 2011).

Currently, there are few toxicological studies of aqueous extracts of *D. hispida* administration during pregnancy. To this effect, the present research was designed to provide more insights into the potential adverse effects of *D. hispida* aqueous extract on pregnant rats during organogenesis period by evaluation on histopathological changes of maternal liver and placenta. In addition, UHPLC-ESI-MS analysis was performed to characterize the steroidal saponins in *Dioscorea hispida* var. *daemona* (Roxb) Prain & Burkill.

2. Materials and methods

2.1. Plant preparation

Dioscorea hispida (synonym: *Dioscorea hispida* var *daemona* (Roxb) Prain & Burkill) fresh tubers were harvested/obtained from Kg Bukit Bakar, Machang, Kelantan. The maturity was about 2–3 years. The tubers were only harvested when half of the tuber appeared on the ground surface, and when they appeared brownish/yellowish in color. The tubers were washed, dried, and grinded to a powdered form. The samples were then extracted with 80 °C water and filtered. The filtrate was collected and sprayed dried. For UHPLC-ESI-MS analysis, 10 mg/mL of dry extract, adequate volume (ca. 2 mL) was passed through a 0.22 μm PTFE membrane filter. The plant was authenticated by Forest Research Institute Malaysia (FRIM) with the voucher specimen number (SBID:0012/08).

2.2. Total polyphenols

The total polyphenols assay was performed using a Folin-ciocalteau assay based on a method of Singleton and Rossi (1965). The method allows determination of oxidised phenols by producing a blue colour from heteropoly phospomolibdate-tungsten anions where the darker the blue colour the more phenols are present.

2.3. UHPLC-ESI-MS analysis

The liquid chromatographic system was a QExactive UHPLC (Thermo Fischer Scientific, USA) comprised of the following components: binary pump, a solvent degasser, an autosampler, and a thermostatically controlled column compartment. Separation was achieved on WATERS C18 (2.1 × 50 mm, 17 μm) column. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) at flow rate 0.4 mL/min. The following solvent composition was used: 0–5 min, 10–20% B; 5–10 min, 20–60% B; 10–13 min, 60–90% B; 14–14.10 min, 90-10% B and 14.1–15 min, 10% B.

2.4. Animals

A total of 30 pregnant Sprague-Dawley rats with a body weight ranging from 180 to 200 g were obtained from the Animal Resource Unit, Medical Research Resource Centre, Institute for Medical Research, Malaysia. Animals were housed in rat standard individually ventilated cages (IVC) under controlled temperature (20 ± 2 °C), humidity (40–60%) with a 12 h light-dark cycle. The rats were provided with standard pelleted food (Specialty Feeds, Australia) and fresh water *ad libitum* throughout the experiment. Ethical approval for this study was obtained from the Animal Care and Use Committee, Ministry of Health Malaysia (ACUC No: ACUC/KKM/02(10/2016).

2.5. Experimental design

Female rats in the pro-oestrous phase were placed into the cage of male (1:1) and left for 24 h. The presence of sperm in the vaginal smear was considered as gestation day 0. The pregnant rats were randomly divided into 5 groups. Group 1 served as negative control while groups 2, 3, 4 and 5 were administered with 250, 500, 1000 and 2000 mg/kg body weight of *D. hispida* aqueous extracts via oral gavage (volume, 10 mL/kg body weight) on gestation day 6 until 20. Animals were observed for any clinical signs and recorded. On day 21, rats were euthanized and caesarean section was performed.

2.6. Fetal and tissue collection and macroscopic examination

The fetuses were removed from gravid uterus, cleaned and examined for any abnormalities. The liver and placenta were removed and weighed. The relative organ weight (ROW) of each organ was then calculated as follows:

$$\text{ROW} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat on caesarean day}} \times 100$$

Both liver and placental tissues underwent macroscopic examination. The tissues were then were kept in 10% formalin solution prior histopathological examination.

2.7. Histopathological preparation and microscopic examination

The tissues were fixed by suspension in 10% formalin solution for a minimum of 24 h. The tissues were trimmed, embedded in paraffin, sectioned and manually stained with haematoxylin and eosin (H&E) according to standard protocols (Besteman et al., 2007; Furukawa et al., 2008). The lesions on the tissues were observed and photographed using Leica microscope with LG LED TV. The morphology of liver and placental tissues was evaluated for any changes including the nucleus morphology and size, nucleus to cytoplasm ratio, and cytoplasm quality and volume. The lesion for liver and placental tissue were examined and recorded. All the procedures were performed according to the guidance and supervision of a veterinary pathologist in University Veterinary Hospital (UVH), UPM Serdang, Malaysia.

2.8. Statistical analysis

All measured parameters were calculated and expressed as mean ± standard error (SE) and median (interquartile range (IQR)). Statistical analysis was performed using SPSS 20.0 software. The relative organ weight and fetal body weight comparison between groups was analyzed using One-Way ANOVA *post-hoc* Tukey test. Number of live and dead fetus per litter was analyzed using Kruskal-Wallis followed by Dunn-Bonferroni pairwise comparisons. The level of the significance was evaluated at p value less than 0.05 ($p < 0.05$).

3. Results

3.1. Total polyphenols

D. hispida aqueous extract yielded 243 ± 2.1 mg gallic acid equivalent (GAE)/100 g of total phenolic content.

Table 1
Mass spectral characteristic of steroidal saponins determined by LCMS-ESI in the extract of *D. hispida*.

Peak No	Retention time Rt (min)	Compound	Formula	Selected ion	m/z observed	Error (ppm)
i.	0.80	Protocatechuic acid	$C_7H_5O_4$	$[M-H]^-$	153.01893	4.541
ii.	2.54	Phloretic acid	$C_9H_9O_3$	$[M-H]^-$	165.05490	1.692
iii.	3.69	4-hydroxy benzoic acid	$C_7H_5O_3$	$[M-H]^-$	137.02383	3.718
iv.	11.49	Dioscin	$C_{45}H_{71}O_{16}$	$[M-H]^-$	867.47981	7.087

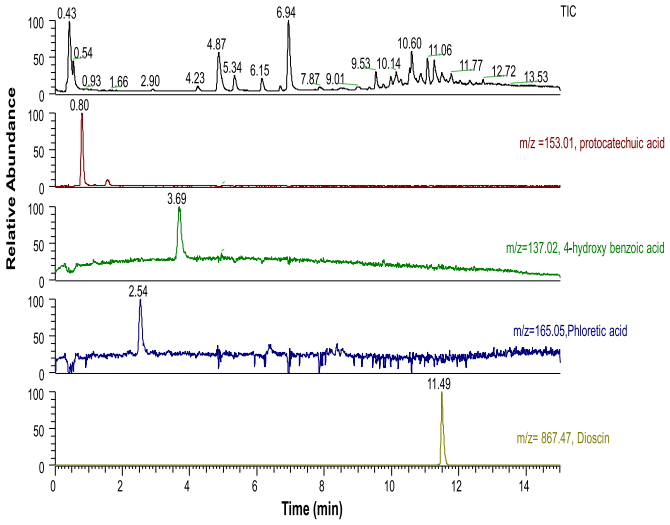

Fig. 1. Chromatograms of the extracts from *D. hispida* analyzed by UHPLC.

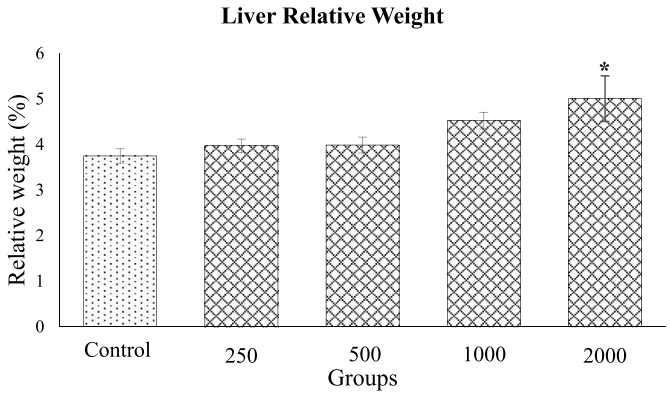

Fig. 2. Relative liver weight of maternal rats administered with *D. hispida* aqueous extract at different concentrations during organogenesis period. Values were presented as mean ± SEM (n = 6). *Indicated significantly different at $p < 0.05$ when compared with control group by One-way ANOVA *post-hoc* Tukey test.

3.2. UHPLC-ESI-MS analysis

In the present study, UHPLC-ESI-MS has been applied to characterize the steroidal saponins in *D. hispida*. A total of four steroidal saponins were detected in which they were tentatively identified based on retention time, accurate molecular ion (m/z), molecular formula with reference to the reference standard and data from the previous study (Table 1). The chromatogram of aqueous extract from the tubers of *D. hispida* is shown in Fig. 1.

3.3. Fetal examination

The average number of lives fetuses was comparable between control, 250, 500 and 1000 mg/kg body weight animals (Table 2). No external abnormalities on fetuses were observed in these groups. Significant changes ($p < 0.05$) were observed in animals treated with 2000 mg/kg body weight extract as 80.6% of the fetuses were found dead and undeveloped due to the immaturity of pregnancy. Only one pregnant rat from the highest dosing group survived up to gestation day 20.

Values were presented as mean ± SEM and median (IQR). $^{\alpha}$ indicates significant differences at $p < 0.05$ when compared with control, 250, 500 and 1000 mg/kg *D. hispida* groups by Kruskal-Wallis, Dunn-Bonferroni pairwise comparisons. $^{\beta}$ indicated significant differences at $p < 0.00$ when compared to control and 250 mg/kg *D. hispida* groups by Kruskal-Wallis, Dunn-Bonferroni pairwise comparisons. * indicates significant differences at $p < 0.05$ when compared to control 250, 500 and 1000 mg/kg *D. hispida* groups by One-way ANOVA *post-hoc* Tukey test.

3.4. Gross examination and relative organ weights

No significant changes ($p > 0.05$) were observed in the gross appearance of maternal liver tissues between groups. However, unformed placentas were found in the animals treated with the highest concentration of *D. hispida* extract (2000 mg/kg body weight). Figs. 2 and 3 show the relative maternal liver and placental weight. There were significant increment ($p < 0.05$) in the relative weight of the maternal rats' liver at 2000 mg/kg body weight concentration of *D. hispida* extract (5.004 ± 0.501) as compared to control group (3.746 ± 0.158). The placental relative weights were increased in group 250, 500, and 1000 mg/kg body weight *D. hispida* extract though these changes were not statistically significant. A significant decrease ($p < 0.05$) on the placental relative weight was only observed in the highest concentration group.

3.5. Histopathological examination

3.5.1. Liver tissue

Fig. 4(A–E) shows the histopathological changes on liver tissues in all groups. The liver of control animals showed large polyhedral

Table 2
Fetal parameters orally treated with *D. hispida* aqueous extract on gestation day 6–20.

Parameters	*D.hispida* aqueous extract (mg/kg body weight)				
	Control	250	500	1000	2000
No. of dead fetuses/litter	0	0	0	0	6.5(13)$^{\alpha}$
No. of live fetuses/litter	14(3)	13.5(1)	13(1)	12.5(6)	0(3)$^{\beta}$
Fetal body weight (g)	5.74 ± 0.05	5.61 ± 0.10	5.29 ± 0.11	4.92 ± 0.40	1.49 ± 0.61*

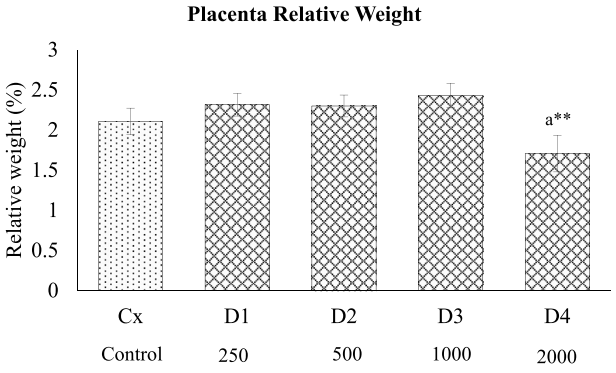

Fig. 3. Relative placenta weight of maternal rats administered with *D. hispida* aqueous extract at different concentrations during organogenesis period. Values were presented as mean ± SEM (n = 6). Values that were significantly different at $p < 0.05$ when compared with control by One-way ANOVA *post-hoc* Tukey test are indicated with * while values marked with [a] were significantly different at $p < 0.05$ when compared with 1000 mg/kg *D. hispida* groups. **including unformed placenta weight.

hepatocytes with prominent round nucleus. Varying degrees of changes and tissue lesions were noted in all *D. hispida* treated groups. Most of the treated livers underwent degeneration as characterized by hepatocyte swelling, dissolution of hepatic cords, cytoplasmic vacuolation, infiltration of inflammatory cells, and presence of external exudates, margination and clumping of nucleus chromatin, cytolysis and karyolysis. Additional features of lesions were demonstrated in animals treated with 2000 mg/kg body weight as fatty changes near the portal tracts, more activated Kupffer cells and spotty necrosis were noted by foci of inflammation cells.

3.5.2. Placental tissue (basal zone)

The histopathological examination of the placenta was focused on two major parts which is the basal and labyrinth zone (Fig. 5A–E). The placental tissue from control group showed normal basal zone structure with intact trophoblastic giant cells, spongiotrophoblast cells, and glycogen cells. However, cellular disrupted and various degrees of basal zone damage were observed in *D. hispida* treated placenta. Fibrin deposition, reduced glycogen cells and nucleus margination of trophoblastic giant cells were observed in 250, 1000 and 2000 mg/kg body weight groups. However, the migration of inflammatory cells was only found in 1000 and 2000 mg/kg body weight animals. In contrast, the basal zone of the group 500 mg/kg body weight, revealed cystic degeneration of glycogen cells which indicated by the presence of large cysts filled with homogenous acidophilic mass and clusters of macrophages and cell debris.

3.5.3. Placental tissue (labyrinth zone)

The histopathological findings of placenta labyrinth zone from control group showed normal morphology which consists of maternal sinusoid with trophoblast cells and septa (Fig. 6A). The labyrinth has more compact architecture in deeper region compared to upper region (Figs. 6A and 7A). The observation of placental labyrinth zone in all *D. hispida* groups was different and varied. The labyrinth zone from animals treated with 250 mg/kg body weight *D. hispida* demonstrated fibrin deposition and cytoplasmic vacuolation of the trophoblast cells in the upper region (Fig. 6B) but in deeper region, there were reduction of trophoblast cells (Fig. 7B). Upper and deeper labyrinth regions of placenta from 500 mg/kg body weight rats were characterized by irregular vessel formation and cytoplasmic vacuolation of the trophoblast cells (Figs. 6C and 7C). In 1000 mg/kg body weight placenta, fibrin deposition, irregular vessel formation and presence of external exudates were observed in upper labyrinth region, while deeper region presented irregular vessel formation with external exudates and reduction in trophoblast cells and septa as shown in Figs. 6D and 7D, respectively. In the group 2000 mg/kg body weight, the upper and deeper labyrinth zone regions have reduced and diffused trophoblast septa with cytolysis and vacuolation of the trophoblast cells but the upper region contain more cellular debris (Fig. 6E) compared to deeper region (Fig. 7E).

4. Discussion

Herbal medicines have been used since time immemorial and become a popular form of alternative therapy in many countries. Despite of the various reported biological properties of *D. hispida*, several studies have reported the toxicity effects of its chemical compounds in

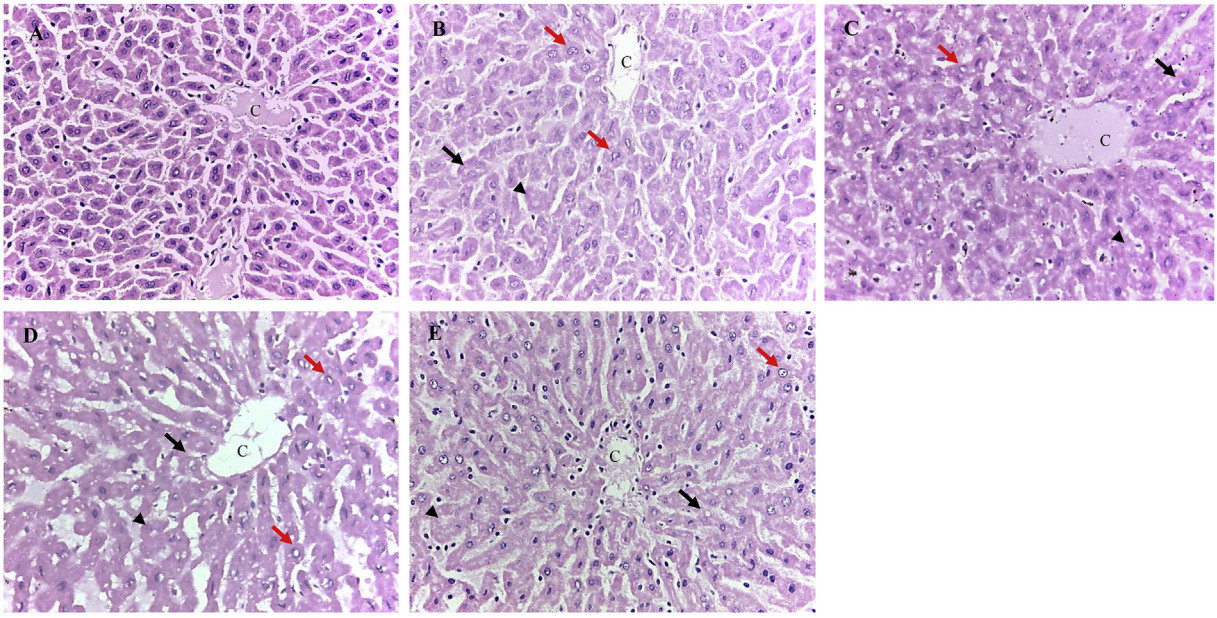

Fig. 4. Photomicrograph of maternal rats' liver collected on GD 21. A is the control while B-E represents groups 250, 500, 1000 and 2000 mg/kg body weight, respectively. Tissues in A (control) showed no significant pathological changes while tissues in B-E showed varying degrees of hepatocytic damage either in the form of degeneration and or necrosis such as cytoplasmic vacuolation (⟶), karyolysis (▶) and nuclear changes (➔). C: Central vein. (H&E, X40).

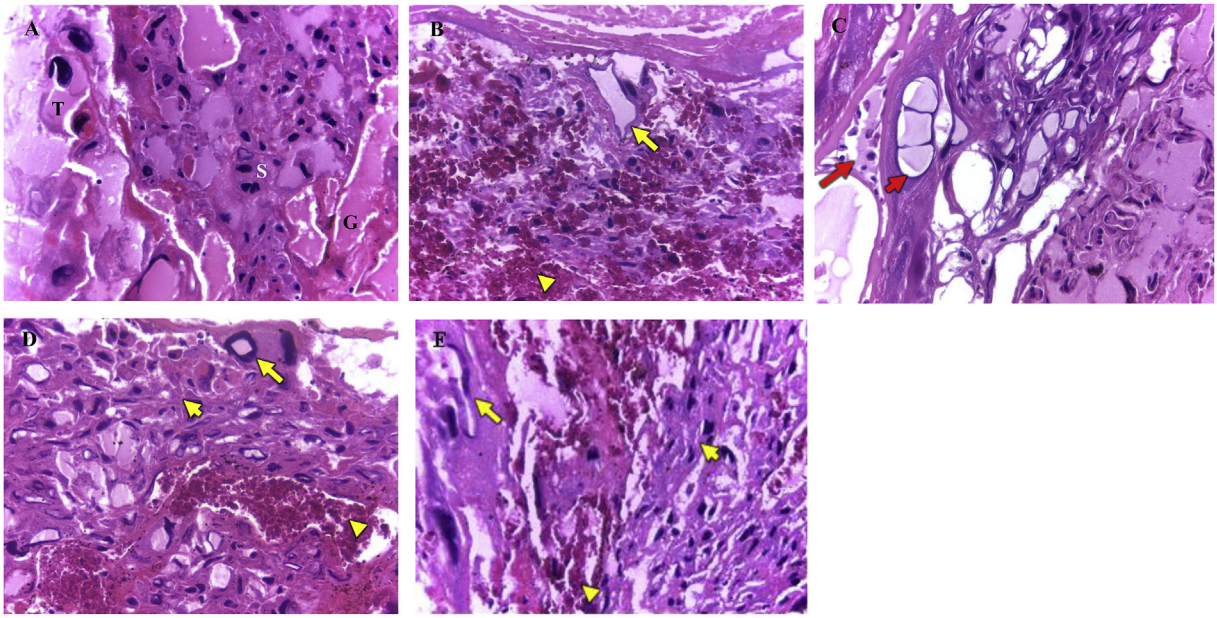

Fig. 5. Photomicrograph of maternal rats' placenta collected on GD 21 (Basal zone). A is the control while B-E represents groups 250, 500, 1000 and 2000 mg/kg body weight, respectively. Tissue in A (control) showed no significant pathological changes while tissues in B-E showed varying degrees of placental tissue damage and disruption of the cellular arrangement. Vacuolated trophoblastic giant cells were observed with margination of nucleus (⇨). Irregular shape of spongiotrophoblasts cells (⇨) with reduced glycogen cell and fibrin deposition (▷) was noted. Formation of large cysts filled with acidophilic mass (➡) and foci of inflammatory cell (➡) was observed in C. T: Trophoblastic giant cells, S: Spongiotrophoblast cells, G: Glycogen cells. (H&E, X40).

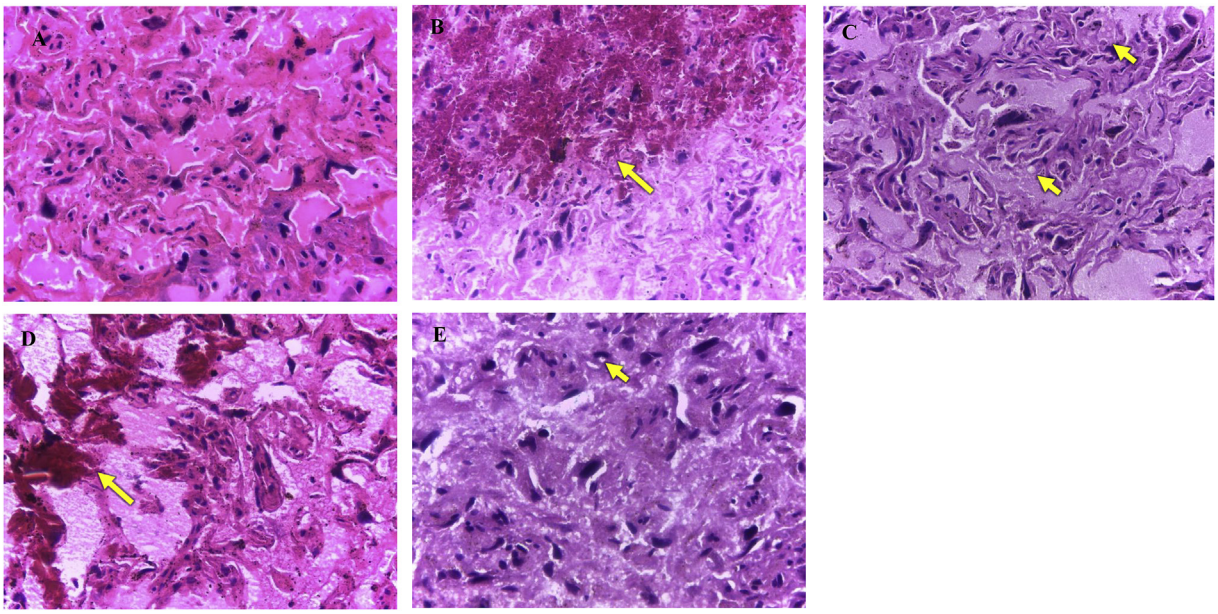

Fig. 6. Photomicrograph of maternal rats' placenta collected on GD 21 (Labyrinth zone, upper region). A is the control while B-E represents groups 250, 500, 1000 and 2000 mg/kg body weight, respectively. B-E shows varying degrees of placental tissue damage, disrupted and irregular vessel formation of the labyrinth zone. Fibrin deposited until the upper region of the labyrinth zone (⇨) in B and D. Cytoplasmic vacuolation of the trophoblastic cells (▷) were observed in C and E. (H&E, X40).

animal models studies (Sasiwatpaisit et al., 2014; Ruyani et al., 2011). In this study, histopathological examination of *D. hispida* on the liver and placenta was performed after 15 days administration during organogenesis to pregnant Sprague Dawley rats.

Liver is the largest internal organ in the body and become the most targeted organ of toxicity due to its function in metabolism. The present study showed an increment in the relative liver weight of treated animals with increasing concentration of *D. hispida* extract. However, a significant change was only observed in the highest group (2000 mg/kg body weight). The histopathological examination of the liver tissues showed that *D. hispida*-treated rats demonstrated some degeneration features such as hepatocyte swelling, dissolution of hepatic cords, cytoplasmic vacuolation, infiltration of inflammatory cells, presence of external exudates, margination and clumping of nucleus chromatin, cytolysis and karyolysis. These injuries could be contributed by the production of reactive oxygen species (ROS) which leads to oxidative stress. Many studies have demonstrated the relationship between ROS species and liver injury induced by hepatotoxins. A study on mice treated with *Dioscorea bulbifera* rhizome extract produced hepatotoxicity effect through oxidative stress (Wang et al., 2010). In another

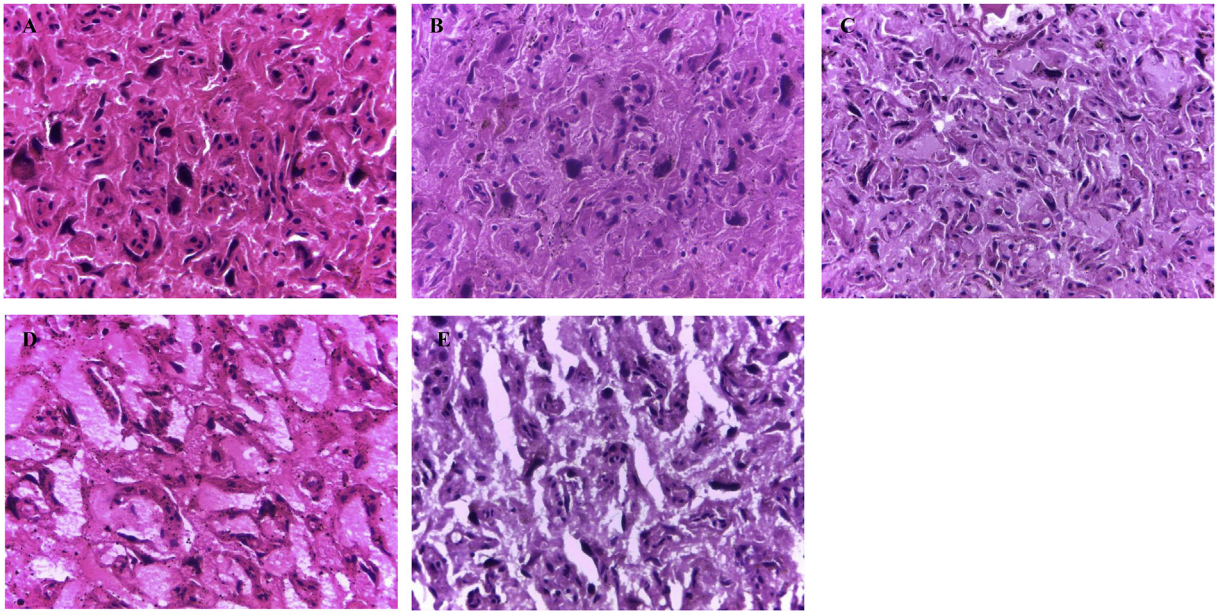

Fig. 7. Photomicrograph of maternal rats' placenta collected on GD 21 (Labyrinth zone, deeper region). A is the control while B-E represents groups 250, 500, 1000 and 2000 mg/kg body weight, respectively. B-E showed varying degrees of placental tissue damage, disrupted and irregular vessel formation of the labyrinth zone. Reduced and irregular vessel formation were observed in D and E. (H&E, X40).

study, *D. hispida* aqueous extract caused alterations on several genes related to hepatotoxic on pregnant rats treated at 2000 mg/kg body weight (Lokman et al., 2017). Conversely, other features of histopathological changes like single-cell necrosis, hepatocyte death cell which usually associated with hepatotoxicity was not observed in all treatment groups. The hepatocytes in all *D. hispida* treated animals displayed only tissues injury but there was no cell death observed. This could results from normal regeneration activity of liver cells. The liver has capacity to regenerate from various types of injuries as facultative liver stem cells have the potential to differentiate into both hepatocytes and biliary epithelial cells and they will proliferate and contribute to regeneration (Alison et al., 2009; Tanaka et al., 2011).

During pregnancy, the placenta is responsible in protecting the embryo and fetus development from xenobiotics. Any alteration induced by toxic agents can cause placental dysfunction resulting in miscarriage or fetal death (Furukawa et al., 2008). Results from this study showed that the number of placenta was reduced with the increase concentration of *D. hispida* extract (unpublished data). The placental relative weights were increased in 250, 500 and 1000 mg/kg body weight rats, as this could be due to cell underwent hypertrophy (Furukawa et al., 2011). These changes however were not dose-dependent. A significant reduction of the placental relative weight was only observed in the highest group (2000 mg/kg body weight) as it was evident by the unformed placenta as this could be due to the adverse effects of *D.hispida extract* on placental development. As previously reported, a disproportionately small placenta may indicate hypoxia or poor nutrient supply to the placenta causing growth restriction in both placenta and fetus (Wallace et al., 2004).

Although spongiotrophoblasts are morphologically different from trophoblast giant cells, both of these cells produce a prolactin-like hormone with lactogens and cytokines during pregnancy to maintain progesterone secretion (Ain et al., 2003). It is reported that trophoblasts and spongiotrophoblasts have higher cell proliferative activity and become a target of toxicity. Histopathologically, all the rats administered with *D. hispida* extract portrayed varying degree of basal zone lesions and cellular disruption compared to control group. Our results showed that *D. hispida* extract at 250, 1000 and 2000 mg/kg body weight causing reduction in the number of trophoblastic giant cells and spongiotrophoblast cells with vacuolation and nucleus margination of trophoblastic giant cells of the basal zone. The giant cell was then stimulated and eliminated the degenerated trophoblast by phagocytosis which leads to the reduction of the number of trophoblast cells (Omer et al., 2014; Kosif et al., 2008). The necrotic cells observed in the labyrinth zone indicated the lack of vascular development and decreased placental-fetal oxygenation (Omer et al., 2014; Witlin et al., 2002). In addition, the present findings also demonstrated that *D. hispida* extract cause fibrin deposition in the basal zone. The fibrin deposition can constrain the maternal blood perfusion to the placenta and will lead to placental necrosis causing fetal illness and death (Omer et al., 2014; Roberts, 2008). This is evident in the low number of live fetuses from the rats treated with the highest concentrations of *D. hispida* extract.

In conclusion, this study has shown that the administration of *D. hispida* aqueous extract at all concentrations induced histopathological changes and lesions in all maternal rats' liver and placenta tissues. Extensive liver and placental tissue degenerations were observed at the highest concentration (2000 mg/kg body weight). To the best of our knowledge, this is the first report demonstrating histopathological findings of liver and placenta induced by *D. hispida*. Therefore, further investigation is required to measure the complete toxicity including the mechanism and identifying the chemical compounds of *D. hispida* that induced such injuries.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.05.046.

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