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Safety evaluation of orally-administered methanol extract of *Muntingia calabura* Linn. leaves: A sub-chronic toxicity study in Sprague Dawley rats

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Abstract*:* *Muntingia calabura* (*M. calabura*), locally known as “kerukup siam” or “buah ceri” belongs to the family Muntingiaceae and has been scientifically demonstrated to exert various pharmacological activities. The objectives of the current study are to evaluate the antioxidant activities and to determine the subchronic toxicity of 90 days orally-administered methanol extract of *M. calabura* (MEMC) in male Sprague Dawley rats. The rats were randomly divided into four groups (n=6). Vehicle control received 8% tween 80 and treatment group received 50, 250 and 500mg/kg of MEMC orally administered daily for 90 days. Blood collection was carried out to obtain the hematological and biochemical profile of the rats. The organs harvested were subjected to histopathological analysis. For the antioxidant test, the extract was subjected to antioxidant study using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH)- and superoxide anion-radical scavenging activity, total phenolic content (TPC) and phytochemical screening. Results obtained show that no adverse effects were observed during the experimental period. Hematological and biochemical analysis also showed no significant changes in this toxicity study. Besides, antioxidant analyses revealed that MEMC has higher DPPH- and SOD-radical scavenges activity as well as higher TPC value. In conclusion, *M. calabura* is safe for consumption and possesses beneficial antioxidant effect.

Keywords: *Muntingia calabura*, methanol extract, subchronic oral toxicity, NOAEL.

INTRODUCTION

Medicine preparation that derived from plant materials can be classified under herbal medicine (WHO), and nowadays there are about 80% of the Asian and African populations depend on traditional medicine as it is the primary method for their health care needs (Unnati *et al*., 2013).

Despite human body is equipped with body defense, sometimes it may fail to combat the oxidative stress. Thus, antioxidant derived from plant can serve as an alternative source to protect our body from free radical attack. Antioxidants are compound with present in the body can interact and stabilize free radicals’ reaction by inhibit or delay the oxidation of other molecules (Velioglu *et al*., 1998).

One of the potential tropical plants that have remarkable biological activities with potential therapeutic applications is *Muntingia calabura* (*M. calabura*). Locally known as ‘kerukup siam’ and ‘buah ceri’, this plant belongs to the family Muntingiaceae. *M. calabura* can be cultivated in countries with warm climate such as Malaysia, Indonesia, and Philippines. Indeed, in Malaysia, *M. calabura* is commonly cultivated as roadside trees (Zakaria *et al*., 2008). Traditionally, Peruvian folklore medicine used various parts of this plant to treat several diseases such as alleviating headache (Verheij & Coronel, 1992), cold (Jensen, 1999), gastric ulcers (Zakaria *et al*., 2006) and swelling of the prostate gland (Zakaria *et al*., 2007).

In order to develop a potent natural-based product (cancer and other diseases), thus many aspects and areas should be taken into consideration. One of the biggest challenges is toxicity of the plant extract. Toxicity can be defined as a degree in which substance can cause adverse effects on living organism. *M. calabura* has been traditionally used by folk medicine practitioners as a remedy to cure several diseases, but little toxicological data is available regarding its safety, thus the aim of this paper is to determine the antioxidant and phytochemical analysis of crude methanolic extract of *M. calabura* (MEMC) as well as to evaluate the sub-chronic toxicity (90 days oral administration) of *M. calabura* extract in male Sprague Dawley rats.

MATERIALS AND METHODS

Plant material collection

The matured leaves of *M. calabura* were collected around

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Universiti Putra Malaysia (UPM) Serdang, Selangor between Jun and July 2012 and were identified by a **botanist** from the Institute of Bioscience, UPM with a voucher specimen number of SK 2200/13.

Extraction of plant materials

The crude MEMC was prepared by drying the collected leaves at room temperature for a week before grinding into small particles in order to remove the water content inside the leaves. The powder form of *M. calabura* was macerated in methanol (1:20; w/v) and the mixture was left in room temperature up to 72 hours and then filtered using cotton wool and filter paper.

Phytochemical screening

The presence of some important phytochemical constituents (i.e., alkaloid, saponin, flavonoid and others) that play a central role for pharmacological activity of MEMC was detected using phytochemical screening test (table 1). The test was carried out by using 5.0 mg of dried powder material together with 100mg of extract (organic).

Pharmacological studies

Antioxidant assays

2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The antioxidant activity of *M. calabura* extract against free radical was measured using the DPPH test as proposed by Blois (1985) with slight modifications. Briefly, 50 µl of MEMC extract (1mg/ml) was added with 50 µl of 1mM DPPH together with 150µl absolute ethanol. The 96-well microtiter plate was shaken for 15 seconds at 500 rpm and incubated in dark room temperature for 30 minutes. The absorbance of reaction mixture was recorded at 520 nm using a microplate reader.

Super oxide anion radical scavenging activity (SOA)

The principle of the assay is to evaluate the scavenging activity of a test solution against superoxide free radical anions according to the methods described by Liu *et al.* (1997) with slight modifications. The sample absorbance was compared against blank samples using ascorbic acid as a control. Nitroblue tetrazolium (NBT) solution was prepared by adding 3.15g of Tris-HCl buffer (16 mM, pH 8), 0.1g $MgCl_2$, 15.0mg 5-bromo-4-chloro-3-indolyl phosphate, and 34.0 mg 4-NBT chloride to 100 mL of distilled water. PMS solution (10µM) was added to start the reaction and the mixture solution was then incubated (25˚C for 5 minutes). The plate was read at 560 nm using a spectrophotometer (Schimadzu UV-Vis 1700).

Determination of total phenolic contents (TPC)

The MEMC sample was prepared at the concentration of 1mg/ml, meanwhile gallic acid was used as the standard (0.5 mg/mL stock). The reaction was started by adding 200µl of extract with 400µl (0.1ml/0.9 ml) of Folin-Ciocalteu reagents. The mixture was allowed to stand at room temperature for 5 minutes. Approximately 400 µl of sodium bicarbonate (60mg/ml) solution was added to the mixture solution and was incubated at room temperature for another 90 minutes. The absorbance was read at 725 nm and the standard calibration curve of gallic acid was plotted (Singleton & Rossi, 1965).

Animal management

Ethical approval was obtained from Universiti Putra Malaysia (ACUC no: UPM/FPSK/PADS/BR-UH/00488). Male Sprague Dawley rats (aged of 5-6 weeks old) were kept in a well-ventilated room at the Animal House, Faculty of Medicine & Health Sciences, Universiti Putra Malaysia. All animals were freely accessible to tap water and fed with the standard pellet diet. In this study, rats were randomly divided into 4 groups of 6 rats (one control group and three treatment groups). Crude MEMC was dissolved in 8% of Tween-80. Rats in treatment group were orally fed with 50, 250 and 500 mg/kg of MEMC for 90 days at a dosing volume of 10 mL/kg of body weight and the control group received 8% of Tween-80 (OECD, 1998).

Table 1: Phytochemical analysis performed on MEMC

Test	Reagent/ solvent	Indicator
Alkaloid test	Mayer's reagent	Alkaloid is detected by the presence of white precipitates
Saponin test	Distilled water	Saponin is detected by the formation stable froth for at least 15 min
Flavonoid test	Ammonia solution	Flavonoid is presence in the sample by the formation of yellow colour in ammonia
Tannin and Polyphenolic compound test	Ferric solution	Formation of blue black colour indicated the presence of hydrolysable tannins, while brownish-green indicated condensed tannins.
Triterpenes/Steroids Test	Liebermann-Buchard reagent	Triterpene is presence in the sample by the formation of reddish colour and steroid is presence by the formation of greenish colour

Hematological and biochemical analysis

Blood sample from rats were collected via cardiac puncture and placed in EDTA tubes. The effects of subchronic oral toxicity test of MEMC was evaluated based on certain hematological parameters; 1) haemoglobin (Hb), 2) mean corpuscular haemoglobin concentration (MCHC), 3) mean corpuscular volume (MCV), 4) monocytes, 5) neutrophils, 6) packed cell volume (PCV), 7) platelet count, 8) red blood cell (RBC) and 9) white blood cell (WBC).

For biochemical assays, blood was collected in plain tubes (without anti-coagulant) and was centrifuged at 3000 rpm for 15 minutes and stored in -80˚C for further analyzed. The serum obtained were analyzed to determine the levels of urea, creatinine (Crea), albumin (Alb), aspartate aminotransferase (AST), alanine amino-transferase (ALT), and alkaline phosphate (ALP).

Histopathological study

After the animals were euthanized, all the vital organs (heart, kidney, liver, lung, stomach and spleen) were cleaned with cold normal saline and fixed in 10% formalin for at least 48 hours. The grossed tissues were subsequently embedded in paraffin wax before sectioned using a rotary microtome (Leica RM2135, Germany) at 4 µm thickness. The sections were stained with Haematoxylin and Eosin (H&E) before being viewed under light microscope.

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism version 5. Data obtained were presented in Mean ± Standard Error of Mean (SEM). The data were analyzed using one-way analysis of variance (ANOVA) and differences between the groups were determined using Dunnet post hoc test with $p<0.05$ as the limit of significance.

RESULTS

Antioxidant activities of MEMC

The capability of MEMC extract to scavenge free radical was determined by the antioxidant assays which were DPPH and SOA assays. For the DPPH assay, at the concentration of 200µg/ml, MEMC extract showed 98.68±0.30% antioxidant activity when compared to control (200 µg/ml ascorbic acid). Meanwhile, MEMC showed 99.32±0.18 % inhibition against superoxide anion free radical in the SOA assay when compared to control, (200 µg/ml ascorbic acid). Lastly TPC value of MEMC extract was 2751.26±10.51 mg GAEs/g extract and result obtained was comparable with the standard solutions of GAE.

Analysis of phytochemical screening of MEMC

As illustrated in table 2, the leaves of *M. calabura* contain flavonoid saponin, tannin and steroid. No presence of triterpenes was detected in this extract.

Table 2: Phytochemical constituents of MEMC

Phytochemical tests	*M. calabura extract* (MEMC)
flavonoid	1+
Saponin	2+
Tannin	2+
Steroid	1+
Triterpenes	-

Scoring: 1) flavonoids, tannins, triterpenes, and steroids: 1+, weakcolor; 2+, mildcolor; 3+, strong color. 2) For saponins: 1+, 1-2cm froth; 2+, 2-3cm froth; 3+, >3cm froth.

90-days sub-chronic toxicity study

Body weight changes during experiment are showed in Fig. 1. In general, all groups have gained in weight with no significant changes when compared with the control group. In addition, there were also no significant different ($p<0.05$) in the weight of internal organ treated with MEMC at all doses when compared to the control group as showed in table 3. Both treated and control groups showed uniformly healthy condition throughout this study.

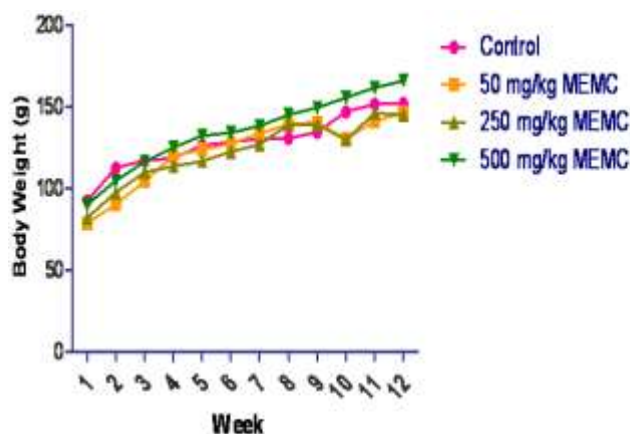

Fig. 1: Body weight of male Sprague-Dawley rats treated with MEMC for 90 days. Groups: 1) control (tween-80) 2) treatments (50, 250 and 500 mg/kg of MEMC).

Effect of subchronic oral administration of MEMC leaves on hematological and biochemical parameters in rats

Data on hematological and biochemical evaluations were summarized in tables 4 and 5, respectively. All hematological parameters showed no significant different with $p>0.05$ compared with control group. Likewise, for biochemical analysis there is insignificant elevation of ALT level in rats treated with 500 mg/kg of MEMC when compared to the control group. However, this parameter remained within normal limit range. Interestingly, there is

Table 3: Organ weight of rats in the subchronic toxicity study of the methanol extract from the leaves of *Muntingia calabura*.

Organ weight (g)	Vehicle control (Tween 80)	50mg/kg MEMC	250mg/kg MEMC	500mg/kg MEMC
Spleen	0.2276±0.02053	0.1925±0.01626	0.1826±0.01320	0.1863±0.01002
Heart	0.3901±0.02831	0.3494±0.02243	0.3455±0.01947	0.3093±0.01098
Lung	0.8265±0.03680	0.7336±0.05594	0.7653±0.03446	0.6798±0.02676
Liver	2.889±0.1912	2.496±0.09839	2.406±0.08437	2.643±0.1558
Kidney	0.8315±0.02757	0.7634±0.03973	0.6568±0.02416	0.6598±0.02142
Colon	1.027±0.1235	0.9902±0.1359	0.7812±0.04510	0.7384±0.04676

Values are expressed as means ± S.E.M

Table 4: Effect of MEMC on hematological profile of Sprague Dawley rats

Parameters	Groups			
	Vehicle control	50mg/kg MEMC	250mg/kg MEMC	500mg/kg MEMC
Eosin (%)	0.1500±0.03416	0.2325±0.06860	0.2600±0.08083	0.1375±0.02955
Hb (g/L)	144.8±6.183	153.8±4.404	142.0±3.391	140.0±8.337
MCHC (g/L)	377.5± 13.23	410.0± 9.806	381.8± 12.00	395.3± 6.047
MCV (fL)	51.50± 0.9574	48.25± 1.109	48.50± 0.2887	49.25± 1.031
Mono (%)	0.4975± 0.1113	0.5275±0.04230	0.5625±0.09455	0.5700±0.04933
Neut (%)	3.200± 0.9521	3.823± 0.7739	3.933± 0.9096	3.495± 0.7879
PCV (L/L)	0.3950± 0.01041	0.3750± 0.002887	0.3800± 0.007071	0.3925± 0.02175
Plts (10^9/L)	1011± 138.7	1160± 19.80	1092± 230.7	1316± 112.8
RBC (10^{12}/L)	7.963±0.4350	7.813±0.1567	7.655±0.1542	8.045±0.4280
WB C (10^9/L)	11.43±2.368	9.125±0.4404	12.43±0.4990	11.30±1.407

Values are expressed as means ± S.E.M

Table 5: Effect of MEMC on biochemical parameters of Sprague Dawley rats

Parameters	Groups			
	Vehicle control	50mg/kg MEMC	250mg/kg MEMC	500mg/kg MEMC
ALT (U/L)	46.58±1.499	53.25±6.513	53.38±1.402	61.23±3.244*
ALP (U/L)	149.0±32.92	157.8±18.72	134.5±5.454	142.5±10.14
AST (U/L)	146.6±29.65	212.1±43.76	191.1±23.65	196.2±41.92
T Bil (umol/L)	1.125± 0.2136	0.7750±0.4110	0.1000±0.0*	0.1000±0.0*
Creat (umol/L)	57.25±3.198	61.50±1.323	63.75±1.315	62.00±1.414
Urea (mmol/L)	7.425±0.7707	6.950±0.8627	8.875±0.4423	8.100±0.1683

Values are expressed as means ± S.E.M
* Significant different as compared to control, $p < 0.05$

significant reduction of total bilirubin for rats fed with 250 and 500 mg/kg MEMC. Other biochemical parameters (i.e. ALP, AST, creatinine and urea) did not show any significant change during the treatment period.

Histophatological study

Fig. 2, 3 and 4 showed the microscopic picture of some vital organs (i.e. liver, kidney, heart, lung, spleen, stomach and colon) of rats treated with MEMC. In general, all the organs did not show any sign of toxicity.

DISCUSSION

Lipid peroxidation is an event that leads to cellular damage. This event caused a shift in the net charge of cells resulting in changes of osmatic pressure, swelling of the cells and eventually cell death. Furthermore, free radicals can attract the inflammatory mediators that cause damage of the cells (Nijveldt *et al*., 2001). Inequality of formation and the reduction of reactive oxygen species (ROS) or reactive nitrogen species (RNS) also can produce the oxidative stress (Kowluru & Chan, 2007). This oxidative stress is believed to act as central key in the pathogenesis and pathophysiological processes of numerous diseases like cardiovascular diseases, diabetes, and cancer (Evans, 2007). According to Kelloff *et al*. (1999), antioxidant derived from dietary product has preventive potential in many diseases such as cancer.

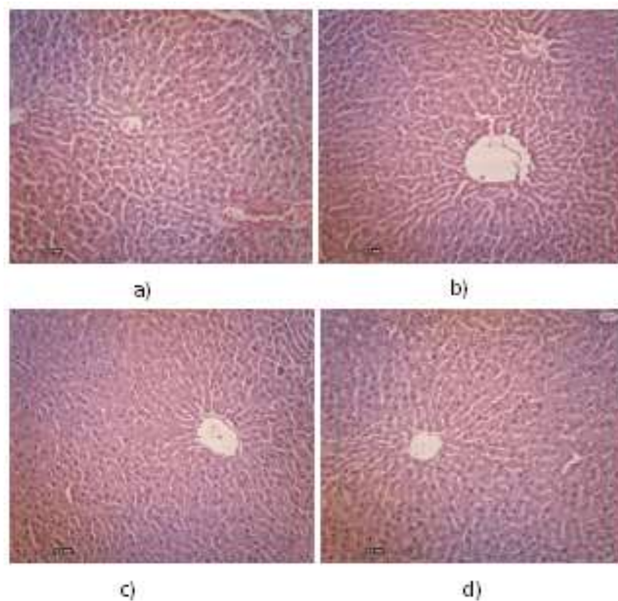

Fig. 2: Photomicrographs of liver histology. a) Control group b) 50mg/kg of MEMC c) 250mg/kg of MEMC d) 500mg/kg of MEMC stained with Hematoxyline and Eosin (100x).

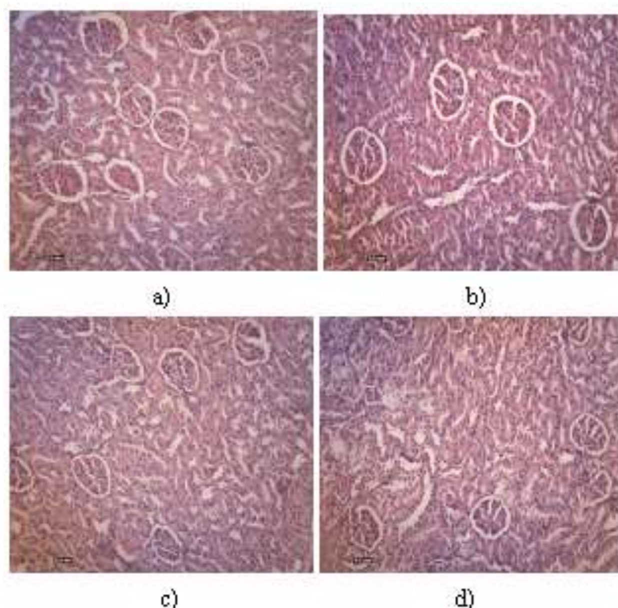

Fig. 3: Photomicrographs of kidney histology. a) Control group b) 50mg/kg of MEMC c) 250mg/kg of MEMC d) 500mg/kg of MEMC stained with hematoxyline and eosin (100x).

In order to measure the antioxidant value present in the extract, two widely antioxidant assays, namely DPPH- and SOA-radical scavenging assays, were conducted. DPPH and SOA assays are based on the capability of sample compound to donate hydrogen and scavenge free radicals. Based on the results obtained, MEMC had considerably high radical scavenging activities in both assays when compared to the standard (ascorbic acid). Apart from that, TPC value of MEMC was measured by the intensity of blue colour that reflects the quantity of phenolic compounds present in the sample. In the present study, MEMC possessed high TPC value. According to Yao *et al*. (2004) methanol offer advantage in extraction process because it evaporates easily in comparison to water and it can inhibit polyphenol oxidase that interfere with the phenolic compounds' extraction.

Phytochemical screening of MEMC showed the presence of several compounds in the extract including flavonoid. Flavonoids have been reported to be involved in: 1) direct scavenging of free radicals; it oxidize free radicals resulting in a more stable and less reactive condition (Nijveldt *et al*., 2001), 2) inhibition of xanthine oxidase activity (Shoskes, 1998), 3) anti-inflammatory activity; quercetin inhibit the cyclooxygenase (COX) and lipoxygenase (LPO) activity, thus, reduce arachidonic acid (AA) formation (Robak & Gryglewski, 1996), 4) inhibit neutrophil degranulation; can cause reduction in AA release by neutrophil and other immune cells [Hoult *et al*., 1994), 5) inhibit metastatis (Caltagirone *et al*., 2000) and 6) angiogenesis process (Fotsis *et al*., 1997).

Moreover, tannins that are present in plant originally have also been considered to be part of the "health-promoting" components (Amarowicz, 2007). They were reported to exhibit anticarcinogenic, antimutagenic, and antimicrobial properties (Amarowicz, 2007). According to Zhang *et al*. (2004) tannins can prevent lipid peroxidation via the inhibition of COX activity.

One of the bioactive compounds that play an essential role as an antioxidant agent is saponin that was normally found in plants, some marine organisms and insects (Thakur *et al*., 2011). Previously, many studies showed the potential of saponin to combat diseases like cancer. Saponin exhibits its pharmacological activity by permeabilising plasma membranes. The amphipathic properties of saponin enable them to penetrate membranes, as they form complex with sterols and cause pore formation (Bach & Rohmer, 2013). In addition, according to Han *et al*. (2013) saponin possessed potential anti-tumor activities via the inhibition of COX-2/PGE2 pathway.

MEMC has been reported to demonstrate various pharmacological effects when tested using several *in vitro* and *in vivo* models. This study was conducted to assess the sub-chronic effect of daily (90-day) administered MEMC in rodents. The rapid administration of doses is aim to study the adverse effects of a test substance after prolonged use. From the results obtained, there were no clinical signs of toxicity upon MEMC administration. Besides, there were no changes in animal behavior, food

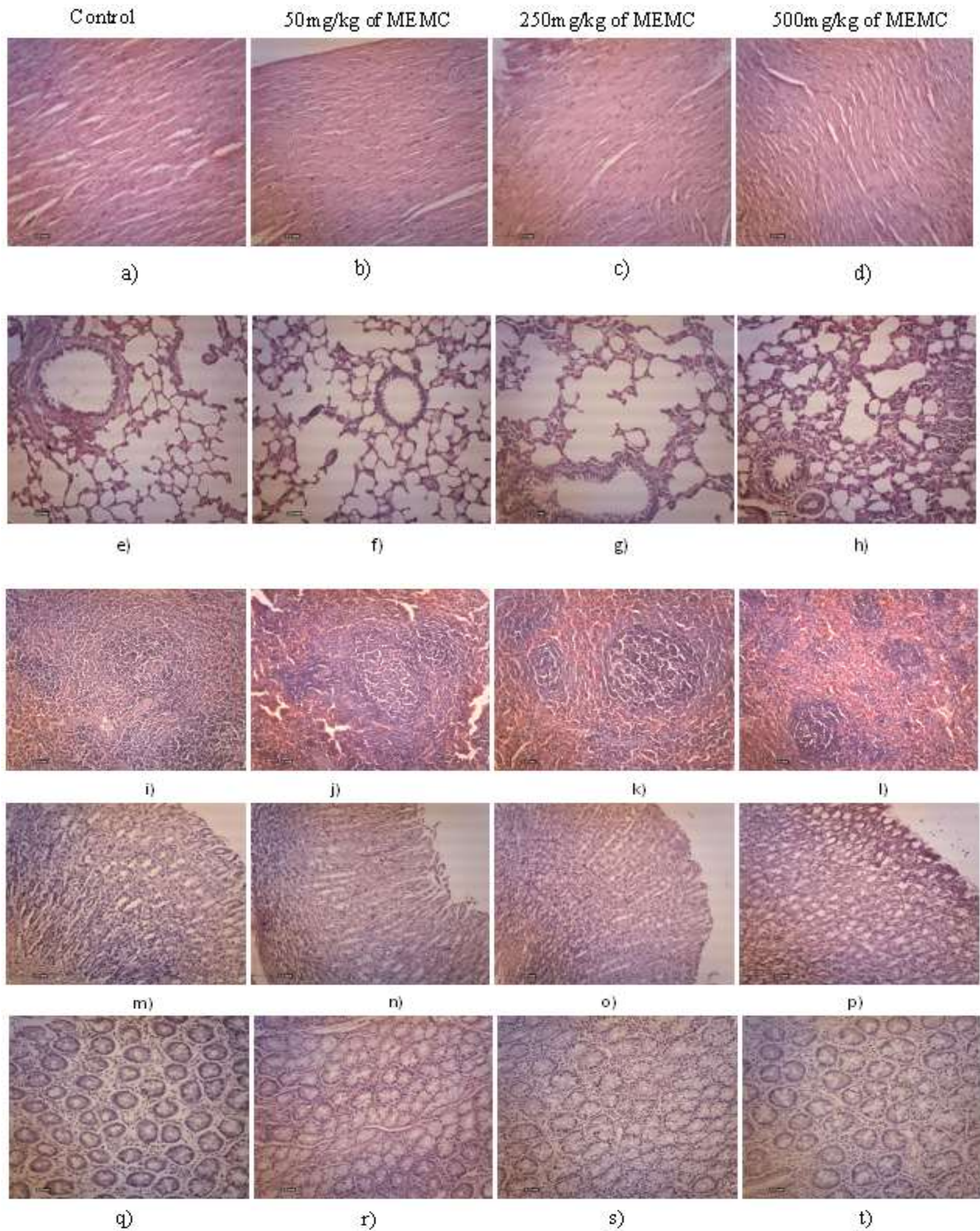

Fig. 4: Photomicrographs of histopathology from representative male Sprague Dawley rats in subchronic oral toxicity: heart; a), b), c) and d), lung; e), f), g) and h), spleen; i), j), k) and l), stomach; m), n), o) and p), colon; q), r), s) and t) stained with hematoxyline and eosin (100x).

and water consumptions, and body weight in all MEMC-treated groups at any dosages tested. According to the Harizal *et al*. (2010), increasing body weights of animals are more closely related to the body fat accumulation rather than the toxic effects of drugs or chemicals.

Evaluations on hematological and biochemical parameters are crucial in order to assess any alterations of natural-based product on hematological system in humans. The hematopoietic system serves as the main target for toxic chemicals and it is a sensitive index for any pathophysiological changes in both humans and animals (Mukinda & Syce, 2007). Data on hematological evaluation of rats treated with MEMC indicated that there were no significant alterations when compared with control rats.

Detoxification occurs at two main organs which are liver and kidney (Arsad *et al*., 2013). Liver is one of the main organs in detoxification process inside body. Besides, it takes part in the regulation of body homeostasis. Apart from that, liver also plays a crucial function for metabolism and elimination of waste metabolites (Kumar *et al*., 2012).

The common parameters used to evaluate liver function are ALT, AST and ALP and any changes regarding these parameters could reflect the status of liver injury (Hor *et al*., 2012). ALT, formerly known as serum glutamic-pyruvic transaminase (SGPT), is mainly found in the liver. Liver injury causes this enzyme to be release into the blood stream. On the other hand, AST is a cytoplasm and mitochondria enzyme, which is formerly known as serum glutamic oxaloacetic transaminase (SGOT). AST enzyme is also found in muscles, heart and other tissues besides liver. Concentration of AST is much higher than ALT in a human body as the cells contain more AST than ALT. ALP is a hydrolase enzyme found in body tissues like bone, intestine and kidney. In the present study, the level of ALT, AST and ALP was normal in all rats treated with MEMC for 90-days. This was further verified by the absence of histopathological changes in the liver (fig. 2). These findings imply that the extract did not induce damage to the liver.

Kidney is the main organ in urinary system. It maintains the homeostasis that involves several processes including filtration, active and passive absorption and secretion (Junqueira & Carneiro, 2005). In acute or chronic renal toxicities, serum urea and creatinine levels are two parameters that are normally determined (Stark, 1980; Arsad *et al*., 2013). As the toxic compounds presence inside the body, the serum level of creatinine and urea are dramatically increases. However, the current results showed that the serum creatinine level in MEMC-treated group have no significant difference when compared to the control animals. On the other hand, increasing level of serum urea is an indicator for toxic effect on the renal tubules, renal parenchyma, or obstruction of the urinary outflow (Evans, 2007). Data on serum urea level indicated that there is no significant difference when compared to the control groups. Histopathological analysis of the kidneys presented normal architecture when compared with the control group, hence, suggesting that MEMC did not cause nephrotoxicity (fig. 3).

Macroscopic examinations of the other organs (heart, lung, spleen, stomach, and colon) of rats treated with various doses of MEMC did not show any changes in colour. In addition, microscopic examinations of these organs also showed normal architecture (fig. 4), suggesting that no toxicity of daily oral administration of the MEMC.

CONCLUSION

The present study demonstrates that *M. calabura* does not exert any toxic effects. There were no toxic effects observed with regards to the behavior, body weight, hematological and biochemical parameters of the rats. Hence, no observed-adverse-effect level (NOAEL) was produced and NOAEL for this extract has been determined to be greater than 500 mg/kg.

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