Efficacy evaluations of *Mimosa pudica* tannin isolate (MPT) for its anti-ophidian properties

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**A B S T R A C T**

**Aim of the study:** Evaluations of the anti-snake venom efficacy of *Mimosa pudica* tannin isolate (MPT) obtained from root of the plant.

**Materials and method:** MPT was investigated in vitro and in vivo for its efficacy against the venom of *Naja kaouthia* snake.

**Results:** In vitro: (1) mice injected i.p. with MPT pre-incubated with *Naja kaouthia* venom at concentrations as low as 0.625 mg/ml showed 100% survival after a 24-h observation period. (2) In the proteomics study, mice injected with MPT pre-incubated with the *Naja kaouthia* venom showed down-regulation of five serum proteins. (3) In the protein–dye-binding study, the percentage of Bradford dye–protein binding showed a reduction relative to the decrease in MPT concentration used to incubate with the venom. In vivo: the results from the animal studies showed that MPT had no in vivo protection against the *Naja kaouthia* venom (0.875 mg/kg) in four different rescue modes and in an oral pre-treatment experiment.

**Conclusion:** The study indicated the promising ability of MPT to neutralize the *Naja kaouthia* venom in vitro experiments but fell short in its in vivo potential. As such, the use of *Mimosa pudica* as therapeutics for snake bites is questionable as all the possible in vivo rescue studies and pre-treatment of the active constituents showed no protection against the affected mice.

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1. Introduction

The promising potential of medicinal plants for the treatment of snake bites has stirred much interest among researchers. Plants have been used to treat snake bites in all parts of the world, far before commercial anti-venoms were invented. People have generally accepted the anti-ophidian properties of plants, without knowing their actual scientific value. Plant extracts, which possess more than one biochemical property, provide extremely rich sources of pharmacologically active compounds. The interaction of such active compounds with the toxins and lethal enzymes of snake venom leads to neutralization or inhibition of toxic activities (Mors et al., 2000; Soares et al., 2005; Sanchez and Rodriguez-Acosta, 2008). In recent times, many researchers have actively researched the efficacy of plants on snake bites at in vitro level but only some have extended their research further into evaluating in vivo potential. Main drawbacks were concern with plant safety as well as how far can their compounds can act efficiently inside the human biological system. It is prudent that all local ethnomedical plants be scientifically evaluated and properly documented in order for adequate knowledge of their efficacy and safety to be obtained.

The plant *Mimosa pudica* L. belongs to the family of Fabaceae, commonly known in English as Sensitive Plant; Touch-me-not Plant or Sleeping Grass while locally it is known as ‘Semalu’. Ethnopharmacologically, the root of this plant has been used for snakebite by traditional and tribal healers (Kirtikar and Basu, 1980; Bhalla et al., 1982; Macmillan, 1999; Pattanaik et al., 2006; Thirumalai et al., 2010) while leaves and stems used against scorpion sting (Pattwari, 1992). According to its traditional use, at the time of the snakebite a root was chewed together with three leaves of *Azadirachta indica*. Alternatively, a warm paste consisting of ground root of *Mimosa pudica* root and 21 number of black pepper can be applied on the snakebite area and bandaged. The roots of
Mimosa pudica was also mentioned to have popularly used against cobra bite by snake charmers and Bejs (traditional quacks) of northeast India (Mahanta and Mukherjee, 2001).

Mimosa pudica has been shown to be effective against snake venoms in different studies. One study in India screened several herbal plants for anti-venom activity against the venom of the Echidnina schistosa, the most toxic of common sea snakes (Fattepur and Gawade, 2007). The investigation revealed positive anti-venom activity in the alcoholic extracts of the Mimosa pudica, Mucuna pruriens, and Andrographis paniculata. In another study using the aqueous extract of the dried roots of the Mimosa pudica, results showed significant inhibitory effects on the Naja naja and Bangarus caeruleus venoms (Meenathisundaram et al., 2009). Researchers Mahanta and Mukherjee (2001) studied the Mimosa pudica root extract for its neutralization ability on the lethality, myotoxicity and inhibition of the toxic enzymes of the Naja kaouthia venom. The aqueous extract of the plant showed significant inhibitory effects in all the activities tested. This was further supported by the study undertaken by Vejayan et al. (2007), in which out of 17 plants screened, only the aqueous extract of the Mimosa pudica showed a 100% ability in neutralizing venom lethality. This would support the use of the Mimosa pudica as a potential anti-venom agent of plant origin against the poisonous venoms of five snakes which can be found in Malaysia.

In this study, attempts to further evaluate the efficacy of the Mimosa pudica tannin isolate were undertaken to determine how far the plant can be used successfully as an anti-ophidian, as reported by Vejayan et al. (2007) and Sia et al. (2011). The study focused on tannin fraction of this plant as it has been established previously no activity was obtained by the fraction containing without any tannin (eluted with 50% methanol of the crude water extract in Sephadex LH-20 packed column) (Sia et al., 2011). A number of studies have reported the use tannins derived from plants against snake venom (Okonogi et al., 1979; Pithayanukul et al., 2005; Jaiswal et al., 2011).

2. Experimental

2.1. Solutions, reagents and chemicals

Freshly prepared solutions and analytical grade chemicals were used in all the experiments. Naja kaouthia monovalent antivenom was purchased from Thai Red Cross Society, Thailand.

2.2. Animals

Male albino mice weighing 20–25 g provided by animal house, School of Science, Monash University, were used for the experiments. The animals were kept under standard condition and experiments were conducted according to the ethics norm approved by the Animal Ethics Committee (AEC) of Monash Australia (AEC No.: SOBCB/MY/2008/36).

2.3. Preparation of Naja kaouthia (NK) venom

NK venom was obtained from Bukit Bintang Enterprise Sdn Bhd, Perlis, Malaysia. The venom was lyophilized, weighed and dissolved in saline (0.85% NaCl/water, w/v), and was agitated gently. The venom sample was used immediately, and was kept in ice throughout the experiments.

2.4. Preparation of Mimosa pudica tannin isolate (MPT)

Mimosa pudica roots were obtained from Bagan Lalang, Sepang, Malaysia. The dried sample was finely powdered. The extract was then 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 with a paper filter (Whatman No. 1), and concentrated using a rotary evaporator at 50 °C, followed by the addition of 3 ml of water. The extract was run through the Sephadex LH-20 column to purify the total tannins, using 50% methanol, followed by 70% acetone (Asquith and Butler, 1985; Hagerman and Butler, 1994). The acetone isolate was collected and rotary evaporated under the same conditions. The final concentrate (MPT) was dissolved with 35% acetone and kept in 4 °C until use.

2.5. In vitro evaluations of MPT

2.5.1. Bradford dye–protein binding assay

The technique was adapted from Kilkowski and Gross (1999) with some modifications. In the first group, NK venom preparations were mixed with equal volume of different concentrations of MPT to give final venom lethality of 2LD50. The mixture was pre-incubated at 37 °C for 20 min. One ml Bradford reagent was then added to 100 μl of the mixture and incubated further for 20 min before the absorption reading was taken at 595 nm. In the second group, the same procedure was repeated with the MPT alone, with varying concentrations. NK venom was run in parallel as controls. The percentage of protein–dye binding was calculated using the following formula and a graph was plotted.

\[
\text{% protein–dye binding} = \frac{\text{Venom}_{\text{abs}} - (\text{MPT Venom}_{\text{abs}} - \text{MPT}_{\text{abs}})}{\text{Venom}_{\text{abs}}} \times 100
\]

2.5.2. Efficacy evaluations in mice of MPT pre-incubated with NK venom

In this in vitro neutralization study, NK venom preparations were mixed with equal volume of different concentrations of MPT to give a final venom lethality of 2LD50. The mixtures were pre-incubated at 37 °C for 20 min and then injected i.p. into the mice. Tannic acid (Sigma) of the same concentration as the MPT, saline (0.85% NaCl/water, w/v) and NK commercial monovalent anti-venom (Thai Red Crescent Society) were done in parallel as controls. Four mice were used per group for each dose.

2.5.3. Proteomics assessment of MPT against venom in mouse serum

2.5.3.1. Treatment of mice. Three treatment groups, with 4 mice in each group, were used in this experiment. The first group received 2LD50 of NK venom preparations alone. The second group received saline (0.85% NaCl/water, w/v) alone. The last group received venom preparations mixed with 0.0625 mg/ml MPT. All treatments were incubated at 37 °C for 20 min before i.p. injection.

2.5.3.2. Serum collection. Using the cardiac puncture technique, blood samples were collected from all three treatment groups after 5 min of treatment. Samples were quickly centrifuged at 3000 rpm for 10 min at 4 °C to separate serum from cells.

2.5.3.3. Two-dimensional electrophoresis (2-DE). The total protein in the samples was estimated using the Bradford microassay technique and calculated to give 20 μg load per IPG strip. The denatured samples were separated by isoelectric focusing in 13 cm Immobiline dry strips (pH 3–10; GE Healthcare). Subsequently, proteins were separated in the second dimension by 15% sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and visualized using silver staining. Differences in protein profiles in the three treatment groups were analysed using PDQuest 8.0 Advance Software. The gel plugs of interest were then sent to a service
provider (Proteomics International, Australia) for identification using MALDI-TOF MS/MS peptide mapping combined with tandem mass spectrometry.

2.6. Evaluation of MPT for protective activity (in vivo) against venom

2.6.1. Administration of venom, followed immediately by MPT treatment

Four groups, consisting of 8 mice per group, were used in this experiment. The control group was injected with venom (mg/kg, i.p.) only. The second and third groups were injected with 2LD50 of NK venom (i.p.) followed immediately with either i.p., i.v., oral or s.c. administration of MPT or tannic acid or saline (0.85% NaCl/water, w/v). The fourth group was injected with 2LD50 of NK venom (i.p.) followed immediately with either i.p., i.v. or s.c. administration of NK monovalent anti-venom. The mortality of the mice was recorded.

2.6.2. Administration of MPT, followed immediately by injection of venom

Four groups of mice, consisting of 8 mice per group, were used in this experiment. The control group was injected with venom (mg/kg, i.p., oral or s.c.) only. The second group was given saline (0.85% NaCl/water, w/v) via either i.p., oral or s.c. The third group was given MPT of different concentrations (10 mg/ml, 2.5 mg/ml, 0.625 mg/ml and 0.03 mg/ml) via i.p., oral or s.c. A lethal dose of 2LD50 venom was injected i.p. after 30 min of oral treatment. The mortality of the mice was recorded.

3. Results

3.1. In vitro Bradford dye–protein binding assay

Fig. 1 shows the percentage of dye–protein signifying the remaining free proteins of NK venom unbound by tannins of MPT. Serially diluted MPT were co-incubated with NK venom for 20 min prior to the addition of the Bradford reagent. The experiment was designed taking into consideration that both the MPT and Bradford reagents will bind with the proteins present in the venom. The amount of free venom proteins present after the MPT treatment was calculated and used to estimate the percentage of protein–dye binding. From the results, it was evident that the percentage of venom neutralized by the MPT increased with higher concentrations of the MPT. MPT at concentrations of 0.068 mg/ml and above showed 100% binding activity against NK venom and the EC50 value was estimated to be 0.002 mg/ml.

3.2. In vitro neutralization of NK venom by MPT

Tables 1 and 2 show the snake venom-neutralizing potential of MPT extracts by in vitro assay. The results are displayed in percentage mortality value. MPT at all concentrations tested (10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml and 0.625 mg/ml) were shown to be 100% potent in neutralizing NK venom. The results corresponded well with the positive control, i.e., the NK commercial antivenom. All the mice injected with anti-venom pre-incubated with NK venom were observed to be alive after the 24-observation period. In contrast, tannic acid (tested at 10 mg/ml) showed 0% potential in neutralizing NK venom, and it was comparable with the negative control, saline (0.85% sodium chloride, w/v). In vitro assay involved pre-incubation of the plant sample with the targeted venom prior to injection into the test mice and the neutralizing potential of the plant was observed by its ability to keep the test mice alive.

3.3. Proteomics assessment of MPT against venom in mice serum

The differences in the protein profiles of serum obtained from the groups involving control mice, venom alone as well as venom pre-incubated with MPT were studied. Using MALDI-TOF MS/MS peptide mapping, a total of five spots were identified to be differentially expressed (>two folds). It could be seen from the results that the serum of the venom-treated mice showed substantial proteomic changes which were then normalized upon co-incubation with MPT. The peptide mass fingerprinting combined with tandem mass spectrometry, of SSP 2505, SSP 4606, SSP 3513 and SSP 3303 (Fig. 2) pointed to serine peptidase inhibitors protein, gel-solin, hemopexin and α2–macroglobulin, respectively. Spot S4407 failed to be identified. The levels of serine protease inhibitor, gel-solin, alpha-2-macroglobulin and hemopexin were found to be up-regulated in the venom-treated mice, compared to the control mice. The results indicated that these four proteins are closely related to the biochemical changes that lead to lethality in mice after NK envenomation.

3.4. Evaluation of MPT for protective activity (in vivo) against venom

Table 3 shows the snake venom-neutralizing potential of the MPT extract by in vivo assay. This experiment was designed to save the test mice first i.p. injected with venom via the immediate application of MPT through different modes of injection – i.p., i.v., oral and s.c. The results are displayed in percentage mortality value. MPT showed 75% mortality when rescued i.p. (Table 3). It failed to save the venom-affected mice, confirming 100% mortality in the 3 other rescue trials via i.v., oral and s.c. (Table 3). Commercially available NK anti-venom was used as the positive control and it showed 100% ability in saving the test mice in all 3 modes of injection tested. Tannic acid of 0.625 mg/ml and the negative control, saline, on the other hand, showed 0% ability to save the mice in all 4 experiments.

3.5. Evaluation of MPT for protective activity (pre-treatment) against venom

Table 4 shows the venom-neutralizing potential of MPT extract by in vivo pre-treatment assays. In this experiment, MPT was administered first via i.p., oral administration or s.c. injection, followed by 2LD50 of venom injection after 5 min of treatment via i.p. injection. The results are displayed in percentage mortality value. MPT at four different concentrations – 10 mg/ml, 2.5 mg/ml, 0.625 mg/ml and 0.03 mg/ml – failed to save the mice, and hence showed 100% mortality in all 3 pre-treatment trials (Table 4). Both tannic acid at
Table 1
Effect of i.p. injection of NK antivenom, tannic acid or saline pre-incubated with NK venom.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean % mortality (lethality/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antivenom</td>
</tr>
<tr>
<td>Sample + NK venom, i.p. injection</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td>Control Saline (0.85% NaCl, w/v)</td>
<td>8/8 (100%)</td>
</tr>
</tbody>
</table>

n = 8 in each group.

Table 2
Effect of i.p. injection of various MPT concentrations pre-incubated with NK venom.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean mortality (lethality/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MPT (mg/ml)</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>MPT + NK, i.p. injection</td>
<td>0/8 (0%)</td>
</tr>
</tbody>
</table>

n = 8 in each group.

Fig. 2. Proteomic pattern of venom injected mice serum. Loading of 20 μg of mice serum protein after 2-dimensional gel electrophoresis at non-linear pH 3–10, stained by silver nitrate and 3-D view of Spot 3513, 4606, 2505, 3303 and 4407 respectively in 3 different groups – control group (S), venom group (V) and MPT-treated group (MPT-V).

Table 3
Effect of treatment of MPT via i.p., i.v., oral and s.c. on lethality of venom injected mice.

<table>
<thead>
<tr>
<th>Treatment mode</th>
<th>Mean mortality (lethality/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NK antivenom</td>
</tr>
<tr>
<td></td>
<td>MPT (0.625 mg/ml)</td>
</tr>
<tr>
<td></td>
<td>Control saline (0.85% NaCl, w/v)</td>
</tr>
<tr>
<td></td>
<td>Tannic acid (0.625 mg/ml)</td>
</tr>
<tr>
<td>Intraperitoneal (i.p.)</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>Intravenous (i.v.)</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>Oral</td>
<td>NA</td>
</tr>
<tr>
<td>Subcutaneous (s.c.)</td>
<td>0/8 (0%)</td>
</tr>
</tbody>
</table>

n = 8 in each group; NA = not applicable for oral route.
0.625 mg/ml and the negative control, saline, showed 0% ability to save the mice in all 3 experiments.

4. Discussion

This study was done using the *Mimosa pudica*, one of the herbal plants traditionally used in phyto-therapeutic treatment by a large proportion of Asians for physical, physiological, and mental ailments (Pushpangadan and Atal, 1984; Amalraj and Ignacimuthu, 2002; Samy et al., 2008; Gomes et al., 2010). Besides, it has been notably used against snake bite in folkloric medicine by traditional healers around the world as well (Kirtikar and Basu, 1980; Bhalla et al., 1982; Macmillan, 1999; Pattanaik et al., 2006; Thirumalai et al., 2010). Representing an efficacy report (both in vitro and in vivo) on the use of MPT as an anti-ophidic agent, this study directed special focus on the efficacy of MPT in protecting venom-challenged mice via the in vivo method.

Results from in vitro Bradford dye–protein binding assay showed that MPT at concentrations of as low as 0.068 mg/ml was able to bind 100% with NK venom proteins in 1 mg/ml of the venom. This means that at dye–protein binding of 100%, all the venom proteins were precipitated/bound to MPT in the sample mixture. The same binding ability was observed in previous studies by Vejayan et al. (2007) and Sia et al. (2011) in a double-immunodiffusion study. Both the studies showed binding capabilities between MPT and venom alongside the formation of a venom sample interaction precipitin line (Vejayan et al., 2007; Sia et al., 2011). The Bradford dye–protein binding obtained showed a reduction in tandem with the decrease in MPT concentrations used for incubation with the venom.

From the results of the in vitro animal study, it was found that pre-incubating MPT with NK venom 20 min prior to treatment yielded 100% survival in the venom-challenged mice. This finding is in accordance with previous in vitro studies done (Mahanta and Mukherjee, 2001; Vejayan et al., 2007; Sia et al., 2011).

Results from the proteomics assessment showed that 5 proteins – serine protease inhibitor protein, gelsolin, hemopexin, alpha-2-macroglobulin and another unidentified protein – were substantially up-regulated by NK venom. These proteins were normalized by co-incubating the venom with MPT prior to injection into the test mice. This shows that the tannin compounds of MPT interacted with NK venom proteins to neutralize the up-regulation of the 5 proteins. Gelsolin, hemopexin, serine protease inhibitor and alpha-2-macroglobulin are acute-phase and inflammatory-related proteins which increase when acute inflammation takes place in mice (Rubin, 1996; Fink, 2009; Kataranovski et al., 2009; Ji et al., 2010). Down-regulation after co-incubating MPT with the NK venom thus shows that MPT neutralizes the venom’s lethal proteins responsible for increasing inflammation and immune responses.

In this study, we proved that the rescue treatment and pre-treatment by MPT failed to save the mice from NK snake venom-induced lethality upon testing with all the technically possible treatment modes. This finding is in contrast with the results obtained in the in vitro study, where the injection into the mice of pre-incubated MPT with the same venom showed 100% neutralization. This could be due to the tannin’s ability to bind non-specifically with NK venom proteins during the incubation period, hence showing 100% ability in neutralizing the toxic effect of the lethal proteins (Mahanta and Mukherjee, 2001; Vejayan et al., 2007; Sia et al., 2011).

A study by Naczek et al. (1996) showed that a statistically significant (p = 0.0001) linear relationship was found between the amount of tannin–protein complex formed and the amount of tannin added to the reaction mixture. Thus, the failure of the rescue and pre-treatment in vivo study might be due to the non-specific binding property of the Mimosa tannin to proteins other than venom proteins. This is not surprising as tannins are known to form soluble and insoluble complexes with proteins (Martin-Tanguy et al., 1977; Hagerman, 1992). Treatment of MPT prior to or after the NK venom injection might have caused the binding of the *Mimosa pudica*’s tannin with macromolecules in the circulatory systems of the mice. In such a case, there would be very little possibility of MPT binding with the venom’s lethal proteins resulting in undiminished availability of venom. These results correlate with the findings of Kokane et al. (2009) which reiterated *Mimosa pudica*’s ability to arrest bleeding and promote wound healing. Their findings also explain the blood thickening effect that we observed during cardiac puncture of the mice after MPT treatment. This effect was not seen in the rescue treatment with NK anti-venom, as the monovalent anti-venom works very specifically against the main lethal proteins of the particular snake venom.

Many studies have shown the positive anti-snake venom action of plant extracts, but most of the studies were done in vitro. Only a few involved in vivo animal studies using rescue experimental designs (Alam and Gomes, 2003; Borges et al., 2005). In papers recording the use of plants against snake venom, the plants were either mixed with venom prior to injection/administration into mice (Mahanta and Mukherjee, 2001; Vasanthi et al., 2003; Pithayanukul et al., 2004, 2005; Meenatchisundaram et al., 2005) or were given to the experimental mice before the venom was administered as pre-treatment (Mors et al., 1989; Asuzu and Harvey, 2003; Ode and Asuzu, 2006; Tan et al., 2009) or the plants were screened for some enzyme inhibition properties only (Oliveira et al., 2005; Pithayanukul et al., 2010). All these experimental designs were not carried out on actual snake bite incidents.

There is a possibility that plant extracts could be effective against snake venom activity in vivo provided the experimental model has been modified to simulate real life experience. However, the plants’ traditional usage presents a drawback. Topical application of plants onto the bitten area, chewing their leaves or bark or drinking the plants’ extracts or decoctions are among the different methods in folk medicine to treat snake bite and this applies to *Mimosa pudica* as well (Samy et al., 2008). Our in vivo animal study was limited by any possibility of mimicking real snake bite scenarios in the laboratory, thus precluding the possibility of topical treatment as one option. Another challenge was the choice of experimental animal used; mouse being the natural predator for snakes means the venom being designed to suit perfectly to kill...
this creature (Daltry et al., 1996; Jorge da Silva and Aird, 2001; Roy et al., 2010).

5. Conclusion

In conclusion, MPT does not show anti-snake venom activity as popularly attributed, at least when tested in mice. While this finding may clarify the supposed efficacy of the Mimosa pudica root tannin as an anti-ophidian agent, further safety evaluation of MPT will provide the answer to the factor(s) leading to this. From the study, it is clear that more in-depth scientific in vivo evaluation is necessary in order to validate the actual anti-ophidian efficacy of the claimed plant substances.

Acknowledgements

The authors are very grateful to Mr. Zainudinn from Bukit Bin-tang Enterprise Sdn Bhd for enabling the mixing and purchasing of all venoms used in this study. The work was conducted utilizing chemicals and consumables supplemented from grants: Malaysian Ministry of Science and Technology (Project Number 02-02-10-SF0033) and Monash University Sunway Campus Internal Grant (514004440000).

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