Journal of Applied Sciences

ISSN 1812-5654

science alert

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OPEN ACCESS Journal of Applied Sciences

ISSN 1812-5654
DOI: 10.3923/jas.2016.477.483

Research Article
Adverse Effects of Tannin Contained in *Mimosa pudica* Root Extract

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Abstract

Background and Objective: *Mimosa pudica* has been used for many traditional healings including snake bites, wound healing, treating bleeding piles, ulcers, diarrhea, anti-inflammatory, anti-diabetic, anti-oxidant and anti-microbial etc. Tannin extracted from plants been beneficial as therapeutics with dividing opinions on its safety. Hence, this study investigated the safety of the total tannin extracted from the root of *Mimosa pudica* plant. **Methodology:** This investigation utilized biochemical markers as well as cytotoxicity and histopatological experiments. Additionally, the total condensed and hydrolysable tannins determined in the particular root extract of the *Mimosa pudica* plant. **Results:** Extract of MPT at the highest dose tested 2.5 mg mL^{-1} elevated significantly the levels of mice serum contained ALT and AST while no observable changes to ALP and GGT levels detected. The cytotoxicity of MPT was found to be with CTC_{50} values of 0.0653 and 0.119 mg mL^{-1} in vero and MDCK cell lines, respectively. The mice organ's histopathology of liver and kidney demonstrated MPT having distinct toxic effects at the highest concentration tested. The total tannin in MPT found to be 3.28 mg TAE g^{-1} dry weight with more hydrolysable type of tannin than condensed tannin 0.31 TAE/dry weight. **Conclusion:** The MPT found to show adverse effects in all experiments conducted at the highest concentration of 2.5 mg mL^{-1}. Hence, the age old doubt regarding the adverse effects of tannin being proven here whereby it was discovered the use of *Mimosa pudica* total tannin is concentration dependent. Therefore, warranted prior caution in determining the safe levels of tannin required before usage.

Key words: Tannin, plant, venom, biochemical enzymes, histopathology, cytotoxicity

Received: July 11, 2016 Accepted: August 10, 2016 Published: September 15, 2016

Citation: J. Vejayan, A. Jamunaa, I. Halijah and S. Ambu, 2016. Adverse effects of tannin contained in *Mimosa pudica* root extract. J. Applied Sci., 16: 477-483.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Mimosa pudica commonly known as a sensitive plant or touch-me-not in English. It is also known as Chui mui, Lajjalu (Hindi), Thottachurunggi (Tamil), Makahiya (Tagalog), Hilahila (Hawaii) and Semalu (Malay). *Mimosa pudica* is used as phytotherapy practiced by a large proportion of the Asian population for the treatment of several clinical complications such as physical, physiological and mental ailments including snake envenomation[1-5]. It has been reported to contain tannin, mimosine (an alkaloid), free amino acids, β-sitosterol, linoleic acid and oleic acid[6]. One activity of the *Mimosa pudica* that has been investigated extensively is its ability against snake venoms. A study on the aqueous extract obtained from the roots of *Mimosa pudica* showed significant inhibitory effect on *Naja naja* and *Bangarus caerulus* venoms[7]. Mahanta and Mukherjee[8] studied on neutralization of lethality, myotoxicity and toxic enzymes of *Naja kaouthia* venom by *Mimosa pudica* root extract. The aqueous extract of the plant showed significant inhibitory effects on all the activities tested. This was further supported by Vejayan *et al*.[9] in which among all the 17 plants screened, only aqueous extract of *Mimosa pudica* showed 100% ability in neutralizing venom lethality. The findings were further strengthened in another *in vitro* study whereby, it was discovered that *Mimosa pudica* was potentially useful as an antivenom agent of plant origin against five poisonous snake venoms found in Malaysia[10]. However, *in vivo* investigations the active fraction containing specifically of tannin was unable in rescuing any mice envenomed with lethal dose of *Naja kaouthia* venom[11]. Hence, tannin in general known to have certain unclear adverse effects, this study evaluated the toxicity of *Mimosa pudica* tannin.

MATERIALS AND METHODS

Plant extract: *Mimosa pudica* (voucher specimen number: HI 347 at the Institute of Biological Sciences, University Malaya) roots were obtained from Bagan Lalang, Sepang, Malaysia. Dried plant sample was finely powdered and the extract was prepared by stirring 4 g of *Mimosa pudica* root powder in 200 mL water for 3 h at room temperature. The crude extract obtained was filtered through filter paper (Whatman No.1) and concentrated using rotary evaporator at 50°C followed by addition of 3 mL of water. The extract was run through Sephadex LH-20 column to purify total tannins using 50% methanol followed by 70% acetone[12,13]. The acetone isolate was collected and rotary evaporated at same condition. The final concentrate (MPT) was dissolved with 35% acetone and kept in 4°C until use.

Animals: Male ICR mice weighing 20-25 g provided by the animal house in Monash University Malaysia. The animals were kept under standard conditions and the experiments were conducted according to the ethics norm approved by the Animal Ethics Committee (AEC) of Monash Australia (AEC No: SOBCB/MY/2008/36).

Cytotoxicity of MPT: Vero and MDCK cells were counted and seeded at 5×10^3 cells per well in flat-bottom 96 well microtitre tissue culture plate followed immediately by increasing dilutions of MPT. Control columns of 6 wells with medium without cells (Blank) and medium plus cells (Negative control) as well as MPT blank were prepared as well. The plate was incubated at 37°C with 5% CO_2 for 20 min prior to addition of promega cell titer 96 aqueous one solution cell proliferation (MTS) assay reagent after treatment period and further incubated for 3 h. Absorbance was read at 492 nm using BioRad Benchmark Plus Multiplate spectrophotometer. Percentage (%) of surviving vero and MDCK cells against MPT concentration (mg mL^{-1}) was plotted and the CTC_{50} values were determined using GraphPad Prism software.

Biochemical enzyme parameters: Blood was collected from mice treated with different concentrations of MPT (200 µL injected intra peritoneally) via cardiac puncture technique and centrifuged at 10,000 rpm for 10 min at 4°C. The serum was used to quantify creatine kinase (CK), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) by a reputable and accredited diagnostic laboratory (Gribbles Pathology Sdn Bhd, Malaysia).

Histopathological analysis: The organ's such as liver and kidneys of mice were analysed for the presence or absence of macroscopic abnormalities following treatment of MPT of varying concentrations. The organs were fixed with 10% formalin (v/v) for 24 h, dehydrated and included into paraffin blocks. Histological cuts of 5 µm were made and stained with hematoxylin and eosin (HE) to be examined under a light microscope.

Determination of tannin contents

Analysis of total phenols: This method was adapted from Makkar *et al*.[14] whereby suitable aliquots of the tannin-containing extract were made up to 0.5 mL with distilled water. Next, 0.25 mL of the folin-ciocalteu reagent was added and after 3 min of incubation an amount of 1.25 mL of the sodium carbonate solution was added. The tubes were vortexed and absorbance was read at 725 nm after 40 min. The amount of total phenols as tannic acid

equivalent was calculated based on calibration curve made using commercial tannic acid (Sigma-Aldrich). Total phenolic content was expressed on a dry matter basis (x%).

Total tannin content assay-removal of tannin from the MPT: A total of 100 mg polyvinyl polypyrrolidone (PVPP; Sigma-Aldrich) was weighed in and added to 1.0 mL distilled water. Next, 1.0 mL of the tannin-containing MPT extract and the PVPP solution was mixed and kept at 4°C for 15 min. It was vortexed again and centrifuged at 3000 g for 10 min and the supernatant was collected. This supernatant has only simple phenolics other than tannins. The phenolic content of the supernatant was measured as mentioned above for total phenol estimation. The content of non-tannin phenols was expressed on a dry matter basis (y%). The x-y is the percentage of tannins as tannic acid equivalent on a dry matter basis.

Determination of condensed tannin: The methodology for this assay was adapted from Porter *et al.*[15] 0.50 mL of the tannin extract diluted with 70% acetone was pipetted into test tube. About 3.0 mL of the butanol-HCl reagent and 0.1 mL of the ferric reagent were added to the tubes and vortexed. Tubes were put in a boiling water bath for 60 min. The tubes were then cooled and absorbance at 550 nm was read. The absorbance of the unheated mixture was used as blank. Condensed tannins (dry matter %) as leucocyanidin equivalent were calculated by the equation:

$$(Abs_{550\,nm} \times 78.26 \times \text{dilution factor})/(\text{dry matter \%})$$

Determination of hydrolyzable tannin: Hydrolyzable tannin was determined by subtracting condensed tannin from total tannin content in MPT isolate.

RESULTS

Cytotoxicity of MPT: Cytotoxicity of MPT was tested using the MTS assay on the vero and MDCK cell lines. The MPT was found to induce over 70% cell death in both cell lines. Dose response studies of MPT on vero and MDCK cells are presented in Fig. 1. The MPT gave CTC_{50} values of 0.0653 and 0.119 mg mL^{-1} in vero and MDCK cells, respectively.

Changes of biochemical enzyme levels in mice induced by MPT: Table 1 shows the changes in five important biochemical enzymes in mice after treatment with three different concentrations of MPT. Results are displayed as mean of four test samples in unit per liter (U L^{-1}) enzyme concentration. The results are compared to the control sample which was injected with saline (0.85% NaCl, w/v). Treatment of MPT at 2.5 mg mL^{-1} increased the alkaline phosphatase, ALP to 287.4 U L^{-1} compared to the control level 283.6 U L^{-1}. The values found to be reduced to 236.0 U L^{-1} with the injection of MPT at concentration of 0.625 mg mL^{-1} and returned to normal at lower concentration of 0.03 mg mL^{-1}. Hence, the effect of MPT on ALP is only marginal at various MPT concentrations.

Control mice showed 178.4 U L^{-1} of AST levels whereas, for the MPT concentrations tested i.e., 2.5 and 0.625 mg mL^{-1}, their AST levels were found to be higher than the control i.e., 344.0 and 299.0 U L^{-1}, respectively. Hence, the AST levels in mice serum found to be markedly elevated when the higher concentrations of MPT were injected into the mice.

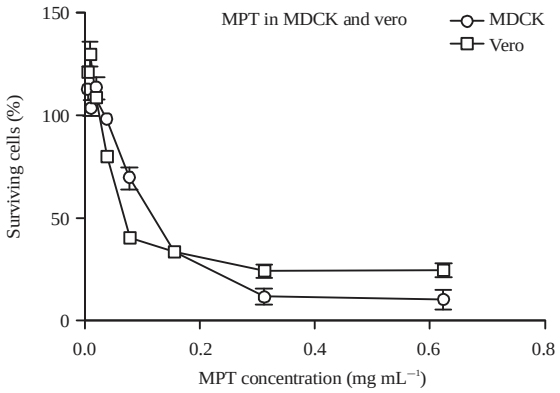

Fig. 1: Cytotoxicity curve of MPT on vero and MDCK cells, n = 3 in each group, experiments were repeated three times. Points are means while bars are SD values

Table 1: Changes of ALP, AST, ALT and CK levels in mice treated with various concentrations of MPT

Biochemical enzyme/group	Control	MPT treated (2.5 mg mL^{-1})	MPT treated (0.625 mg mL^{-1})	MPT treated (0.03 mg mL^{-1})
Enzyme concentration (U L^{-1})				
Alkaline phosphatase (ALP)	283.6	287.4	236	280
Gamma-glutamyl transferase (GGT)	<3	ND	<3	<3
Aspartate aminotransferase (AST)	178.4	344	299	39.2
Alanine aminotransferase (ALT)	40.4	106.3	65	8.25
Creatine kinase (CK)	36	ND	ND	102.7

n = 4 in each group, results shown in mean and SD values, p<0.005 and ND: None detectable

Tests for ALT levels showed that the control mice contained 40.4 U L^{-1} of the enzyme and it increased almost two fold to 106.3 U L^{-1} with 2.5 mg mL^{-1} of MPT. The AST level in MPT of 0.625 mg mL^{-1} was 65.0 U L^{-1} higher than control as well.

Treatment of MPT at even the lowest concentration of 0.03 mg mL^{-1} increased the creatine kinase, CK level to 102.7 U L^{-1} compared to the control of only 36.0 U L^{-1}. The CK values for both MPT with concentrations of 2.5 mg mL^{-1} and even that of 0.625 mg mL^{-1} were found to be too high for detection.

Histopathology of MPT on animal liver and kidney: The histopathological assessment of liver was performed for control and MPT. Figure 2 and 3 shows histopathological changes in liver and kidney of mice after administration of MPT (0.625, 1.25 and 2.5 mg mL^{-1}) under H and E staining. Mice in the control (Saline) group showed normal, well defined histological structures without any signs of vascular or inflammatory changes.

In the 2.5 mg mL^{-1} concentration of MPT treatment, there was moderate to severe vascular and sinusoidal congestion as well as inflammatory changes with extensive necrosis in comparison with the control group. However, at lower doses of MPT 1.25 mg mL^{-1}, there were mild vascular changes. Mild inflammatory changes that were even less severe than those observed after 1.25 mg mL^{-1} administration were observed in 0.625 mg mL^{-1} MPT group (Fig. 2). Distinct changes such as presence of inflammation and congestion were observed in all groups with maximum severity in 2.5 mg mL^{-1} MPT, followed by 1.25 and 0.625 mg mL^{-1} in descending order of morphology with necrosis of hepatocytes with focal regenerative changes was seen in 1.25 mg mL^{-1} MPT. Some of the tissues showed heavy areas of congestion with dilated vascular spaces and parenchymal extravasation of RBCs (Table 2).

Normal histology of the glomerulus and tubules was found in kidney tissue of mice in control (Saline) group. Mice treated with 0.625 and 1.25 mg mL^{-1} showed mild vascular changes in the kidneys. At 2.5 mg mL^{-1} MPT however, some tubular necrosis was noted in addition mild vascular and inflammatory changes with signs of vascular congestion and glomerular atrophy (Fig. 3). The morphological changes reduced in extent in 1.25 mg mL^{-1} MPT and still further in

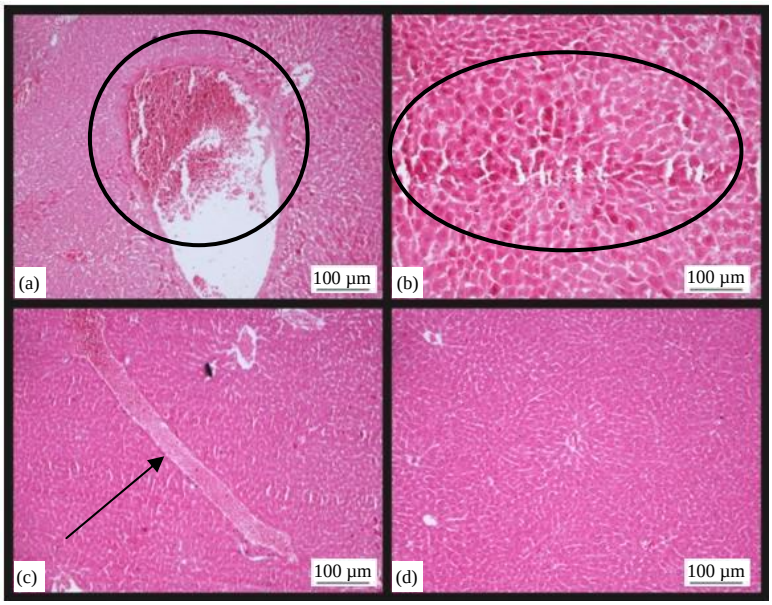

Fig. 2(a-d): Histopathological changes in mice liver after administration of MPT (H and E staining), photomicrographs of liver sections after administration of MPT (0.625, 1.25 and 2.5 mg mL^{-1}), (a) Photomicrograph of liver in 2.5 mg mL^{-1} MPT at 100x showing a large dilated central vein filled partially with stasis of blood (black circle) surrounded by zones of few necrotic hepatocytes, (b) Photomicrograph of liver in 2.5 mg mL^{-1} MPT at 100x showing hepatocytes undergoing coagulative necrosis surrounded by regenerative hepatocytes (black circle), (c) Photomicrograph of liver in 1.25 mg mL^{-1} MPT at 100x showing a large congested blood vessels filled with RBCs surrounded by zones of few oedematous sinusoids (arrow) and (d) Photomicrograph of 0.625 mg mL^{-1} MPT at 100x showing liver parenchyma with healthy hepatocytes arranged in normal architecture

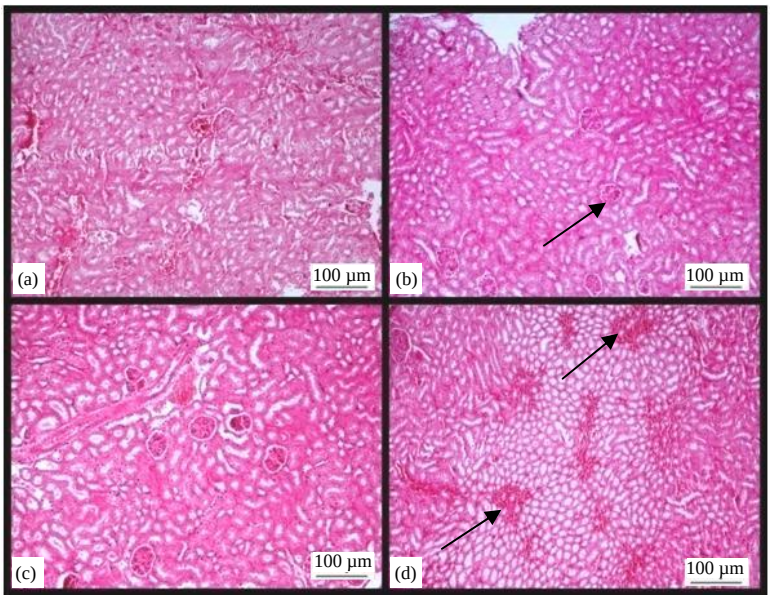

Fig. 3(a-d): Histopathological changes in mice kidney after administration of MPT (H and E staining), photomicrographs of kidney tissue sections after administration of MPT (0.625, 1.25 and 2.5 mg mL^{-1}), (a) Photomicrograph of 2.5 mg mL^{-1} MPT at 100x showing kidney with numerous glomeruli, some of which show moderate degree of atrophy and focal areas of congestion between the tubules, (b) Photomicrograph of 1.25 mg mL^{-1} MPT group at 100x showing kidney with numerous glomeruli, some of which show mild degree of atrophy (black arrow), (c) Photomicrograph of 0.625 mg mL^{-1} MPT at 100x showing kidney with numerous glomeruli with good architecture and tubules arranged in order with mild congestion and haemorrhage and (d) Photomicrograph of 0.625 mg mL^{-1} MPT at 100x showing kidney with numerous tubules arranged in order with mild congestion (as indicated in arrows) and haemorrhage

Table 2: Histological grading on liver and kidney of mice treated with MPT

Sample	Inflammation (liver)	Necrosis (liver)	Congestion (liver)	Inflammation and atrophy (kidney)	Necrosis (kidney)	Congestion (kidney)
MPT 2.5 mg mL^{-1}	++	++	+	+	+	+
MPT 1.25 mg mL^{-1}	+	+	++	-	-	+
MPT 0.625 mg mL^{-1}	+	+	+	-	-	-
Control (Saline)	-	-	-	-	-	-

-: No change, +: Mild changes, ++: Moderate changes and +++: Severe changes

0.625 mg mL^{-1}. As such, in both kidneys and liver 0.625 mg mL^{-1} MPT showed almost mild changes to nearly normal architecture.

Total phenolic, condensed tannin and hydrolyzable tannin contained in MPT: The amount of total phenolic content and total tannin content in MPT was determined with the folin-ciocalteu reagent and the results were expressed as milligrams of tannic acid equivalent per gram of dry weight (mg TAE g^{-1} dry weight). The total phenolic content was calculated from a linear dose-response regression calibration curve of standard tannic acid solution (0-0.25 mg mL^{-1}). Table 3 shows estimation of total phenolic, tannin, condensed and hydrolysable tannins in MPT expressed in tannic acid equivalent TAE g^{-1} of MPT dry weight. The amount of total phenols in MPT was determined to be 5.34 mg TAE g^{-1} of MPT dry weight. The assay was coupled with polyvinyl polypyrrolidone (PVPP-binding tannin phenolics) and the total tannin content was calculated from the total phenol by subtracting the amount of non-phenolic content of MPT. The amount of tannin in MPT was determined to be 3.28 mg TAE g^{-1} of MPT. The total condensed tannin and total hydrolysable tannins were estimated to be 0.31 mg TAE g^{-1} of MPT and 2.97 mg TAE g^{-1} of MPT, respectively.

Table 3: Total phenolic, tannin, condensed and hydrolysable tannin in MPT

Chemical analysis	MPT (mg TAE g^{-1} dry weight)
Phenolics	5.34
Non-tannin phenol	2.06
Tannin phenol	3.28
Condensed tannin	0.31
Hydrolysable tannin	2.97

DISCUSSION

Mimosa pudica has been used in the traditional medicine to treat several diseases such as leprosy, dysentery, vaginal and uterine complaints as well as snake bites[1-3]. In a study related to snake venom though *in vitro* investigations gave promising results however, for *in vivo* animal study (i.e., pre and rescue treatments) failed to safe the test mice envenomed with $2LD_{50}$ of *Naja kouthia* snake venom[11]. Previous study has demonstrated that the toxicity of this plant or its extract has not been well reported. This study assessed its safety using biochemical markers as well as cytotoxicity and histopathology tests. In any herbal ingestion, it is crucial regardless of the efficacy, the body interacts with the herbal or any foreign constituents in an attempt to get rid of the substances if it is harmful toxins or convert them instead into useful cellular components. A failure in eliminating from the body is signified by changes of certain enzymes levels or/and other cell components. The enzymes commonly assessed included aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK) and alkaline phosphatase (ALP). Increased levels of these specific hepatic enzymes marker after chemical or immunological intoxication, compared to normal mice will indicate considerable hepatocellular damage and resulting leakage of cytosolic contents into the systemic circulation[16].

Extract of MPT at 2.5 mg mL^{-1} significantly increased the levels of serum AST and ALT. This is diagnostic of hepatocellular damage as seen in disorders that cause the death of numerous liver cells (extensive hepatic necrosis) such as acute viral hepatitis A or B, pronounced liver damage inflicted by toxins as from an overdose of acetaminophen (paracetamol) and prolonged collapse of the circulatory system (shock) when the liver is deprived of fresh blood bringing oxygen and nutrients[17].

The I.p. administration of MPT to mice also produced dose dependent multiple organ toxicities including the kidneys and the liver. The liver known to be a key organ in the metabolism and detoxification of xenobiotics is vulnerable to damage induced by a huge variety of chemicals. Kidneys are particularly vulnerable to toxic agents given their high rate of perfusion and their ability to concentrate a range of substances in the tubular lumen. The physiological alteration observed in the experimental organ tissues may account for the histopathological changes[18,19]. At the liver, distinct changes such as presence of inflammation and congestion observed in 2.5 mg mL^{-1} MPT treated group. At this highest dosage given necrosis of hepatocytes with local regenerative changes was seen and in some tissues heavy areas of congestion with dilated vascular spaces and parenchymal extravasation of red blood cells was visible. Notable pathological changes seen in the kidneys however, it was only mild to moderate level of inflammation and congestion involving the renal glomerulus and renal tubules and pelvis region. Few glomeruli were atrophic and some tubules had casts which were seen scattered for the 2.5 mg mL^{-1} treated group. Similar to the liver cells, the morphological changes reduced in the 1.25 mg mL^{-1} treated group and was further reduced in 0.625 mg mL^{-1} treated group. Both kidneys and liver of 0.625 mg mL^{-1} treated mice showed mild changes to nearly normal architecture. The results of the biochemical markers evaluated in this study easily demonstrated adverse hepatoxic effects at high concentration of MPT unlike the histopathological changes in the mice organs investigated that showed only mild to moderate architecture changes. It was also evident the histological changes such as necrosis in hepatic lobules and inflammatory infiltration of lymphocytes and macrophages around the central vein, were simultaneously improved as the concentration of MPT decreases.

Cytotoxicity evaluation on MPT revealed it exerted cytotoxic effects on both cultured vero and MDCK cells. This was observed in the decrease in the number of surviving cells after the treatment with MPT based on MTS cell viability assay. These effects were observed to be dependent on MPT concentration.

The results in determining the total phenolic compounds revealed the root of *Mimosa pudica* is composed mainly of hydrolysable tannins. The total tannin compounds of MPT were estimated to be 3.28 mg TAE g^{-1} dry weight while, condensed tannin and hydrolysable tannin were determined to be 0.31 and 2.97 mg TAE g^{-1} dry weight, respectively. The condensed tannin generally being accepted to be better in complexing with free proteins rather than the hydrolysable tannins as it is less conjugated[20]. On the contrary hydrolysable tannins are reported to be highly toxic to non-ruminants, the toxicity was observed to be lower in ruminant which is highly attributable to acid or enzymatic hydrolysis in the livestock's rumen and excretion of phenolics as glucoranides in urine[20].

CONCLUSION

Results suggested *Mimosa pudica* tannin to be mostly unsafe in all experiments conducted at 2.5 mg mL^{-1} concentration. The contributor of the toxicity most likely is due to the high hydrolysable tannin. Herbal preparations derived from *Mimosa pudica* root extracts are cautioned of

its adverse effects if to be ingested or applied internally at high concentration. A prior determination of its or for that matter any other source of total tannin's safe levels suggested before attempting to evaluate or screen tannins biological benefits.

ACKNOWLEDGMENT

The study was conducted utilizing chemicals and consumables supplemented from grant: Malaysian Ministry of Science and Technology (Project Number 02-02-10-SF0033) and UMP Internal Grant (RDU1603134).

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