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Plant extract activities against the fibroblast cell lysis by honey bee venom

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Bee venom stings are still health problems in tropical areas. Local people use herbs as their first choice to relieve envenomation symptoms. In this study, the aqueous extracts of 22 plant species were screened for their activity against fibroblast cell lysis after *Apis mellifera* Linn. (Apidae) bee venom treatment. The venom was preincubated with plant extracts for 30 min and then added to confluent fibroblast cells for 30 min. More than 30% viable cell was obtained after treatment with venom preincubated with 0.706 mg/ml extracts of *Andrographis paniculata* (Burm. F.) Nees (Acanthaceae). Around 50% viable cells were obtained from extract treatments without venom preincubation. *Barringtonia acutangula* (L.) Gaertn. (Lecythidaceae), *Ipomoea aquatica* Forssk. (Convolvulaceae) and *Sapindus rarak* DC. (Sapindaceae) showed moderate activities (10-30% viable cells) with various activity of cell toxicity (17-52% viable cells obtained from the control). Extraction with 0, 50 and 90% ethanol solution gradually decreased activity suggesting the hydrophilic properties of the ingredients. *A. paniculata* and *I. aquatica* were confirmed to have neutralizing activity *in vivo*. Two serum enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) increased indication of acute liver dysfunction after 2 h of swarm attack in anesthetized mice. These two plant extracts were able to reduce ALT, not AST, levels in experimental mouse serum. *A. paniculata* and *I. aquatic* showed high efficiencies as bee-venom antidote with low toxicity.

Key words: *Apis mellifera*, ALT assay, AST assay, bee venom, plant extract.

INTRODUCTION

Hymenoptera stings including those of honey bees (*Apis mellifera*) remain a serious health problem especially in tropical countries (Fitzgerald and Flood, 2006; Uawonggul et al., 2007; Vetter and Visscher, 1998; Warrell, 1993; Prado et al., 2010). In Thailand, although there are no official records of envenomation, swarm attacks by bees from unintentional disturbance or accidentally stepping near their nest frequently appear as news in the mass media. For bee venom composition, melittin has been reported as the most abundant active component possessing powerful cell lytic activity, especially on red blood cell membrane resulting in hemolysis and release of haemoglobin (Ownby et al., 1997; Habermann, 1972). Other components are phospholipase A, hyaluronidase (spreading factor), vasoactive amines and unidentified proteins. Until now, antagonists and/or antidotes against these components

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are still under investigation.

Envenomation by *A. mellifera* rarely causes a serious case, and commonly dose not require intensive medical attention, except in those cases with immediate hypersensitive reactions or swarm attacks with more than 30 stings at one time. Pain, redness and swelling are well known as ordinary local symptomatic symptoms. Although many victims need not receive care, because the venom can be neutralized by their own immunity, some require symptomatic treatments since no anti-serum is available. Symptoms after massive bee envenomation are quite serious. Hemolysis, coagulopathy, thrombocytopenia and rhabdomyolysis including liver dysfunction have been reported as "delayed toxic reactions" (Kolecki, 1999).

However, for mild reactions, local medicinal plants are traditionally believed to be active as antidotes and are main choices for therapy. For herb treatment, plant extracts were originally prepared by grinding in water and/or local alcohol beverage as vehicles to prepare aqueous extract or alcoholic extracts. The extracts including solids have to be applied directly to the stung area to reduce the symptoms. However, some plants are recommended to be taken orally for higher efficiency of treatment, especially those believed to be safe enough (from their experience) such as *Andrographis paniculata*, *Barleria lupulina*, *Ipomoea aquatica*, *Impatiens balsamina* and *Jussiaea repens*. In old Thai drug recipes, nearly 100 plant species are listed as animal- or insect-bite antidotes (Daduang and Uawonggul, 2008). After analyzing, only 4 species are believed to be specific as bee venom antidote. More than 50 plants are believed to be used for general anti-insect venom purposes, without any scientific studies supporting their effectiveness.

In this study, plants recorded as insect-bite antidotes have been tested for their effectiveness in anti-bee venom activity with both *in vitro* and *in vivo* studies in order to clarify antidote activity efficiency.

MATERIALS AND METHODS

Bee venom

A. mellifera was purchased from Kasorn Honey Farm, located near Khon Kaen University, Khon Kaen, Thailand. Venom milking was manually performed using special apparatus as described previously (Eskridge et al., 1981). Briefly, upper and lower wire frames were constructed. The upper connect to one electrode of the power supply whereas the lower connect to the other electrode. The bee nest was shaken above the lower frame. After the bees fall down onto the lower frame, the upper frame was put down in close conjunction. A voltage of 100 volts was applied for 1 m/s at 10 Hz frequency using a Grass stimulator model S88. While shocking, bees released their venom onto the wire. The venom was pooled and kept at 4°C.

Plant samples and plant extracts

Plant samples (Table 1) were collected from northern and northeastern parts of Thailand and prepared as described previously (Uawonggul et al., 2006). Briefly, samples were chopped into small pieces, ground, extracted with 0, 50 or 90% ethanol in water at room temperature at a ratio of 1 g per 1 ml solvent and filtered through gauze layers, mimicking the traditional methods of healers. The extracts were kept at -65°C and thawed in a 37°C water bath before use. The extracts had been aliquoted for freeze drying using a speedvac concentrator. Freeze dried materials were weighed and the concentrations of soluble and insoluble fine particles were measured.

Chick embryonic fibroblast cell primary cultures

Cell cultures were prepared as described previously (Uawonggul et al., 2006). Briefly, after embryos were removed, chopped, and incubated with collagenase in HBSS, cells were suspended with DMEM supplemented by 5% fetal calf serum, 100 U/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate at 37°C in a 98% humidified atmosphere with 5% CO_2. Cells reached their confluency within 3 days, then they were transferred to 24-well plates. Upon reaching confluence again in the next two days, they were used for *in vitro* cell lytic assay.

In vitro cell lytic assay

Plant extracts with dilutions varying from 1:50 to 1:400 were preincubated with DMEM lacking venom (as "controls"), or with 0.6 µg/µl venom in DMEM (as "tests") at 37°C for 30 min. After medium aspiration, confluent chick embryonic fibroblast primary cells were treated with "controls", or "tests" for 30 min. Viable cells were then harvested using 10 units/ml collagenase, stained with 1% trypan blue in PBS (1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, 135 mM NaCl and 2.5 mM KCl) and counted by hemocytometers under low magnifying power microscopes.

$$\text{Percentage of viable cells} = \frac{\text{Number of cells left in "test" or "control"}}{\text{Total cell number}} \times 100$$

Experiments were done in triplicate and plotted as dilution curves. Plant extracts at concentrations of 0.706 mg/ml, after freeze drying, were preincubated with "controls" or "tests" at 37 °C for 30 min before cell counting and analyzing as described above. For ethanol extraction, ethanol had to be completely vaporized prior to adding to water and suspended before adding to the cells.

In vivo venom neutralization assay

Three Swiss Webster female mice, 20-25 g body weight were taken care of under standard treatments. They were anesthetized by 2.5 mg/kg body weight ketamine prior to being stung by a swarm of 10 bees. For test mice, 30 min before swarm attacking, they were fed by oral route with 2 g/kg body weight of plant extract dissolved in 0.2 ml PBS buffer, pH 7.4. After stinging, the mice were left for 2 h, terminated and blood collected for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assay. The assays were made by routine procedures at 37°C on a Cobas 6000 analyzer with commercial test kits from Roche Diagnostics. All statistical analyses were performed using SPSS (version 15.0). Mean levels of liver enzyme were compared between study groups using one way ANOVA. The results were considered statistically significant when $P < 0.05$.

Table 1. Plant species used for anti-bee venom screening.

Scientific name	Family	Local name	Organs used
Andrographis paniculata (Burm. f.) Nees	Acanthaceae	Fa tha laai joan	Leaf
Barleria lupulina Lindl.	Acanthaceae	Salet phangphon tua poo, Chong ra ar	Leaf
Barringtonia acutangula (L.) Gaertn.	Lecythidaceae	Kra don num, Jik na	Stem bark
Clerodendrum indicum (L.) Kuntze	Verbenaceae	Tao yai mom	Leaf
Clinacanthus nutans (Burm.) Lindau	Acanthaceae	Salet phangphon tua mea, Paya yo	Leaf
Coccinia grandis (L.) Voigt	Cucurbitaceae	Tam lueng	Leaf
Cucurma longa L.	Zingiberaceae	Kamin chun	Rhizome
Curcuma cf. *zedoaria*	Zingiberaceae	Wan Paya Ngoo Tua Mea	Rhizome
Gynura pseudochina (L.) DC.	Asteraceae	Wan maha garn	Leaf
Ipomoea aquatica Forssk.	Convolvulaceae	Pak bung	Leaf
Ipomoea pes-caprea .Br.R (.Linn)	Convolvulaceae	Pak bung tha lay	Leaf
Justicia gendarussa .f .Burm	Acanthaceae	San pra hom	Whole plant
Ludisia discolor .Rich .A (Ker Gawler)	Orchidaceae	Wan ron thong	Rhizome
Mesua ferrea L.	Guttiferae	Bun nak	Leaf
Momordica cochinchinensis (Lour.) Spreng	Cucurbitaceae	Fuk khao	Root
Muehlenbeckia platyclados Meissn (.Muell.b.F)	Polygonaceae	Takhab hin	Whole plant
Parameria laevigata (Juss.) Moldenke	Apocynaceae	Krua sude	Leaf
Passiflora edulis Sims	Passifloraceae	Lin mungkorn	Leaf
Rumex sp.	Polygonaceae	Pak gard som	Whole plant
Sapindus rarak DC.	Sapindaceae	Pra kam dee kwai	Leaf
Trigonostemon reidioides Craib	Euphorbiaceae	Lod thanong	Root
Vitex trifolia L.	Verbenaceae	Kon tee so ta lay	Root

RESULTS

Medicinal plants

Plants used in this study were clear-cut in 3 categories: (1) those generally recognized as bee venom and/or other venom antidotes in traditional records, (2) those available in the area of the Northern and Northeastern Part of Thailand, and (3) those clearly identified with scientific names. Thus, 22 plant species from 14 Families were selected. All plants were extracted by 0% ethanol (as aqueous extract), 50 and 90% ethanol. Aqueous extract (without ethanol) mimicked traditional methods by healers with the least possibility of affecting the efficacy of extracts. However, 50% extraction was intended to mimic traditional methods that sometimes using local traditional alcohol beverages (with about 30-50% ethanol concentration; Daduang and Uawonggul, 2008), whereas 90% ethanol was the method to try to extract nearly all extractable components from the plants.

In vitro cell lytic assay

Twenty-two extracts were used for this study. Nine extracts were reported as results from 2 fold dilution before cell treatment whereas 13 plant extracts were reported as end-point determination. Results showed that 0.6 µg/µl of bee venom completely lysed cells within 20 min in a 98%

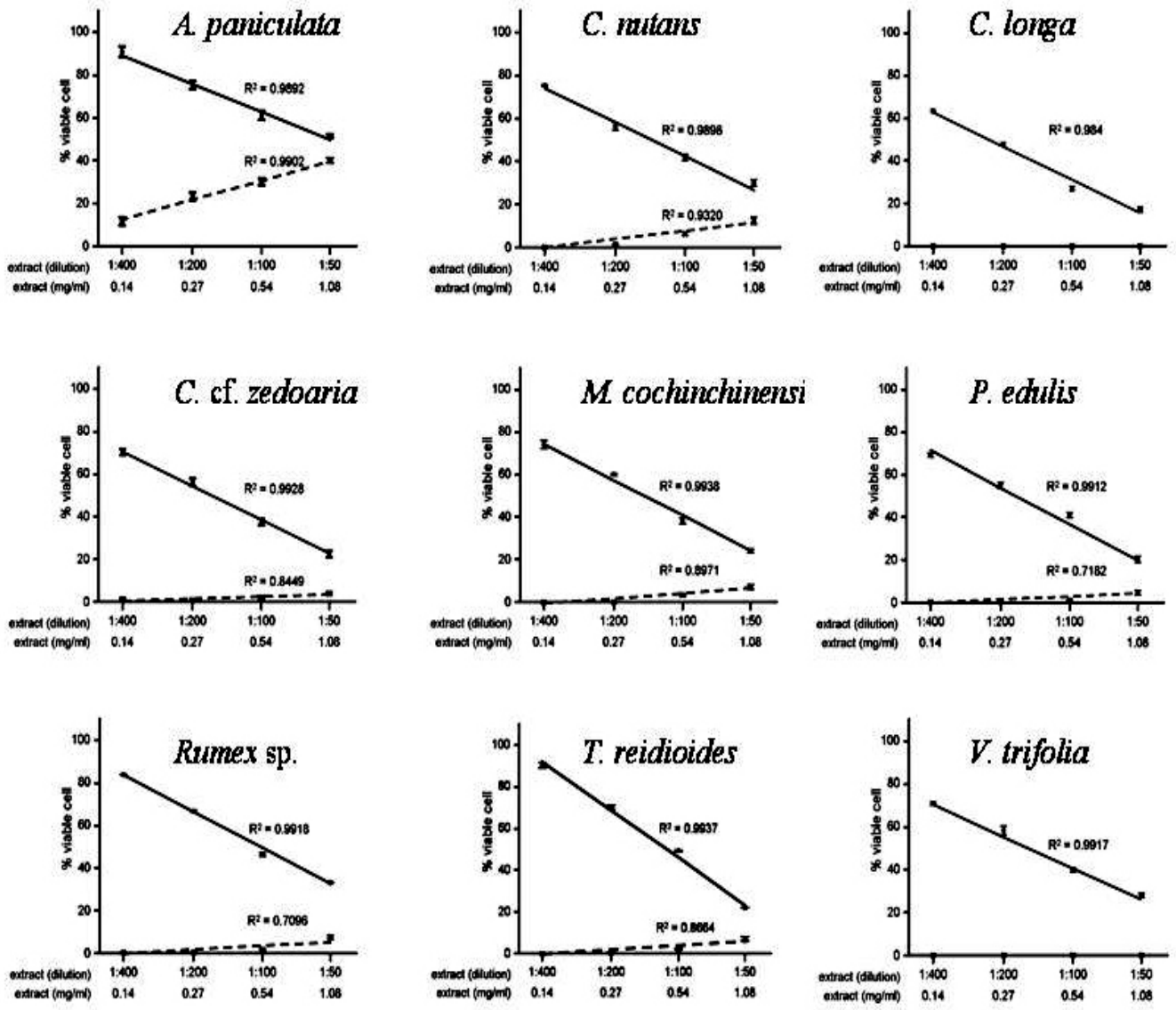

Figure 1. Viability of fibroblast cells after 30-min treatment with controls (dash line) or with plant extracts (as indicated in each panel) preincubated with *A. mellifera* honey bee venom (solid line). Plant extracts at various concentrations were 30 min preincubated with DMEM (as controls), or with 0.6 µg/µl venom diluted in DMEM (as tests) at 37 °C. Confluent cells were treated with "controls", or "tests" for 30 min before viable cell counting.

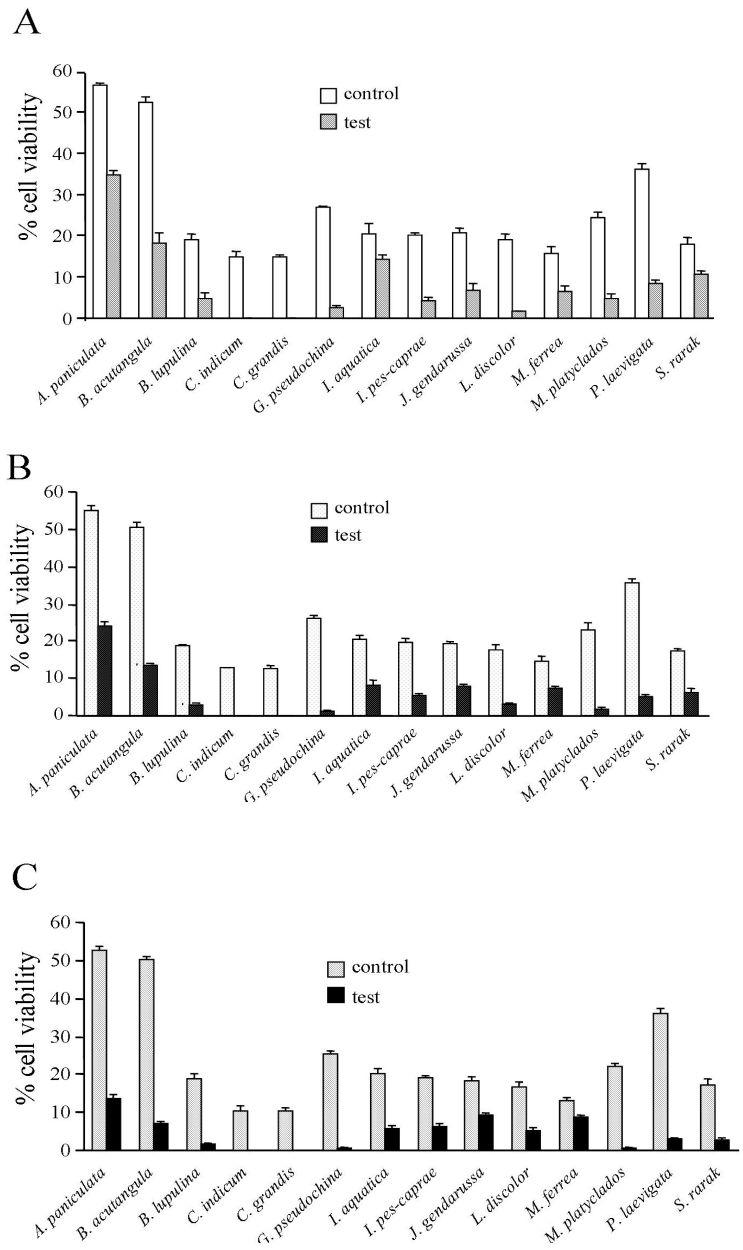

Figure 2. Viability percentage of fibroblast cells after 30-minute treatment with plant extracts as "control", or 0.706 mg/ml plant extracts preincubated with *A. mellifera* venom as "test". Plant samples were extracted with 0% (A), 50% (B) and 90% (C) ethanol.

humidified 37°C incubator without any viable cells observable. Results from nine plant extract incubations were plotted using log[dilution] along X axis versus percentage of viability on the Y axis (Figure 1). The coefficient of linear regression (R^2) of the straight lines approached 1.00, indicating the high reliability of the assay. Aqueous extract of *A. paniculata* exhibited high anti-bee venom activity (> 30% viable cell obtained from the "test") with low cytotoxic effects (> 50% viable cell obtained from the "control") at 0.706 mg/ml plant extracts. *Barringtonia acutangula*, *I. aquatica* and *Sapindus rarak* showed moderate activities (10-30%) with various activities of cell toxicity (17-52%) whereas low (< 10%) or no activities were obtained from the 18 remaining aqueous extracts.

Fourteen plant extracts representative of high, moderate and low activities were selected for further extraction using 50 and 90% ethanol solution (Figure 2). However, their activity gradually decreased while the concentration of ethanol increased. For example, activity

Table 2. Effects of bee venom against hepatotoxicity in mice through serum transaminase in *in vivo* venom neutralization assay.

Treatment	ALT (U/L)	ALT (U/L)	AST (U/L)	AST (U/L)
	Mean±SD	Median	Mean±SD	Median
control	26±10.09[a]	26.5	199±48.08[a]	193
bee venom	193±86.12[b]	191.5	1044±292.20[b]	1013
A. paniculata	44±8.92[a]	45.5	211±57.47[a]	228
I. aquatica	43±9.48[a]	40	152± 28.50[a]	152
bee venom ± *A. paniculata*	118±71.05[c]	111	938± 352.15[b]	1025
bee venom ± *I. aquatica*	113±36.32[c]	112.5	1059± 69.29[b]	1108

Level of significance was at $p < 0.01$ using one way ANOVA. The significant difference was represented by different superscript alphabet as a, b or c

of *A. paniculata* decreased from about 34 (0% ethanol), (Figure 2A) to 24 (50%), (Figure 2B) and 13 (90%), (Figure 2C). Among active ingredients clarified as chemicals for therapeutics, most of the components in the samples are believed to dissolve in 90% ethanol solution. After ethanol extract incubation, their activity gradually decreased while concentration of ethanol increased. This indicates the hydrophilic properties of the active ingredients. Their molecular structures were affected by ethanol. Moreover, herb preparation using alcohol beverage as vehicles during drug preparations among traditional healers may be misunderstood. These results contrast to the belief in the traditional healing world.

In vivo venom neutralization assay

Arthropod envenomation affects the responses of many systems. Kidney, muscle and liver functions are affected (Prado et al., 2010). Many reported liver dysfunction after bee swarm attacks. França et al. (1994) reported the elevation of many serum enzymes including AST and ALT in five males, after being attacked by swarming African honey bees (*A. mellifera scutellata*) in Brazil. Grisotto et al. (2006) reported the increase of AST, whereas serum ALT remained stable, in rats, 24 h after injection with 0.5 mg/kg bee venom. In order to clarify the activity of the plants, *in vivo* experiments using animals have been performed. Two plants had different activities, *A. paniculata*, with high activity, and *I. aquatica*, with moderate activity, from *in vitro* cell lytic assay, were further ingested orally by bee swarmed mice. Plant extracts were intentionally applied to the experimental mice by oral administration, since dose absorbed by topical preparation was uncertain. Actually traditional healers always treat patients with both application of plant extract to the stung site and oral administration. As described by Daduang and Uawonggul (2008), many plant extracts, believed to be safe enough for human, were administrated both orally and topically to assure the efficiency of herb extracts to relieve envenomation effects. For safety, these two plants are widely well-known to be non-toxic, especially *I. aquatica* is a common vegetable of daily cooking.

Neutralization was indicated by ALT and AST serum level determination (Table 2). After anesthetic mice were swarm attacked for 2 h, bee venom significantly increased ALT and AST level from 26 and 199 U/L to around 193 and 1044 U/L, respectively. Plant extracts alone did not show significantly acute toxicity effects on the liver (44 and 211 for *A. paniculata* and 43 and 152 U/L for *I. aquatica*, respectively). ALT values decreased to 118 and 113 U/L in the test groups with herb treatments. However, these plants showed insignificant results to decrease AST value after 2 h of bee attacks, 938 U/L for *A. paniculata* and 1059 U/L for *I. aquatica* but no elevation of creatinine (data not shown). As basically known, ALT is specifically found from liver cell damage whereas AST can be released from muscle cell, red blood cell and liver cells after damage of these organs. Thus, bee venom had an acute effect on liver cell membrane permeability resulting in ALT and AST secretion but no acute renal failure in experimental mice. Reduction in level of ALT after herb treatment showed the efficiencies of herbs as antagonists against bee venom action on liver function.

Plants with anti-venom activity

Plants with activity against venom actions have been summarized from literature previously reported including this study (Table 3). Antidote activity is expressed in 3 levels, high, moderate and low with different definition corresponding to methods of each study. Four plants *A. paniculata*, *B. acutangula, I. aquatica* and *S. rarak* have activity in this study corresponding to old drug records. Only *B. lupulina* and *C. nutans* are specifically recommended for bee venom treatment in the old records, but expressed low activity in this study. However, the remaining exhibited low or no activity in this study.

DISCUSSION

Bee envenomation is still serious. Devastation of forest results in the decrease of bee habitat. Their adaptation to living surrounding us promotes the accident of bee sting

Table 3. Conclusion of herb activities for snakebite and insect-bites

Plant species	Anti-animal bite activities recommended by Daduang and Uawonggul (2008)	Anti-snake venom activities reported by Daduang et al. (2005)[1]	Anti-scorpion venom activities reported by Uawonggul et al. (2006)[2]	Anti-bee venom activity reported in this study[4]
A. paniculata	Snake and insect-bite	High	High	High
B. lupulina	Snake and insect-bite especially for ant, bee, centipede, mosquito and scorpion	High	No	Low
B. acutangula	Insect-bite, snakebite	-	High	Moderate
C. indicum	Insect-bite	-	No	No
C. nutans	Insect-bite especially for ant, bee, catfish, centipede, hornet, jellyfish, millipede, mosquito, scorpion and wasp	Moderate	Moderate	Low
C. grandis	Snakebite	-	No	No
C. longa	Insect-bite especially for fire ant	High	No	No
C. cf. *zedoaria*	Snakebite	High	No	Low
G. pseudochina	Insect-bite	High	Low	Low
I. aquatica	Snake and insect-bite especially for centipede	-	Moderate[3]	Moderate
I. pes-caprea	Jellyfish	-	Low	Low
J. gendarussa	Insect-bite	-	Low	Low
L. discolor	Insect-bite	-	Low	Low
M. ferrea	Insect-bite	-	Low	Low
M. cochinchinensis	Insect-bite	-	Low	Low
M. platyclados	-	-	Low	Low
P. laevigata	-	-	Low	Low
P. edulis	-	-	Low	Low
Rumex sp.	Insect-bite	-	Moderate	Low
S. rarak	Insect-bite	-	Moderate	Moderate
T. reidioides	Insect-bite	-	Low	Low
V. trifolia	Jellyfish	-	Low	No

[1] Plant extracts with dilution at 1:12.5 for anti-*N. n. siamensis* venom screening. High was more than 50% inhibitory effect obtained; moderate, 20-50%; low, less than 20%; no activities, 0% activity. [2] 0.706 mg/ml plant extracts were used for cell lytic test for anti-*H. laoticus* venom screening. High was more than 30% viable cell obtained; moderate, 10-30%; low, less than 10%; no activities, 0% activity. [3] For *I. aquatica*, 0.406 mg/ml plant extracts were used for cell lytic test for anti-*H. laoticus* venom screening. [4] 0.706 mg/ml plant extracts were used for cell lytic test for anti-*A. mellifera* venom screening. High was more than 30% viable cell obtained; moderate, 10-30%; low, less than 10%; no activities, 0% activity.

including swarming attacks. Therapy, if needed, is symptomatic. Traditional therapy is still popular from generation-to-generation knowledge transfer. Thus, in this study, 22 plant species, most reported in the old recipes as venom antidotes, were collected for investigation of their potency against cell lysis from *A. mellifera* bee venom in the laboratory using an *in vitro* cell lytic assay method. Two plants with different activities were further studied *in vivo* by neutralization assay. The study intended to use advanced techniques to clarify traditional knowledge and make the possibility of finding out any new chemicals for envenomation therapeutics.

However, only 1 from 22 species showed high activity against bee venom with more than 30% viable cells obtained after cell treatment with the venom pre-incubated with plant aqueous extracts. Three plants showed lower activities as bee venom antidotes whereas 18 plants did not exhibit significant activities.

A. paniculata, generally known as insect bite antidote, showed highest activity in this assay. It has been reported to be highly active against *Heterometrus laoticus* scorpion venom *in vitro* cell lytic assay, as well (Uawonggul et al., 2006), implying its multi-action against many kinds of venom possessing different mechanisms of action. It is known that scorpion venom has cell lytic activity from its clear potassium channel blocker activity, whereas bee venom mainly takes action by phospholipase, to degrade phospholipid components on cell membrane. Many reports show its actions to relieve many health problems in the world of traditional healers without relating activities. For example, a variety of fever-related illnesses, digestive problems, dysentery, cholera, diabetes, upper respiratory infection, influenza, bronchitis, gonorrhoea, dyspepsia, helmintic and antipyretic symptoms are reported as targets, as well as to activate liver function and assist against jaundice (Kligler et al., 2006). However, no suitable theories can explain these phenomena.

B. lupulina, with local name “Salet Phangphon Tua Poo” meaning “Male Mongoose Saliva”, is widely reputed as an anti-elapid snakebite treatment in the tropical areas of Southeast Asia and South America (Kanchanapoom et al., 2001; Lans et al., 2001). Although high activity has been seen after anti-elapid venom assay, it shows no activity against scorpion venom (Uawonggul et al., 2006) and activity was quite low in this study. However, it is reported as an anti-inflammatory and analgesic agent (Suba et al., 2005). Thus, therapeutic efficiencies were possibly from anti-inflammatory action.

B. acutangula, I. aquatica and *S. rarak* showed moderate activity (10-30% of cell viability) in this study. These 3 species have been reported as having significant activity against *H. laoticus* venom, as well. Additionally, *B. acutangula* is quite well-known in the local healer for snakebite therapy in Thailand, but until now, no suitable studies have given scientific support.

C. nutans, although well-known as anti-snake venom among traditional healers with local name “Salet Phangphon Tua Mea” meaning “Female Mongoose Saliva”, does not show any activity against neuromuscular transmission block by *Naja naja siamensis* cobra neurotoxin in isolated rat phrenic-nerve diaphragm preparations (Cherdchu et al., 1977). It shows moderate activity in anti-*N. n. siamensis* venom analysis using modified ELISA technique (Daduang et al., 2005). Although it is specifically recommended for bee venom in the old Thai recipes, in this study, contrary to anti-scorpion venom activity, it exhibited low activity.

Curcuma zedoaria cf.*,* one of the most famous anti-king cobra venom antidotes, being used among people in the “King Cobra Village” in Khon Kaen Province, Thailand, was investigated. It local name is “Wan Paya Ngoo Tua Mea” meaning “Queen of Snakes rhizome” implying its potential to be an anti-snake venom antidote. However, local people recognize it as a general antidote for venom from many venomous animals. Although it possesses a high possibility to be a snakebite antidote (Daduang et al., 2005), it showed completely negative results both as anti-scorpion venom and anti-bee venom agents in this study. Toxins in elapid are apparently approved to be neurotransmission blockers at the acetylcholine receptors whereas those of scorpion and bee venom are completely different. This study indicates that therapies have to be different for toxin from different animal venoms. *Mesua ferrea* is reported to be anti-inflammatory (Hutt and Houghton, 1998) and an analgesic drug (Hassan et al., 2006). The efficiency of treatment from this plant in traditional records may arise from its many modes of action.

In this study, herbs alone did not show any acute effects to ALT and AST values after orally administrated to experimental mice. ALT, not AST, values were decreased after *A. paniculata* and *I. aquatica* extract treatments to swarm attacked mice suggesting that although AST and ALT could be indications for hepatocellular necrosis, AST was ordinarily not only from liver, but heart muscle, striated muscle, pancreas including many organs which release the enzyme as original sources, as well. Damage to these organs results in increases in the level of AST in blood circulation. Thus, it can be concluded that extract from *A. paniculata* and *I. aquatica* had a tendency to neutralize toxicity of bee venom in mouse liver.

For herb treatment for animal bites or stings, people always used one herb for venom from different species. With their poor knowledge, they recognized one kind of herb to be universal at relieving symptoms from venom of many animal species. Much evidence shows that venom can be neutralized by the defense mechanism of the body, without any effects from herb treatment, resulting in misunderstanding of those plants as an antidote (Daduang and Uawonggul, 2008). Since the main components in venom are peptides and proteins with the most delicate structure, pH or any uncomplicated factors

can have any effect, resulting in confusion of their actions. Many times, data from the traditional healers is obvious.

Traditional healers prefer to keep this knowledge with them for their own profit. Data is always lost without any records after their passing away. Advanced scientific experiments are necessary to clarify these plant activities.

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