THE POTENTIAL OF *MIMOSA PUDICA* (MIMOSACEAE) AGAINST SNAKE ENVENOMATION

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VEJAYAN, J., IBRAHIM, H. & OTHMAN, I. 2007. The potential of *Mimosa pudica* (Mimosaceae) against snake envenomation. In this study, neutralization of the lethal effects of *Naja naja kaouthia* venom was investigated by co-incubating the venom with water extracts of various plants. The mixture of venom-extract was then injected intraperitoneally (i.p.) into mice. From the 17 plants screened, only *Mimosa pudica* (Mimosaceae) showed 100% ability in neutralizing the $2LD_{50}$ lethality of this venom. This plant was also found to show 50% or more ability in neutralizing the $2LD_{50}$ toxicity of other venoms, namely, *Ophiophagus hannah, Bungarus candidus, B. fasciatus* and *Calloselasma rhodostoma*. Its active fraction (MP188ECT3) also showed interaction towards *N. n. kaouthia* venom. This was based on results of the 2-D electrophoresis (2-DE) established by using immobilized pH gradient 3–10 on this venom. Some spots representing proteins were found missing upon combining the venom with 0.7 mg of the fraction prior to 2-DE. Among these are isomers of a lethal protein identified by MALDI-TOF MS as phospholipase A_2 which have been significantly found to disappear in the treated gel compared with the 2-DE gel of the venom only. Results of the double immunodiffusion study showed binding capabilities between MP188ECT3 fraction and venom preparations with the formation of a venom-antivenom precipitin line. These results demonstrated the potential use of *M. pudica* as an antivenom agent of plant origin against five poisonous snake venoms found in Malaysia.

Keywords: Anti-snakebite, medicinal plant, 2-D electrophoresis, venom

VEJAYAN, J., IBRAHIM, H. & OTHMAN, I. 2007. Potensi *Mimosa pudica* (Mimosaceae) dalam menahan bisa ular. Dalam kajian ini, peneutralan kesan bisa ular *Naja naja kaouthia* diselidik dengan cara mengeramkan bisa tersebut dengan pelbagai ekstrak akueus tumbuhan. Campuran ini kemudiannya disuntik secara intraperitoneum (i.p.) ke dalam tikus. Daripada 17 tumbuhan yang dikaji, hanya *Mimosa pudica* (Mimosaceae) menunjukkan kebolehupayaan 100% meneutralkan kesan $2LD_{50}$ bisa ular ini. Tumbuhan ini juga mempunyai kebolehan 50% atau lebih dalam meneutralkan kesan $2LD_{50}$ bisa ular *Ophiophagus hannah, Bungarus candidus, B. fasciatus* dan *Calloselasma rhodostoma*. Selain itu, keputusan elektroforesis dua dimensi (2-DE) menunjukkan bahawa pecahan aktif (MP188ECT3) tumbuhan ini bertindak balas dengan bisa *N. n. kaouthia*. Imej 2-DE bagi bisa ini dihasilkan dengan menggunakan kecerunan pH pegun 3–10. Hasil pencerapan imej ini menunjukkan ketiadaan beberapa tompok yang mewakili protein selepas bisa ini dicampur dengan 0.7 mg pecahan aktif tersebut. Antaranya ialah beberapa isomer bagi protein yang boleh membawa maut. Isomer ini dikenal pasti melalui MALDI-TOF MS sebagai fosfolipase A_2 dan didapati hilang pada imej 2-DE yang diperlakukan dengan pecahan aktif berbanding dengan imej 2-DE bagi bisa saja. Keputusan kajian imunoresapan berganda menunjukkan pecahan MP188ECT3 mampu menjerap pada bisa. Ini dapat dilihat daripada pembentukan garis mendakan bisa-penawar. Keputusan yang diperoleh menunjukkan potensi *M. pudica* sebagai satu agen antibisa terhadap lima jenis ular berbisa yang dijumpai di Malaysia.

INTRODUCTION

During the year 1958 to 1980, there were 55 000 cases of snakebites recorded in hospitals in Malaysia (Tan 1991). In Kangar District Hospital in Perlis alone there were 284 snakebite cases reported from January 1999 to December 2000, of which 9.8% involved foreign tourists (Jamaiah *et al.* 2004). The common form of treatment for snakebites is the use of antivenom serums. Its effectiveness, however, has been marred by its narrow cross-reactivity against heterologus snake venoms, a common problem faced especially when using monovalent antivenoms to treat

inaccurate diagnosis of biting species (Tan 1991). Inaccurate diagnosis results from complications in identifying the biting snake species by physicians. This is evident as only 68 cases of the total 284 snakebite cases in Perlis were positively identified, of which 50 were common cobras (*Naja naja*), 16 Malayan pit vipers (*Calloselasma rhodostoma*) and two were sea-snakes (Jamaiah *et al.* 2004).

Many plants are used in traditional medicine as active agents against various effects induced by snakebites. A total of 578 species of higher plants from 94 families have been cited in literature as being active against snakebite (Mors 1991). However, only a few attempts have been made to correlate scientifically the nature of these plant-natural products with anti-snake venom properties. This, together with acute adverse reactions showed by antivenom serums have prompted us to undertake this study in search of an alternative snakebite treatment from plant origin.

MATERIALS AND METHODS

Plant samples

The plants used in this study and the parts taken for analysis are listed in Table 1. The plants were authenticated by the Institute of Biological Sciences, University of Malaya, Kuala Lumpur where voucher specimens were also deposited. Mimosine (Sigma Chemical Co.), a commercially obtained constituent found in *Mimosa pudica* plant, was also screened for antivenom activity.

Venoms

Venoms were extracted from various snake species, namely, *Naja naja kaouthia*, *Ophiophagus hannah*, *Bungarus candidus*, *B. fasciatus* and *Calloselasma rhodostoma* found in the Perlis Snake Farm, Malaysia. The venoms were lyophilized before being stored at -20 °C.

Animals

Male albino mice weighing 20–25 g provided by the Animal House, Faculty of Medicine, University of Malaya were used for the experiments. The animals were kept under standard conditions and experiments were conducted according to the ethical norms approved by the Ethic Committee of the university (No: BIO/IO/05/2003/IO(R)).

Plant extraction

We modified the methods by Mahanta and Murkherjee (2001) for the extraction of plants. Fresh plants were cut into small pieces and dried in a convection oven at a temperature not exceeding 50 °C. After drying, the plant materials were finely ground using a blender. Plant extracts were prepared by stirring 4 g of the dry powdered plant material in 200 ml of water for 3 hours at room temperature. The crude extracts obtained were first filtered through muslin cloth and further clarified by filtration through filter paper (Whatman No. 1). Extracts were then concentrated using a rotary evaporator set at 50 °C to get rid of the water completely and this was followed by the addition of 10 ml water.

Inhibition of lethality of *N. n. kaouthia* venom by various plants

Lethal dose (LD_{50}) of *N. n. kaouthia* venom was determined using four groups of mice, eight per group. The venom was administered intraperitoneally (i.p.) and the number that died in each group within 24 hours was recorded. The LD_{50} was calculated by the method of Miller & Tainter (1944). For the venom inhibition study, $2LD_{50}$ of a mixture consisting of 200 µl venom sample was injected i.p. into each mouse. The procedure involved 100 µl of $4LD_{50}$ preparations of venom which were pre-incubated with 100 µl plant extract at 37 °C for 60 min prior to injection.

Inhibition of *M. pudica* extract on various venoms

Since *M. pudica* extract showed 100% ability in neutralizing $2LD_{50}$ lethality of *N. n. kaouthia* venom, it was then screened for inhibition of lethality towards the rest of the venoms, i.e. *O. hannah, B. candidus, B. fasciatus* and *C. rhodostoma*. Plant extract was co-incubated with venom prior to the administration into animals using the same method for screening the inhibition of *N. n. kaouthia* venom above. The assay was done using $2LD_{50}$ of the venoms that gave total lethality to the control mice.

Two-dimensional electrophoresis (2-DE)

The interaction of MP188ECT3, the active fraction of *M. pudica,* towards *N. n. kaouthia* venom was evaluated with the use of 2-DE. MP188ECT3 fraction was obtained from *M. pudica* root extract that was fractioned through a series of bioassay-guided fractionation beginning with solid phase extraction using C18(EC) and C8(EC) columns and then followed by further fractionation with Sephadex LH-20 gel loaded column. In order to determine the dry weight, 100 µl of this fraction was freeze-dried in an eppendorf microcentrifuge tube that was previously weighed. For the treated sample 2 mg of *N. n. kaouthia* venom (186 µg protein) was weighed and reconstituted with 100 µl of deionised water and 100 µl (0.7 mg) of the MP188ECT3 fraction. On the other hand, for untreated sample 2 mg of *N. n. kaouthia* venom (186 µg protein) was weighed and reconstituted with 200 µl of deionised water.

Both the untreated and treated samples were then incubated in a water bath for an hour at 37 °C. On completion, the samples were centrifuged at 10 000 rpm for 10 min before performing 2-DE. Briefly, the denatured venoms were separated by isoelectric focusing in 18 cm Immobiline dry strips (pH 3–10; Amersham Biosciences, Sweden). Subsequently, proteins were separated in the second dimension by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized using Coomassie blue staining (0.025% w/v Coomassie brilliant blue G in methanol:water:acetic acid 50:45:5).

MALDI-TOF MS peptide mapping of 2-DE protein spots

In-gel digestion of selected spots obtained from 2-DE gels after the final destaining process was performed according to Shevchenko *et al.* (1996). The Coomassie stained spots were excised and 50 µl of acetonitrile was added to each gel piece and left for 15 min to dehydrate. To rehydrate it, the supernatant was replaced with 25 µl of 25 mM NH_4HCO_3 for 10 min. These steps of rehydration followed by dehydration were repeated to give a total of three washes. Finally the spots were dried for 10 min using a Speedvac concentrator.

In-gel tryptic digestion

The dried spots were then re-suspended in 10 µl of 10 ng/µl trypsin in 25 mM NH_4HCO_3 and incubated overnight for complete digestion at 37 °C. On completion, the gel was once again dehydrated by adding acetonitrile for 15 min. Finally the gel piece was removed and the supernatant was kept in -20 °C until further use.

Sample preparation

An equal amount of tryptic digested samples were mixed together with a matrix solution consisting of α-cyano-4-hydroxy cinnamic acid in acetonitrile acidified with trifluroacetic acid (1 mg/ml). Thereafter 0.4 µl of this mixture was spotted on the slide and air-dried.

Mass spectrometry

Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) of peptide mixture was obtained on an Ettan MALDI-TOF-Pro mass spectrometer with a delayed ion source and nitrogen laser of 337 nm. The acceleration voltage was set to 20 kV with positive ion reflectron mode. The low mass rejection was activated. The instrument was calibrated externally with peptide samples of adrenocorticotropic hormone and (Ile7) angiotensin III.

Database queries

Proteins were identified using the monoisotopic masses with the internet search program ExPaSy (http://www.expasy.org/tools/aldente/). The search parameters were Swiss-Prot for database, other vertebrata for species, 1 for missed cleavages, trypsin for enzyme and MH^+ for ion mode.

Double immunodiffusion

The interaction of *M. pudica* and MP188ECT3 towards *N. n. kaouthia* venom was evaluated with the use of double immunodiffusion technique (Ouchterlony & Nilsson 1978).

Precoating of glass plates

A total of 0.5 g agar was boiled in 100 ml water by using microwave oven. The agar solution was allowed to cool to 50 °C and then 2 ml of it was pipetted onto each microscope slide placed on a levelling table. The agar was then allowed to solidify and then left overnight at room temperature or for four to six hours at 40 °C until completely dry.

Preparation of microscope slides

Agar (1 g) was boiled in 100 ml water using microwave. The 1% agar solution was then allowed to cool to 45 °C and then 3.5 ml of it was pipetted onto a precoated slide. The slides were left to solidify at room temperature after which four holes were punched out by using a gel puncher (see Figure 1).

The plugs of agar were removed from each well by using a Pasteur pipette attached to a vacuum line. The test was conducted by pipetting 16 µl of *N. n. kaouthia* venom (10 mg/ml) and 16 µl of plant crude extract (100 mg/ml) into wells 1 and 2 respectively followed by incubation overnight at room temperature in a humid chamber. For its fraction MP188ECT3 at a concentration of 10 mg/ml was used. Slides were examined for immunoprecipitin lines.

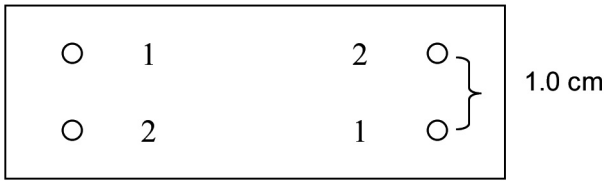

Figure 1 Schematic representation of microscope slide

Staining precipitin lines

The gel was washed in five changes of 100 ml 0.85% NaCl over a period of 48 hours. Then the gel was dried either by leaving it for 16 hours at room temperature or for two to six hours at 40 °C. The gel was then immersed in Coomasie blue stain for 30 min to 1 hour until stained bands were visible. The gels were destained in three to four changes of destaining solution (water:acetic acid:methanol 87:8:5) and then left to dry in air at room temperature.

RESULTS

The LD_{50} of *N. n. kaouthia* venom was calculated to be 0.35 ± 0.01 mg/kg. In the experiment to determine venom inhibition of various plant extracts, we observed that *M. pudica* extract had 100% ability in neutralizing the $2LD_{50}$ toxicity of *N. n. kaouthia* venom in mice (Table 1). The rest of the extracts had 50% or less neutralizing ability. Mimosine, however, failed to show any activity against the venom which clearly suggested that it was not the active component of this plant responsible for the antivenom effect.

Table 1 *Naja naja kaouthia* venom inhibition by various plant extracts

Voucher No.	Plant extracts	Plant part used	Mice	
			Dead/Total	Mean weight ± SD (g)
HI 1347	*Mimosa pudica*	Root	0/8	22.2 ± 2.0
HI 1348	*Curcuma zedoaria*	Rhizome	8/8	23.8 ± 1.5
HI 1367	*Zingiber officinale*	Rhizome	8/8	22.7 ± 1.3
HI 1346	*Terminalia catappa*	Leaf	5/8	23.1 ± 1.5
HI 1343	*Phyllanthus urinaria*	Leaf	7/8	22.5 ± 1.6
HI 1351	*Curcuma xanthorrhiza*	Rhizome	7/8	23.6 ± 1.4
HI 1371	*Barleria lupulina*	Leaf	4/8	23.0 ± 1.7
HI 1350	*Curcuma mangga*	Rhizome	7/8	23.6 ± 1.2
HI 1349	*Curcuma aeruginosa*	Rhizome	4/8	22.5 ± 1.2
HI 1370	*Curcuma rubescens*	Rhizome	8/8	22.4 ± 1.2
HI 1369	*Curcuma inodora*	Rhizome	8/8	23.3 ± 1.2
HI 1372	*Melaleuca cajuputi*	Leaf	6/8	23.8 ± 1.5
HI 1368	*Allium sativum*	Seed	8/8	21.5 ± 1.5
HI 1344	*Andrographis paniculata*	Leaf	8/8	22.5 ± 1.5
HI 1373	*Ipomoea pes-caprae*	Leaf	8/8	23.0 ± 1.3
HI 1374	*Myristica fragrans*	Seed	8/8	23.8 ± 1.0
HI 1345	*Oenanthe javanica*	Leaf	8/8	20.8 ± 1.0
–	Mimosine	–	8/8	23.0 ± 2.1

In the screening of *M. pudica* extract towards the inhibition of lethality of various other venoms, we found that LD_{50} values of the venoms were 1.68 ± 0.42, 0.12 ± 0.01, 2.99 ± 0.44 and 7.59 ± 0.76 mg/kg for *O. hannah*, *B. candidus*, *B. fasciatus* and *C. rhodostoma* respectively. We also observed that *M. pudica* showed 50% and more ability in neutralizing the $2LD_{50}$ toxicity of the various venoms in mice (Table 2).

Figure 2 showed the untreated 2-DE gel (B) comprising various bioactive protein spots of *N. n. kaouthia* venom being separated based on their chemical (isoelectric point, pI) and physical characteristics (molecular weight, M_w). In comparison, the 2-DE results of the *M. pudica* fraction (MP188ECT3)-treated gel (A) showed a number of protein spots missing due to the ability of the fraction to bind directly towards some protein spots to form precipitin.

A total of 113 spots were selected and carefully picked from the 2-DE gel of the untreated control and were then tryptic-digested, extracted and analyzed with MALDI-TOF MS. Among them only 38 produced quality MALDI-TOF MS spectra. The remainder of the spots failed to give any spectra. These spots were annotated and displayed in Figure 3. A total of 14 spots were linked specifically to *N. n. kaouthia* upon identification using peptide mass fingerprinting (Table 3). Some proteins were mapped to more than one spot, for example, cobra venom factor (Q91132). This protein was identified in three spots. Figure 2(A) showed the complete disappearance of six distinctive spots, namely, NN10, NN11, NN12, NN13, NN45 and NN46 (in the rectangular box in Figure 2B) in comparison with the control gel. These spots were consequently identified to belong to isomers of phospholipase A_2 family of enzymes by means of the internet accessible ExPaSy (protein MS database). We could not establish the identity of other spots especially those at the high molecular weight region although they were found to completely disappear as well.

In the double immunodiffusion technique, a precipitin line was clearly detected between wells containing 10 mg/ml of *N. n. kaouthia* venom and 100 mg/ml of *M. pudica* extract (Figure 4). This result was also shown by its fraction MP188ECT3 at a concentration of 10 mg/ml (Figure 5).

Table 2 Various venoms inhibition by *M. pudica* extract

	Mice	
Venoms	Dead/Total	Weight ± SD (g)
Ophiophagus hannah	2/8	22.2 ± 2.0
Bungarus candidus	1/8	23.8 ± 1.5
Bungarus fasciatus	1/8	22.7 ± 1.3
Calloselasma rhodostoma	4/8	23.1 ± 1.5

DISCUSSION

It has been reported that the aqueous extract of *M. pudica* dose dependently inhibited toxic enzymes (hyaluronidase and protease activities), myotoxicity and lethality of venoms from various Indian snakes, namely, *N. naja, Vipera russelii* and *Echis carinatus* (Mahanta *et al.* 2001, Girish *et al.*

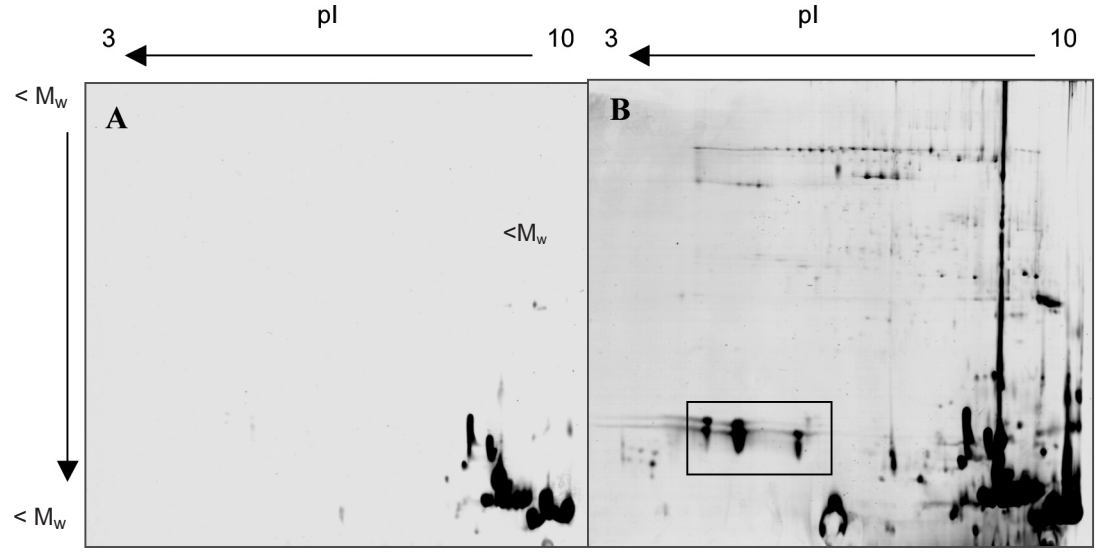

Figure 2 The 2-DE results showing a number of protein spots missing in the *M. pudica fraction* (MP188ECT3) treated gel (A) in comparison with the untreated control gel (B). Note: pI = isoelectric point; M_w = molecular weight.

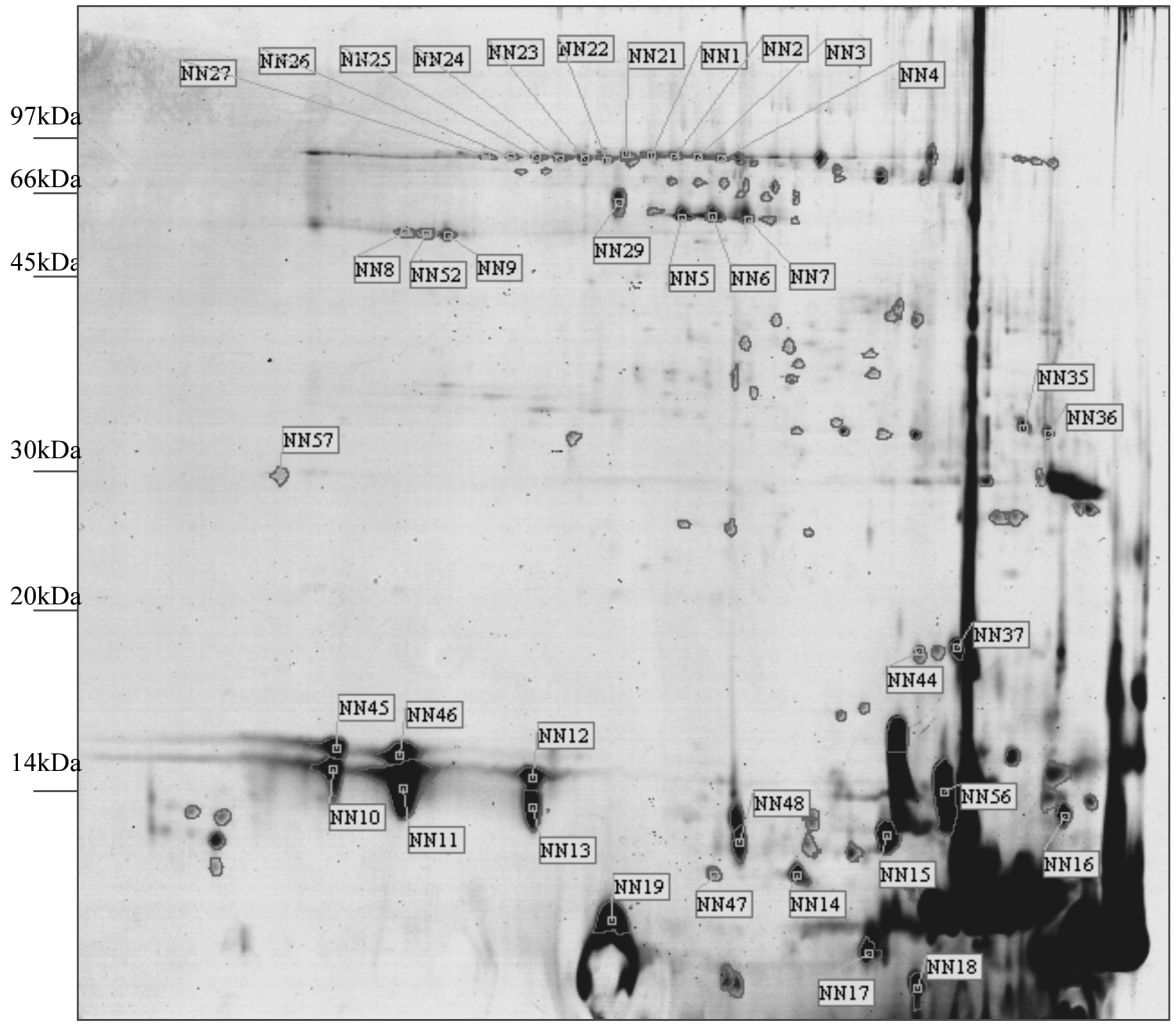

Figure 3 2-DE of *N. n. kaouthia* venom. Coomassie blue staining showing the 38 annotated spots of the venom. The spots were detected and annotated by using Image Master 2D Platinum software.

2004). Certain plants used in this study have also been reported to be snakebite antidotes based on studies by other workers. For instance, two different doses (2 or 4 g/kg) of *A. paniculata* polar extract were given to mice i.p. 30 min prior to administration of an LD_{50} (320 µg/kg) of cobra venom (*N. naja*). There was a difference in the time until death of test groups depending on the doses, i.e. after 47 min for the former and 52 min for the latter as compared with the control group which died within 7 min (Nazimudeen *et al.* 1978). We cannot make any comparison of these findings with our study as we did not evaluate the prolongation time of death of the mice. In another study, complete protection was found when an aqueous extract of fresh rhizome of a *Curcuma* sp. was given either via subcutaneous administration (s.c.) or i.p. in comparison with mice treated with venom alone (Cherdchu *et al.* 1978, Cherdchu & Karlsson 1983). The reason for differences in the results obtained between this report and our study is unclear but it could be due to the different *Curcuma* plant species used; no species identification was reported in the former and the plant was only named as 'Wan Ngu' (found native in Thailand).

Not all ethnopharmocological claims of the ability of plants in protecting against snakebite envenomation are proven. In one study, a herb known as *Clinacanthus nutans* reputed in Thailand and Malaysia to be snakebite antidote showed absence of antagonism activity against *N. n. siamensis* venom in both *in vitro* and *in vivo* experiments (Cherdchu *et al.* 1977).

In our study the MP188ECT3 fraction was found to bind with certain protein components of the venom and precipitated them. This allowed the deactivation of potent venom

Table 3 Protein from *N. n. kaouthia* venom identified by MALDI-TOF-MS peptide mapping

Spot label	Swiss-prot accession no.	Protein name	Mw (kD)	pI	Peptide masses matched	Sequence coverage
NN5	P82942	Hemorrhagic metalloproteinase kaouthiagin	44	6.7	826.652, 880.552, 981.508 1099.585, 1274.619, 1692.714	13
NN8	Q91132	Cobra venom Factor beta chain	44	5.3	991.404, 1032.455, 1176.543 1231.563, 1293.503, 1337.612 1444.661, 1489.715, 1730.807	20
NN9	"	"			991.404, 1032.455, 1176.543 1231.563, 1293.503, 1337.612 1444.661, 1489.715, 1730.807	23
NN52	"	"			1032.509, 1176.639, 1337.675 1648.712	11
NN10	P00597	Phospholipase A_2 isozyme 2	13	5.2	1157.566, 1205.631, 1697.689 1842.660	39
NN11	"	"	"	"	812.459, 1157.679, 1188.675 1205.708, 1610.861, 1697.861 1842.996	67
NN12	P00596	Phospholipase A_2 isozyme 1	13	4.9	1697.968, 1769.943, 1842.995	35
NN13	"	"	"	"	900.443, 1188.619, 1232.683 1248.657, 1697.952, 1769.923	47
NN45	"	"	"	"	900.507, 1232.659, 1842.764 2182.917	39
NN46	"	"	"		900.587, 1842.733	17
NN14	P01391	Long neurotoxin 1	8	8.6	1315.668, 1547.938	34
NN15	P82463	Muscarinic toxin-like protein 2	7	8.2	995.619, 1011.587, 1151.703 1319.716, 1576.922, 1613.858 2188.212, 2344.355	85
NN16	P82885	Thaicobrin	12	9.2	913.551, 1078.661, 1495.925 1536.046, 1811.139, 2270.485	77
NN17	P82464	Muscarinic toxin-like protein 3	8	8.1	1177.751, 2510.584	45

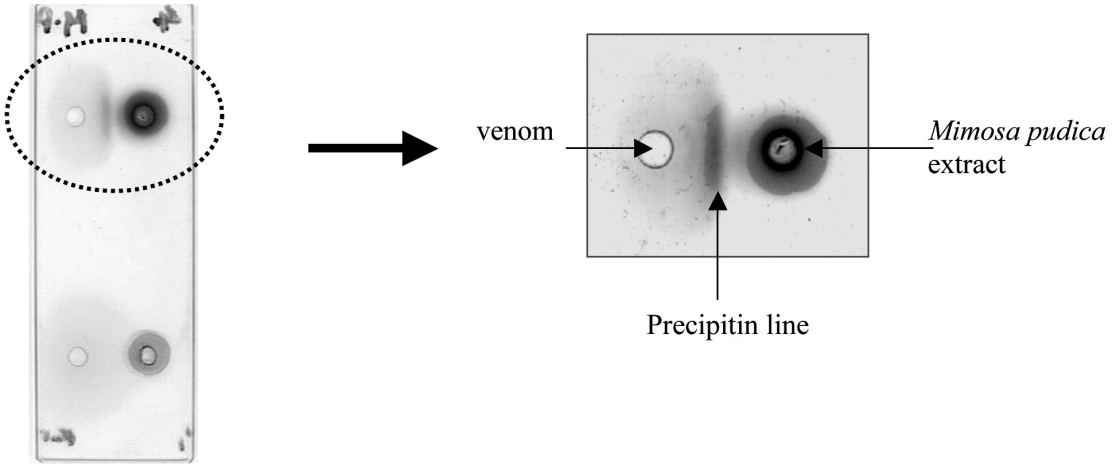

Figure 4 Precipitin line formed between *M. pudica* extract (1.5 mg) and *N. n. kaouthia* venom (0.15 mg)

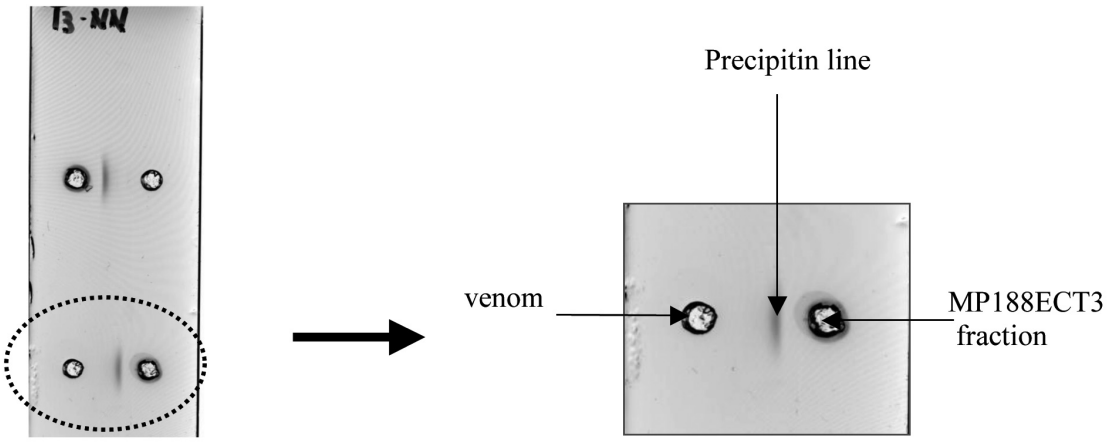

Figure 5 Precipitin line formed between MP188ECT3 fraction (0.15 mg) and *N. n. kaouthia* venom (0.15 mg)

components responsible for life-threatening conditions. Presumably, the missing protein spots of the MP188ECT3 fraction-treated 2-DE gel of *N. n. kaouthia* venom could be important functional proteins of this venom. Based on results of the MALDI-TOF MS peptide mapping a number of lethal proteins were in fact identified, e.g. hemorrhagic metalloproteinase kaouthiagin, isomers of phospholipase A_2, long neurotoxin 1 and muscarinic toxin-like proteins. Though it was not possible to identify many of the spots it was, however, possible to identify isomers (isozyme 1 and 2) of phospholipase A_2. In snakebite phospholipase A_2 enzymes, besides functioning in the digestion of the prey, also exhibit potent toxic effect such as neurotoxic, direct hemolytic, anticoagulant, myonecrotic or hemmorhagic activities (Rosenberg 1986, Soares & Giglio 2003).

We also investigated the binding capabilities of *M. pudica* and its fraction MP188ECT3 with *N. n. kaouthia* venom using the double immunodiffusion technique. Results showed clear precipitin line forming at the juncture at which high concentration of the venom and antivenom (*Mimosa* extract or its fraction) met and bound together during the diffusion on opposite directions. These results demonstrated the binding ability of *M. pudica* crude extract as well as its fraction MP188ECT3 towards *N. n. kaouthia* venom.

The root of *M. pudica* has been reported to contain approximately 10% tannin (Duke 1985). Most of the biological properties of tannins are linked to their ability to form complexes with macromolecules, particularly with proteins (digestive and other enzymes, fungal or viral proteins). Perhaps these astringent characteristics may explain the anti-snake venom activity of the aqueous extract of *M. pudica* root as the dried venom is known to contain mainly proteins (70–90%) and the protein components include enzymes and non-enzymatic proteins or polypeptide (Tu 1977). This may also explain why Mimosine, a non-tannin compound, failed to show any activity against this venom. Hence, there is a need to determine the homogeneity of the fraction and consequently to elucidate the chemical structure of the compound with the use of LCMS and NMR. The present study has demonstrated the potential of this plant as an antivenom agent of plant origin against five poisonous snake venoms and due efforts will be made to obtain pure constituent from its fraction MP188ECT3.

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