

Efficacy of tannins from *Mimosa pudica* and tannic acid in neutralizing cobra (*Naja kaouthia*) venom

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Abstract: In the present study, the effectiveness of *Mimosa pudica* tannins (MPT) in neutralizing the lethality of *Naja kaouthia* venom was compared with commercially derived tannins. Preincubation of MPT with *N. kaouthia* venom maintained 100% survival of mice after 24 hours. The mouse group in which there was no preincubation, no protection against the effects of the venom was observed. *M. pudica* tannin was found to be more effective in neutralizing the lethality of *N. kaouthia* venom when compared to commercial tannic acid. Two protein spots were missing in the two-dimensional gel electrophoresis (2-DE) of the MPT treated mouse indicating the down-regulation of venom proteins. The results from this study indicated that tannins obtained from *M. pudica* are better than tannic acid in neutralizing the lethality of *N. kaouthia* venom *in vitro*. However, further investigations are required to establish that *M. pudica* has potential for treating *N. kaouthia* snakebites.

Key words: plant extract, snake venom, therapy, tannin, phospholipase A₂.

INTRODUCTION

Snakebites represent a public health problem in numerous countries (1). In Malaysia, the common cobra (*Naja kaouthia*) is responsible for most bites, which display varied clinical pictures, the most prominent one being neurotoxicity (2). In some cases, the bites produce significant local swelling and extensive tissue necrosis that may lead to the development of long term complications, such as chronic ulceration, infection and osteomyelitis (3).

Antivenom therapy is the only specific treatment for systemic snake envenomations. However, antivenom does not provide enough protection against some toxic enzyme effects induced by snake venoms (4). Antivenom treatment, in some cases, is not be able to prevent local tissue necrosis and may also predispose the

victim to the risk of hypersensitivity reactions such as anaphylaxis and serum sickness due to equine and ovine allergens present in the serum (2, 5).

In several countries, the development of antivenoms is costly and the supply is limited (3). Nevertheless, in many regions numerous plants have been successfully used as snake venom antidotes (6, 7). A preliminary study screened 17 plants for anti-snake venom activity and showed that only *Mimosa pudica* can significantly neutralize the lethal effects of five common species of venomous snakes (8). A work by Mahanta and Mukherjee (9) has demonstrated the anti-snake venom potential of this plant. Tannins comprise a group of polyphenolic compounds widely distributed in the plant kingdom that present the unique ability to form complexes with macromolecules, particularly proteins (10).

Since snake venoms consist mainly of proteins (70-90%) that are responsible for a wide range of clinical signs and symptoms, this study aims to analyze the biological properties of tannins regarding anti-snake venom activity.

MATERIALS AND METHODS

Plant Material

Mimosa pudica plants were collected from the field in Bagan Lalang beach, Malaysia. Only *M. pudica* roots were cut into small pieces and dried in a convection oven at 50°C. The dried roots were finely ground to powder using a blender. The crude extract was prepared using 4 g of powdered root in 200 mL of deionized water. Subsequently, the sample was concentrated using a rotary evaporator and then redissolved by the addition of 1.5 mL of water. This sample was then subjected to tannin isolation using Sephadex (LH-20) chromatography as described in our previous work (8). It was estimated that each 100 µL of isolate contained 0.7 mg of dry weight material. The commercial source of tannin was Fluka BioChemika (Germany).

Venom and Antivenom

Lyophilized *Naja kaouthia* venom obtained from the Snake and Reptile Farm, Perlis, was freeze dried and stored at -80°C until further use. The commercial source of snake antivenom was the Thai Red Cross, Bangkok, Thailand.

In Vitro and In Vivo Animal Studies

Male adult ICR mice (8-week-old) weighing between 20 and 25 g were used in the experiments. The LD₅₀ of *N. kaouthia* was determined by using

the graphical method of Miller and Tainter (11). Various dilutions of *N. kaouthia* venom were obtained by dissolving the lyophilized venom in saline (0.85% NaCl/water, w/v). An initial high dose of snake venom was administered to ensure 100% mortality in mice. From this, the dose was gradually adjusted by a decrease of 0.1 mg/kg until no death was observed. This was the dose range used for LD₅₀ determination.

As for the anti-snake venom assay, four groups of mice (each group had eight mice) were employed (Table 1). All injections (200 µL) were given by the intraperitoneal (IP) route. Treated mice were observed over 24 hours for mortality.

In the *in vivo* experiment (rescue treatment), mice received treatment solutions and venom by separate routes. The IP injection of snake venom was immediately followed by IV injection into the tail vein of treatment solutions.

In the *in vitro* experiments, *M. pudica* tannin, tannic acid and monospecific *N. kaouthia* antivenom were given to mice. The aliquots of the treatment solution were mixed with equal volumes of snake venom (total volume was 200 µL) and IP injected into each mouse.

Two Dimensional Gel Electrophoresis (2-DE)

Mouse serum from two different treatment groups was utilized for comparison. One group was treated with a preincubated mixture of venom and tannin from *M. pudica*, while the other was treated with *N. kaouthia* venom only. *M. pudica* tannin was incubated (37°C) with *N. kaouthia* venom for ten minutes before the injection into each mouse. In both treatments, an injection (IP) of 200 µL was administered. Immediately after inoculation, mice were sacrificed with ether

Table 1. The different treatment groups of mice for both *in vitro* and *in vivo* studies

	Group	Number of mice	<i>In vitro</i> treatment (IP only)	<i>In vivo</i> treatment (IP followed by IV)
Experimental group	1	8	2 LD ₅₀ venom + <i>M. pudica</i> tannins	2 LD ₅₀ venom + <i>M. pudica</i> tannins
	2	8	2 LD ₅₀ venom + tannic acid	2 LD ₅₀ venom + tannic acid
Control group	3	8	2 LD ₅₀ venom	2 LD ₅₀ venom
	4	8	2 LD ₅₀ venom + antivenom	2 LD ₅₀ venom + antivenom

IV: intravenous, IP: intraperitoneal.

and blood was collected via cardiac puncture and stored at -80°C . Protein concentration was determined using the Bradford assay.

For the first dimension electrophoresis, IPG strips (Immobiline Dry Strip[®], GE Healthcare-Bio-Sciences AB, Sweden, pH 3-10, 13 cm) were used. The first dimension isoelectric focusing (IEF) was run on IPGphor[®] (GE Healthcare-Bio-Sciences AB, Sweden) at 200 V for one hour, 1000 V for one hour and 8000 V for 3.5 hours. After the run, the strips were kept at -80°C . The proteins were separated during the second dimension by 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Fast staining of the gels was carried out using Coomassie Brilliant Blue. The gel images were scanned into a computer and the differences in protein expression between the *M. pudica* tannin-treated and venom-treated gels were analyzed by ImageMaster[®] 2D Platinum software (developed by the Swiss Institute of Bioinformatics in collaboration with GeneBio and GE Healthcare-Bio-Sciences AB, Sweden).

RESULTS

The crude water extract was separated into two fractions: MP_{MeOH} eluted with 50% methanol (without any activity) and MPT eluted with 70% acetone (Figure 1).

Venom doses ranging from 0.7 to 1.1 mg/kg were injected into each group of mice. The data obtained is shown in Table 2. Using these data (probit analysis), the 2 LD₅₀ of *N. kaouthia* venom were calculated to be 0.92 ± 0.10 mg/kg.

Preincubation of 2 LD₅₀ (1.84 mg/kg) of *N. kaouthia* venom with *M. pudica* tannins was able to completely neutralize the snake venom since the survival rate of mice was 100% after 24 hours of observation (Table 3). Preincubation of the venom with commercial tannic acid showed only a mouse survival rate of 12.5%. Tannins obtained from *M. pudica* were eight times more effective in neutralizing the lethal effects of *N. kaouthia* venom compared to the tannic acid. As expected, all mice treated with the venom-antivenom mixture survived after 24 hours.

Results of the *in vivo* experiment (rescue treatment) showed that administration of *N. kaouthia* venom and *M. pudica* tannins by separate routes failed to inhibit the lethality in mice. The same result was observed in mice treated with tannic acid. Only the serum with antivenom was

able to fully protect the mice against *N. kaouthia* venom-induced lethality after 24 hours.

The analysis of scanned images of the 2-DE gels using the ImageMaster 2D[®] Platinum software, to compare the protein expression in sera from mice subjected to two different treatments, revealed two missing spots in the lower left region of the 2-DE serum map of mice treated with *M. pudica* tannins (Figure 2).

The labeled protein spots from each gel were then visualized in a three-dimensional perspective (Figure 3). Each identified protein spot on the gel showed up an individual peak. The analysis confirmed that two protein spots had been down-regulated due to the protein spots NN2 and NN4 that were missing from the 2-DE map of mice treated with *M. pudica* tannin and venom (no peaks).

DISCUSSION

The aim of the present study was to compare the efficacy of tannins obtained from *M. pudica* and commercial tannic acid on *N. kaouthia* venom neutralization. Both *in vitro* study (preincubation of plant extract with venom) and *in vivo* study (rescue treatment) were carried out to test the efficacy of both sources of tannins. Monovalent *N. kaouthia* antiserum was used as control. As tannins are known to precipitate proteins, it was expected that activities of some of the toxic enzymes of *N. kaouthia* would be inhibited (10).

The current results revealed that preincubation of *M. pudica* tannins with *N. kaouthia* venom (*in vitro* study) before their inoculation in mice was able to maintain 100% survival after 24 hours, which corroborates previous *in vitro* studies (8). On the other hand, the *in vivo* test of *M. pudica* tannins – administration of venom first followed immediately by the plant extract using separate injection routes – failed to rescue mice from the lethal effects of *N. kaouthia* venom.

The *in vitro* test of commercial tannic acid showed only 1/8 or 12.5% protection in mice. The tannic acid also failed to show any inhibition of lethality in the rescue experiment. Based on the study results, we found that *M. pudica* tannins are more effective than tannic acid in neutralizing 2 LD₅₀ of *N. kaouthia* venom *in vitro*, but less efficient than antivenom.

In the 2-DE study, two spots representing venom proteins were missing in the 2-DE map

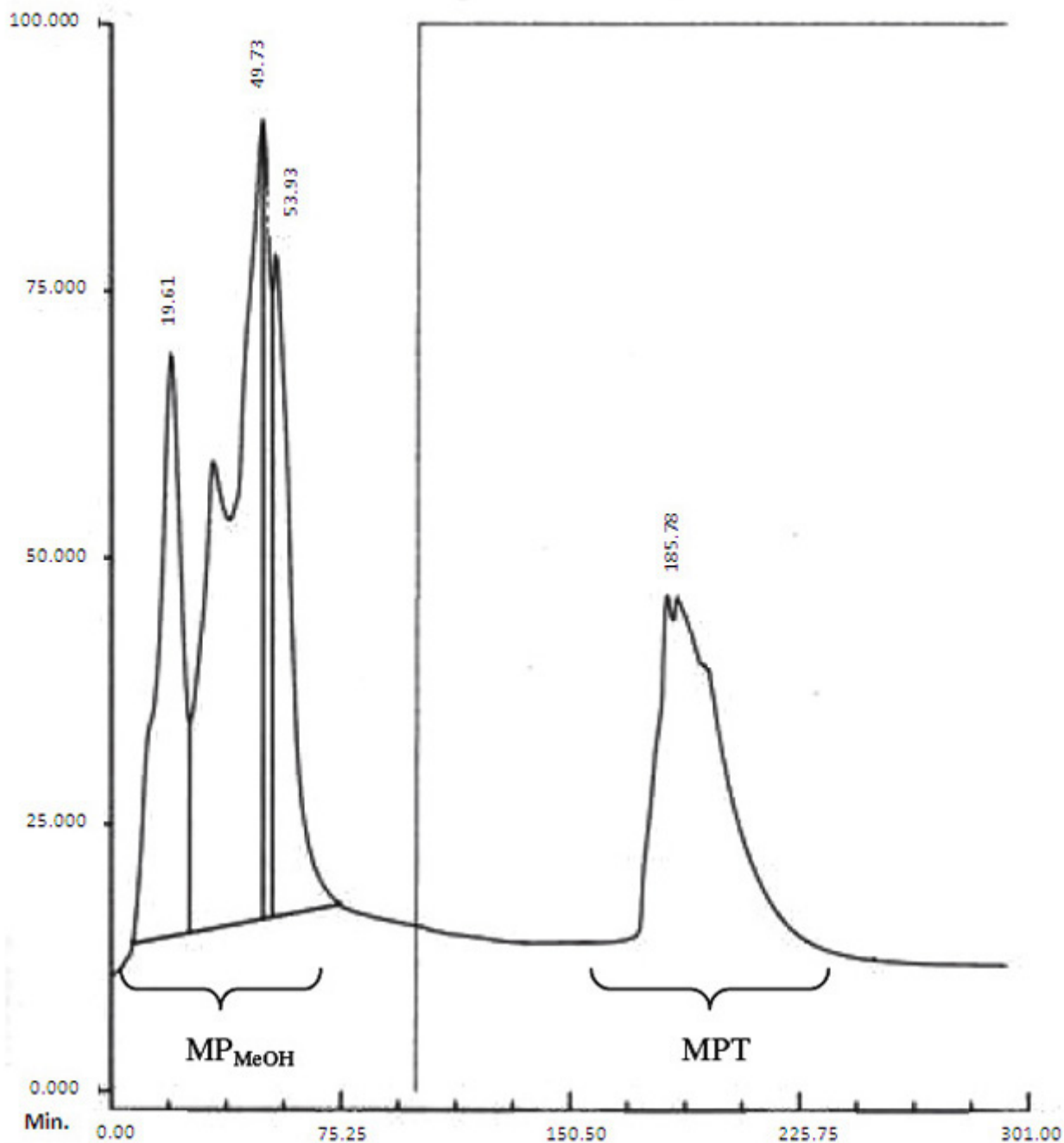


Figure 1. The HPLC spectrum obtained through fractionation of the crude extract using Sephadex LH-20 packed column (dimension – 30.0 cm x 1.0 cm ID). The peaks were detected at 344 nm.

Table 2. Results of mortality data in mice envenomed with *Naja kaouthia* venom, obtained according to Miller and Tainter method (11)

Group	Dose (mg/kg)	Log dose	Dead	Survived	Dead (%)	Corrected (%)	Probit
1	0.70	-0.15	0	8	0	3.13	3.12
2	0.80	-0.10	1	7	12.5	12.5	3.85
3	0.90	-0.05	3	5	37.5	37.5	4.68
4	1.00	0.00	7	1	87.5	87.5	6.16
5	1.10	0.04	8	0	100	96.9	6.88

Table 3. Results of the *in vitro* experiment

	Treatment	Dead/total	Protection (%)
Experimental group	2 LD ₅₀ (1.84 m/kg) venom + <i>M. pudica</i> tannins	0/8	100
	2 LD ₅₀ venom + tannic acid	7/8	12.5
Control group	2 LD ₅₀ venom	8/8	N/A
	2 LD ₅₀ venom + antivenom serum	0/8	100

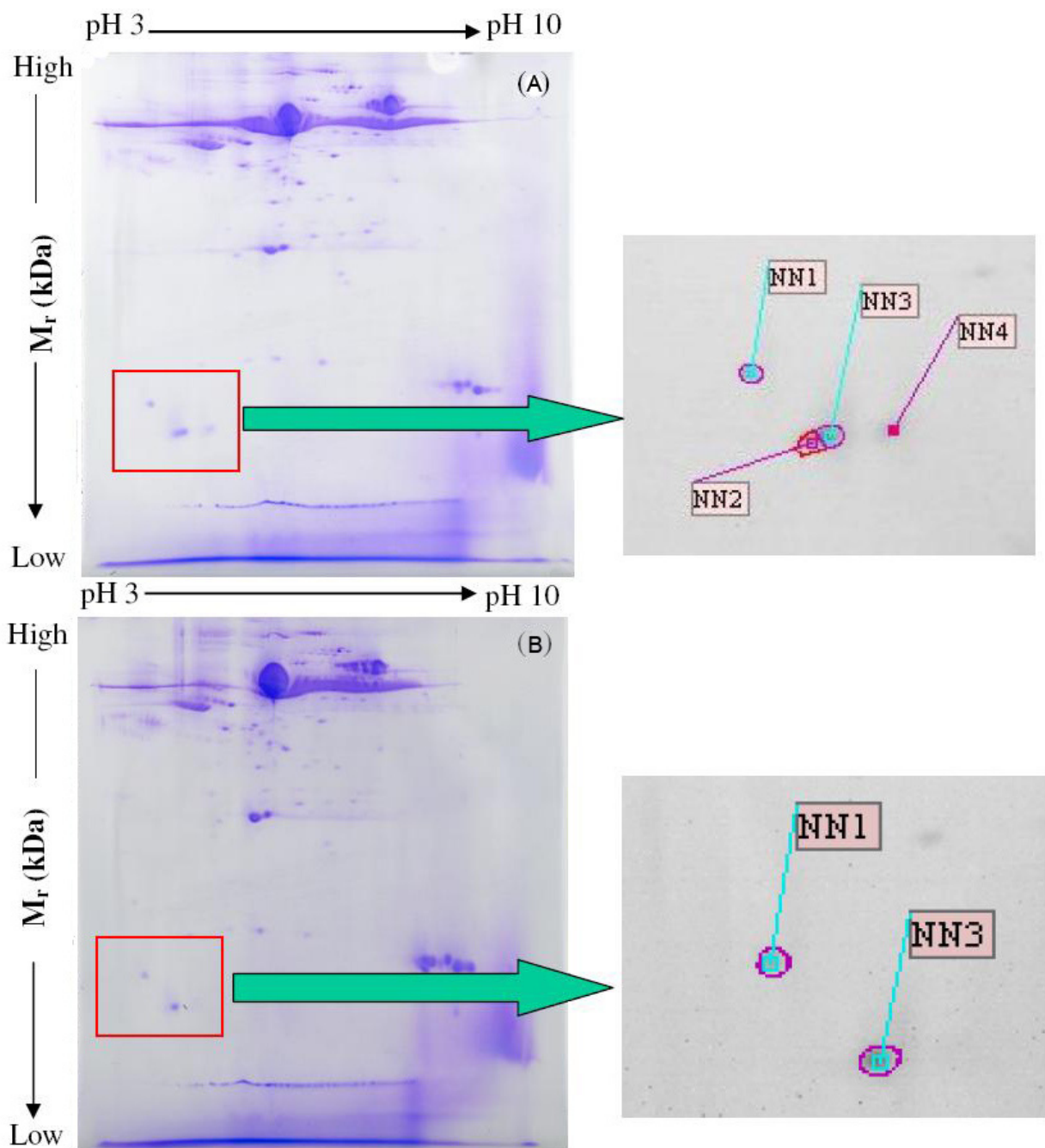


Figure 2. Expression of protein spots between mice treated with (A) snake venom and (B) *M. pudica* tannins plus venom. Spots designated NN2 and NN4 were missing from the 2-DE map of *M. pudica* tannin treated mouse sera.

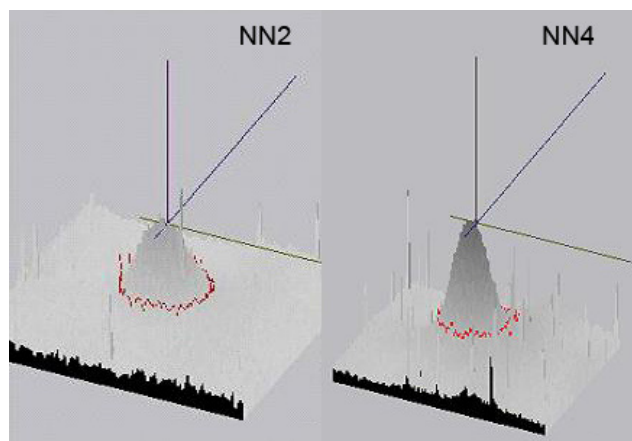


Figure 3. The three-dimensional images of NN1 and NN3 spots clearly showing individual peaks. No peaks were detected for NN2 and NN4 using the ImageMaster 2D® Platinum software.

of mice treated with *M. pudica* tannins. There are two types of plant tannins, namely hydrolysable and condensed tannins (10). Some plants, including *Pentace burmanica* and *Areca catechu*, that produce mainly condensed tannins are able to inhibit snake venom activities (12). In our study, the commercial tannic acid employed comprised the hydrolysable and gallotannin types, and it was established that *M. pudica* tannins were more effective in neutralizing the snake venom. We hypothesized that the roots of *M. pudica* contained a higher rate of condensed and hydrolysable tannins.

In our study, we also demonstrated that the rescue treatment failed to save mice from lethality induced by snake venom. This finding does not agree with several studies that found positive results by using *in vitro* neutralization methods of plant extracts for anti-snake venom assays. However, one work showed similar results regarding tannins of *Musa paradisiaca* extract that presented *in vitro* neutralization activities, but not *in vivo* (13). This can be attributed to the fact that tannins are able to non-specifically bind to *N. kaouthia* venom proteins and precipitate them, thus provoking the anti-lethal effects. Nevertheless, in *in vivo* studies tannins may interact with plasma proteins from the blood circulation. Therefore, there is a decrease in binding to *N. kaouthia* venom proteins and, hence, tannins become less efficient as anti-venom.

From the 2-DE results obtained, it was shown that tannin compounds of *M. pudica* root extract interacted with *N. kaouthia* venom proteins and produced down regulation of the

two protein spots. We emphasize the importance of two missing spots in a particular region of the gel based on two previous studies, the protein spots on that region of the gel were identified as isoenzymes of phospholipase A₂ when compared with the protein database (8, 14).

By comparing the results of the 2-DE gel with the results of the preliminary study by Vejjayan *et al.* (8), it was demonstrated that the missing protein spots on the *M. pudica* treated gel were the isomers of PLA₂ enzymes. Phospholipases A₂ constitute major components of snake venoms and exhibit a wide range of pharmacological actions such as neurotoxicity; cardiotoxicity; myotoxicity; anticoagulant, hemorrhagic, edematous, hemolytic, convulsive and hypotensive activities (15, 16). By showing that PLA₂ enzymes have been down-regulated, this explained why mice that received venom previously incubated with *M. pudica* tannins were fully protected from the lethal effects of *N. kaouthia* venom.

Based on the present observations, *M. pudica* demonstrated potential for an antagonist to *N. kaouthia* venom at *in vitro* tests.

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CONFLICTS OF INTEREST

There is no conflict.

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ETHICS COMMITTEE APPROVAL

The present study was approved by the Joint Committee of the IMU/RC and IMU/EC, International Medical University. The Ethics Committee/IRB reference number is: 4.6/02/2007

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