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Original Article

HEPATOPROTECTIVE ACTIVITY OF *MERREMIA BORNEENSIS* AGAINST CARBON TETRACHLORIDE (CCl_4)-INDUCED ACUTE LIVER DAMAGE IN RATS: A BIOCHEMICAL AND HISTOPATHOLOGICAL EVALUATION

MOHAMMAD IQBAL[1*], MUHAMMAD DAWOOD SHAH[1,2], SENTY VUN-SANG[1], RIANA BINTI AWANG SAMAN[1]

[1]Biotechnology Research Institute, [2]Borneo Marine Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia
Email: driqbalmohammad@gmail.com

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ABSTRACT

Objective: The pathogenesis of various liver injuries involves oxidative damage. This research was planned to examine the effects of *Mereemia borneensis* extract on hepatic oxidative damage caused by carbon tetrachloride (CCl_4) in rats.

Methods: Sprague Dawley rats were exposed to *M. borneensis* (125 and 250 mg/kg b. wt.) once daily for 14 d followed by two doses of CCl_4 (1.2 ml/kg b. wt.). After 2 w, the rats were sacrificed and hepatoprotective analysis was done.

Results: Orally administration of CCl_4 enhances serum transaminase (ALT; alanine transaminase and AST; aspartate transaminase), γ-glutamyl transpeptidase, lipid peroxidation, reduction in glutathione, catalase, glutathione reductase, glutathione peroxidase, quinone reductase and glutathione S-transferase. Pretreatment of rats with *M. borneensis* at 125 and 250 mg/kg body weight significantly reduced levels of ALT, AST, γ-glutamyl transpeptidase and lipid peroxidation of CCl_4 treated rats. Pretreatment with *M. borneensis* against rats treated with CCl_4, hepatic enzymatic and non-enzymatic antioxidant molecules have increased significantly. A decreased histopathological change in the liver is further evidence of the protective effect of *M. borneensis*.

Conclusion: Our data suggest that *M. borneensis* can be a potential hepatoprotective agent in preventing or treating degenerative diseases that involve oxidative stress.

Keywords: *Merremia borneensis*, Oxidative stress, Carbon tetrachloride, Hepatoprotective activity, Histopathology

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INTRODUCTION

A rise in the generation of reactive oxygen species (ROS) including oxygen free radicals are caused by oxidative stress, which is responsible for different degenerative diseases including inflammation, carcinogenesis, Parkinson's disease, neurodegenerative diseases, retinal degeneration, Alzheimer's disease, mutagenesis, diabetes mellitus and liver disease [1, 2].

Carbon tetrachloride (CCl_4) is the most widely used chemical inducer for experimental hepatic damage, involves hepatic necrosis by bioactivation of microsomal cytochrome P450-dependent monooxygenase system, resulting in the generation of ROS and trichloromethyl free radicals which trigger protein damage and lipid peroxidation [3, 4]. Studies have been shown that a high amount of polyphenolic compounds extracted from plants may prevent liver damage due to the presence of an antioxidant, antifibrotic and immunomodulatory [5, 6].

Merremia borneensis, is a creepy plant and it belongs to the family Convolvulaceae. The young leaves of the plant are brown but at maturity, turn to green colour. The leaves are big with eight veins, intervened with each other. A productive tree has wide leaves of up to one foot wide and the diameter of each stem can reach up to two inches. The plant is distributed in Malaysia and other South Asian countries. The plant is used by local people for the treatment of different diseases, including breast cancer [7]. There is, however, no scientific evidence concerning the hepatoprotective function of plant's leaves. Therefore it is necessary to conduct further research for the hepatoprotective potential of *M. borneensis* leaves and to verify the claim using a model of hepatic injury induced by CCl_4 in Sprague Dawley rats.

MATERIALS AND METHODS

Reagents and chemicals

Carbon tetrachloride (CCl_4), bovine serum albumin (BSA), trichloroacetic acid (TCA), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), β-nicotinamide adenine dinucleotide phosphate reduced (NADPH), 2,6-dichloroindophenol, 1-chloro-2,4-dinitrobenzene (CDNB), sulfosalicylic acid (SSA), thiobarbituric acid (TBA), flavin adenine dinucleotide (FAD), hydrogen peroxide (H_2O_2), tween 20, tris HCl, glutathione reductase, oxidized and reduced glutathione, L-γ-glutamyl-p-nitroaniline, sodium azide, ethylene diamine tetraacetic acid (EDTA) and glycyl glycine, D,L-alanine, α-ketoglutarate, D,L-aspartate, 2,4 dinitrophenyl hydrazine (DNPH), sodium hydroxide were obtained from either Aldrich, USA or Sigma Chemical Company, St Louis, MO, USA. The other solvents and chemicals used were either industrial grade or commercially available with the highest purity.

Plant material

The leaves of *M. borneensis* were collected from the campus of Universiti Malaysia Sabah, Malaysia. The plant was identified based on the morphological features and from the database present in the library by a Botanist from Institut Biologi Tropika dan Permulihan (IBTP), Universiti Malaysia Sabah, Malaysia. The plant samples were air-dried and then grind to a fine 1-mm mesh powder [8].

Extract preparation

The powdered leaves (100 g) were exhaustively extracted with (80%) ethanol from soxhlet and concentrated in a rotary evaporator to dryness under reduced pressure to yield dried ethanol extract, which was 14% of the starting material [9].

Experimental animals

In this study, adult female Sprague Dawley rats purchased from Penang, Malaysia (Tes Jaya Laboratory Services) were used. The rats are 4-8 w old, weighing between 150–200 g. They were put up in plastic cages in groups of 3 rats per cage and kept with a 12 h

light/dark cycle in a well-maintained room, at 25±1 °C and 50±5% humidity. One week prior to the experiment, the rats were acclimatized with free access to food and water.

Experimental protocol-CCl_4 induced hepatotoxicity

Total of 24 Sprague Dawley rats were randomly grouped into four experimental groups of six rats each. Group I served as normal control and was given only normal saline. While group II administered with 1.2 ml/kg b. wt. of CCl_4 in the 1:1 ratio with corn oil as a vehicle. Group III and group IV received 14 d of daily *M. borneensis* (125 and 250 mg/kg b. wt. respectively) extract treatment and CCl_4 (1.2 ml/kg b. wt.) treatment. All the animals were sacrificed by cervical dislocation 24 h after administration of the CCl_4.

Post-mitochondrial supernatant (PMS) preparation

The standard protocol for preparing tissue homogenate for biochemical assay following a method defined by Iqbal *et al.* [10] which is adapted from Mohandas *et al.* [11]. The liver was removed and cleaned free of any debris then immediately perfused with 0.85% w/v cold sodium chloride. The liver was mashed in a cold solution of 0.1 M, pH 7.4 phosphate buffer with 1.17% w/v potassium chloride. The homogenate was then centrifuged in a refrigerated centrifuge at 2,000 x g, 4 °C for 15 min to remove the nuclear debris. The obtained aliquot was centrifuged at 10,000 × g, 4 °C for 30 min to get PMS, that used to evaluate reduced glutathione content and malondialdehyde as well as enzyme source.

Biochemical assays

The procedure by Jollow *et al.* [12] were adapted to measure liver reduced glutathione by measuring the yellow colour production at wavelength 412 nm through a spectrophotometer. The results were read as reduced glutathione micromoles per gram of tissue.

Hepatic lipid peroxidation in post mitochondrial supernatant was done by calculating the production rate of TBARS using technique as reported by Iqbal *et al.* [13] modified from Buege and Aust [14]. The results were stated as the quantity of malondialdehyde found in the supernatant of each sample and evaluated by a spectrophotometer at 37 °C, the wavelength of 535 nm. The result calculated using a molar extinction coefficient of 1.56 X 10^5 M/cm.

Glutathione peroxidase activity was assessed using the Mohandas *et al.* [11] procedure, as referred by Iqbal *et al.* [13]. Enzyme activity was calculated using a molar extinction coefficient of 6.22 x 10^3 M/cm as nmol NADPH oxidized/min/mg protein.

The activity of glutathione reductase was carried out following the method of Carlberg and Mannervik [15], which elaborated in Iqbal *et al.* [13]. The action of the enzyme was quantitated by calculating the disappearance of NADPH at a wavelength of 340 nm and measured as nmol NADPH oxidized/min/mg protein using a coefficient of molar extinction of 6.22 × 10^3M/cm.

The activity of catalase was determined by the Claiborne [16] method as reported by Iqbal *et al.* [13]. The absorbance changes were reported at wavelength 240 nm, in which the enzyme activity was measured and calculated using a molar extinction coefficient of 6.4 x 10^3M/cm as nmol H_2O_2 consumed/min/mg protein.

A modified method by Athar and Iqbal [17] from Habiq *et al.* [18] were employed using a substrate known as 1-chloro 2,4 dinitrobenzene to determine the glutathione S-transferase activity. At wavelength 340 nm, the changes in absorbance were recorded and calculated using a molar extinction coefficient of 9.6 x 10^3M/cm as nmol CDNB conjugate formed/min/mg protein.

The modified protocol of Benson *et al.* [19] by Iqbal *et al.* [20] was used in the determination of quinone reductase activity. The enzyme reaction was quantified based on the disappearance of 2,6-dichlorophenolindophenol at wavelength 600 nm with 30-s intervals for 3 min. The reaction of the enzyme was estimated using the molar extinction coefficient of 2.1 × 10^4M^{-1} cm^{-1} as nmoles of 2,6-dichlorophenolindophenol reduced/min/mg protein.

Serum transaminases determination

The assessment to determine serum marker AST and ALT were adapted from Reitman *et al.* method [21]. In short, 0.2 ml serum and 0.5 ml of the AST and ALT substrate (2 mmol α-ketoglutarate and 200 mmol D, L-alanine (ALT) or 200 mmol D, L-aspartate (AST)) was placed in a 37 ° C water bath for 15 min. The reaction was contained after incubation by adding 0.5 ml of 1 mmol DNPH. After 20 min, each tube was added 5 ml of 0.4 NaOH and the reaction was measured at optical density 510 after 30 min using water as the blank.

Histological determination

For histological, livers from all rats were removed and cleaned with ice-cold saline, and a small cross-section of the liver was obtained and fixed in 10% neutral formalin buffered solution. For microscopy examination, the liver section was embedded in wax, cut into 6 µm thick section and stain with hematoxylin and eosin stain (H and E). The tissues were examined under a microscope randomly and without knowledge of the animal or group.

Protein determination

Liver protein contents were measured using BSA as standard using Aitken *et al.* [22] method.

Data analysis

Data analysis was conducted employing the statistical kit SPSS 17.0 (SPSS Inc., Chicago IL). Analysis of variance (ANOVA) followed by testing with LSD was conducted to evaluate the statistical difference among groups. The values are means±SEM for six rats in each group. *P*-value<0.05 was considered as significant.

RESULTS

Protective effect of *M. borneensis* extract on glutathione metabolism

The level of glutathione is significantly depleted in CCl4 treated group when compared to the untreated control group. Rats administered *M. borneensis* along with CCl4 showed dose dependant rise in the GSH level when compared to the CCl4-treated group (fig. 1). Treatment with CCl4 alone resulted in the depletion of hepatic glutathione by 35% as opposed to the control group. However, 23% and 35% recovery were noted in the pretreatment group with *M. borneensis* extract at 125 and 250 mg/kg body weight, respectively against CCl4 treated group.

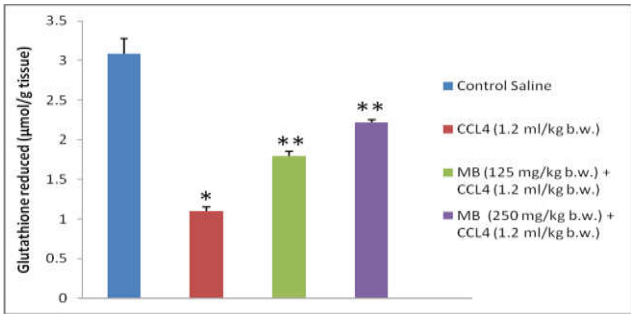

Fig. 1: Effect of pretreatment of rats with *M. borneensis* extract on glutathione (GSH) levels in the liver in CCl_4 induced liver damage. Each value represents mean±SEM of six animals. *Indicates significance at p<0.05 compared to the control group. ** Indicates significance at p<0.05 compared to CCl_4 group

Protective effect of *M. borneensis* extract on lipid peroxidation

As shown in fig. 2, CCl_4 treatment enhances the level of lipid peroxidation by 56% compared to the normal control group. Treatment with *M. borneensis* extract caused reduction in the level of lipid peroxidation by 80% and 66% at a dosage of 125 and 250 mg/kg body weight respectively in contrast to CCl_4 treated group.

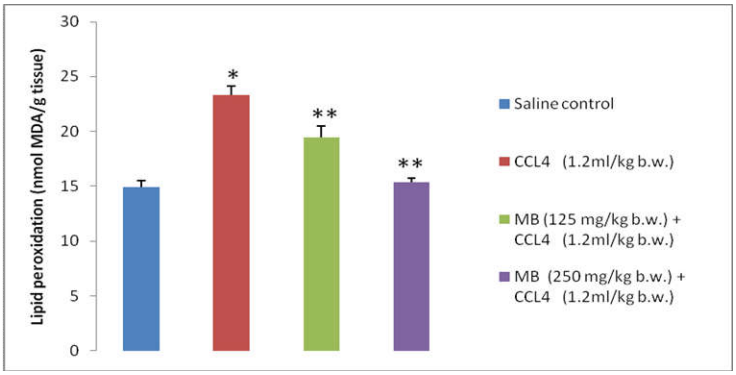

Fig. 2: Effect of pretreatment of rats with *M. borneensis* extract on lipid peroxidation levels in the liver in CCl_4 induced liver damage. Each value represents mean±SEM of six animals. *Indicates significance at p<0.05 compared to the control group. ** Indicates significance at p<0.05 compared to CCl_4 group

Protective effect of *M. borneensis* on hepatic antioxidant profile

Treatment of CCl_4 shows a reduction in the activities of hepatic antioxidant enzymes (table 1) such as catalase, glutathione peroxidase and glutathione reductase by 37%, 31% and 22%. However, treatment with *M. borneensis* extracts caused the recovery of the antioxidant enzymes by 15%, 15%, and 10% at the lower dose of 125 mg/kg body weight and 28%, 25% and 19% at the higher dose of 250 mg/kg body weight respectively, when compared with the group only treated with CCl_4.

Table 1: Effect of pretreatment of rats with *M. borneensis* extract on catalase, glutathione peroxidase and glutathione reductase

Treatment groups	Catalase (nmol H_2O_2 Consumed/ min/mg protein)	Glutathione peroxidase (nmol NADPH oxidized/min/mg protein)	Glutathione reductase (nmol NADPH oxidized/min/mg protein)
	mean±SEM	mean±SEM	mean±SEM
Saline control	328.61±13.1	34.77±1.9	25.52±0.21
CCl_4 (1.2 ml/kg b. wt.)	207.34±16.9*	23.92±0.6*	19.91±0.21*
MB (125 mg/kg b. wt.)+CCl_4 (1.2 ml/kg b. wt.)	255.93±11.6**	29.35±0.5**	22.46±0.21**
MB (250 mg/kg b. wt.)+CCl_4 (1.2 ml/kg b. wt.)	298.36±4.1**	32.54±1.2**	24.63±0.24**

Each value represents mean±SEM of six animals. *Indicates significance at p<0.05 compared to the control group. ** Indicates significance at p<0.05 compared to CCl_4 group.

Protective effect of *M. Borneensis* on hepatic secondary metabolizing enzymes

Table 2 shows that CCl_4 treatment causes a decrease in the activity of glutathione S-transferase and quinone reductase by 43% and 76% as compared to the saline control group. At a lower dose 125 mg/kg body weight, *M. borneensis* treatment increased the activity of glutathione S-transferase and quinone reductase by 19% and 11% and 30% and 18% respectively at the higher dose of 250 mg/kg body weight in contrast to CCl_4 treated group.

Table 2: Effect of pretreatment of rats with *M. borneensis* extract on glutathione S-transferase and quinone reductase

Treatment groups	Glutathione S-transferase (nmol CDNB conjugate formed/min/mg protein)	Quinone reductase (nmol of dichloroindophenol reduced/min/mg protein)
	mean±SEM	mean±SEM
Saline control	35.09±0.66	8.59±0.05
CCl_4 (1.2 ml/kg b. wt.)	20.32±0.55*	2.07±0.06*
MB (125 mg/kg b. wt.)+CCl_4 (1.2 ml/kg b. wt.)	26.71±0.66**	2.97±0.10**
MB (250 mg/kg b. wt.)+CCl_4 (1.2 ml/kg b. wt.)	30.58±1.08**	3.60±0.13**

Each value represents mean±SEM of six animals. *Indicates significance at p<0.05 compared to control group. ** Indicates significance at p<0.05 compared to CCl_4 group.

Protective effect of *M. borneensis* on serum markers (ALT and AST)

The data of oral administration of *M. borneensis* on hepatic serum makers intoxicated with CCl_4 is presented in table 3. In contrast to the saline control group, treatment leads to serum ALT and AST level increase about 136% and 87%, respectively. Whereas treatment with *M. borneensis* extract at both doses resulted in 94%-92%, and 82%-87% reduction in the values of serum ALT and AST respectively as compared with CCl_4 treated group.

Table 3: Effect of pretreatment of rats with *M. borneensis* extract on alanine transferase and aspartate transferase

Treatment groups	ALT (µmole pyruvate/min/dl) mean±SEM	AST (µmole pyruvate/min/dl) mean±SEM
Saline control	0.05±0.003	0.07±0.007
CCl_4 (1.2 ml/kg b. wt.)	0.093±0.004*	0.17±0.004*
MB (125 mg/kg b. wt.)+CCl_4 (1.2 ml/kg b. wt.)	0.086±0.002**	0.16±0.002**
MB (250 mg/kg b. wt.)+CCl_4 (1.2 ml/kg b. wt.)	0.081±0.009**	0.14±0.010**

Each value represents mean±SE of six animals.*Indicates significance at p<0.05 compared to the control group. **Indicates significance at p<0.05 compared to CCl4 group.

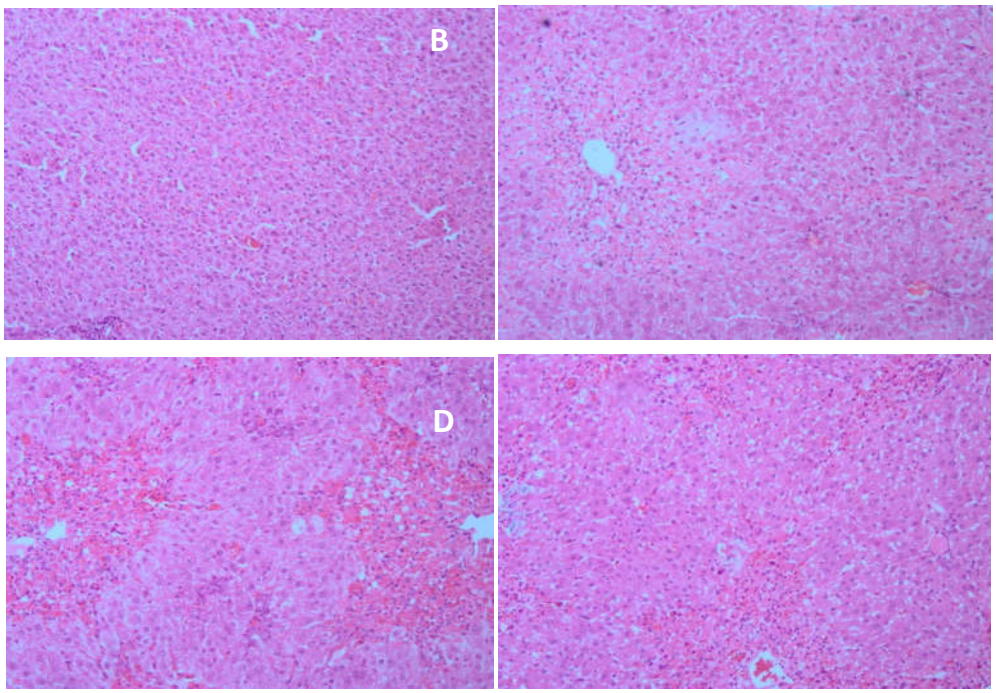

Fig. 3: Effect of *M. borneensis* on liver histopathological alterations in rats. (A) Saline treated control (B) CCl_4 (1.2 ml/kg b. wt.) (C) MB (125 mg/kg b. wt.)+CCl_4 (1.2 ml/kg b. wt.) (D) MB (250 mg/kg b. wt.)+CCl_4 (1.2 ml/kg b. wt.). Specimens stained with hematoxylin and eosin (a, b, c and d) X 20)

Protective effects of *M. borneensis* on liver histopathology

The saline control group shows normal liver architecture as indicated in fig. 3 A. CCl_4 treated group produced in focal necrosis, dense portal infiltration and inflammation (fig. 3B). Whereas rats treated with M. borneensis showed a minimum grade of necrosis and inflammation (fig. 3 C and D).

DISCUSSION

Most commonly used hepatotoxins in hepatic disease experimental studies and screening the plant extract is CCl_4 [23]. The damages caused by CCl_4 are, as a result of the generation of trichloromethyl radicals [23]. These radicals further result in peroxidative degradation of the membranes consisting of polyunsaturated fatty acids [24].

Reduced glutathione plays a vital role as an endogenous antioxidant and in the maintenance of intracellular redox homeostasis [25]. Studies have been shown that GSH conjugation is essential in eliminating toxic metabolites, which mainly cause hepatic pathology [26]. The treatment of CCl_4 causes significant depletion of glutathione level. Whereas, the pretreatment of M. borneensis reduces the toxicity of CCl_4. The restoration of GSH level may play a role in the mechanism of liver protection by M. borneensis against CCl_4 toxicity.

Lipid peroxidation is the main cause of hepatotoxicity caused by the chained reaction of CCl_4 free radical *derivatives* and is accountable in damaging the cell membrane due to the formation of free radical derivatives. The antioxidant activities of some substances play an important role in liver protection [27]. In the current study, elevation in the levels of MDA (the end product of lipid peroxidation) indicates enhanced lipid peroxidation. Pretreatment with M. borneensis significantly reversed these effects. There is a possibility that antioxidants do affect the mechanism of hepatoprotection of M. borneensis.

In our recorded study, the level of serum markers has significantly elevated in the serum after CCl_4 treatment, as previously reported [28, 29]. Yet, increased levels of the above enzymes are significantly reduced by pretreatment with *M. borneensis*, indicating that the extract reduced hepatic damage, which is further supported by the less amount of histopathological injury.

The antioxidant enzymes are vital for the detoxification of ROS [30]. The decreased activities of hepatic antioxidant enzymes in CCl_4 induced rats have been reported previously [31]. In our study, CCl_4 treatment decreased the activities of the catalase, glutathione peroxidase and glutathione reductase. Administration of *M. borneensis* significantly increased the level of the above enzymes in a dose-dependent manner. The recovery in their activity with the extract may be due to decreased oxidative load. The plant extract may act by scavenging the reactive oxygen metabolites due to the occurrence of different antioxidant compounds [32].

CCl_4 administration has also lowered the level of phase 2 enzymes, glutathione S-transferase and quinone reductase. Quinone reductase

protects the tissue by the elimination of toxic compounds and also stops the semiquinones formation by reduction of one-electron, thus reduces free radical generation from the auto-oxidation of semiquinones [19, 33]. The level of phase 2 enzymes is restored by *M. borneensis*.

The toxicity of CCl_4 has caused such extensive vascular degenerative changes and centrilobular necrosis in hepatocytes. Treatment with different doses of ethanolic extract of leaves of *M. borneensis* produced slightly degenerative changes and lowered the necrotic zones in the hepatocytes, indicating its hepatoprotective efficiency.

CONCLUSION

These results thus suggest that significantly decreased level of hepatic GSH and lipid peroxidation (MDA) along with normalizing activities of antioxidant enzymes and plasma markers (ALT, AST) suggest that *M. borneensis* extract has protective effects against CCl_4-induced hepatotoxicity by reducing the oxidative stress. A reduction in the histopathological alternation in the liver is further supporting the biochemical findings.

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AUTHORS CONTRIBUTIONS

Muhammad Dawood Shah, Senty Vun-Sang and Riana Binti Awang Saman conducted the experiments and prepared the manuscript. Dr. Mohammad Iqbal-Helped in designing and conduction the experiment.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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