ORIGINAL ARTICLE

Effect of *Dioscorea hispida* var. Daemona (Roxb) Prain & Burkill on Oxidative Stress and DNA Damage in the Liver of Pregnant Rats

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ABSTRACT

Dioscorea hispida **var. daemona (Roxb) Prain & Burkill is an intoxicating yam which is commonly known as 'ubi gadong' in Malaysia and traditionally consumed in various part of the world. However, the tuber of this plant is poisonous as it contains toxic compound, dioscorine. The aim of this study was to evaluate the hepatoxicity effect of** *D. hispida* **aqueous extract (DHAE) by measuring the level of oxidative stress and DNA damage in the liver of pregnant Sprague Dawley (SD) rats. Twenty pregnant rats were randomly divided into four groups (n=5) consisting of control, low 500 mg/kg, medium 1000 mg/kg and high 2000 mg/kg group that were administered with different concentrations of** *D. hispida* **by oral gavage for 15 days from gestation day (GD) 6 until 20. At GD 21, the liver was collected and oxidative stress (reactive oxygen species (ROS) level, H_2O_2 concentrations, superoxide dismutase (SOD) lipid peroxidation malondialdehyde (MDA) and DNA damage were measured. No significant changes on ROS level and H_2O_2 concentration in 1000 and 2000 mg/kg body weight DHAE except for the lowest concentration group ($p<0.05$) when compared to control whereas the SOD activity was comparable with untreated rats. Increase of MDA level and DNA damage was observed in all treated groups. In conclusion, DHAE did not increase oxidative stress in maternal rats' liver however may induces DNA damage. Further study is required to confirm these findings through a longer DHAE administration to understand the mechanism involves in its effects.** (*Int J Biomed Sci* 2020; 16 (3): 30-36)

Keywords: *Dioscorea hispida*; reactive oxygen species (ROS); Malondialdehyde (MDA); Superoxide dismutase (SOD); DNA damage; oxidative stress; comet assay

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INTRODUCTION

During pregnancy, physiological changes occurs to support fetal growth and development which includes the alterations on maternal hepatic functions. Liver is the primary organ that involves in metabolism of lipid, cholesterol, bile acid and glucose and detoxification of drugs and therefore become a major target organ of many chemicals and drugs to induce hepatotoxicity. The use of herbal plants during pregnancy and their effects on liver toxicity have been reported in both human and animal studies (1) .

In Malaysia, *Dioscorea hispida* var. daemona (Roxb) Prain & Burkill is popularly known as Ubi Gadong and its tuber has been consumed as a staple food. In some states of Malaysia like Kelantan and Terengganu, *Dioscorea hispida* (*D. hispida*) is consumed by villagers due to high content of carbohydrate despite of its potential to produce toxicity effects. Traditionally, the tuber was soaked in running water for a few days to remove the toxic compounds. Various studies have been conducted for its beneficial uses including hypoglycemic activity for diabetic patients or any other associated diseases like obesity and diabetes due to its resistant starch that slows the absorption of glucose uptake (2, 3). In addition, pharmacological investigations have demonstrated that *Dioscorea* species possess anthelmintic activity (4), antioxidant activity (5), anti-inflammatory activity (6), antitumor activity (7) and hypoglycemic activity (8).

Besides its pharmacological activities, *D. hispida* is also well known as a poisonous plant due to its toxic substances (9). Few studies have shown that the consumption of *D. hispida* causes poisoning symptoms and irregularities to various organs including liver (10, 11). Dioscorine (alkaloid) (12) and diosgenin (saponin glycoside) (4) are the main isolated compounds found in various species of *Dioscorea* (13). Dioscorine which was initially isolated from *D. hispida* in 1937 is known to be poisonous (14) and believed to cause dizziness and nausea (15) and able to activate fatal paralysis of the nervous system (16). In addition, dioscin, is an isomer of dihydrodioscorine resembling picrotoxin that could cause seizures (14).

Notwithstanding the toxicity effects of *D. hispida*, other compounds like diosgenin which was first discovered from *Dioscorea tokoro Makino* by Fujii and Matsukawa in 1935 has become a great interest in pharmaceutical industry in developing therapeutic drugs due to its pharmacological potential against numerous diseases including metabolic disease (diabetes, obesity, dyslipidemia and hypercholosterolemia) inflammatory diseases and cancer (17-19).

In a previous study, it was reported that *D. hispida* consumption at 2000 mg/kg body weight in rats altered several genes which affected the liver functions. (11). Therefore, the aim of this study was to further evaluate the mechanism involved in hepatotoxicity effects of *D. hispida* aqueous extract (DHAE) through assessment of oxidative stress and DNA damage in pregnant Sprague Dawley (SD) rats.

MATERIALS AND METHODS

Chemicals

OxiSelect in vitro ROS Assay Kit (Green Fluorescence), Oxiselect Superoxide Dismutase Activity Assay, Oxiselect TBARS Assay Kit (MDA Quantification) and OxiSelect Comet Assay Kit (3-Well Slides) were purchased from Cell Biolabs, Inc. (San Diego, CA).

Preparation of *Dioscorea hispida* aqueous extract

D. hispida tuber were collected from Machang, Kelantan, Malaysia. Samples were washed, dried, and grinded to a powder form prior to extraction with water. The plant was authenticated at Herbarium of Forest Research Institute of Malaysia (FRIM) with the voucher specimen number of SBID 008/14. Standardization of *D. hispida* against dioscine was performed using liquid chromatographic system, QExactive UHPLC (Thermo Fischer Scientific, USA). Total polyphenols and UHPLC-ESI-MS analysis were carried as described by Hussin *et al*. (2019) (20).

Study design

Twenty healthy nulliparous females and five fertile males of SD rats with body weight from 180 – 250g obtained from the Animal Resource Unit, Medical Resource Research Centre, Institute for Medical Research (IMR). The animals were divided into 4 groups consisted of a negative control group 0 mg/kg and three treatment groups which received 500 mg/kg, 1000 mg/kg and 2000 mg/kg body weight (BW) of DHAE.

Animal handling

The rats were housed in polypropylene cages, lined with wood shaving at controlled temperature of 20 to 26°C, with 40 to 60% humidity under 12 hours of light and dark cycle. The rats were acclimatized for 7 days and given commercial rat diet (Specialty Feeds, Australia) and water *ad libitum*. The female rats which were in the pro-oestrous phase were placed with male rats (1:1) and left overnight. Vaginal smear was performed the next morning on each rat and mating was confirmed by the presence of sperm

which designated as gestation day (GD) 0. From GD 6 to GD 20, the rats were administered with DHAE once daily by oral gavage. The rats were examined for any clinical signs of maternal toxicity i.e vaginal bleeding, diarrhea, piloerection, changes in locomotion inside the cage, dull fur, urination or maternal deaths once daily after 1-hour of *D. hispida* administration (21). At GD 21, carbon dioxide inhalation and caesarean hysterectomy was immediately performed to all SD rats. Liver tissues were harvested, weighed, recorded and kept immediately at -80°C prior to analysis.

Reactive Oxygen Species (ROS) Level Assay

Liver tissues were resuspended in 50 mg/mL phosphate buffer saline (PBS) and homogenized on ice using mortar and pestle. The samples were centrifuged at 10,000 rpm for 10 minutes in 4°C. The supernatant of the sample (approximately 50 µL) and hydrogen peroxide (H_2O_2) standard was collected and added into 96-well plate, followed by addition of 50 µL of catalyst in each well and incubated for 5 minutes at room temperature. 100 µL of dichlorodihydrofluorescein (DCFH) solution was added into each well. The microplate was incubated at room temperature for 45 minutes. The ROS activity was measured spectrophotometrically at 485 nm excitation/ 520 nm with POLARStar Omega Reader.

Superoxide dismutase (SOD) determination

Liver tissues were homogenized with 5-10 mL of cold 1x lysis buffer and centrifuged at 12000 x g for 10 minutes. Xanthine/Xanthine Oxidase reagent was then added to supernatant and absorbance was read at 490 nm with microplate reader (POLARstar Omega). The activity of SOD was determined by inhibition percentage of chromogen.

Lipid peroxidation Malondialdehyde (MDA) assay

About 0.10 g of liver tissues was weighed and perfused in 1.5 mL of PBS containing 20 mM ethylenediaminetetraacetic acid (EDTA) and resuspended at 50 mg/mL in PBS containing 1X butylated hydroxytoluene (BHT). Then, the tissues were homogenized with 500 µL of 1X BHT by using mortar and pestle on ice then centrifuged at 10,000 x g for 5 minutes. The tissue lysate supernatant was then collected and assayed directly for its TBARS level after a brief incubation at 95°C and then read on PROmega microplate at 532 nm. MDA content was calculated by comparing the value with the predetermined MDA standard curve.

DNA Comet Assay

Liver tissues were collected and centrifuged at 3000 rpm and resuspended in 1 mL of ice cold PBS containing 20 mM EDTA (without Ca^{2+} and Mg^{2+}) for 5 minutes. The suspension was spread on the slide and kept at 4°C for 15 minutes. Then, the slide was transferred to a glass container filled with pre-chilled lysis buffer. The slide was immersed in the buffer for 60 minutes, transferred to another container filled with pre-chilled alkaline solution and then immersed for 30 minutes at 4°C in the dark. The slide was carefully transferred from alkaline solution to electrophoresis chamber containing pre-chilled alkaline solution for 25 minutes at 4°C, 18 V, 300 mA. The slide was washed 3 times in deionized water for 2 minutes at 4°C then immersed in 70% ethanol for 5 minutes before drying it at room temperature. When the slide and gel was completely dried, Vista Green Dye was added and incubated at room temperature for at least 15 minutes. The slide was viewed under Confocal Laser Microscope and analyzed using Open Comet 1.3 using Image J software.

Statistical Analysis

All mean ± SEM (standard error of mean) values were calculated and statistical analysis was done using SPSS version 18.0 (SPSS Inc., Chicago, USA). The data were analyzed using one-way analysis of variance (ANOVA). The difference was considered significant when *P* value was less than 0.05 ($p<0.05$).

RESULTS

Effects of *D. hispida* aqueous extract on ROS level and H_2O_2 concentration

Oxidative stress activities of DHAE on maternal rat's liver are shown in Fig. 1 and Table 1. ROS levels in the 500 mg/kg, 1000 mg/kg, and 2000 mg/kg groups were higher compared to 0 mg/kg and the value was significantly different ($p<0.05$) for 500 mg/kg group where H_2O_2 concentration in 500 mg/kg, 1000 mg/kg, and 2000 mg/kg was higher compared to 0 mg/kg and the value was significantly different ($p<0.05$) for 500 mg/kg group where H_2O_2 concentration of 500 mg/kg group increased by 59.51% from 0 mg/kg. However, the value of H_2O_2 concentration decreased as the dosage of treatment increased.

Effect of *D. hispida* aqueous extract on MDA level

MDA level of maternal rat's liver was increased in a dose-dependent manner. However, the increment is only evident and the highest in 2000 mg/kg group as shown in

Table 1 and Fig. 2 where treatment increased 26.7% compared to 0 mg/kg group. However, the difference was not statistically significant (p>0.05).

Effect of *D. hispida* aqueous extract on SOD inhibition activity (%)

The average percentage of SOD activities in 500 mg/kg, 1000 mg/kg and 2000 mg/kg group were slightly lower compared to 0 mg/kg. Based on Fig. 2 and Table 1, the inhibition activities declined by 5.44%, 5.03% and 1.14% for 500 mg/kg, 1000 mg/kg and 2000 mg/kg group, respectively. However, decreased in SOD inhibition activities were not statistically significant.

Effect of *D. hispida* aqueous extract on DNA Damage

Illustration of treated and untreated cell is as depicted in Fig. 3. The OTM measurements (Table 1) of 500 mg/kg, 1000 mg/kg and 2000 mg/kg group were increased compared to 0 mg/kg group showing that the DNA damage increased as the concentration of DHAE increased. However, the OTM measurements were not statistically significant (P>0.05).

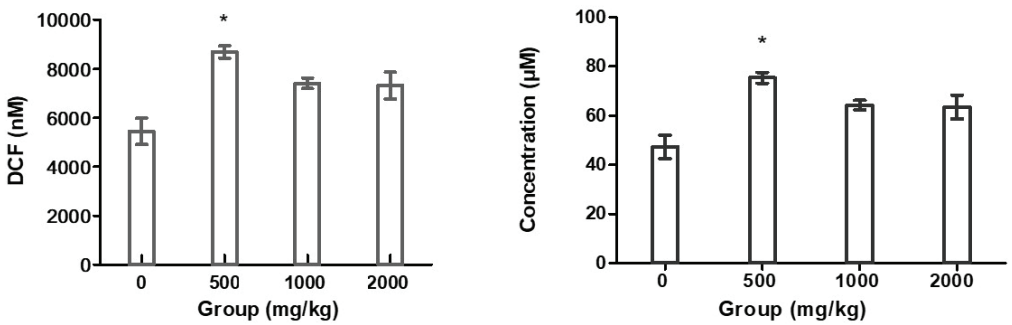

Figure 1. Level of ROS and H_2O_2, in liver tissue of maternal SD rats for control 0 mg/kg, 500 mg/kg, 1000 mg/kg and 2000 mg/kg group. The value was expressed as mean ± SEM (n=5). *Significantly different from control (0 mg/ml) group (p<0.05).

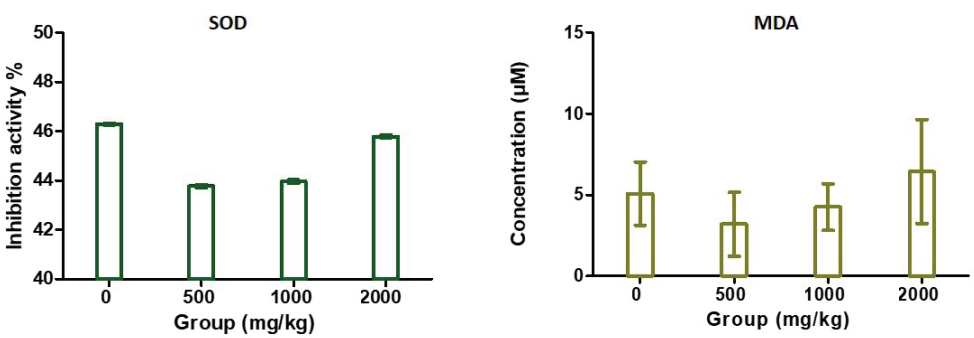

Figure. 2 SOD inhibition activity and MDA level in liver tissue of maternal SD rats for control 0 mg/kg, 500 mg/kg, 1000 mg/kg and 2000 mg/kg group. The value was expressed as mean ± SEM (n=5). *Significantly different from control (0 mg/ml) group (p<0.05).

Table 1. Oxidative stress and DNA damage of *D. hispida* in the liver of pregnant rats

D. hispida concentration	ROS production (DCF nm)	H_2O_2 Concentration (µM)	SOD activity (inhibition %)	MDA Concentration (µM)	DNA Damage (OTM) (µm)
0 mg/kg	5442.54 ± 541.27	47.29 ± 4.71	46.29 ± 0.03	5.08 ± 1.95	39.12 ± 10.06
500 mg/kg	8681.22 ± 252.62[a]	75.43 ± 2.19[a]	43.77 ± 0.05	3.20 ± 1.97	47.06 ± 11.75
1000 mg/kg	7404.3 ± 220.91	64.33 ± 1.92	43.96 ± 0.07	4.25 ± 1.44	48.17 ± 15.01
2000 mg/kg	7311.03 ± 550.02	63.52 ± 4.78	45.76 ± 0.06	6.43 ± 3.20	51.33 ± 11.84

[a]Significance value p<0.05.

DISCUSSION

The consumption of herbal medicine during pregnancy is increasing worldwide. The safety assessment on mother and fetuses is necessary to ensure the effectiveness of the herbal plants is more than their potential toxicity effects. Previous studies have demonstrated that the administration of DHAE at 2000 mg/kg body weight altered several genes related to hepatotoxic effects i.e Btg2, Gsr, L2hgdn, S100a8, Slc17a3, Bhmt, Cd68 and Cyp1a2 (11) which can cause histopathological changes in the liver (20). We conducted this study to further investigate the hepatotoxicity activities of DHAE through the production of reactive oxygen species (ROS), lipid peroxidation, superoxide dismutase activity and DNA damage in the liver tissues of pregnant rats. The present study showed that the extract did not induce any hepatotoxicity effects through oxidative stress when administered daily from GD-6 to GD-20 however may induce DNA damage in the liver of maternal rats.

During late pregnancy, microsomal enzyme activity in the liver i.e cytochrome P450, CYP3A and CYP2E1 is decreased resulting in the increment of oxidative stress and the high concentration of ROS production in the liver (22). This oxidative stress causes hepatic damage by provoking alteration of biological molecules such as DNA, proteins, lipids and, notably, modulating biological pathways associated with genes transcription, protein expression, cell apoptosis, and hepatic stellate cell activation. The current findings have shown that the concentration of ROS and H_2O_2 in the liver obtained from maternal rats was higher in 500 mg/kg group compared to control animals, however these changes were not dose-dependent as the concentration of ROS and H_2O_2 decreased with increment of DHAE concentration. In the body, respiration of mitochondria generates the ROS and become the major ROS generation in the cells. The primary sources of endogenous ROS production are the mitochondria, plasma membrane, endoplasmic reticulum, and peroxisomes (23).

The endogenous antioxidants specifically SOD, glutathione peroxidase and glutathione reductase play important roles in scavenging the ROS. SOD is one of the enzymatic endogenous antioxidants which convert the reactive oxygen metabolites O^{2-} into H_2O_2 which is further catalyzed to H_2O by catalase or glutathione peroxidases (24). Our findings have shown that the SOD activities were not corresponding to the increment of H_2O_2 production which may indicate that the conversion of O^{2-} to H_2O_2 could be due to other mechanisms. Moreover, SOD inhibition was reduced as DHAE concentration increased which may attenuate ROS and H_2O_2 level. It was reported that besides endogenous antioxidants, other compounds such as polyphenol, vitamin and minerals are known to act as exogenous antioxidant (25). Decreased of ROS and H_2O_2 activities in 1000 and 2000 mg/kg body weight DHAE could be due to the hepatoprotective effects of DHAE in the liver. The present of multiple compounds in *D. hispida* and its active metabolites may act synergistically to scavenge reactive oxygen metabolites at the higher concentrations. It was reported that the chemical compounds like dioscin and diosgenin may contribute to hepatoprotective effects through adjustment of mitochondrial function (26) and reduction of endoplasmic reticulum stress and oxidative stress (27), respectively.

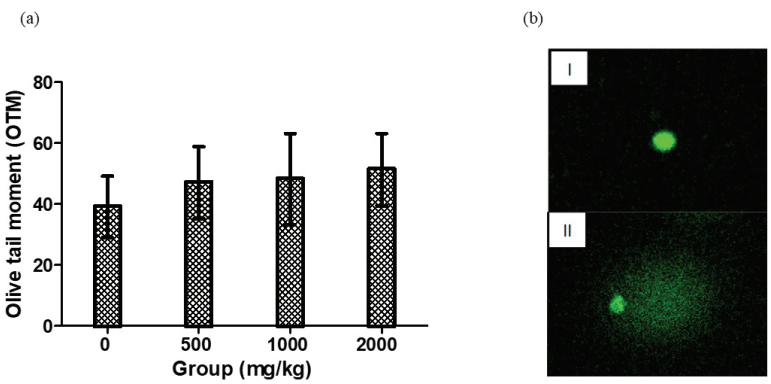

Figure 3. (a) Olive tail moment (OTM = Tail DNA% × Tail moment length) in liver tissue of maternal SD rats for 0 mg/kg, 500 mg/kg, 1000 mg/kg and 2000 mg/kg group. The value was expressed as mean ± SEM (n=5); (b) Representative image of untreated (I) and *D. hispida* treated (II) observed under Leica Confocal Laser Microscope.

Production of ROS in the liver may inflict direct damage to lipid that cause lipid peroxidation. Lipid peroxidation level was measured by the presence of MDA formation which is used as a biomarker, as a result of oxygen radical reaction with polyunsaturated fatty acids (PUFA) which is mostly reactive against protein and DNA (28). In this study, MDA level was increased against DHAE concentration and demonstrated to be dose-dependant. Similar finding was observed on mice treated with Diobulbin B, a bitter compound isolated from *Dioscorea bulbifera* L. demonstrated high liver MDA production after 12 days administration at the 64 mg/kg body weight (29). DHAE may cause liver injury at higher concentration as some of the compounds might contribute to this effect. Our findings have also shown an elevation of olive tail moment (OTM) proportional to the concentration of the extracts. DNA integrity was measured through single cell gel electrophoresis (SCGE) to investigate occurrence of DNA damage due to DHAE. It has also been reported that free cyanide can disrupt the mitochondrial respiratory chain leading to an increase production of ROS (29) that could activate an apoptotic mechanism of cell death resulting from intracellular oxidative stress (30). The ROS causes several types of damage including modification of DNA bases, single- and double-DNA breaks, damage to deoxyribose sugar and damage to the DNA repair systems. Dioscin have been shown to have anti-cancer activity apart from lipid-lowering and hepatotoprotective effects by promoting ROS accumulation and inducing DNA damage (30). Accumulation of ROS can lead to lipid peroxidation and DNA damage which leads to increase of MDA level and OTM length. In our previous study, histopathological changes and lesions were evident in maternal rats' liver in which we believed caused by the production of reactive oxygen species (20).

Current findings demonstrated that DHAE did not increase oxidative stress in maternal rats' liver however may induces DNA damage. Further studies on the prolonged administration of DHAE need to be conducted as to clearly determine the potential hepatotoxicity or hepatoprotective effect and its mechanism.

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CONFLICT OF INTEREST

Author Elda Nurafnie Ibnu Rasid, Author Abdul Rahim Azlan, Author Nur Fatihah Mamat Daud, Author Tengku Aideed Shah, Author Norizah Awang , Author Wan Mazlina Md. Saad and Author Hussin Muhammad have no conflict of interest.

ETHICAL APPROVAL

This study was approved by Animal Care and Use Committee (ACUC), Ministry of Health Malaysia (Approval No. ACUC/KKM/02(10/2016).

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