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Safety assessment and oxidative stress evaluation of myricetin derivative-rich fraction from *Syzygium malaccense* in C57BL/6J mice

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Abstract

Myricetin derivatives from *Syzygium malaccense* leaf extract are known to have numerous therapeutic efficacies but there is no documented evidence corroborating its safety. Therefore, the present work aimed to evaluate the safety profile of myricetin derivative-rich fraction (MD) from *S. malaccense* leaf extract through single and repetitive oral administration in C57BL/6J mice. In the acute toxicity study, mice were orally administered with MD at single doses of 25, 150, 500, and 1,500 mg/kg. Subsequently, a modified sub-chronic toxicity assessment was performed by administering 150 mg/kg MD orally for 16 weeks. In both acute and sub-chronic toxicity studies, there were no lethal effects and behavioural signs of toxicity observed. The body weight, food, and water intakes of mice were normal throughout the respective experimental periods. As compared to the respective control groups, MD caused a significant improvement in serum uric acid and aspartate aminotransferase levels. The histopathological analysis of MD-administered mice did not show any inflammation or cell death. The MD-treated mice showed significantly reduced protein carbonyl and lipid hydroperoxide levels in urine, liver, and kidney tissues. Taken together, the no-observed-adverse-effect level of MD was up to 1,500 mg/kg, and considered safe for oral consumption over relatively long durations with oxidative stress attenuating properties.

Introduction

Since time immemorial, plant and their resources have played an important role as a traditional remedy for various disorders. According to the World Health Organization (WHO), there is a high demand for natural products among developing countries (Yuan *et al.,* 2016). However, the use of herbal-based medicines as complementary therapeutics has elicited some concerns due to their unknown toxic effects (Vieira *et al.,* 2016). Therefore, toxicity studies are essential to identify the possible toxic adverse effects of medicinal plants and their phytoconstituents. To examine the safety of medicinal plants, animal models and clinical trials have been conducted in the recent years (Bostan *et al.,* 2017). To date, several studies have reported on the numerous medicinal properties of active phytoconstituents which include antioxidant, antimicrobial, anticancer, antidiabetic, and anti-obesity properties (Forni *et al.,* 2019).

The human body contains enzymatic and non-enzymatic antioxidants that counteract the oxidative stress caused by oxidants. Numerous phytoconstituents such as flavanones, isoflavones, flavonols, anthocyanidins, and phenolic acids can prevent oxidative stress caused by free radicals; this is achieved via several mechanisms such as scavenging of reactive oxygen species (ROS), activation of antioxidant enzymes, metal chelation, inhibition of oxidases, and controlling uric acid levels (He *et al.,* 2017).

Syzygium malaccense (L.) Merr. & L.M. Perry is a plant that belongs to the Myrtaceae family, and originates from Malaysia. The local names of *S. malaccense* are "Malay apple" or "Red Jambo" (Batista *et al.,* 2017). Previous studies have shown that the leaf extracts of *S. malaccense* possess promising antioxidant and antihyperglycemic properties. To date, the safety and biological efficacies of *S. malaccense* have yet to be explored in detail. Flavonoids or bioflavonoids are derived from the Latin word *flavus* meaning yellow, and ubiquitously found in many plants. According to Arumugam *et al.* (2014), the major flavonoids present in leaf extracts of *S. malaccense* are myricetin derivatives (MD).

Myricetin is a flavonol widely present in the

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human diet through foods such as tea, fruits, red wine, vegetables, and medicinal plants. It consists of benzene rings A and B with hydroxyl groups at carbon positions of 3, 5, 7, 3', 4', and 5'. The presence of multiple hydroxyl groups facilitates the antioxidant reactivity of myricetin (Semwel *et al.,* 2016). Examples of myricetin derivatives are myricetin-3-O-rhamnoside, myricetin-3-O-glucoside, myricetin-3-O-arabinoside, myricetin-3-O-galactoside, and myricetin-3-O-rutinoside (Arumugam *et al.,* 2014; Semwel *et al.,* 2016).

Numerous evidence has suggested that myricetin derivatives possess various biological efficacies such as anticancer (Devi *et al.,* 2015), antioxidant, antihyperglycemic (Arumugam *et al.,* 2016), anti-inflammatory (Hou *et al.,* 2018), and antimalarial (Rudrapal and Chetia, 2017) properties. To the best of our knowledge, *in vivo* biosafety of myricetin derivative-rich fraction from *S. malaccense* leaf extracts has yet to be reported. Therefore, in the present work, the short-term and long-term effects of this extract on toxicity and oxidative stress were evaluated using the C57BL/6J mouse model.

Materials and methods

Materials

Standard mice chow (Altromin Maintenance Diet 1324) was purchased from Altromin, Lage, Germany. Formaldehyde solution and paraffin wax were purchased from Leica Surgipath, United States. All reagents were of standard grade.

Preparation of myricetin derivative-rich fraction (MD)

Fresh *S. malaccense* leaves were collected from a plantation located in Tangkak, Johor Bahru, Johor, Malaysia on 20th December 2016. The identification of *S. malaccense* leaves was confirmed by the Herbarium Department, Forest Research Institute of Malaysia (FRIM) (Sample ID: PID220712-15). The leaves were cleaned, dried at 40°C, and ground to a fine powder using a mechanical grinder. The powdered leaves (50 g) were subjected to ethanol extraction at a ratio of 1:20 (w/v) for 24 h at 37°C in a rotary shaker, as described in a previous study (Arumugam *et al.,* 2014). The ethanolic extract was filtered, and the filtrate was rotary evaporated at 40°C. Then, the ethanolic extract was further purified using a liquid-liquid extraction method. Water and *n*-hexane were used as solvents. The myricetin derivative-enriched aqueous fraction (0.8 g/50 g leaf powder) was separated before being dried using a rotary evaporator and freeze dryer. The presence of myricetin derivatives in the extract was confirmed using HPLC and LCMS analyses as described in a previous study (Arumugam *et al.,* 2014).

Ethics approval and experimental animals

The animal protocols were evaluated and approved by the Institutional Animal Care and Use Committee (IACUC), University of Malaya (M/20022018/03012018-01/R). The C57BL/6J strain was selected for the safety assessment. Six-week-old C57BL/6J male mice (19 - 30 g) were purchased from the Animal Facility Unit, Jeffrey Cheah School of Medicine and Health Science, Monash University Malaysia, Selangor. The animals were acclimatised for 2 w, and maintained under standard laboratory condition (25 ± 1°C) with free access to food and water. The toxicity study was carried out at the Laboratory Animal Centre (LAC), University of Malaya, Kuala Lumpur, Malaysia. Experimental procedures for the toxicity study were performed under the guidelines of the Organization for Economic Cooperation and Development (OECD), OECD 423 for acute toxicity study and OECD 408 for sub-chronic study with certain modifications.

Acute toxicity study

The mice were randomly divided into five groups of six mice each (normal saline, 25, 150, 500, and 1,500 mg/kg MD). The highest dose used in the acute toxicity study was 1,500 mg/kg MD due to poor solubility at high doses. All mice were starved for 4 h with free access to water prior to the administration of the solubilised test samples. The test samples were dissolved in normal saline (vehicle), and administered via the epigastric route using a gavage needle. The control group received normal saline instead. The volume of the dose was fixed at 10 mL/kg body weight of mice. The animals were closely observed for 30 min, 1, 2, 4, 8, 24, and 48 h for toxicity symptoms. The clinical signs of toxicity and general characteristics of mice such as mortality, skin texture, eye colour, state of faeces, urination, sleep and gait, lethargy, lacrimation, and piloerection were monitored. The initial and final body weights, as well as food and water intakes were noted throughout the experimental period. Apart from that, mice urine was collected before treatment (Day 1) and after treatment (Days 7 and 14). The urine was centrifuged at 12,500 *g* for 10 min at 4°C, and the supernatant was kept at -80°C until the biochemical assays were performed. On the 15th day, the mice were anaesthetised with isoflurane after withholding food for 4 h. Blood was drawn from all

mice via cardiac puncture, collected in serum collection tubes, and centrifuged at 3,750 *g* for 10 min. The serum was analysed for sodium, potassium, chloride, urea, uric acid, alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) using an automated clinical chemistry analyser (ADVIA 2400, Siemens Healthcare) at the Clinical Diagnostic Laboratory Unit, University Malaya Medical Centre, Kuala Lumpur, Malaysia. Both liver and kidneys were immediately harvested for oxidative stress and histopathological analysis. To evaluate tissue oxidative stress, organs were immediately perfused *in situ* with ice-cold saline. The organs were homogenised using ice-cold phosphate buffer saline (PBS), and the concentration of the homogenates was fixed at 10% (w/v) and 0.1 g of tissues in 1 mL of ice-cold PBS. The homogenised samples were stored at -80°C.

Sub-chronic toxicity study

The long-term effects of MD were tested in mice for a duration of four months (16 w). A dose of 150 mg/kg (10% of the highest dose tested in acute toxicity study, 1,500 mg/kg MD) was selected as the targeted dose for the sub-chronic toxicity evaluation. The mice were randomly divided into two groups (n = 6). Group 1 served as the control, and was given normal saline (vehicle), while Group 2 was administered MD at 150 mg/kg b.wt. via the epigastric route using a gavage needle. To minimise physical stress in mice, treatment was administered at 2-d intervals. The physical condition of mice was closely monitored throughout the 16-w treatment. Their body weight, as well as water and food intakes were recorded. The urination and faecal state of the mice were closely observed every day. Urine was collected on weeks 1, 4, 8, 12, and 16 to examine the urinary oxidative indices and antioxidant levels. On the last day of week 16, the mice were fasted overnight, and anaesthetised with isoflurane. Blood was drawn via cardiac puncture and collected in serum collection tubes. The serum was analysed to examine kidney and liver function profiles. Vital organs (liver and kidneys) were excised immediately following blood collection for histopathological and oxidative stress analyses. For tissue oxidative stress evaluation, the collected organs were perfused *in situ* and homogenised as described in the acute toxicity study.

Histopathological examination

Tissues of the liver and kidney were dissected, fixed in 10% (v/v) formalin, and routinely processed. Paraffin-embedded tissue sections (4-µm thick) were prepared and dried overnight before being stained with haematoxylin and eosin (Ni and Wang, 2016).

Oxidative indices measurement

The protein carbonyl content in urine and tissue homogenates were determined according to the method outlined by Kanagasabapathy *et al.* (2013). An advanced oxidation protein products (AOPP) reagent solution was freshly prepared by mixing PBS, 50% glacial acetic acid, and 1.16 M potassium iodide at a ratio of 81:15:4. The reagent solution (200 µL) was added to 18 µL of the sample (urine or tissue homogenate), and the absorbance was measured at 340 nm. AOPP concentration was determined based on the chloramine-T standard (0 to 500 µM). The results were expressed as µM of chloramine-T for urine samples, and µmol of chloramine-T per g tissue for tissue homogenates.

The lipid hydroperoxides (LHP) level in urine and tissue homogenates were determined according to the method described by Esterbauer and Cheeseman (1990) with certain modifications. Freshly prepared 375 µL of 10.3 mM 1-methyl-2-phenylindole (MPI) and 225 µL of 5 M hydrochloric acid were added to 150 µL of the sample (urine or tissue homogenate). The solution mixture was incubated at 45°C for 40 min in a water bath. Then, it was centrifuged at 12,500 *g* for 10 min. The absorbance of the resulting supernatant was measured at 586 nm. Different concentrations of 1,1,3,3-tetraethoxypropane (TEP) solution (0 to 20 µ M) was used as a standard for lipid hydroperoxide quantification, and the result was expressed as µM of TEP.

Antioxidant activity measurement

The ferric-reducing antioxidant power (FRAP) levels in urine and tissue homogenates were measured based on the method described by Chandramathi *et al.* (2009). The FRAP reagent was freshly prepared by mixing 300 mmol/L acetate buffer, 10 mmol/L 2,4,6-tripyridyl-S-triazine (TPTZ) in 40 mM hydrochloric acid, and 20 mM iron(III)chloride hexahydrate ($FeCl_3.6H_2O$) at a ratio of 10:1:1. As a control, different concentrations of ferrous sulphate monohydrate ($FeSO_4.H_20$) in the range of 0 - 1000 µM were used. Ten microliters of the sample (urine or tissue homogenate) were added to 300 µL of FRAP reagent. Then, the reduction of ferric tripyridyltriazine ($Fe^{III}TPTZ$) complex by the non-enzymatic antioxidants in samples was measured spectrophotometrically at 593 nm after 4

min, and the result was expressed as μM of iron sulphate.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Each value represented a minimum of six mice (n = 6) in a group. In the acute toxicity study, one-way analysis of variance (ANOVA) was used to determine significant differences in the data, and this was followed by Dunnett's multiple comparison test (DMRT) between groups. T-test was used to analyse differences between the means in sub-chronic toxicity study. The p values of less than 0.05 were considered as statistically significant. Data analysis and graphs were created using Graph Pad Prism Version 8.0 (GraphPad Software Inc., California, USA).

Results and discussion

The toxicological evaluation of a pharmacological agent is an important assessment as it is usually performed before the agent is commercialised. The acute toxicity study is the initial step in the toxicological assessment of unknown substances. It also serves as a method of examining the adverse effects that occur in a short period (14 days) via single administration. However, the long-term effect of phytomedicine can only be examined through multiple administration, and may provide crucial data for clinical applications (Abotsi *et al.,* 2011). Among laboratory animals, mice were chosen to be used in the present work as they are known to exhibit fast responses and reactions. Their small size enables cost-effective and high throughput studies (Vandamme, 2014). The C57BL/6 mouse strain is known to be the most widely used strain in major biomedical research fields.

Acute toxicity study

The mean lethal dose (LD_{50}) value is an important parameter in the acute toxicity assessment. It is also an initial step in the screening of the safety profiles of chemical and pharmacological agents. In the present work, the oral LD_{50} value of MD was found to be above 1,500 mg/kg b.wt. This indicated that MD could be relatively safe for consumption and would not cause any form of hepatotoxicity complications since it was within the range of 1,000 – 5,000 mg/kg b.wt. (Lorke, 1983). These results concurred with previous findings by Schmeda-Hirschmann *et al.* (1987), whereby the maximum dose of myricitrin and myricetin enriched extract from *Eugenia uniflora* leaves (4200 mg/kg) was not toxic to BALBc mice.

All mice did not show any abnormalities, and they were active throughout the experimental period. Dermatitis is a common syndrome inherited by C57BL/6J mice, and this can be triggered by an environmental factor as well as food substances that the mice may be exposed to (Biase *et al.,* 2019). In the present work, a single oral administration of MD did not show any dermatitis symptoms in the mice; their skin remained smooth throughout the experimentation period. Generally, irritation and allergies cause lacrimation and half-closed eyes in mice. However, a single oral administration of MD did not trigger these symptoms in the mice, and their eyes remained normal throughout the study period. These findings suggested that MD did not induce an allergic reaction in mice, and was well accepted by the body immune system.

Urination and the state of faeces are important parameters that are observed in toxicity studies (Nfozon *et al.,* 2019). All mice in the present work had no diarrhoea, and all had normal faeces. Not only that, the sleep and gait of mice were also normal following MD administration. Besides, the single high dose of MD (1,500 mg/kg b.wt.) did not affect their activeness and movement throughout the study period. Moreover, the body weight of MD-administered mice showed a gradual increase after the first week and second week of treatment (Table 1). No significant changes were found in the food consumption and water intake of mice (Table 1).

The kidneys play a significant role in the regulation of homeostasis within the body by balancing electrolytes such as potassium, sodium, and chloride. In the present work, both potassium and chloride of the all MD-administered groups were not significantly altered as compared to the control group. However, the single oral administration of MD at 1,500 mg/kg b.wt. showed a significant reduction in serum sodium level as compared to the control group. This could be due to high water intake (Table 1) that leads to the dilution of sodium concentration in the blood (Joo and Kim, 2013). Meanwhile, the serum uric acid levels of the all MD-administered mice and urea level of 1,500 mg/kg MD group were significantly lower as compared to the control group (Table 2), which reflected the potential renal protective effect of MD. Besides, the serum AST levels of MD-administered mice in the 150, 500, and 1,500 mg/kg groups were found to be significantly lower as compared to the control group (Table 2). The reduction in serum urea and liver enzymes upon single-dose administration concurred with previous acute toxicity studies

Table 1. Effects of MD on the body weight, and food and water intakes of mice in acute and sub-chronic toxicity studies.

Study	Treatment group	Treatment period	Body weight (g)	Food intake (g/day/mouse)	Water intake (mL/day/mouse)
Acute toxicity	Normal saline	1st day	26.67 ± 1.63	4.00 ± 0.63	4.83 ± 0.68
		7th day	26.67 ± 1.63	4.33 ± 0.82	5.00 ± 0.71
		14th day	27.83 ± 1.47	4.33 ± 0.52	4.83 ± 0.41
	25 mg/kg MD	1st day	25.67 ± 1.75	4.36 ± 0.50	5.00 ± 0.63
		7th day	26.67 ± 1.75	4.19 ± 0.70	5.25 ± 0.69
		14th day	26.83 ± 1.47	4.24 ± 0.62	5.00 ± 0.63
	150 mg/kg MD	1st day	26.67 ± 1.37	3.83 ± 0.75	5.03 ± 0.66
		7th day	27.33 ± 1.03	4.06 ± 0.65	5.33 ± 0.52
		14th day	27.33 ± 1.51	4.19 ± 0.40	5.67 ± 0.41
	500 mg/kg MD	1st day	26.00 ± 2.37	4.17 ± 0.41	5.25 ± 0.52
		7th day	26.67 ± 1.75	4.31 ± 0.54	5.33 ± 0.75
		14th day	26.83 ± 1.47	4.67 ± 0.52	5.42 ± 0.49
	1,500 mg/kg MD	1st day	26.17 ± 2.32	4.37 ± 0.50	5.67 ± 0.52
		7th day	26.83 ± 2.04	4.64 ± 0.50	5.75 ± 0.69
		14th day	27.83 ± 1.94	4.50 ± 0.55	5.58 ± 0.59
Sub-chronic toxicity	Normal saline	Week 1	24.17 ± 0.75	4.00 ± 0.45	3.92 ± 0.74
		Week 4	26.00 ± 0.63	4.17 ± 0.52	3.83 ± 0.52
		Week 8	27.83 ± 0.98	3.83 ± 0.41	3.67 ± 0.75
		Week 12	29.67 ± 0.52	4.00 ± 0.55	4.00 ± 0.48
		Week 16	31.17 ± 0.41	4.08 ± 0.38	3.75 ± 0.69
	150 mg/kg MD	Week 1	24.17 ± 0.98	3.83 ± 0.26	3.67 ± 0.61
		Week 4	25.50 ± 1.05	4.00 ± 0.32	3.94 ± 0.14
		Week 8	27.33 ± 1.21	3.86 ± 0.29	4.06 ± 0.33
		Week 12	28.50 ± 1.05	3.92 ± 0.20	4.08 ± 0.58
		Week 16	30.33 ± 0.52	3.92 ± 0.38	3.91 ± 0.37

Values are mean ± standard deviation (SD). MD = myricetin derivative-rich fraction. There was no significant difference.

(Saleem *et al.,* 2017; Aouachria *et al.,* 2017). Besides, histopathological studies of liver and kidney of the control group (administrated with normal saline) and MD-administered groups did not show inflammation or cell death, which indicated the non-toxic effect of MD on the kidneys and liver (Figure 1A).

Oxidative stress is implicated in numerous acute and chronic pathological processes. Imbalance and high oxidative stress are reflected by free radical attacks on biological molecules such as proteins, lipids, and DNA of tissues and cells. Therefore, urine, serum, plasma, and homogenised tissue samples are commonly used in examining oxidative stress levels in rodent research (Longini *et al.,* 2017). In the present work, the changes in oxidative indices and non-enzymic antioxidant levels were measured in both urine and tissue homogenates.

Advanced oxidation protein products (AOPP) is one of the essential biomarkers of oxidative stress as proteins are the major targets of

Table 2. Serum biochemistry profiles of mice in acute and sub-chronic toxicity studies.

	Acute toxicity					Sub-chronic toxicity	
	Normal saline	**25 mg/kg MD**	**150 mg/kg MD**	**500 mg/kg MD**	**1,500 mg/kg MD**	**Normal saline**	**150 mg/kg MD**
Kidney function profile							
Sodium (mmol/L)	151.00 ± 1.73	149.33 ± 1.16	151.33 ± 0.58	149.00 ± 4.58	148.67 ± 2.08*	144.67 ± 0.58	145.00 ± 1.00
Potassium (mmol/L)	6.33 ± 0.25	5.50 ± 0.53	5.43 ± 0.64	6.07 ± 0.78	5.50 ± 0.62	7.29 ± 0.48	7.45 ± 0.45
Chloride (mmol/L)	110.50 ± 1.50	111.67 ± 0.58	111.33 ± 2.08	110.67 ± 3.22	109.00 ± 2.65	107.33 ± 1.53	106.50 ± 2.12
Urea (mmol/L)	8.57 ± 1.07	7.60 ± 0.27	7.53 ± 0.65	7.30 ± 0.70	6.17 ± 0.46*	8.63 ± 0.12	7.80 ± 0.17*
Uric acid (µmol/L)	170.00 ± 8.00	133.50 ± 3.50***	137.00 ± 1.00***	120.27 ± 6.75****	113.00 ± 5.00****	184.50 ± 2.50	144.17 ± 3.01**
Liver function profile							
ALP (U/L)	78.00 ± 12.00	74.67 ± 8.51	69.00 ± 5.00	57.67 ± 17.50	76.00 ± 7.00	59.33 ± 5.77	52.50 ± 5.50
ALT (U/L)	30.33 ± 5.77	30.00 ± 11.79	39.67 ± 6.51	46.00 ± 10.00	42.00 ± 17.44	81.17 ± 6.00	63.83 ± 3.25*
AST (U/L)	185.00 ± 21.00	150.00 ± 16.00	133.67 ± 13.50*	84.00 ± 17.52**	89.67 ± 17.90**	343.33 ± 17.95	247.00 ± 11.27*

Values are mean ± standard deviation (SD); *significant at $p < 0.05$, ** significant at $p < 0.01$, and *** significant at $p < 0.001$, and **** significant at $p < 0.0001$ against the normal saline group; MD = myricetin derivative-rich fraction; ALP = alkaline phosphatase; ALT = alanine aminotransferase; and AST = aspartate aminotransferase.

free radicals while lipid peroxidation is the process of oxidative degradation of lipids by reactive oxygen species. Ferric-reducing antioxidant power (FRAP) measures the total non-enzymic antioxidant level; this is estimated by measuring the ferric-reducing ability of targeted samples. The urinary AOPP level of all mice before the administration of MD was not significantly different ($p > 0.05$). At the end of week 1 (7th day of treatment), the urinary carbonyl content of MD-administered mice was remarkably lower ($p < 0.05$) than that of the control group. The urinary AOPP level remained significantly lower ($p < 0.05$) than the control group by the end of week 2 (14th day of treatment) in the 150 and 500 mg/kg MD-administered groups (Table 3). The lipid hydroperoxide levels of 500 and 1,500 mg/kg MD groups were found to be significantly lower ($p < 0.05$) than that of the control group at the end of week 1. At the end of week 2, the urinary lipid hydroperoxide levels of all MD treated mice showed no significant difference as compared to the control group (Table 3). Meanwhile, the urinary FRAP levels of 150 and 500 mg/kg MD-administered groups measured at the end of first and second weeks were significantly lower ($p < 0.05$) as compared to that of the control group (Table 3).

In addition, the protein carbonyl content in liver and kidney tissues of 150 and 500 mg/kg MD-administered groups were significantly lower than the control group, whereas only the protein carbonyl content in kidney tissue of 1,500 mg/kg MD group was significantly lower ($p < 0.05$) than the control group (Table 3). However, the lipid hydroperoxide levels in both liver and kidneys were not significantly different after a single administration of MD (Table 3). Besides, the FRAP level in the liver and kidneys of both 150 and 500 mg/kg MD-administered groups showed a significant increase as compared to the control group. However, in the 1,500 mg/kg MD-administered group, only the kidney tissue showed significantly higher FRAP level ($p < 0.05$) than the control group (Table 3). The protein carbonyl and lipid hydroperoxide levels in urine and tissue homogenates were ameliorated in accordance with the elevated FRAP level, thus confirming the potent antioxidant effect of MD.

The results obtained from the acute toxicity study suggested that the oral administration of the highest dose of MD (1,500 mg/kg b.wt.) was not toxic, and that MD showed an ability to ameliorate oxidative stress in mice.

Sub-chronic toxicity study

One of the sensitive indices of toxicity after the administration of toxic substances is a reduction in body weight gain (Hayelom *et al.,* 2012). The changes in body weight of mice in modified sub-chronic toxicity study are depicted in Table 1. There was no significant difference in body weight between the MD-administered group and the control group throughout the experimental period, suggesting that the exposure to MD facilitated normal growth without any physiological changes. According to the previous findings, MD were able to maintain a healthy body weight in the management of dyslipidaemia, cardiovascular risk, as well as hypertension (Su *et al.,* 2016; Wang *et al.,* 2017).

The oral administration of MD at 150 mg/kg b.wt. caused no significant changes in food and water intakes as compared to the control group throughout the experimental period ($p > 0.05$) (Table 1). This suggested that MD consumption supported normal digestion without suppressing feeding and drinking urges.

The liver and kidneys are primary organs that are easily affected by toxic compounds and drugs. Additionally, the kidneys are the primary defenders against toxic substances that enter the bloodstream. Potential causes of nephrotoxicity include the environment, medications, organic solvents, and heavy metals (Orr and Bridges, 2017; Perazella, 2018). Nephrotoxicity causes an impairment in the urine and dysregulation of blood electrolytes. Therefore, kidney and liver damages can be examined by analysing serum biochemistry profiles (Bailey *et al.,* 2004).

In the present work, the repeated administration of MD maintained the level of sodium, potassium, and chloride as compared to the control group (Table 2), suggesting that MD did not result in adverse effects on the regulation of electrolytes within the body. Uric acid is an end-product of purine metabolism which is transported via the blood to the kidneys, and excreted through urine. The control of uric acid is important to prevent complications such as gout, kidney stones, as well as hyperuricemia. In the present work, the repetitive administration of MD significantly lowered serum uric acid level (Table 2). The reduction in uric acid level could be attributed to the inhibition of xanthine oxidase, an enzyme involved in the hydroxylation of xanthine. A similar result was reported by Mo *et al.* (2007), whereby oral administration of 50 and 100 mg/kg of myricetin for three days resulted in a reduction in liver uric acid levels in hyperuricemic mice.

The liver plays a vital role in breaking down substances that enter the body such as drugs, herbs, and supplements (Singh *et al.,* 2016). Studies have proven that excessive use or high doses of drugs and

Table 3. The oxidative indices and antioxidant measurement in urine and tissue homogenates of mice in acute toxicity study.

Group	Sample		AOPP (µM)	AOPP (µmol/g tissue)	LHP (µM)	FRAP (µM)
Normal saline	Urine	1st day treatment	219.12 ± 10.96		11.76 ± 1.23	943.79 ± 30.84
		7th day treatment	253.57 ± 12.34		12.81 ± 0.73	975.03 ± 17.26
		14th day treatment	244.25 ± 9.35		11.63 ± 1.06	983.48 ± 17.79
	Tissue	Liver		1.98 ± 0.14	0.81 ± 0.13	589.90 ± 64.45
		Kidney		0.92 ± 0.10	0.95 ± 0.40	654.35 ± 13.29
25 mg/kg MD	Urine	1st day treatment	221.16 ± 10.09		12.36 ± 1.08	968.91 ± 27.88
		7th day treatment	214.68 ± 11.58**		11.45 ± 0.83	944.22 ± 30.95
		14th day treatment	223.20 ± 11.87		12.53 ± 1.10	951.20 ± 16.64
	Tissue	Liver		1.70 ± 0.20	0.77 ± 0.10	706.01 ± 82.95
		Kidney		0.80 ± 0.10	0.98 ± 0.46	664.96 ± 28.96
150 mg/kg MD	Urine	1st day treatment	217.77 ± 10.32		11.81 ± 0.91	956.69 ± 24.34
		7th day treatment	219.31 ± 10.98**		11.60 ± 0.61	926.57 ± 26.50*
		14th day treatment	221.65 ± 10.14*		12.45 ± 1.08	940.90 ± 14.46*
	Tissue	Liver		1.40 ± 0.23*	0.69 ± 0.11	723.36 ± 65.98*
		Kidney		0.66 ± 0.10*	0.83 ± 0.27	738.98 ± 27.78**

500 mg/kg MD	Urine	1st day treatment	214.68 ± 12.47		11.84 ± 1.25	942.06 ± 21.13
		7th day treatment	222.09 ± 11.50**		11.12 ± 0.68*	927.62 ± 20.91*
		14th day treatment	218.26 ± 10.25*		12.47 ± 1.03	949.65 ± 13.09*
	Tissue	Liver		1.39 ± 0.32*	0.78 ± 0.44	748.67 ± 75.29*
		Kidney		0.68 ± 0.08*	0.91 ± 0.44	731.82 ± 34.40*
1,500 mg/kg MD	Urine	1st day treatment	220.73 ± 10.68		12.24 ± 1.60	950.52 ± 26.75
		7th day treatment	223.07 ± 11.79*		10.87 ± 0.71*	929.28 ± 19.17*
		14th day treatment	224.06 ± 13.10		12.15 ± 1.32	954.90 ± 29.81
	Tissue	Liver		1.53 ± 0.40	0.74 ± 0.19	707.37 ± 81.33
		Kidney		0.76 ± 0.10*	0.84 ± 0.53	748.24 ± 61.68*

Values are mean ± standard deviation (SD); *significant at $p < 0.05$, **significant at $p < 0.01$ against the normal saline group. MD = myricetin derivative-rich fraction; AOPP = advanced oxidation protein product; LHP = lipid hydroperoxides; and FRAP = ferric reducing antioxidant power.

medications could lead to hepatoxicity and impaired liver function (Neuman, 2019). Generally, serum liver enzymes including aspartate aminotransferase (AST), alkaline phosphatase (ALP), and alanine aminotransferase (ALT) are measured as indicators of hepatocellular effects. AST and ALT are important enzymes produced by the liver to facilitate the metabolism of amino acids. Keeping these enzymes at normal levels is crucial to maintain a healthy state of the liver. In the present work, the repetitive administration of MD significantly ($p < 0.05$) ameliorated ALT and AST levels in mice (Table 2). Matić *et al.* (2013) reported that the administration of myricetin and myricetin-enriched *Cotinus coggygria* extract lowered serum AST and ALT levels in pyrogallol-induced-toxicity affected mice, thus conferring hepatoprotection. A histopathological examination of liver and kidneys revealed that repeated administration of MD did not cause inflammation in the liver and kidney tissues, suggesting that MD had no cytotoxic effects on the vital organs of mice (Figure 1B).

In the sub-chronic toxicity study, multiple administrations of MD were able to significantly ($p < 0.05$) attenuate protein carbonyl and lipid hydroperoxide levels in urine between weeks 4 and 16 (Figures 2A and 2B). This could be possibly attributed to the antioxidant efficacies of MD-rich fraction. Besides, the urinary FRAP level was significantly reduced between weeks 8 and 16 (Figure 2C). This could be attributed to the reduced uric acid level excreted in the urine as a result of reduced serum uric acid level (Table 2). Uric acid is known to have a ferric-reducing property; thus, a reduction in uric acid level typically correlates with an attenuation in FRAP level (Kumarasamy *et al.,* 2017). The repeated oral administration of MD at 150 mg/kg b.wt. for 16 weeks showed a significant ($p < 0.05$) reduction of protein carbonyl content and lipid hydroperoxide level in both liver and kidneys as compared to the control group (Figure 2D and 2E). Meanwhile, the FRAP level in the liver and kidneys of 150 mg/kg MD administered mice was found to be significantly ($p < 0.05$) higher than that of the control

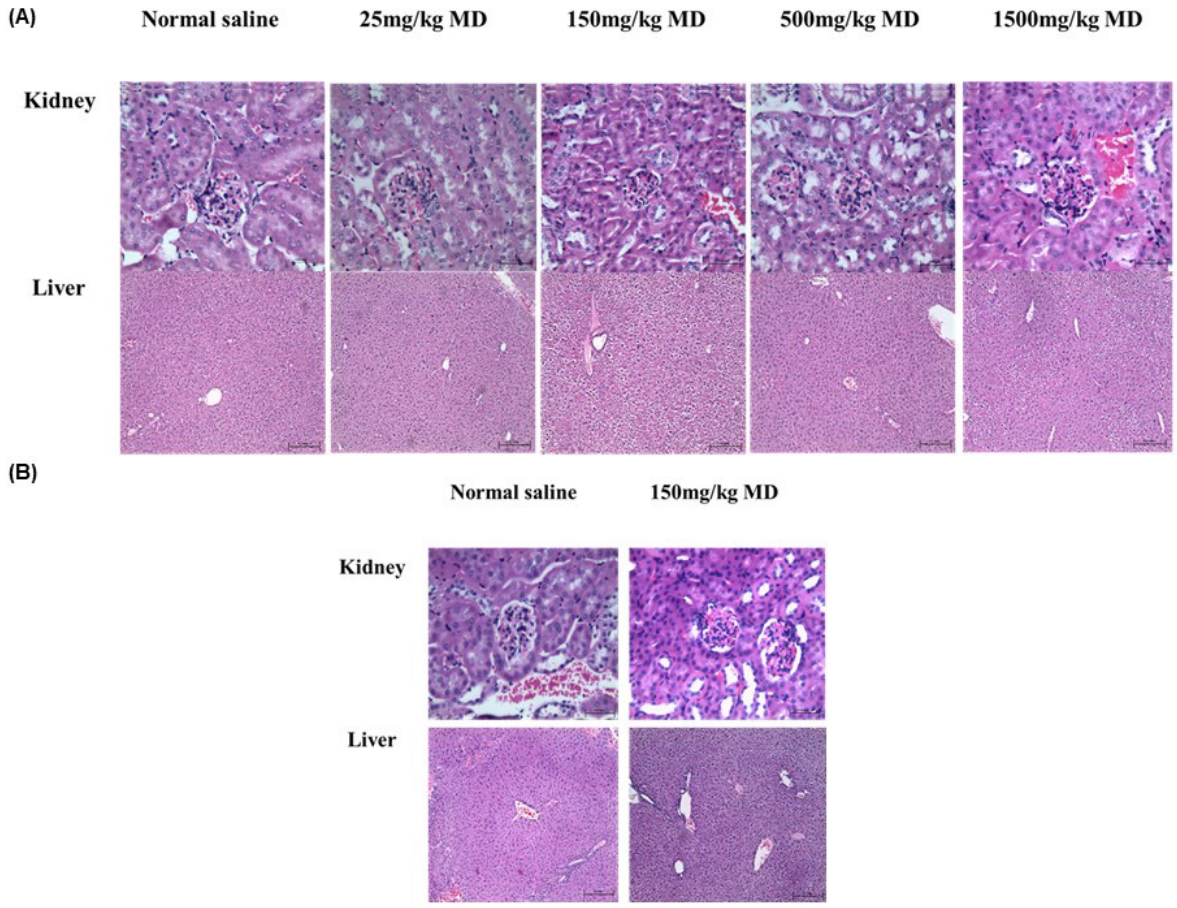

Figure 1. Histopathological (haematoxylin and eosin staining) analysis of organs administered with MD in (A) acute toxicity and (B) sub-chronic toxicity studies. Original magnification = liver (×10 obj.), and kidney (×40 obj.). Scale bar = liver (0.2 mm), and kidney (0.05 mm). MD = myricetin derivative-rich fraction.

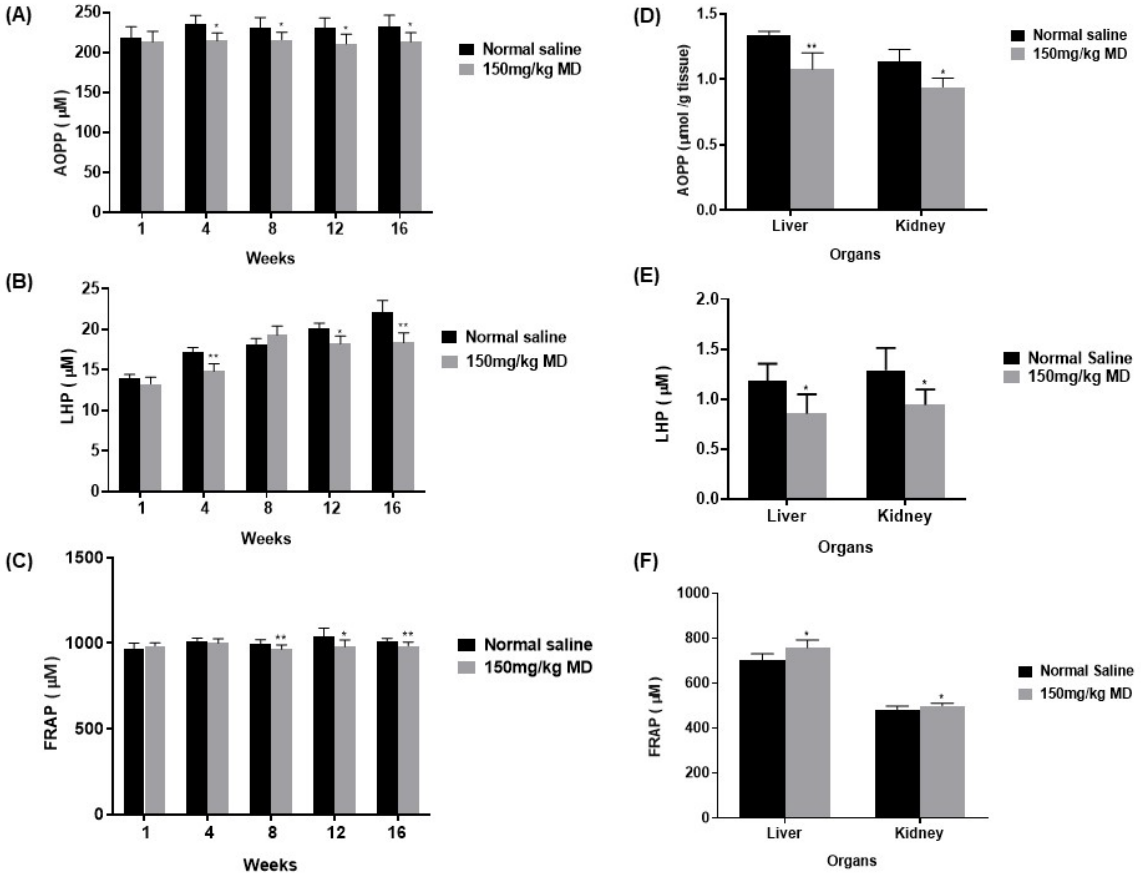

Figure 2. The effect of MD on advanced oxidation protein products (AOPP): (A) and (D) lipid hydroperoxides (LHP); (B) and (E) ferric reducing antioxidant power (FRAP); (C) and (F) levels in urine and tissue homogenates of mice in sub-chronic toxicity study. Values are mean ± standard deviation (SD); * significant at $p < 0.05$ and ** significant at $p < 0.01$ against the normal saline group. MD = myricetin derivative-rich fraction.

group (Figure 2F), thus indicating the high antioxidant capacity of MD.

An overwhelming body of information has been reported on the antioxidant activity of myricetin, and it has been proven that the antioxidant activity of myricetin is likely due to the hydroxyl present in its B-ring structure. The hydroxyl group in the C-4′ position can react with lipid peroxide radical, CH_3OO, to attenuate lipid peroxidation (Semwel *et al.,* 2016). Meanwhile, the presence of a double bond between the C2 - C3 position, catechol like structure in ring-B, and the 3-hydroxyl functional groups of myricetin contribute to the reduction of Cu and Fe ions (Mira *et al.,* 2002).

The attenuation of oxidative indices in urine and tissue homogenates could be attributed to the metabolites of MD which are formed upon intestinal microbial digestion. As reported by Du *et al.* (2014), myricitrin can be dehydroxylated to quercetin-3-O-rhamnoside and subsequently deglycosylated to quercetin. Furthermore, myricitrin can also be deglycosylated to aglycon myricetin. The antioxidant activities of quercetin and myricetin have been reported in previous studies (Guven *et al.,* 2019).

Conclusion

In conclusion, the findings of the present work indicated that the myricetin derivative-rich fraction (MD) from *Syzygium malaccense* leaf extract was safe and well-tolerated up to a single high dose of 1,500 mg/kg; thus, the no-observed-adverse-effect level of MD was 1,500 mg/kg. Both acute and sub-chronic toxicity assessments demonstrated that MD was able to improve oxidative stress and serum biochemistry profiles in mice without causing toxic effects. Therefore, it would be safe for oral consumption.

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