ฤทธิ์ต้านพิษงูเห่าโดยสารสกัดโลดทะนงแดงและเมล็ดหมากในหนูถีบจักร

นางสาวอรุณรัตน์ ศรีทำมา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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NEUTRALIZATION OF NAJA KAOUTHIA VENOM BY TRIGONOSTEMON REIDIOIDES CRAIB. AND ARECA CATECHU LINN. EXTRACT IN MICE

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy in Pharmacology

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Thesis Title	Neutralization of *Naja kaouthia* venom by *Trigonostemon reidioides* Craib. and *Areca catechu* Linn. extract in mice
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Field of Study	Pharmacology
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อรุณรัตน์ ศรีทำมา : ฤทธิ์ต้านพิษงูเห่าโดยสารสกัดโลดทะนงแดงและเมล็ดหมากในหนูถีบจักร (NEUTRALIZATION OF *NAJA KAOUTHIA* VENOM BY *TRIGONOSTEMON REIDIOIDES* CRAIB. AND *ARECA CATECHU* LINN. EXTRACT IN MICE) อาจารย์ที่ปรึกษา : รศ.ดร. พรเพ็ญ เปรมโยธิน, อาจารย์ที่ปรึกษาร่วม : นฤมล พักมณี, 84 หน้า. ISBN 974-17-5743-3

วัตถุประสงค์ของการศึกษานี้เพื่อทดสอบฤทธิ์ของสารสกัดโลดทะนงแดงและเมล็ดหมากในการยับยั้งพิษที่ก่อให้เกิดการตายและการทำลายกล้ามเนื้อของพิษงูเห่าไทย (*Naja kaouthia*) ในหนูถีบจักร ในการศึกษาฤทธิ์การทำลายพิษงูเห่า ใช้สารสกัดน้ำที่ได้จากโลดทะนงแดงหรือเมล็ดหมากหรือที่ผสมกัน และกรองก่อนใช้ นำมาผสมกับพิษงูเห่า และแช่ไว้ในอุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 1 ชั่วโมง ก่อนที่จะให้หนูถีบจักรโดยการฉีดเข้ากล้าม พบว่าสารสกัดจากเมล็ดหมากหรือสารสกัดจากโลดทะนงแดงผสมกับเมล็ดหมาก มีฤทธิ์การทำลายพิษงูเห่า สารสกัดจากเมล็ดหมากในขนาด 0.2 มิลลิกรัม/ตัวหนู สามารถป้องกันการตายได้ 100 เปอร์เซ็นต์ เมื่อให้พิษงูเห่าในขนาดที่ทำให้ตายทั้งหมด (LD_{100}, 8 ไมโครกรัม/ตัวหนู) สารสกัดของสมุนไพรผสม (โลดทะนงแดง:เมล็ดหมาก) ในขนาด 2.4:0.8 มิลลิกรัม/ตัวหนู สามารถยืดเวลาการรอดชีวิตของหนูถีบจักรที่ได้รับพิษงูเห่าในขนาด LD_{100} โดยทำให้การรอดชีวิตเพิ่มขึ้นจาก 0 เปอร์เซ็นต์ เป็น 66.67 เปอร์เซ็นต์ สารสกัดโลดทะนงแดงไม่มีฤทธิ์การทำลายพิษงูเห่า

ในการศึกษาฤทธิ์ต้านการตายจากพิษงูเห่า ใช้สารสกัดน้ำ (กรอง/ไม่กรอง) และสารสกัดเอทานอลที่ได้จากสมุนไพรเดี่ยวหรือสมุนไพรผสม ให้โดยการป้อนในหนูถีบจักร ที่เวลา 1, 2 หรือ 3 ชั่วโมง หรือ ก่อน ทันที หรือ หลังทันทีที่ฉีดพิษงู (ในขนาด 6 ไมโครกรัม/ตัวหนู) พบว่าสารสกัดน้ำจากสมุนไพรผสม (ไม่กรอง) เท่านั้นที่มีฤทธิ์ต้านการตายจากพิษงูเห่า เมื่อให้ในขนาด (โลดทะนงแดง:เมล็ดหมาก) 0.6:0.2 มิลลิกรัม/ตัวหนู และ 1.2:0.4 มิลลิกรัม/ตัวหนู ทำให้เปอร์เซ็นต์การรอดชีวิตเพิ่มขึ้นจาก 6.25 เปอร์เซ็นต์ ของหนูควบคุม เป็น 18.75 เปอร์เซ็นต์ และ 31.25 เปอร์เซ็นต์ ตามลำดับ โดยให้สารสกัดสมุนไพรผสม 1 ชั่วโมง ก่อนการให้พิษงูเห่า และยังพบว่าสารสกัดน้ำนี้ในขนาด 1.2:0.4 มิลลิกรัม/ตัวหนู ทำให้การทำงานของเอ็นไซม์ ครีเอทีน ฟอสโฟไคเนส ที่ถูกกระตุ้นให้เพิ่มขึ้นด้วยพิษงูเห่า (4 ไมโครกรัม/ตัวหนู) มีค่าลดลงจาก 2632 ± 498 Units/L ไปเป็น 585 ± 139 Units/L โดยสรุปสารสกัดสมุนไพรผสมของโลดทะนงแดงและเมล็ดหมาก สามารถยับยั้งการตายและการทำลายกล้ามเนื้อจากพิษงูเห่าได้

ภาควิชา	เภสัชวิทยา	ลายมือชื่อนิสิต.....
สาขาวิชา	เภสัชวิทยา	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา	2546	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEY WORD : *NAJA KAOUTHIA* VENOM / ANTI-SNAKE VENOM / *TRIGONOSTEMON REIDIOIDES* CRAIB. / *ARECA CATECHU* LINN. / MEDICINAL PLANTS

ARUNRAT SRITHAMMA : NEUTRALIZATION OF *NAJA KAOUTHIA* VENOM BY *TRIGONOSTEMON REIDIOIDES* CRAIB. AND *ARECA CATECHU* LINN. EXTRACTS IN MICE. THESIS ADVISOR : ASSOCIATE PROFESSOR PORNPEN PRAMYOTHIN, Ph.D., THESIS COADVISOR : NARUMOL PAKMANEE, M.Sc. 84 p. ISBN 974-17-5743-3

The aim of this study was to investigate the inhibitory effects of *Trigonostemon reidioides* Craib. and *Areca catechu* Linn. on lethality and myotoxicity of *Naja kaouthia* venom in mice. In neutralization study, the filtrate of water extracts from each plant or mixed-plants was preincubated with *N.kaouthia* venom at 37°C, 1 hr prior to intramuscular injection to mice. It was found that water extract from *A.catechu* or mixed-plants showed neutralization activity. *A.catechu* extract at dose 0.2 mg/mouse can protect 100 % of mice receiving the LD_{100} dose (8 μg/mouse) of *N.kaouthia* venom. Mixed-plant extract (*T.reidioides*:*A.catechu*) at a dose ratio of 2.4:0.8 mg/mouse prolonged the survival time of mice receiving LD_{100} of venom. It increased % survival of mice from 0% to 66.67%. The extract of *T.reidioides* did not have the neutralization activity.

In anti-lethal activity study, water extract (with/without filtration) and ethanol extract from each plant or mixed-plants were administered orally at 1, 2 or 3 hr or immediately before or immediately after injection of *N.kaouthia* venom (6 μg/mouse). When given 1 hr prior to the venom injection, only the unfiltered water extract of mixed-plants (*T.reidioides*:*A.catechu*) at a dose ratio of 0.6:0.2 mg/mouse and 1.2:0.4 mg/mouse increased % survival of mice from 6.25% of control to 18.75% and 31.25%, respectively. This water extract at dose 1.2:0.4 mg/mouse also decreased creatine phosphokinase (CPK) activity induced by *N.kaouthia* venom (4 μg/mouse) from 2632 ± 498 units/L to 585 ± 139 units/L. In conclusion, preparation of *T.reidioides* and *A.catech*u can inhibit lethality and myotoxicity of *N.kaouthia* venom.

Department	Pharmacology	Student's signature ……
Field of study	Pharmacology	Advisor's signature……
Academic year	2003	Co-advisor's signature……..

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LIST OF ABBREVIATION

AChR	=	Acetylcholine recepter
A.catechu	=	*Areca catechu* Linn.
N.kaouthia	=	*Naja kaouthia*
T.reidioides	=	*Trigonostemon reidioides* Craib.
g	=	gram
cm^2	=	centimeter square
μg	=	microgram
kg	=	kilogram
mg	=	milligram
min	=	minute
ml	=	milliliter
L	=	liter
w/v	=	weigh by volume
/	=	per
%	=	percent
sp.	=	specie
i.v.	=	intravenous
i.m.	=	intramuscular
i.d.	=	intradermal
i.p.	=	intraperitoneal
LD_{50}	=	fifty percent lethal dose
LD_{100}	=	one hundred percent lethal dose
hr	=	hour
s.c.	=	subcutaneous
C	=	degree Celsius

LIST OF ABBREVIATION (CONTINUED)

CPK	=	creatine phosphokinase
SE	=	standard error
ethanol 28^{o}	=	28 percent ethanol
U	=	Unit
g	=	centrifugation force unit (gravity)
RBC	=	red blood cell

CHAPTER I
INTRODUCTION

Snakebite is one of the most important public health problems of tropical countries including Thailand. Approximately 7,000 cases of snakebites are reported annually. The actual number of bites may be much higher because many events were not reported.

There are 175 species and subspecies of snakes native to Thailand, that 56 are very venomous (Cox, 1991). The most medically important venomous snakes in Thailand are in 2 families: Elapidae and Viperidae. Among the family Elapidae, there are three species of the genus *Naja* (cobras): *Naja kaouthia* (monocle cobra), *Naja siamensis* (spitting cobra), *Naja sumatrana*; two species of the genus Bungarus (kraits): *Bungarus candidus* (Malayan krait) and *Bungarus fasciatus* (Banded krait); and the king cobra (*Ophiophagus hannah*) of the genus Ophiophagus. Other Elapidae snakes in Thailand include sea snakes and Asian coral snakes of the genus *Calliophis*. They have potent venoms but rarely bite humans. Family Viperidae is divided in two subfamilies: Viperinae has one specie, *Daboia russelli siamensis* (Siamese Russell's viper); and Crotalinae has two species: *Calloselasma rhodostoma* (Malayan pit viper) and *Trimeresurus albolabris* (green pit viper) (Lawan Chanhome et al., 1998).

Snake venoms are complex mixtures. Most of the constituents are proteins, but low molecular weight compounds such as peptides, nucleosides and metal ions are also present. The death of the prey is due to respiratory or circulatory failure caused by various neurotoxins, cardiotoxins, coagulation factors and other substances acting alone or synergistically.

The cobra is a common poisonous snake found throughout Thailand. It is once an important cause of death from snakebite in Thailand. The cobra is considered very dangerous and produces systemic poisoning because rapid action of neurotoxin causes respiratory paralysis and death. Their toxins are composed of neurotoxins,

cardiotoxins, enzymes and proteins (Sivamogsthem and Tejasen, 1973). In addition to the respiratory crisis, local reaction of the bitten site is also a serious problem. Though not life threatening, the local reaction may prolong the duration of hospitalization and it may increase morbidity in some cases (Prapai Pongprasit et al., 1988).

Antivenom is the most recommended and the mainstay of treatment for snake envenomation. The most effective of antivenom is monospecific. A species diagnosis must be made before the right treatment can be chosen. Frequently, it is difficult to get the right diagnosis. In addition, clinical signs of snake envenomation are complicated so, sometimes medical staff misinterpreted and gave inappropriate antivenom (Sornchai Looareesuwan, Chaisin Viravan and Warrell, 1988). Furthermore, the antivenom carries a risk of severe adverse reaction. Antivenom reaction occurred usually more than 20% by developing an early or late reactions (Warrell, 1999: 52-53) that could be lethal. Besides, antivenom sometimes does not provide enough protection against venom-induced hemorrhage and nephrotoxicity, which is an important cause of death (Sutherland, 1977; Corregan et al., 1987; Gilon, Shalev and Benbassat, 1989; Warrell, 1989) and local tissue damage (Leon et al., 2000). Skin testing is necessary before antivenom is administered.

Traditional treatment of snakebite has been used worldwide. Many medicinal plants are recommended against snakebite. Lists of such plants can be found in literature (Houghton and Osibogun, 1993; Singh, Rughubanshi, and Sing, 2002; Kshirsagar and Singh, 2001). Many Thai medicinal plants were recommended such as *Sansevieria metallica* (Agavaceae), *Eucharis grandiflora* Planch. (Amaryllidaceae), *Curcuma aeruginosa* Roxb. (Zinguberaceae), *Boesenbergia petiolata* Sirirugsa (Zingiberaceae), *Trigonostemon reidioides* (Euphorbiaceae), *Crinum rubra* (Amaryllidaceae), *Typhonium trilobatum* (Araceae), *Cleome viscose* Linn. (Capparidaceae), *Clinacantus nutans* Burm., *Curcuma sp.* (Zingiberaceae) (มาลี บรรจบ และคณะ ,2543; สมหมาย กระจ่างลิขิต และคณะ, 2525; พระเทพวิมลโมลี, 2522). Only two plants were tested scientifically: *Clinacanthus nutans* Burn. and *Curcuma sp.* (Zingiberaceae) (Cherdchu et al., 1977; Cherdchu and Karlsson, 1983; Panee Tejasen, Amphawan Chantaratham and Duangta Kanjanapothi ,1969, 1969; Panee Tejasen and

Pairojana Sapavajit, 1970; Panee Tejasen and Laddawan Sunyapridakul, 1970; Amphawan Chantaratham and Panee Tejasen, 1970; Panee Tejasen and Chatchawadee Thongtharb, 1978).

The combined preparation of root of Lot thanong daeng (*Trigonostemon reidioides* Craib.) and betel nut (*Areca catechu* Linn.) has been used against snakebite by folk healer in Surin province. Physician at Kabchoeng Hospital in Surun province has used this preparation to treat patients with snakebite for more than 10 years. However, their actions against snakebite are still unclear scientifically. Sombat Prabhawicha (สมบัติ ประภาวิชา, 1998) reported that water extract from root of Lot thanong daeng prolonged survival time when administered orally after 5 minutes of snake envenomation in mice.

Chemical constituents in Lot thanong daeng root were reported previously. They are a mixture of steroid palmitate (β-sitosteryl palmitate, stigmasteryl palmitate, campesteryl palmitate and cholesteryl palmitate), a mixture of long chain acid (C_{16}-C_{35}), a mixture of steroid (β-sitosterol, stigmasterol and campesterol), acetyl aleuritolic acid, trigonostemone (1,1,7-trimethyl-3,6,9-trimethoxy-2-phenanthrenone), 5-hydroxy-6,7-dimethoxycoumarin, 5,7-dihydroxy-6-methoxy coumarin, a mixture of long chain amide (C_{44}-C_{48}), a mixture of steroid glycoside, 5α-stigmastane-3,6-dione and water soluble constituents such as sugars, amino acids and chloride salts (Theerawut Wangamnauyporn, 1998; วิภา เชิดชูสกุลชัย, 2530). Sitosterol and stigmasterol were reported to have anti-snake venom activity. They are able to neutralize the lethal dose of South American rattlesnake venom (Mors et al, 1989) and inhibit myotoxicity of crotalid venoms (Melo et al., 1994).

Areca catechu Linn. belongs to the family Palmaceae. It is commonly known as Areca Palm, Betel Palm, Betelnut Palm, and locally known in Thai as Mak, Makmia. This plant is widely distributed all over the tropics. It is also cultivated and spontaneously grown in some places. Nuts contain alkaloids namely, arecoline, arecaine, arecaidine, guvacoline, guvacine, and traces of choline. Tannins, gallic acid, gum, oily matter, and a number of amino acids are among the constituents found in the nuts (Dar and Khatoon, 1997). Recently, Pakatip Ruenraroengsak (2002) reported that seed of *Areca catechu* Linn. contains high tannin content. It contains both hydrolysable and

condensed tannins. These tannins could inhibit lethal activity of snake venom in mice, inhibit acetylcholinesterase activity and protect necrosis in rats.

Therefore, the aim of this study was to investigate the inhibition effects of the preparation from root of Lot thanong daeng (*Trigonostemon reidioides* Craib.*)* and Mak seed (*Areca catechu* Linn.) on lethality and myotoxicity of *Naja kaouthia* venom in mice.

CHAPTER II
LITERATURE REVEIW

1. Snakes and venomous snakes

Snakes are cold-blooded vertebrate. They are classified in class Reptilia, subclass Synaptosuria, order Squamata, suborder Serpentes. About 3,300 species are recognized and classified in 11 snake families. Venomous snakes are identified in only five families: Elapidae, Hydrophiidae, Viperidae, Crotalidae, and Colubridae. There are 175 species and subspecies of snakes native to Thailand, depending on the acceptance of proposed new species and subspecies. All of these, 62 are nonvenomouses, 50 are mildly venomous, and 56 are very venomous. The distinctions between mildly venomous and venomous are variable due to envenomation by a rear-fanged. The 175 species and subspecies in Thailand are classified within 4 superfamilies, 8 families, 15 subfamilies, and 67 genera (Cox, 1991).

2. Important venomous snakes in Thailand

The most important venomous snakes in Thailand are 2 families, Elapidae and Viperidae.

Family Viperidae

The snakes in this family have long fangs which are normally folded up against the upper jaw but are erected when the snake strikes. They are divided into 2 subfamily, the typical vipers (Viperinae) and pit viper (Crotalinae). The Crotalinae have a special sense organ, the pit organ, to detect their warm-blooded prey. This is situated between the nostril and the eye.

Subfamily Viperinae: Only one species is in Thailand, *Vipera russelli siamensis* (Siamese Russell's Viper). This snake is motionless and relies on camouflage to avoid detection but will hiss loudly and strike quickly when threatened. The venoms are complex and composed of several toxins, which are responsible for hemolysis, procoagulant activity, rhabdomyolysis and neurotoxicity. Pain is moderate at the bite site and later at regional lymph nodes. Rare neurotoxic symptoms includes drowsiness,

syncope, visual disturbances, and pituitary necrosis. Bite-site symptoms of swelling and necrosis are less severe than with pit viper bites. Common sites of bleeding are fang marks, gums, nose, skin, the gastrointestinal tract, kidneys and central nervous system. Renal failure is multifactorial. Renal damage may require peritoneal dialysis or hemodialysis (Lawan Chanhome et al., 1998; Cox, 1991). Their venom is quite toxic and potentially lethal. Mortality from their bites is fairly high.

Subfamily Crotalinae: The snakes in this subfamily have a prominent loreal pit located between the nostril and the eye that leads to a thermosensitive organ used in finding prey. There are thirteen species and subspecies native to Thailand, but the important venomous snakes are in two species, *Calloselasma rhodostoma* (Malayan Pit Viper) and *Trimeresurus albolabris* (green pit viper). Like Russell's viper, the venom has hemotoxic activity. The bite site is painful. There is severe necrosis. Hemorrhagic blisters are common, petechiae, gingival bleeding and hematuria are seen, but renal failure is rare. Morbidity is high, but mortality among hospital-treated patients is rare in Thailand. The green pit viper venom contains cytotoxins, hyaluronidase and substances that cause coagulopathies with defribination. Clinical manifestations consist of pain and early edema at the bite site. This spreads to the entire limb, cephalic and peripheral, and can be followed by ecchymosis, hemorrhagic blistering, and coagulopathies. Renal failure and central nervous system hemorrhages are rare but have been reported. Bites by these snakes are common and may be seen within Bangkok. The mortality should approach zero, but morbidity can be high (Lawan Chanhome et al., 1998; Cox, 1991).

Family Elapidae

Snakes in this family have short permanently erect fangs. This venom is highly toxic and often fatal to man. Elapidae venoms contain potent neurotoxins and also, like many other snake venoms, several varieties of proteins, both enzymes and nonenzymes. The main toxic constituent is neurotoxin which is the major cause of death from respiratory paralysis. There are 6 species of Elapidae in Thailand. Among these, there are three species of the genus *Naja* (cobras): *Naja kaouthia* (monocle cobra), *Naja siamensis* (spitting cobra), *Naja sumatrana*; two species of the genus Bungarus (kraits): *Bungarus candidus* (Malayan krait) and *Bungarus fasciatus* (Banded krait); and the king cobra (*Ophiophagus hannah*) of the genus Ophiophagus. Other Elapidae snakes in

Thailand include sea snakes and Asian coral snakes of the genus *Calliophis*. They have potent venoms but rarely bite humans (Lawan Chanhome et al., 1998; Cox, 1991; Piboon Jintakune and Lawan Chanhome, 1995).

3. Snake Venom

Snake venoms are complex mixtures, produced by a specialized sero-mucous gland and inoculated by a specialized apparatus. Most of the constituents are proteins, with low molecular weight compounds such as peptides and nucleosides, metal ions are also present. The death of the prey is due to respiratory or circulatory failure caused by various neurotoxins, cardiotoxins, coagulation factors, and other substances acting alone or synergistically. The various enzymes injected into the prey start the digestion of the tissue. Snake venoms and other toxic secretions contain a large number of pharmacologically highly active substances with a specific mode of action (Meier, 1991).

Snake venoms are either colorless or yellowish, depending on the amount of *l*-amino acid oxidase present, an enzyme with riboflavin as part of its prosthetic group. The most important pharmacologically active constituents are proteins. There are two groups to be distinguished: enzymes and nonenzymic polypeptides (Meier, 1991).

Over 90% of the solid snake venom components are proteins or peptides possessing toxic or biological effects. The nonprotein fraction of snake venoms consists of inorganic anions and cations of low-molecular weight substances like amino acids, small peptides, lipids, nucleosides and nucleotides, carbohydrate, and amines (Bieber, 1979; Devi, 1968; Stocker, 1991).

The venom composition may vary depending on age, (Meier, 1986, Moreno et al., 1988) geographic origin, (Meier et al., 1985; Stocker, Fischer, and Brogli, 1986) and individual snake, (Taborska and Kornalik, 1985) as concluded from toxicity determinations and measurements of enzyme activities.

3.1 Proteins and polypeptides in snake venoms

A) Enzymes in snake venoms

Most of the proteins in venom are water-soluble enzymes. Many of these enzymes are hydrolases and possess a digestive role such as proteinases, exo- and endopeptidases and phosphodiesterases. At least 26 enzymes have been detected in snake venoms. Of these enzymes, 12 are found in all venoms, venom contents differ significantly related to snake species (Table 1). Enzymes may be subjected to great variations depending on the condition of snake (e.g., age, nutrition, sex, living space or circumstances). Enzyme levels of viperid and crotalid venoms are in the range 80-90% of total dry matter, whereas the corresponding range for elapid venoms is 25-70% (Iwanaga and Suzuki, 1979).

Like in the majority of animal protein, structure, immunological properties and biological behavior of snake venom enzymes are species specific. Accordingly, the interaction of venom protein and their targets in prey organism depends on specific features of both snake and prey species.

B) Nonenzymic snake venom proteins

1) Neurotoxins

The venom of many snake species contains agents that affect nerve functions of the prey animal, causing cramps, convulsions, or paralysis. Snake venom neurotoxins can be grouped into two major categories:

- **Postsynaptic neurotoxins**

These toxins found predominantly in elapid venoms. These neurotoxins are low-molecular weight basic protein. They were divided in two types by the basis of their sizes and disulfide linkages. Short neurotoxins contain 60-62 amino acid residues and 4 disulfide cross-linked bridges and most of long neurotoxins consist of 70-74 amino acid residues and 5 disulfides bridges or sometime 4 disulfide bridges. The mode of action of both types is the same. They block neuromuscular transmission by binding specifically with high affinity to the nicotinic acetylcholine receptor in the postsynaptic membranes of skeletal

muscles, prevents the binding of chemical neurotransmitter acetylcholine and thereby blocks the excitation of muscle. However, short neurotoxins are generally more potent on mammalian tissue than long neurotoxins (Namiranian and Hider, 1992). This block at the neuromuscular junction leads to flaccid paralysis. This action similar to *d*-tubocurarine, so called curare-like or curaremimetic or curariform toxins or α-neurotoxin. Whereas the block of d-tubocurarine is easily reversible by physostigmine, the effect of most α-neurotoxins is virtually irreversible or slowly reversible.

- Presynaptic neurotoxins

The presynaptic neurotoxins are mostly toxic phopholipase A_2 and they exert the catalytic function of this type of enzyme. The significance of phospholipid clevage for the neurotoxic effect is not yet fully understood. All of these neurotoxins found in elapid and some viperid snake venoms have a basic phospholipase A_2 in common that may be complexed with acidic, basic or neutral protein units. The mode of action is two types :

(a) highly toxic phospholipase A_2 which inhibit transmitter release from nerve terminals

(b) toxins which enhance transmitter release (dendrotoxins)

b) Cytotoxins

Cytotoxins are toxic polypeptides consisting of 60 to 62 amino acid residues with four intramolecular disulfide bonds. The pharmacological actions of cytotoxin comprise hemolysis, cytolysis, depolarlization of muscle membrane, and specific cardiotoxicity.

Table 1. Enzymes found in snake venoms

Enzymes found in all snake venoms
Phospholipase A_2, L-Amino aicd oxidase, Phosphodiesterase, 5′- Nucleotidase, Phosphomonoesterase, Deoxyribonuclease, Ribonuclease, Adenosine triphosphatase, Hyaluronidase, NAD-nucleosidase, Arylamidase, Peptidase
Enzymes found in crotarid and viperid venoms
Endopeptidase, Arginine ester hydrolase, Kininogenase, Thrombinlike enzyme, Factor X activator, Prothrombin activator
Enzymes mainly in elapid venoms
Acetylcholinesterase, Phospholipase B, Glycerophosphatase
Enzymes found in some venoms
Glutamic-pyruvic transaminase, Catalase, Amylase, β-Glucosaminidase, Lactate dehydrogenase, Heparinaselike enzyme

From S. Iwanaga and T. Suzuki (1979)

c) Myotoxins

In severe cases of envenomation, myonecrosis can cause permanent tissue damage, producing loss of fingers and toes, legs and arms. But in less severe cases, the muscle can be regenerated. Myotoxins are snake venom polypeptides that induce skeletal muscle contracture or produce local myonecrosis or myoglobinuria. Myonecrosis, although common in most cases of snake envenomation, is most pronounced with Crotalidae and Viperidae venoms and can also be observed with Hydrophiidae and Elapidae envenomation.

d) Cardiotoxins

Cardiotoxins are single-chain polypeptides. Chemical and structural related to the neurotoxins. All of these toxins are highly basic polypeptides consisting of about 60-62 amino acid residues with four disulfide linkages in the molecules. They have little affinity for nicotinic AChR but act on cell membrane. Cardiotoxins have lytic effects on a wide range of cells, so they have other names as direct lytic factor, cytotoxin, membrane-disruptive polypeptide or membrane toxin. These have many effects including systolic contractions, hemolysis, cystolysis, and muscle depolarization (Harvey, 1991).

Other nonenzymic snake venom proteins act as proteinase inhibitors or represent structure analogues of proteinase inhibitors, bradykinin-potentiating peptides, choline esterase inhibitor, phospholipase inhibitors, nerve growth factors, lectins, proteins affecting platelet functions and proteins acting on the complement system.

3.2 Nonproteinous snake venom components

The nonprotein portion of the venom is much smaller than the proteins. In general, it is biologically less active. Included in the nonprotein fraction are metal ions, inorganic anions, and some small organic molecules such as peptides, lipids, nucleosides, carbohydrates and amines (Stocker, 1991).

Since all snake venoms contain multiple components with different mechanisms of action, the pathogenesis developing after a bite is of a vary complex nature. It is not

only dependent on the qualitative composition, but also on their quantitative distribution of different components in a particular venom.

4. Cobra

4.1 General Information

Cobra is a common snake throughout Africa and Asia. Africa species are generally larger than those from Asia and have narrow hoods. Furthermore, each species is fairly distinct and there is little controversy regarding their classification. The cobras of Southeast Asia are generally smaller than their African counterparts and can spread their hoods to a greater width. Southeast Asian cobras, however, have few distinct external characteristics. There is considerable variation in both color and pattern, even among individual specimens from the same geographic area. Thai members of Genus ***Naja*** range from one to two meters in average length. They eat a wide variety of food including other snakes, frogs, lizards, birds, and small mammals. Thai members of genus ***Naja*** appear to categorize within three groups. **First**, *Naja kaouthia* or monocled cobra or Siamese cobra and the Suphan Cobra forming one group. They are rather large snake and the Suphan Cobra is absent of a hood pattern. *Naja kaouthia* is abundant throughout Thailand. It is found near human habitation where there is abundant supply of rodents, ducklings, and chicks. It is the most dangerous snake and important cause of death from snakebite (Payom Buranasin, 1993; Sornchai Looareesuwan et al., 1988; Mukda Trishnananda et al., 1979). The Suphan Cobra has been described under the names *Naja naja kaouthia* and *Naja kaouthia suphanensis*. This type may have a color variation of the true *Naja kaouthia* and therefore, not a new species or subspecies. It may ultimately prove to be a new subspecies, but currently the name *Naja kaouthia suphanensis* is not scientifically valid. The Suphan Cobra is found in the lowland of the Central Region, especially in the provinces of Ang Thong and Suphan Buri. **The second** group includes three closely related spitting cobras: the Black Spitting Cobra, the Black and White Spitting Cobra, and the Isan Spitting Cobra. The Black Spitting Cobra and the Golden Spitting Cobra, of Group 3, have been described in various publications as *Naja atra* and *Naja naja atra. Naja naja atra* has not been recorded in Thailand and is not a spitting cobra. The Black Spitting Cobra is reported to

be most numberous in the provinces of Chon Buri, Suphan Buri, and Kanchana Buri. The Black and White Spitting Cobra has been refered to as *Naja naja sputatrix.* It is a rather common snake in the West and Central Region, especially in the provinces of Ang Thong, Suphan Buri, Kanchanaburi, and Tak. It is also found in the South and Southeast. The Isan Spitting Cobra has been refered to as *Naja siamensis*, a name not yet officially recognized. It is found in the provinces of the Northeast and Central Region that border the Norteast. **The third group** is formed of one single species, *Naja sumatrana* or Golden Spitting Cobra. It has been described in other names of *Naja atra* and *Naja naja atra*. It is a rather small cobra and has yellow or yellow-green body. It is the least common of the Thai cobras. The rage is restricted to the South, where it has been found in the upland areas of Surat Thani, Nakhon Ratchasima, and Phatthalung Provinces (Cox, 1991).

4.2 Cobra Venom Composition

Cobra venom is a mixture of many different proteins. The most toxic components are neurotoxins, which are single chain proteins of about 60 or 70 residues. The neurotoxins are low molecular weight and diffused rapidly into the bloodstream. These toxins bind to nicotinic acetylcholine receptors, and they are the cause of death by paralyzing respiratory muscle. Neurotoxins are the principle lethal toxins in cobra venoms, therefore the crude venoms produce the same neurotoxic effect as pure neurotoxins. The crude venoms are a mixture of various proteins and contain other lethal toxins. Cardiotoxins are the next most toxic components, and hence the crude venoms provoke cardiotoxic as well as neurotoxic effect (Tu, 1977). Neurotoxins and cardiotoxins of cobra venoms are structurally similar, but they have different pharmacologic effects. Cardiotoxins contain 60 to 62-amino acid residues that are similar to neurotoxins. They do not bind to cholinoceptors. Cardiotoxins cause the dropping in systemic blood pressure followed by bradycardia resuting in cardiac arrest (Lee and Lee, 1991). The lethality of cardiotoxins is greatly increase in the presence of phospholipse A_2, which display a synergistic action (Tu, 1977). However, the effects of cobra venoms are not restricted to neurotoxins and cardiotoxins. The venoms also cause local myonecrosis, although they do not produce local hemorrhage. Cobra

venom contains a variety of enzymes and hemolytic components, but the enzymes found are lesser than that in venoms of Viperidae and Crotaridae. They possess caseinolytic, plasma protease, phospholipase A_2, adenosine monophosphatase and acetylcholineesterase activities, with significant quantitative differences (Mukherjee and Maity, 2002). Unlike krait venom, which contains both pre and postsynaptic toxins, the neurotoxins of cobra venoms are postsynaptic acting (Tu, 1977).

4.3 Symptom and Pathology of Cobra bite

Before going to the stage of paralytic, preparalytic symptoms, apart from the local symptoms of severe pain and swelling, may be occurred, of these including: headache, vomiting, drowsiness, loss of consciousness, vasomotor sing such as pallor, sweating, weak to absent pulse and hypotension. The recognition of the preparalytic symptoms and sings of envenomation assumes to be a great importance because, if the antivenin is given in time at this stage, it may effectively prevent paralysis developing or may limit its extent. These symptoms may appear within minutes of the bite, so they may occur at the same time of muscle paralysis (Reid, 1964).

4.3.1 Local poisoning

Local poisoning of cobra bite is a serious problem. Cobra venom also contains a potent cytotoxin, which is responsible for pain and rapidly spreading of swelling and tissue necrosis. The severity varies from mild to severe necrosis of skin and subcutaneous tissue. Local symptoms are pain, swelling with or without blistering and necrosis (Prapai Pongprasit et al, 1988; Homma and Tu, 1971). Pain, then varying degrees of swelling and later necrosis, are outstanding features of local poisoning. Pain may be started immediately after the bite and remained for 10 days depending on the extent of necrosis. Swelling usually stared two to three hours after bite and reached a maximum in 24 to 48 hours and may persist for up to 18 days (Reid, 1964; Mukda Trishnananda, 1979; Campbell, 1979; Prapai Pongprasit et al, 1988; Homma and Tu, 1971).

Local necrosis is now accepted as the most common sequelae to an effective cobra bite and once was classified as peculiar to cobra bite.

Approximately fifty percent of the victims bitten by the cobra face the problem of local tissue necrosis, which is difficult to treat (Narumol Pakmanee et al., 1993) and antivenom can not prevent, unless it was administered intravenously within 30 minutes after envenomation (Prapai Pongprasit et al, 1988). After cobra bite is a dusky discoloration around the bite marks, extending in area and deepening in colour each day. About the third or fourth day the gray-black area becomes encircled by a red raised rim and sometimes sanguineous blisters developed on the middle of the dusky area. Then was often evident: incision released red-yellow material and revealed necrosis of subcutaneous tissue (Reid and Tu, 1964; Warrell et al, 1976). The area of skin necrosis may vary from a few cm^2 up to 600 cm^2 (Reid, 1964). Most of the patients developed local necrosis around the bite marks, usually 4 days after the bite (Mukda Trishnananda et al, 1979). While, patients were discharged one to three days after the bite, so that local necrosis may well have been overlooked, and often patients comeback to hospital with severe local necrosis.

The healing process needs long time in hospital may be 1 to 2 months, some cases required skin graft, and often occurred of permanent tissue loss, morbidity or amputation (Prapai Pongprasit et al, 1988).

4.3.2 Systemic symptoms

The earliest symptom of systemic poisoning is drowsiness, starting one to five hours after the bite. Difficulty in opening the eyes, speaking, opening the mouth, moving the lips, and swallowing followed three to four hour later. The susceptibility of various muscles to neurotoxins varies considerably. The most susceptible are extrinsic eye muscles and elevator of eyelids while superficial facial muscle and diaphram are resistant. The general weakness was usually the last symptom to develop, followed by paralysis of the muscle in severe cases (Reid, 1964; Mukda Trishnananda et al., 1979). The outstanding feature of systemic poisoning is paralysis of the muscles due to rapid action of neurotoxin at the myoneural junction. Respiratory paralysis may occur within 3-4 hours in severe cases and is the important cause of death with or without complicating

shock, septicaemia and renal failure (Sornchai Looareesuwan et al, 1988). Restlessness, irregular breathing, and mental confusion usually develope before respiratory paralysis, indicating the early significant clinical sings of impending respiratory failure. It is important for clinicians to regcognize the early signs of systemic poisoning and the warning signs of respiratory failure.

5. Quantity of venom injected at a bite

This is very variable, depending on the species and size of snakes, the mechanical efficiency of the bite, whether one or two fangs penetrated the skin and whether there are any repeated strikes. The snake may be able to control whether or not venom is injected. For whatever reason, a proportion of bites by venomous snakes do not result in the injection of sufficient venom to cause clinical effects. About 50% of bites by Malayan pit vipers and Russell's viper, 30% of bites by cobras and 5-10% of bites by saw-scaled vipers do not result in any symptoms or signs of envenoming. Snakes do not exhaust their store of venom, even after several strikes, and they are no less venomous after eating their prey.

Although large snakes tend to inject more venom than the smaller of the same species, the venom of smaller, younger viper may be richer in some dangerous components, such as those affecting haemostasis (Warrell, 1999).

The severity of snake venom poisoning not only depends on the venom amount injected, but also on a number of variables, such as bite site, venom quality, the age, weight, and health of the victim bitten, and the medical treatment.

6. Treatment of Snake Envenomation

6.1 First aid treatment

First aid treatment is carried out immediately or very soon after the bite, before the patient reaches a dispensary or hospital. Most of the traditional, popular, available and affordable first aid methods have proved to be useless or even frankly dangerous. These methods include making local incision or pricks/punctures ("tattooing") at the site of the bitten limb, attempts to suck the venom out of the wound, use of (black) snake stones, tying tight bands (tourniquets) around the limb, cryotherapy, electric shock,

topical instillation or application of chemical. Most of studies have shown tourniquets did not prevent or adequately delay the spread of venom from the bite site (warrell, 1999; Pe et al., 1987). The two most important principle of first aid for snakebite are immobilization of the bitten limb and rapid transport of the patient to medical care. Bites by cobras, king cobras, kraits or sea snakes may delayed absorption of venom from the site of the bite by pressure immobilization which bundle of crepe bandage around the entire bitten limb, starting distally around the fingers or toes and moving proximally, to include a rigid splint. Compression bandage or a tight tourniquet should not be released until the patient is under medical care in hospital because of it may result in the dramatic development of severe systemic envenomation. This method was extremely painful and very dangerous if the tourniquet was left on for too long (more than 40 minutes), as the limb might be damaged by ischaemia. Many gangrenous limbs resulted. Pressure immobilization is recommended for bites by neurotoxic elapid snakes, including sea snakes, but should not be used for viper bites due to its danger by increasing local effects of necrotic venom. However, it is not recommened for bite by cobra whose venom cause local necrosis (Warrell, 1999).

6.2 Conventional treatment

The antivenom is the most recommened and the mainstay of treatment for snake envenomation. Monovalent or monospecific antivenom neutralizes the venom of only one species of snake. Polyvalent or polyspecific antivenom neutralizes the venom of several different species of snakes. However, antivenom treatment caries a risk of severe adverse reactions and in most countries it is costly and may be in limited supply. It should therefore be used only in patients in whom the benefits of antivenom treatment are considered to exeed the risks. Indications for antivenom vary in different countries. Antivenom treatment is recommended if and when a patient with proven or suspected snakebite develops one or more of the following signs:

Systemic envenoming

- Haemostatic abnormalities: spontaneous systemic bleeding (clinical), coagulophathy (**20WBCT or other laboratory**) or thrombocytopenia (<100x10^9/litre) (**laboratory**).
- Neurotoxic signs: ptosis, external ophthalmoplegia, paralysis etc. (**clinical**)
- Cardiovascular abnormalities: hypotension, shock, cardiac arrhythmia (clinical), abnormal ECG.
- Acute renal failure: oliguria/anuria (clinical), rising blood creatinine/urea (laboratory).
- (Haemoglobin-/myoglobin-uria) dark brown urine (clinical), urine dipsticks, other evidence of intravascular haemolysis or generalized rhabdomyolysis (muscle aches and pains, hyperkalaemia) (clinical, laboratory).
- Supporting laboratory evidence of systemic envenomation.

Local envenoming

- Local swelling involving more than half of the bitten limb (in the absence of a tourniquet).
- Swelling after bites on the digits (toes and especially fingers).
- Rapid extension of swelling (for example beyond the wrist or ankle within a few hours of bites on the hands or feet).
- Development of an enlarged tender lymph node draining the bitten limb.

(Warrell, 1999)

Antivenom treatment should be given as soon as it is indicated. It may reverse systemic envenoming even when this has persisted for several days or, in the case of haemostatic abnormalities, for two or more weeks. If there is only local envenoming, antivenom may be effective when it is given within the first few hours after the bite.

6.3 Problem of Antivenom Treatment

Antivenom remains the only agent widely used in snakebite treatment. However, it carries with many problems.

1) The most effective antivenom is the monospecific. A specie diagnosis must be made before the right treatment can be chosen. Frequently, patients cannot recognize or bring the snakes to hospital, so it is difficult to make right diagnosis. From data of the Thai Ministry of Public Health, culprit snakes cannot be identified in about 80% of envenomation (Songsumard, 1995). In addition, clinical signs of snake envenomation are complicated, sometimes the medical staff misinterpreted and gave inappropriate antivenom (Sornchai Looareesuwan et al., 1988).

2) Antivenom reactions

Antivenom reactions occur usually more than 20% by developing react either early (within a few hours) or late reactions (5 days or more) after being given antivenin (Warrell, 1999).

Early anaphylactic reactions: usually develop within 10-180 minutes after starting antivenom. The patient begins with itching (often over the scalp) and develops urticaria, dry cough, fever, nausea, vomiting, abdominal colic, diarrhoea and tachycardia. A minority of these patients may develop severe life-threatening anaphylaxis such as hypotension, bronchospasm and angio-oedema. Although, severe anaphylactic shock is rare, it is the cause of death at high risk (Gilon et al., 1989). Skin testing is necessary before antivenom is administered.

Late (serum sickness type) reactions: develop 1-12 (mean 7) days after treatment. Serum sickness reactions are less dangerous and less frequently reported. Clinical features include fever, nausea, vomiting, diarrhoea, itching, recurrent urticaria, arthralgia. Myalgia, lymphadenopathy, periarticular swellings, mononeuritis multiplex, proteinuria with immune complex nephritis and rarely encephalopathy. Pateints who suffer early reactions and are treated with adrenaline, antihistamines and corticosteroid are less likely to develop late reactions.

Pyrogenic (endotoxin) reactions: usually develop 1-2 hours after treatment. Symptoms include shaking chills (rigor), fever, vasodilatation and a fall in blood pressure. Fabrile convulsion may be precipitated in children. These reactions are caused by pyrogen contamination during the manufacturing process. (Warrell, 1999; Gilon et al., 1989; Chippaux and Goyffon, 1998)

3) Antivenom development in animal, as horse or sheep, is time consuming, expensive and requires special storage condition (Chippaux and Goyffon, 1998).

4) Antivenom sometimes does not provide enough protection against venom-induced hemorrhage and nephrotoxicity, which it is the important cause of death (Sutherland, 1977; Corregan et al., 1987; Gilon et al., 1989; Warrell, 1989) and local tissue damage (Leon et al., 2000)

5) Antivenom is administered via intravenous, it needs technician to administered (Chippaux and Goyffon, 1998).

6.4 Traditional treatment

Traditional treatment of snakebite has been used throughout in the world, especially in rural areas. The form of traditional treatment are varied in each area such as tourniquet, drink urine (mostly the pateint's own) or other drinking to induce vomiting, topical treatment is using scarification, potassium permanganate, paraffin and brestmilk (Newman et al., 1997). The popular traditional remedy is the use of medicinal plants at bite site, taken orally or by routes (Otero, Nunez, Barona et al., 2000).

Plants have long been used for treatment of envenomation by snakebite. They take a major part of treatment by traditional healer in many societies. Many plants have the reputation of being useful against snakes and snakebite in most countries worldwide. Lists of such plants can be found in literature (Houghton and Osibogun, 1993; Singh et al., 2002; Kshirsagar and Singh, 2001). However, these lists are only gleaning information not scientifically proved. Traditional healers used medicinal plants in different forms by different route of administration with variety in numbers of plants in each time of treatment. The most popular route are drinking and topical application. The oral form may be prepared by infusion, decoction, maceration and chewing. The dose is varied depending on the severity of patients. Other forms including the external bath and steam application. More than one form are always used (Otero, Fonnegra, Jimenez, et al. 2000; Kshirsagar and Singh, 2001; Mebs, 2000).

7. Tests to determine antivenom activity

7.1 In vivo whole animal testing

The protection of whole animal against a dose of venom afforded by extracts or compounds is the method that approximates most closely to the field situation. This method has been used by many workers, beginning with the Herculean work in India in 1931. It is highly unlikely that such a project could or would be undertaken these day due to cost and equally important, the ethical considerations of using large numbers of animals.

Most recent work has been carried out in mice to test total crude extracts which is previously summarized (Houghton and Osibogun, 1993). In most cases determined the lethal dose of plant extracts and incubation time prior to the injection of venom. The survival rate with or without extract was determined. Any significant decrease in mortality was taken to imply the protection against venom by compounds present in plant extract.

A few studies have been carried out when the extract was given prior to or after the injection of venom. The latter procedure is the most analogous to snakebite cases occurring among general population.

Some work has also been carried out using traditional Chinese medicine preparation for the treatment of snakebite (Martz, 1992). These preparations are often a mixture of several plants extracts, therefore, it is impossible to determine which ingredients that neutralize the effects of venom or exert other beneficial effects such as a reduction in inflammation.

7.2 Testing using isolated organ preparations

The use of isolated tissues for testing biological activity dose not require large doses or long periods as experiments in whole animals. After effect was produced it can be removed by washing out and the preparation can be re-used for a series of experiments. In addition, a more quantitative approach can be used to measure the effects. The tests have been developed correspond to the types of venoms and consist of measurements on nerve-muscle preparations, isolated muscles and studies on blood-clotting procedures.

The Cobra venom and their constituents impaired neuromuscular transmission have been extensively studied using the nerve-muscle preparations from the neck of chick (biventer cervicis) and abdomen of rat (phrenic nerve-hemidiaphagm). Indirect stimulation of these preparations is inhibited by venom components. Consequently any plant extracts containing antivenom activity may reduce or even reverse these inhibitory effects. Isolated rat uterus was used by Calixto et al. (1985) to demonstrate the decrease in response to bradykinin released by venom of *Brothrops jararaca* in the presence of a 50% ethanolic extract of rhizomes of *Mandevilla velutina* (Apocynaceae).

Another use of isolated tissue was described in a test for agents protected against the myotoxins of crotalid venom (Mores et al., 1989). The rate of release of creatine kinase enzyme from superfused limb muscles was measured. Venom causes damage to the sarcoplasmic membrane resulting in a high rate of release of enzyme.

Tests of hemorrhagic toxicity can produce by measuring the change in optical density of skin at the site of injection of venom in sacrificed mice.

7.3 Tests using enzymes

The development of enzyme-based assays has permitted automated testing for enzyme-inhibition or enzyme-activation. This technique is applicable only where a particular enzyme can be linked to a disease state. Since the action of snake venoms is diverse and several enzyme systems may be involved, it has not been used for any general screening procedures. Besides, enzyme assays have the disadvantage that any activity displayed in vitro in such a test may not bear very much relation to effects in the whole animal.

8. Plants proven to be active in anti-snake venom and their active compounds

A general problem in the description of any antidotal action of plant ingredients is the complexity of the effects caused even by single venom components. However, the essential effects which would have to be controlled are those animal experiments and plant protective effect reported. In such studies, the drug is prepared using traditional formulae. Snakebite most often is mimicked by injections of the snake venom. However, in some studies, the antidote is administerd after *in vitro* preincubation (*in vitro*

deactivation) or in others it is administerd *in vivo* either before or after the administration of the venom.

The study of plant against snakebite has moderately reported. Otero, Nunez, Jimenez et al. (2000) studied 74 ethanolic extracts of plants used by traditional healers for snakebites in the northwest region of Columbia against lethal effect of *Bothrops atrox* venom. The result showed that seven plant extracts demonstrated 100% neutralizing capacity within 48 h and 5 plant extracts showing partial neutralization when the extracts were preincubated with snake venom before injection in mice. When the extracts were independently administered by oral or i.p. route 60 min before an i.m. venom injection, 4 extracts had partial and significant neutralizing capacity and one extract was partially effective when it was administered either i.v. 15 min before or i.p. 5 min after an i.m. venom injection.

Otero, Nunez, Barona et al. (2000) reported that 31 of 75 extracts of plants used by traditional healers for snakebites, had moderate or high neutralizing ability against the haemorrhagic effect of *Bothrops atrox* venom. After preincubattion of several doses of every extracts (7.8-4000 μg/mouse) with six minimum haemorrhagic doses (10 μg) of venom was i.d. injected into mice. When the extracts were independently administered by oral, i.p. or i.v. route either before or after an i.d. venom injection, neutralization of haemorrhage dropped below 25% for allextracts. In these, two plant extracts, *Brownea rosademonte* (Caesalpiniaceae) and *Pleopeltis percussa* (Polypodiaceae) were able to inhibit the proteolytic activity of *B. atrox* venom on casein.

Preliminary study used the extract from *Diodia scandens* Gronov ex L. (Rubiaceae) on toxic effects of saw scaled viper (*Echis carinatus*) which is the most common snake in the savanna of Nigeria by Onuaguluchi (1989). The water-soluble fraction of the aerial parts of plant was used to pretreat albino mice i.p. 30 min before administration of venom. Using a venom dose of 2 mg/kg i.p. this fraction reduced the mortality from 50% to 10% at a concentration of 1.5 mg/kg i.p. If 4 mg/kg of the venom were given i.p. death was delayed for more than 4 hr compared with 10 min in the control group.

Diodia scandens has previously been studied by Mittal et al. (1981) who reported its used as an antidote against (unknown) snake species. Some antihistamine and antiserotonin activities were found in the aqueous extract.

Andrographis paniculata (Acanthaceae) is common throughout the plains of India. Nazimudeen et al. (1978) reported its use as a snakebite antidote. After extraction with 90% ethanol of the air-dried whole plant, the water-soluble fraction was used in their study. Mice were treated with this fraction 4 g/kg or 2 g/kg, i.p. 30 min prior to the administration of an LD_{50} (320 μg/kg) of cobra venom (*Naja naja*). The time until death of the animals was determined. The control group died within 7 min after envenomation, the test groups died after 47 min (2 g/kg) and 52 min (4 g/kg). Although the cause of death (respiratory failure due to neuromuscular blockade) did not alter, the extract did show a life-prolonging effect.

Pereira et al. (1991) reported on oral pretreatment of mice against twice the lethal dose (5 mg/kg s.c.) of *Bothrops jararaca* venom. Of the 18 species of 13 plant families was tested, extracts of *Phylanthus klotzschianus* (Euphorbiaceae), *Casearia sylvestris* (Flacourtaiceae) and *Apoleia leiocarpa* (Leguminosae) conferred 100% protection up to 48 hr after administration. Extracts of other species, such as *Periandra pujalu* and *Periandra mediterranea* (Papilionideae), appeared to be less effective, and their protective action decreased with time. In each case the control group had no survivors.

Yuliang et al. (1979) reported on the Yunnan snakebite drug. The water-soluble extract of traditional several plants was given orally to mice 40 min prior to the injection into the front paw of *Naja naja* venom or *Agkistrodon halys* venom that prolonged the survival time of treated mice. In 1985, Liang investigated the water-soluble active principle of the Guangdong snakebite drug said to be active against *Naja naja* envenomation. The composition of the drug was descriped by Wu (1981) and Ma et al. (1982). They found that Guangdong snakebite drug consists of two herbs, *Passiflora cochinchinensis* Spreng (Passifloraceae) and *Citrus grandis* (L) Osbeck (Rutaceae). A mixture of plant constituents was given s.c. up to 18 min after administration of *Naja naja* venom. The mortality was alleged to decrease from 85% to 52%.

Methanol extracts from the stem of *Schumaniophyton magnificum* Harms (Rubiaceae) contain schumaniofoside, a chromone alkaloidal glycoside (Akunyili and Akubue, 1986, 1987). The stem-bark is crushed and the juice squeezed into an incision made at the site of the bite. The authors found that this compound reduced the lethal effect of black cobra (*Naja melanoleuca* Hall.) venom in mice. Protective effects were observed when the compound was given s.c. 1 min after administration of an LD_{50} of venom dissolved in saline by the same rout. The mortality deceased from 50±8.9% to 15.0±8.4%. There was a time-dependent effect. When administered 60 min after the venom schumanniofoside offered no protection.

Okonogi et al. (1979) summarized their results on the detoxifying effect of Persimmon tannin extracted from *Diospyros kaki* Thunb. (Ebenaceae), which is used as a folk remedy against Mamushi (*Agkistrodon halys blomhoffi*) bites in Japan. From fresh unripe fruits, a fraction was isolated which contained a tannin (Persimmon tannin). The antidotal effect of the pure persimmon tannin was tested using the venom of *Laticauda semifasciata* Reinwardt (Erabu sea snake) and its anti-haemorrhegic effect was determined using *Trimeresurus flavorviridis* Hallowell (Habu land snake). Both were compared with tannic acid prepared according to the Japanese pharmacopeia (the use of a 5% solution of tannic acid for emergency treatment of snakebite had been previously proposed by Okokogi et al. (1970). Mice were treated with preincubated mixtures of *Laticauda semifasciata* venom and persimmon tannin. Under these conditions, the venom was inactivated. Venoms of Sakisshima-habu (*Trimeresurus elegans*) and Mamushi (*Agkistrodon halys blomhoffi*) were used to demonstrate the detoxifying effect of persimmon (*Diospyros kaki* Thunb.) tannin. Male albino rabbits were treated i.m. with 120 μg/0.2 ml of Habu and Mamushi venom each containing varying amounts of persimmon tannin, and were observed for 24 hr. At tannin concentrations of 0.25% or greater, bleeding, necrosis and the swelling ratio of muscular tissue were completely inhibited.

More et al. (1989) reported on the *in vitro* neutralization of lethal and myotoxic activities of South American rattlesnake venom by extracts and constituents of *Eclipta prostrata* L. (Asteraceae). The herbaceous plant is know as 'erva botao' in Brazil. It is noteworthy that it has the same reputation in China. Fresh aerial parts of the plant

yielded wedelolactone, sitosterol and stigmasterol. The solutions were mixed with 3-4 LD_{50} (0.08 mg/kg) of *Crotalus durissus terrificus* venom dissolved in saline and preincubated for 30 min before being injected into the thigh (0.2 ml/10g) of Swiss mice. Wedelolactone (0.8 mg/animal), sitosterol (2.3 mg/animal) and stigmasterol (2.3 mg/animal) decreased the mortality to 0/8, 2/8, and 1/8 mice, respectively. The protection conferred by *Eclipta prostrata* against the myotoxicity effects of *Crotalus durissus terrificus* venom was investigated by means of isolated skeletal muscle preparations. Release of creatine kinase into the medium was almost completely inhibited in muscle exposed to 150 μg/ml of venom in the presence of 8.5 μg/ml of an aqueous extract of *Eclipta prostrata.* Other experiments were performed by preincubation of 0.25 μg/g of venom and 250 μg/g of aqueous extract for 15 min and subsequent i.m., injection of mixture. No increase in creatine kinase plasma levels was observed, while the control group showed a maximum of more than 1500 U/litre after 8 hr.

Aristolochia Shimadai Hay. (Aristolochiaceae) radix is known as ' Ma-tou-ling' and is one of the most important Chinese drugs. Besides its use for emetic and expectorant purposes it has been used in folk medicine for the treatment of snakebite. The inactivation of Formosan snake venoms *in vitro* by crude extracted of *Ariatolochia radix* has been described by Tsai et al. (1975). The dried root was extracted with ethanol. Fifteen per cent aqueous solutions of Aristolochia crude extracts and a solution of venom were mixed together and allowed to stand for 1 hr at 37° C before injection. Crotalid venoms (*Trimeresurus gramineus, Trimeresurus mucrosquamatus* and *Agkistrodon acutus*) and elapid venoms (*Bungarus multicinctus* and *Naja naja atra*) were used. The crude extracts of *Aristolochia radix* appeared to be effective against crotalid venoms but not against those of elapids.

Eryngium creticum is widely distributed in Jordan. The extract of this plant was reported to prolong the survival period of the tested animals against the venoms of *L. quinquetriatus* and *C. cerastes*). Phytochemical analysis of *E. creticum* was reported. Nine compounds were isolated and identified, they are: deltion, marmesin, quercitol, 3-(β-D-glucopyranosyloxymethyl)-2,4,4-trimethyl-2,5-cyclohexadien-1, 1-(β-D-glucopyranosyloxy-3-methoxy-5-hydroxy benzene), β-sitosterol, β-sitosterol-β-D-

glucopyranose, manitol, dulcitol. Extracts of fresh *E. creticum* inhibited the haemolytic activity of *C. cerastes* venom completely compared with 79% inhibition using extracts of dried plant leaves. Cold extracts of both fresh and dried plant roots gave 100% inhibition of snake and scorpion venom activities. However, ethanol extracts of the leaves and roots enhanced RBC haemolysis rather than inhibiting venom activities on red blood cells (Alkofahi et al., 1997).

Abubakar et al. (2000) reported the extract of the leaves of *Guiera senegalensis* was found to detoxify venom from two common northern Nigerian snake species, *Echis carinatus* and *Naja nigricollis*, in separate experiments. There was a remarkable reduction in the mortality of albino mice after i.p. administration of reconstituted venom incubated with the extract. The extract of *G. senegalensis* contains high amounts of tannins. This might be the plausible mechanism of the detoxifying action of the plant extract and its success against snake envenomation.

Mahanta and Mukherjee (2001) studied neutralization of lethality, myotoxicity and toxic enzymes of *Naja kaouthia* venom by *Mimosa pudica* root extracts. Roots of *Mimosa pudica* are popularly used against cobra bite by snake charmers and traditional quacks of northeast India. This study indicates that the aqueous extract of root *M. pudica* is effective in neutralizing the lethality, myotoxicity and tested toxic enzymes (protease, phospholipase A_2, Acetylcholinesterase) of *Naja kaouthia* venom. The methanolic/ethanolic extracts faile to inhibition of lethality myotoxicity and phospholipase activity of venom. Significant neutralization of toxic enzymes of *Naja kaouthia* venom by water extracts of *Mimosa pudica* roots might lead to inhibition of lethality of venom.

Recently, Alam and Gomes (2003) reported root extracts of *Vitex negundo* Linn. and *Emblica officinalis* Gaertn. possess potent snake venom neutralizing capacity. The methanolic extracts of these plants antagonized the *Viper russellii* and *Naja kaouthia* venom induced lethal activity both in *in vitro* and *in vivo* studies. The methanol extract was dissolved in saline before used. To assess the *in vitro* antagonism, after preincubation of several doses of venom with fix dose of plant extract was injected i.v., into male albino mice. To assess the *in vivo* antagonism, 5 LD_{50} of venom was injected s.c., into mice following immediately with various doses of plant extract given orally.

Viper venom-induced haemorrhagic, coagulant, anticoagulant and inflammatory activity (*in vitro* and *in vivo*) was significantly neutralized by both plant extracts.

Although many plants may not neutralize the venom itself, they may be still used to treat snakebite because they can alleviate some of symptoms or responses. The fear and panic could be alleviate by tranquilizing compounds and this may account for some of reputation enjoyed by *Rauvalfia serpentina* (Apocynaceae) since this plant is well-known as a source of the tranquilizing alkaloid reserpine. Plant extracts which cause a general stimulation of the immune system might also have some beneficial effect. Several plants contain compounds which have been shown to have an immunostimulant effect (Table 3). Another important biological activity which might also contribute to relieving the effects of snakebite is the stimulation of the immune system, which would lead to a more rapid removal of the venom. A general anti-inflammatory effect of a plant extract might also be useful in alleviation of symptoms. A selection of such plants is listed in Table 2.

Mucuna pruriens (Leguminosae) is medicinal plant that used by traditional healers in Nigeria. The seed is prescribed as a prophylactic oral antisnake remedy by traditional practitioners and it is claimed that when seeds are swallowed intact, the individual is protected for one full year against any snake bite. Aguiyi et al. (1997) reported extract of *M. prurein* seed (MP101UJ) has activity against elapidae venom and viperidae venom (Guerranti et al., 1999). Guerranti et al. (2001) reported MP101UJ has protective activity against *Echis carinatus* venom *in vivo* test. The protective effect was evident when the mice were injected with MP101UJ almost 24 hr before the injection of *E. carinatus* venom and this effect also showed when MP101UJ was injected 7 and 14 days before the *E. carinatus* venom injection. There was no protection when the mice were injected with a preincubated mixture of *E. carinatus* venom and MP101UJ. The protection so many days after injection of MP101UJ, may be due to the formation of a specific antibody, as demonstrated by Aguiyi et al. (1999), or to a protein-protein binding between a MP101UJ factor(s) and molecule(s) of mouse blood or tissue. The effect of MP101UJ on prothrombin activation was examined both direct and indirect effect that plant extract increased the procoagulant activity of *E.carinatus* venom. The main cause of death after the bite of *E.carinatus* is haemorrhage. This may suggest that

the protective effect of MP101UJ or serum of immunized mice is the increase in procoagulant activity of *E.carinatus.* In the same, study of Guerranti et al. (2002) showed that water extract of *M. prureins* seed has antivenin activity against *Echis carinatus* venom. This activity involved immune mechanism. After administration of *M. prureins* proteins the rise of antibodies responsible for protection was observed.

Elaeodendron balae Kosterm (Celastraceae) is a tree growing in the southeast of Sri Lanka. The root has been used externally to cure swelling and is also believed to be effective against snakebite. A parallelism between the traditional use against snakebite and analgesic and/or anti-inflammatory activity has been repeatedly observed (Goncalvez et al., 1990).

Calixto et al. (1985) have studied the selective inhibition by crude extracts of *Mandevilla velutina* (Apocynaceae) of bradykinin action on isolated rat uterus. The rhizomes were extracted by traditional method, i.e. in 50% ethanol and in water 1:3 (w/v). The dried filtrate was resuspended in saline. Using isolated rat uterus preparation, isotonic contractions were recorded. Preparations exposed to the extract (0.05-4 mg/ml) for 20 min showed a concentration-dependent shift to the right of the concentration-response curve to bradykinin. The crude extract did not affect the uterine tone. The onset of anti-bradykinin activity was < 10 min and the effect could be reversed by washing the preparations for 30-60 min. At the highest concentration (4mg/ml), the rightward displacement was about 60-fold.

Gymnema sylvestre (Asclepiadaceae) root is used as an antidote for snakebite in Indian folk medicine. Kini and Gowda (1982) investigated the inhibitory effect of potassium gymnemate on *Naja naja* ATPase. The sigmoidal curve obtained suggests that the enzyme is allosteric in nature. The inhibitor is of competitive type and shows co-operativity in binding with ATP.

Other plants have analgesic properties, thus lessening the pain of the bite, these include *Papaver somniferum* (Papaveraceae) and members of the Solanaceae which contain tropane alkaloids. It is also important to bear in mind that a plant may contain compounds with sedetive or tranquilizing effects which might lessen the panic induced in many victims of envenomization. Examples of such plants are the species of *Rauvolfia*

and members of the Valerianaceae, such as *Valeriana officinalis*, which have been used for centuries as sedatives.

Table 2. Plants used to treat snakebite shown to have anti-inflammatory effects

Species	Family
Anacardium occidentale	Anacardiaceae
Argenone mexicana	Papaveraceae
Boswellia serrata	Burseraceae
Brunfelsia uniflora	Solanaceae
Capparis spp.	Capparidaceae
Casearia sylvestris	Flacourtiaceae
Cyperus rotundus	Cyperaceae
Dolichos labalab	Leguminosae
Ficus carica	Moraceae
Morus alba	Moraceae
Prosopis spicigera	Leguminosae
Santolina chamaecyparissus	Compositae
Securidaca longepedunculata	Polygalaceae
Stachytarpheta dichotoma	Verbenaceae
Terminalia spp.	Combretaceae
Withania somnifera	Solanaceae
Zanthoxylum spp.	Rutaceae

from Houghton and Osibogun (1993)

Table 3. Plants and constituents with immunostimulant effects

Species	Family	Immunostimulant Constituent(s)
Aristolochia spp.	Aristolochiaceae	Aristolochic acid I
Stephania tetradra	Menispermaceae	Cepharanthine
Tylophora ovata	Asclepiadaceae	Tylophorine
Many species	Compositae	Sesquiterpene Lactones
Echinaceae angustifolia	Compositae	Polysaccharides

from Houghton and Osibogun (1993)

9. Compounds responsible for anti-venom activity

Active substances from plants reputed to neutralize or inhibit the effects of snake venoms are as following:

9.1 Steroids

Sitosterol (β-sitosterol) is the most abundant of phyto-steroids. The compound occurs either as such or in the form of its glucoside ('sitosterolin'), frequently accompanied by it mono-unsaturated analogue, **stigmasterol**, also either free or as its glucoside. The plants reputed for antisnake venom activity in which sitosterol or its glucoside and it analogue, stigmasterol, in varying proportion was reported by Mors et al. (2000). These substances form stable molecular addition compounds with many organic molecules, including aliphatic hydrocarbons, alcohols, ethers, carboxylic acids and aromatics.

Sitosterol and stigmasterol isolated from *Eclipta prostrata* (Asteraceae) showe inhibited myotoxicity of crotalid venom. Although sitosterol and stigmasterol are less effective than wedelolactone and crude extract. They are interact synergistically with wedelolactone (Mors et al., 1989). These substances

also neutralize lethal dose of South American rattlesnake (*Crotalus durissusterrificus*) in mice (Melo et al., 1994). Sitosterol increased percent survival of mice when administered by oral 1 hr prior to envenomation of *Bothrops jararaca* snake (Pereira et al., 1994).

These compounds may be found in other plants which reputed for anti-snake venom activity including: *Achillea millefolium*, *Aegle marmelos*, *Aristolochia serpentaria*, *Caesalpinia bonduc*, *Calendula officinalis*, *Cissamperos glaberrima*, *Cocculus hirsutus*, *Cynanchum paniculatum*, *Euphorbia hirta*, *Gloriosa superba*, *Marsypianthes chamaedrys*, *Ocimum basilicum*, *Ophiorrhiza mungos*, *Oldenlandia diffusa*, *Pluchea indica*, *Pothomorphe umbellate*, *Prestonia coalita*, *Serenoa repens*, *Sophora subprostrata* and *Taraxacum officinale* (Mors et al., 2000).

9.2 Pentacyclic triterpines (free or glycosides)

Pentacyclic triterpines with anti-snake venom activity are abounded. In the following, important examples will be listed by Mors et al. (2000), as well as the anti-snake venom plants in which they occur. Example of these are oleanoleic acid, lupeol, ursolic acid, taraxerol, α-amyrin, β-amyrin, friedelin, betulinic acid, betulin, bredemeyeroside and gymnemagenin. For many pentacyclic triterpenes, anti-inflamatory and anti-hepatotoxic activities have been reported. Of the mention compounds are oleanoleic and ursolic acid.

Among these, **bredemeyeroside B**, a new triterpenoid saponin, has been isolated from the roots of *Bredemeyera floribunda* Willd. (Polygavaceae) which used as a remedy for the treatment of snakebit in Brazilian folk medicine. In laboratory tests the Bredemeyerosides showed snake venom antidote activity (Daros et al., 1996; Pereira et al., 1994). In addition, More et al. (2000), reported that bredemeyeroside protected lethality of mice from *Brothrops jararaca* venom when administered by gastric intubation 1 hr prior to envenomation.

Pereira et al. (1994) reported that **β-amyrin** increased percent survival of mice when administered by oral 1 hr prior to envenomation of *Bothrops jararaca* snake.

These triterpenoids and their glycosides possess receptor binding activities has recently been demonstrated. Several compounds of this class, of the β-amyrin type, were tested in direct receptor binding assays, as well as their interaction with a number of specific binding sites.

9.3 Phenolic compounds

Phenolic compounds are important constituents of anti-snake venom plants. They will be subdivided into the following categories: hydroxybenzoic acids, cinnamic acid derivatives, coumarins, curcuminoids, flavonoids, and polyphenols (vegetable tannins).

9.4 Hydroxybenzoic acids and their methyl ethers

2-Hydroxy-4-methoxy benzoic acid was identified as a snake venom neutralizing factor in the Indian anti snake venom plant *Hemidesmus indicus* (Alam, Auddy, and Gomes, 1994). Other components are: 3,4-hydroxybenzoic acid or **protocatechuic acid** which is equally represented in anti-snake venom plants. Examples are *Fogopyrum cymosum* and *Cryptolepis sinensis*, both from China; *vanillin* (4-hydroxy-3-methoxy-benzaldehyde) is one of the many constituents identified in *Marsdenia cundurango*; **gentisic acid** (2,5-dihydroxy-benzoic acid), is present in the rhizomes of the yellow gentian (*Gentiana lutea*), an anti-snake venom plant of old employed in many region of Europe; *3,4-dihydroxybenzaldehyde*, has been identified in the rhizomes of the Chinese snake venom antidote (*Perilla ternate*); **monomethy ether of 2,6-dihydroxybenzoic acid**, is a component of the bulbs of *Gloriosa superba*, used against snakebite in India; **methyl ether of p-hydroxybenzoic acid** (anisic acid), occurs in the Nort-African anti-snake venom plant *Ruta montana*.

9.5 Cinnamic acid derivatives

Among the cinnamic acid derivatives, **caffeic acid** and its relatives merit special attention. Other members of the series, like *o*-coumaric and ferulic acid, are also of importance. Examples of anti-snake venom plants in which caffeic

acid is present are several species of *Polygonum*, *Prestonia coalita*, *Strychnos nux-vomica*, *Taraxacum officinale*, as well as a number of plants in which free caffeic acid accompanies chlorogenic acid, where it is present as an ester of quinic acid.

Caffeic acid is a substituent in many biologically active molecules. Most important examples are the verbascosides, rosmarinic acid and the many caffeic acid derivatives present in *Echinacea species*— plants known since ancient times for their anti-snake venom activity by North American Indians.

Several oligomers of caffeic acid were isolated from two African anti-snake venom plants—*Berkleya spekeana* and *Echinops amplexicaulis*. These compounds proved to be antidotes against snake venoms by oral and parenteral administration (Agoro, 1978).

9.6 Chlorogenic acid

Chlorogenic acid is caffeic acid which esterified with quinic acid, has been identified in many anti-snake venom plants such as *Achillea millefolium, Arctium lappa* and *Citrullus colocynthis*. This substance, isolated from *Vernonia condensate* Baker., showed increase percent survival of mice when administered by orally 1 hr prior to envenomation of *Brothrops jararaca* snake (Pereira et al., 1994).

9.7 Curcumimoids

Three diarylheptanoids—**curcumin**, demethoxycurcumin and bis demethoxycurcumin-make up the yellow dye of the rhizomes of turmeric, *Curcuma longa*, and other *Curcuma species*. In the first scientific work on the subject, the extract of the plant was shown to inactivate almost completely the neurotoxin of the cobra, *Naja naja siamensis* (Cherdchu and Karlsson, 1983). Many elements have been shown to form chelates with the curcumimoids.

Unsaturated ketone of turmeric, **ar-turmerone** was shown to inhibit the lethal action of rattlesnake venom (Ferreira et al., 1992)

9.8 Coumarins

Coumarin occurs often in considerable amounts in anti-snake venom plants. Examples are *Dipteryx odorata, D. punctata*, *Liatris squarrosa*, several *Mikania species* and *Torresea cearensis*. Coumarin showed increase survival mice from *Brothrops jararaca* snake venom when administered 1 hr orally before envenomation (Pereira et al., 1994; Mors et al., 2000).

All other coumarins are oxygenated at C-7, being therefore derivatives of umbelliferone, which occurs in *Aegle marmelos*, *Daphne mezercum* and *Ipomoea batatas*. This latter species produces also **scopoletin**, abundant as well in several species of *Brunfelsia* and in *Heterothalamus psiadioides*. The other compounds in this group as herniarin and ayapin, are isolated from the Amezonian anti-snake venom plant *Eupatorium triplinerve* which shows to exhibit considerable hemostatic activity (Bose and Sen, 1941). The molecular machanism of coumarin is little understanding. A number of investigations in plants have demonstrated the inhibition of various enzymes by coumarin.

9.9 Flavonoids

Flavonoids have been held responsible for anti-inflammatory, anti-hepatotoxic, anti-hypertensive, anti-arrhythmic, hypocholesterollemic, anti-allergic, anti-tumor, many other activities and enzymes inhibiting activity. Flavonoids have been shown to inhibit phospholipase A_2, important toxic components of snake venoms (Alcaraz and Holt, 1985) and quercetin which is a potent inhibitor of lipoxygenase.

Some of the simplest flavonoids can be found in the genus *Primula*. A good example is primatin, a constituent of the Indian anti-snake venom plant *Primula denticulata*.

Quercetin and several of it glycosides are the flavonoids most often encountered in anti-snake venom plants. **Rutin** was described the protective action, already fifty years ago, with anti-histamine against vascular effects caused by *Bothrops atrox* venom (Mors et al., 2000).

Other flavonoids and anti-snake venom plants in which they occur, either free or as glycosides such as hesperetin, naringenin, galangin, kaempferol, luteolin, diosmetin, quercitrin and isoquercitrin.

Isoflavonoids as a group show many biodynamic properties. A few show anti-snake venom activity such as tectoridin, iridin, and derricidin.

9.10 Pterocarpans

The isolation of two prenylated pterocarpans provided the most recent interest in snake venom antidotes: **cabenegrin A-I** and **A-II** (Nakagawa et al, 1982). These compounds were identified in the anti-snake venom remedy 'Espec′ifico Pessoa', a plant extract produced and sold in the northeast of Brazil. More recently, a similar compound, which was called **edunol**, was isolated from the Mexican anti-snake venom plant *Brongniartia podalyrioides* and showed to neutralize the lethal action of the venom of *Bothrops atrox*.

9.11 Coumestans

Eclipta prostrata is well known as an anti-snake poison both in China and in Brazil. Several compounds—flavonoids, phytosterols and one coumestan, **wedelolactone**- were identified in the extracts of the plant by different author. Among the components, wedelolactone, stigmasterol and sitosterol were found to be the main ones capable of neutralizing the lethal effect, on mice, of South American rattlesnake (*Crotarus durissus terrificus*) venom (Mors et al., 1989).

Wedelolactone was shown to exert several well defined pharmacological actions: anti-myotoxic, anti-haemorrhagic, antiproteolytic, antiphospholipasic (Melo et al. 1994).

9.12 Aristolochic acids

The roots of Aristolochia species are famous remedies against snakebite. The names 'Virginia snakeroot' and 'serpentary' (*Aristolochia serpentaria*) allude to this reputation. Most of the chemically studied Aristolochia species produce in their roots peculiar organic nitro-compounds, having a

phenanthrene nucleus: the aristolochic acids and aristolactams. The interaction of these (probably the most abundant aristolochic acid) with edema-inducing enzymes from Indian Viperidae has been studied in detail (Tsai et al., 1980). With the aid of a circular dichroism study, Vishwanath et al. (1987) found that aristolochic acid forms a 1:1 complex with phospholipase A_2, acting like a non-competitive inhibitor of the enzyme. The chelating properties of nitro-compounds with the nitro-group in appropriate vicinity to an acidic hydrogen atom, producing an intramolecular hydrogen bridge, are well known. From their observations with the aid of circular dichroism and spectral observations in the near ultraviolet, the Indian authors concluded that the aristolochic acid-enzyme association causes a significant change in the secondary structure of the protein.

9.13 Vegetable tannins

The first scientific approach to a natural anti-snake venom remedy, in modern times, concerns not a micromolecule but a much larger polyphenol. Japanese workers reported about the detoxifying action of persimmon tannin (from the unripe fruits of *Diospyros kaki*) as an acknowledged medicine against snake venom envenomation, in Japan (Okonogi and Hattori, 1978; Okonogi et al., 1979). The chemical structure of this tannin was studied by Matsuo and Ito (1979). Two ellagitannins from *Euphorbia hirta*, Eufhorbins A and B had their complicated structures elucidated (Yoshida et al., 1988).

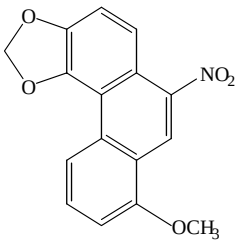
Among the mechanisms of action of all compounds considered, the one of vegetable tannins is probably the best understood, due to the exhaustive work at Sheffield about the chemical nature of protein-tannin interaction. Also, the enzyme inhibiting activity of tannins are well known. Most remarkably, of the seven botanical genera mentioned examples of tannin containing medicinal plants, three include well known anti-snake venom species, viz., *Paeonia*, *Agrimonia* and *Rubus.*

9.14 Polysaccharides

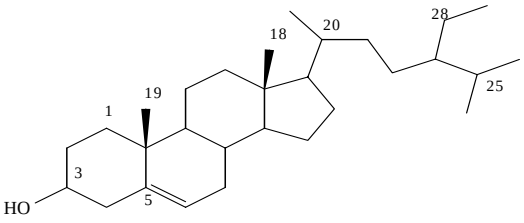
Polysaccharides have been shown to exhibit mainly antiinflammatory and immunomodulating activities. These properties can also be extended to anti-snake venom actions.

The bark of *Casearia sylvestris* is known as such a remedy throughout Brazil. Its aqueous extract yielded, besides sitosterol and stigmasterol, a mixture of polysaccharides, which could be resolved into five distinct units, three being neutral and two of acidic nature (Mors et al., 2000). *Calendula officinalis*, an anti-snake venom plant already mentioned to contain steroids and triterpenes, has its wound healing action attributed to the presence of an immunostimulanting heteroglycan (Varlen et al., 1989).

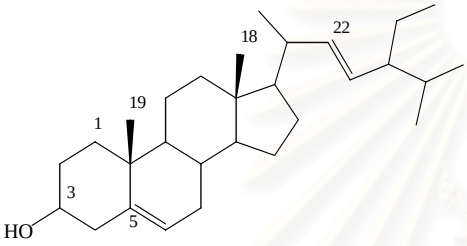
Figure 1. Structure of some compounds responsible for anti-snake venom activity



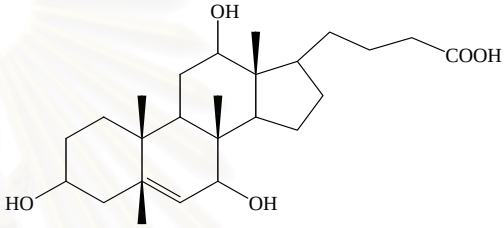
Aristolochic acid I



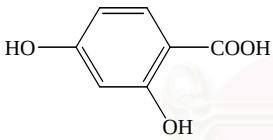
β-sitosterol



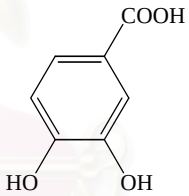
Stigmasterol



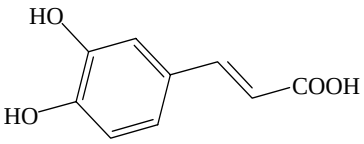
Cholic acid



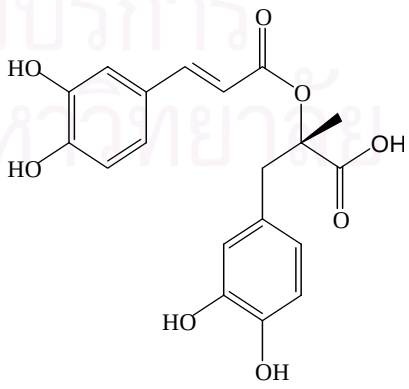
2,4- dihydroxybenzoic acid



Protocatechuic acid

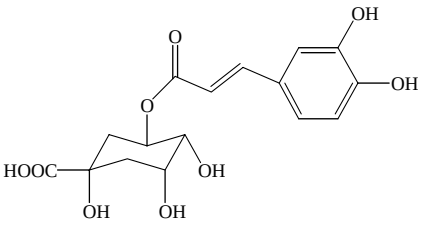


Caffeic acid

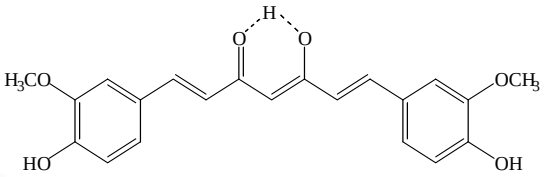


Rosmarinic acid

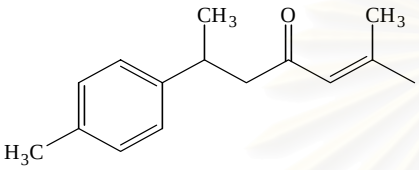
Figure 1. Structure of some compounds responsible for anti-snake venom activity (continue)



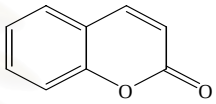
Chlorogenic acid



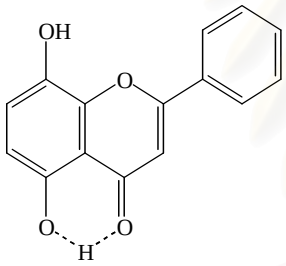
Curcumin



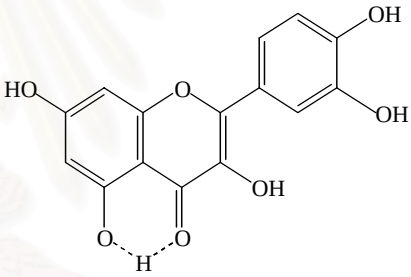
Ar-turmerone



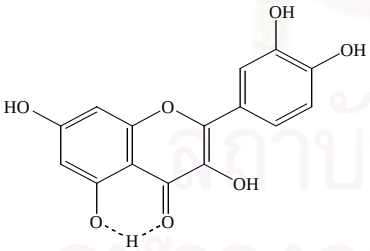
Coumarin



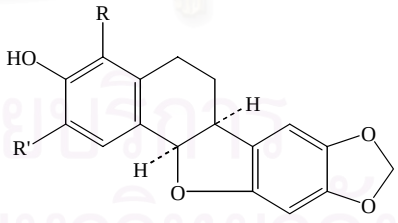
Primetin

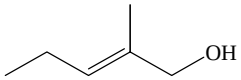


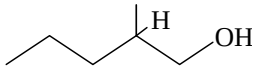
Quercetin



Rutin



Carbenegrin I, R =  R' = H

Carbenegrin II, R =  R' = H

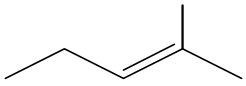
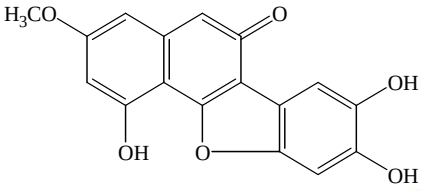
Edunol, R =  R' = H

Figure 1. Structure of some compounds responsible for anti-snake venom activity (continue)



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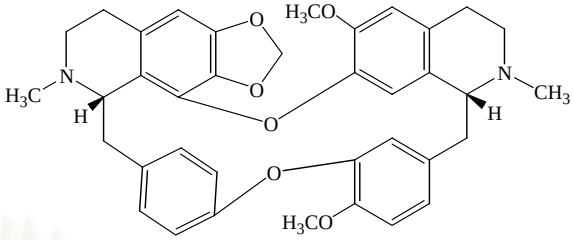
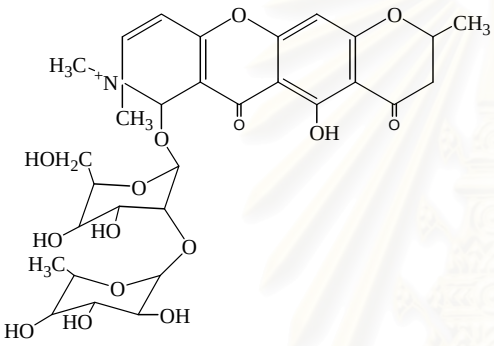
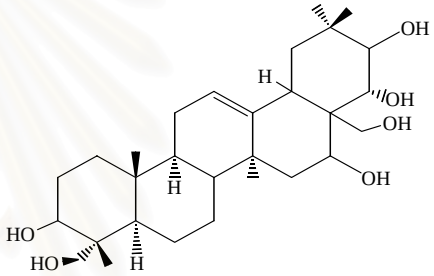


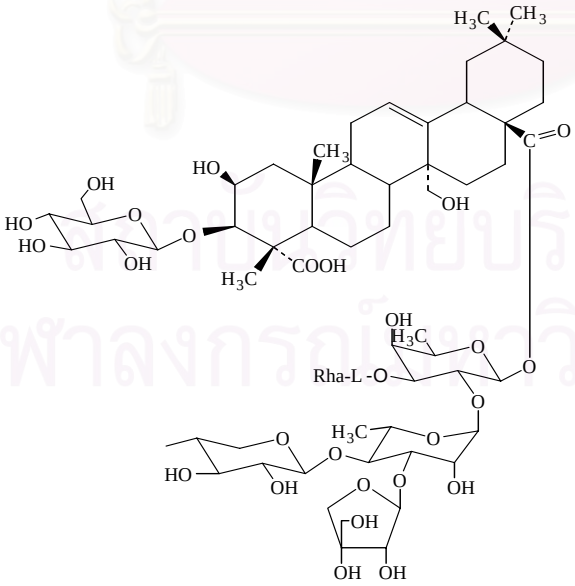
Cepharanthine



Schumaniofioside



Gymnemic acid



Bredemeyeroside B

10. Studies of plants against snakebite in Thailand

Many Thai medicinal plants were recommended against snakebite such as *Sansevieria metallica* (Agavaceae), *Eucharis grandiflora* Planch. (Amaryllidaceae), *Curcuma aeruginosa* Roxb. (Zinguberaceae), *Boesenbergia petiolata* Sirirugsa (Zingiberaceae), *Trigonostemon reidioides* (Euphorbiaceae), *Crinum rubra* (Amaryllidaceae), *Typhonium trilobatum* (Araceae), *Cleome* viscose Linn. (Capparidaceae), *Clinacantus nutans* Burm. *Curcuma sp.* (Zingiberaceae) (มาลี บรรจบ และคณะ ,2543; สมหมาย กระจ่างลิขิต และคณะ, 2525; พระเทพวิมลโมลี, 2522). However, a few of plants were studied in scientifically.

Panee Tejasen, Amphawan Chantaratham and Duangta Kanjanapothi (1969) studied the antagonistic effect of a medicinal plant, local called 'Wan Ngu' (*Curcuma sp*., Zingiberaceae). The native use its rhizome for treatment of snakebite given by orally against cobra venom (*Naja naja siamensis*). The anesthetized mongrel dogs was used to determine the antagonistic effect of Wan Ngu on the toxic effect of cobra venom on the cardiovascular and respiratory system. After the administration of fifty percent extract of rhizome from Wan Ngu, there are slight hypotention and tachycardia but no signs of bronchospasm at the beginning of first stage, and no bradycardia. It can increase the survival of the experimental animals when LD_{100} of cobra venom is administered.

In the next study (Panee Tejasen et al., 1969) fifty percent of Wan Ngu extract was given after cobra venom, it can prolong the life of anesthetized dogs. It brings heart rate and blood pressure return to normal within 15 minutes after LD_{100} of venom administration with slightly change of respiratory rate and depth. In addition, it can promote the survival of 100% of the white mice treated with LD_{50} and $4LD_{50}$ of cobra venom.

Panee Tejasen and Pairojana Sapavajit (1970) studied the comparative effects of antivenine and Wan Ngu against cobra venom on the cardiovascular and respiratory system. The result showed both Wan Ngu and antivenin antagonized the cardiovascular and respiratory system from LD_{100} of cobra venom.

Panee Tejasen and Laddawan Sunyapridakul (1970) reported that 50% aqueous extract of Wan Ngu administered subcutaneously at the same site of cobra venom

injection within 30 minutes permitted survival of 100% in dogs. The administration of Wan Ngu extract via other routes or later than 30 minutes after cobra envenomization permitted survival of less than 100%.

Amphawan Chantaratham and Panee Tejasen (1970) reported that 50% aqueous extract of Wan Ngu completely antagonized the neuromuscular blocking action of cobra venom when administered (i.v.) prior or mixed with venom in dogs. However, when administration after cobra venom the effect is decreased.

One hundred percent of Wan Ngu extract, 100% of fresh leaves of Slaed Pang Porn extract (*Clinacanthus nutans* Burn.), Hydro-adreson (corticosteriod) and antivenin decrease mortality rate of rats using LD_{100} of cobra venom from 100% death to 60±5.77%, 63±3.34%, 67±3.34% and 6±5.77% respectively. However, venom in dose higher than LD_{100}, did not decrease the death rate even with the maximum effective dose of Wan Ngu, Slaed Pang Porn extract or corticosteriod. Wan Ngu extract and Slaed Pang Porn extract were administered orally within 15 minutes after cobra venom injection and repeated twice at one hour interval, but hydro-adreson administered by s.c., route. The antivenom was administered s.c., within 15 minutes and repeated 3 times at on hour interval (Panee Tejasen and Chatchawadee Thongtharb, 1978).

Cherdchu and Karlsson (1983) reported on the proteolysis-independent cobra neurotoxin inhibiting activity of *Curcuma sp.* (Zingiberaceae). An aqueous extract of fresh rhizome was prepared. Phrenic nerve-hemidiaphram preparations were made from adult female albino rats, and the contractile response of the muscle-nerve preparation determined. When purified *Naja naja siamensis* neurotoxin (NTx) was added to the nerve-muscle preparations, a 95.8% inhibition of neuromuscular activity was observed, and both the indirectly evoked and the directly evoked contractions of the hemidiaphram muscle were significantly potentiated. When NTx and the aqueous extract were added simultaneously to the neuromuscular preparation, inhibition was found to be less than 40%. When the same amount of NTx was incubated with the muscle-nerve preparation for 105 min with subsequent washing and reincubating in the presence of aqueous *Curcuma sp.* extract, no recover of neuromuscular transmission was found. *In vivo* experiments were performed by treating Swiss albino mice with doses of cobra venom of 0.75 and 2.0 mg/kg, mixed with aqueous extract. Complete

protection was found when the mixture was given either s.c., or i.p., while mice treated with venom alone died after 138 min (s.c.) or 130 min (i.p.). When the venom and *Curcuma sp.* extract were administered independently by different routes, no prolongation of survival time was observed, showing the *in vitro* deactivation.

Recenlty, Pakatip Ruenraroensak (2002) studied anti-snake venom activities of tannin from Thai medicinal plants. Seven Thai medicinal plants were quantitatively evaluated for their tannin contents and types. The extraction process used aqueous ethanol (50%). Four extracts of high tannin contents were selected and tannic acid was included for comparison. Neutralizing capacities of these plants and tannic acid against snake venom activities were quantitatively determined in *Naja kaouthia* venom by *in vitro* method. Anti-lethal activity and anti-necrotizing activity were determined in mice and rats. The neutralization occurred by precipitating the venom proteins with various concentrations of the plant extracts or tannic acid. It was found that the extracts of *Pentace burmanica* Kurz (PB), *Pithecellobium dulce* Benth. (PD) and *Areca catechu* Linn. (AC) both contained hydrolysable and condensed tannins while the extract of *Quercus infectoria* Olivier. (QI) contained only hydrolysable tannins. The crude extracts containing high tannin contents could completely precipitate venom protein whereas those extracts with the lower tannin contents could partially precipitate the venom protein. Extracts containing condensed tannins could inhibit lethal activity and acetylcholinesterase activity at much lower tannin concentrations than tannic acid and extract with solely hydrolysable tannins. All of the plant extracts and tannic acid could protect the animals from the occurrence of necrosis.

11. *Trigonostemon reidioides* Craib. and *Areca catechu* Linn.

The preparation of root from Lot thanong daeng (*Trigonostemon reidioides* Craib.*)* and nut seed (*Areca catechu* Linn.) has been used against snakebite by folk healer in Surin province. Doctor at Kabchoeng Hospital in Surin province has been using them for treatment patients with snakebite in hospital for more than 10 years. However, the scientific study is very little, especially against snakebite.

11.1 *Trigonostemon reidioides* Craib.

Trigonostemon reidioides Craib. belongs to Euphorbiaceae family and found predominantly in north eastern of Thailand. Its Thai name is generally called Lot thanong daeng, and has many local names according to local area such as Lot thanong (Ratchaburi, Prachin Buri, Trad), Khao yen noen (Prachuap Khiri Khan, Ratchaburi), Thanong and Rak thanong (Nakhon Ratchasima), Du bia and Du tia (Phetchaburi), Nang saeng (Ubon Ratchasima), Thanong daeng (Prachuap Khiri Khan), Nat kham (Northern), Hua ya khao yen noen (Ratchaburi) (Tem Smitinand, 2001).

T. reidioides is a small undershrub 0.5-1.5 m high, all parts pubescent. Leaf is simple, alternate, oblong or oblonglanceolate, 2-4 cm wide, 7-12 cm long, finely, pubescent on both surfaces. Inflorescence in auxillary or ramiflorous panicle, unisexual, monoecious flowers in pink or purple. Fruit is capsule, 3 lobed subglobose (Wongsatit Chuakul et al., 1998).

This plant is believed to be one of medicinal plants and is used as drug from the ancient. In Thai medicinal books recorded that root grind with water can be taken as emetic for food poisoning, especially from mushroom and shells, or use as antiasthmatic, laxative, treatment of bloody and mucous sputum or stools; topically apply to abscesses, sprains, swelling and bruises. In addition, as folk medicine using together with seed of *Areca catechu* Linn. by grinding with water for take and with lemon juice for application to treat against snake envenomation ((Wongsatit Chuakul et al., 1998).

The chemical constituents in of Lot thanong daeng root were previously reported. The chemical constituents included a mixture of steroid palmitate (β-sitosteryl palmitate, stigmasteryl palmitate, campesteryl palmitate and cholesteryl palmitate), a mixture of long chain acid (C_{16}-C_{35}), a mixture of steroid (β-sitosterol, stigmasterol and campesterol), acetyl aleuritolic acid, Trigonostemone (1,1,7-trimethyl-3,6,9-trimethoxy-2-phenanthrenone), 5-hydroxy-6,7-dimethoxycoumarin, 5,7-dihydroxy-6-methoxy coumarin, a mixture of long chain amide (C_{44}-C_{48}), a mixture of steroid glycoside,5α-stigmastane-3,6-dione

and water soluble fraction constituents as sugars (glucose, fructose, arabinose and rhamnose), amino acids (glycine, alanine, isoleucine, and γ-aminobutyric acid) and chloride salts (Theerawut Wangamnauyporn, 1998; วิภา เชิดชูสกุลชัย, 2530). Sitosterol and stigmasterol were reported to have anti-snake venom activity. They were able to neutralize lethal dose of South American rattlesnake venom (Mors et al, 1989) and inhibit myotoxicity of crotalid venoms (Melo et al., 1994).

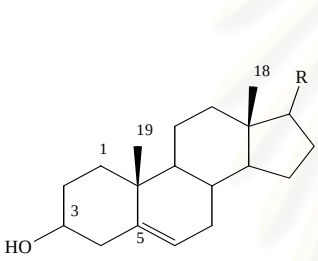
11.2 *Areca catechu* Linn.

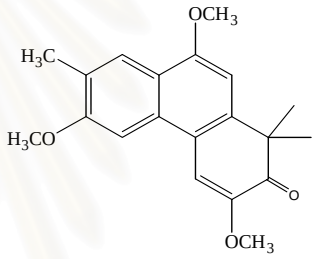
Areca catechu Linn. belongs to the family Palmaceae. It is commonly known as Areca Palm, Betel Palm, Betelnut Palm, and locally known in Thai as Mak, Makmia. This plant is widely distributed all over the tropics. It is also cultivated and spontaneously grown in some places. The leaves are pinnate, 1-2 m long. The fruits are ovoid 5-7 cm long with persistent perianth segment at their base. The seeds, known as betel nut or areca nut, are bluntly and rounded conical, about 1.5-3 cm high and 2-3 cm wide at their base (Tem Smitinand, 2001). The seed contains a number of different alkaloids but chiefly arecoline. The minor alkaloids are arecaine, arecaidine, arecolidine, guvacoline, guvacine, and traces of choline. Tannins, gallic acid, (+)-catechin, phenolic compounds, gum, oily matter, and a number of amino acids are among the constituents found in the nuts (Dar and Khatoon, 1997; Thaweephol Dechatiwongse Na Ayudhya, Yenchit Techadamrongsin and Warunee Jirawattanapong, 1993). The seed possesses antibacterial and antifungal activities, it has been used externally to stop bleeding and as an astringent (Thaweephol Dechatiwongse Na Ayudhya et al, 1993). Arecoline is a potent muscarinic receptor agonist (Goto et al., 1997) that rapidly crosses the blood-brain barrier and induces a range of parasympathetic effects. Recent research suggests that betel nut alkaloids possessing potent muscarinic cholinomimetics may be therapeutic in schizophenia (Sullivan, 2000). Other pharmacological effects of arecoline are the increase in cerebral metabolism and blood flow, and vasodilator (Goto et al., 1997).

Traditional medicine is one of constituents in nervine tonic and possesses stimulant and astringent properties. The dry nut increases salivation, lower perspiration, sweetens the breath, strengthens the gums and antidepressant (Dar and Khatoon, 1997).

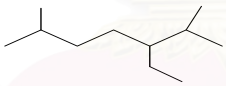
Recently, Pakatip Ruenraroengsak (2002) reported Areca nut has high tannin content. It contained both hydrolysable and condensed tannins. These tannins could inhibit lethal activity of snake venomin mice, inhibit acetylcholinesterase activity and protect necrosis in rats.

Figure 2. Structures of some chemical constituents from the root of *Trigonostemon reidioides* Criab.



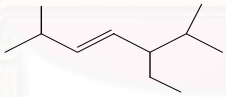


β- sitosterol, R =

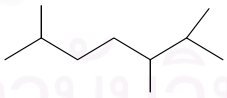


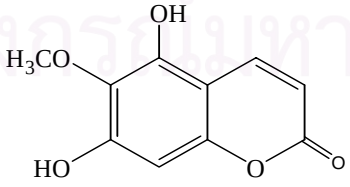
Trigonostemone

Stigmasterol, R =



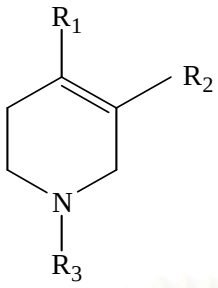
Campesterol, R =



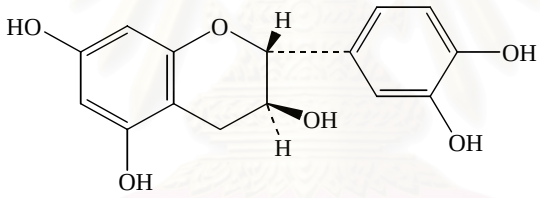


5,7-dihydroxy-6-methoxy coumarin

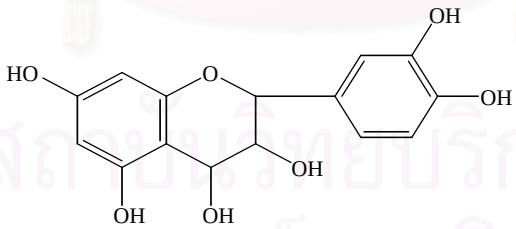
Figure 3. Structures of some chemical constituents from the seeds of *Areca catechu* Linn.



	R_1	R_2	R_3
Arecoline	H	$COOCH_3$	CH_3
Arecaidine	H	COOH	CH_3
Guvacoline	H	$COOCH_3$	H
Guvacine	H	COOH	H
Arecolidine	OCH_3	OCH_3	CH_3



Catechin



Leucocyanidin

CHAPTER III
MATERIALS AND METHODS

1. Chemicals and Biochemicals

Venom Lyophilized *Naja kaouthia* venom were obtained from Queen Saovabha Memorial Institute, Thailand Red Cross Society, Bangkok, Thailand and was preserved in 2-8° C for future use. It was dissolved in 0.9% saline and was frozen until used. Venom concentration was expressed in terms of dry weight.

Plant The roots of *Trigonostemon reidioides* Craib. and dry areca nuts (*Areca catechu* Linn.) were bought from Kabchoeng Hospital. The voucher specimens were identified by Faculty of Pharmacy, Mahidol University. The dried roots of *Trigonostemon reidioides* Craib. and dried areca nut (*Areca catechu* Linn.) were ground into small pieces and made into powder. The powder kept in desiccators at room temperature before use.

Water extraction The powder of *Trigonostemon reidioides* Craib. and areca nuts (*Areca catechu* Linn.) in ratio 3:1 were dissolved in 50 ml distilled water for 5 minutes. This solution was fleshly prepared before use.

Ethanol extraction The powder of mix-plants *Trigonostemon reidioides* Craib. and areca nuts (*Areca catechu* Linn.) in ratio 3:1 (600 g+ 200 g) , *Trigonostemon reidioides* Craib. alone 600 g and areca nuts (*Areca catechu* Linn.) alone 200 g, were extracted with ethanol 95% by refluxing (60-80°C for 24 hr) in a soxhlet apparatus. The filtrates were concentrated at 60°C on water-bath and drying at vacuum. The dried residues were kept in desiccators at room temperature. The dried residues were suspended with ethanol 28° and distilled water. The solutions were fleshly prepared before use.

Animal Swiss albino mice, either male or female, weight 18-20 g were obtained from National Laboratory Animal Center, Mahidol University, Thailand.

2. Methods

2.1 Median lethal dose (LD_{50})

Median lethal dose (LD_{50}) is defined as the least amount of venom (μg dry weight) injected intramuscularly to animals and resulted in the 50% death of animals within 24 hr. Swiss albino mice weighing 18-20 g, either male or female were used in this experiment. The venom solution having doses 3-12 μg/mouse were prepared in 0.9% saline. The 0.1 ml venom solution was injected intramuscularly (i.m.) to mice. Eight mice were used for each test dose. Control animals were injected with 0.9% saline only. The percent death of animals was recorded at 24 hr after the injection. The LD_{50} was calculated by probit analysis.

2.2 Neutralization of lethal venom effect

The lethal dose (LD_{100}) of *Naja kaouthia* venom was pre-incubated with plant solutions at 37°C for 60 minutes. Male Swiss albino mice weighed 18-20 g were used. The *N.kaouthia* venom was prepared with 0.9% saline to final concentration of 8 μg/mouse. The powder of mix-plants (*T. reidioides* 1.5 g: *A. catechu* 0.5 g) was dissolved with 50 ml distilled water. The solution was stirred for 5 min and filtered. The *N. kaouthia* venom (8 μg/mouse) was pre-incubated with 0.02 ml, 0.04 ml and 0.08 ml of the mix-plants filtrate in 0.9 % saline to final volume of 0.1 ml. The pre-incubation 0.1 ml was injected intramuscularly to left thigh of mice. The doses of mix-plants (*T. reidioides*: *A. catechu*) were 0.6:0.2, 1.2:0.4 and 2.4:0.8 mg/mouse (crude plant). The single plant also used. The powder of *T. reidioides* alone 1.5 g and *A. catechu* alone 0.5 g, each powder were dissolved with 50 ml distilled water and filtered. The 0.02 ml filtrated of each plant was pre-incubated with *N. kaouthia* venom (8 μg/mouse) in 0.9 % saline to final volume of 0.1 ml at 37° C for 1 hr. The dose of *T. reidioides* was 0.6 mg/mouse and *A. catechu* was 0.2 mg/mouse. The percent survival was observed at 24 hr. Control group was pre-incubated snake venom with 0.9% saline to a final volume of 0.1 ml. Six mice were used in each group.

2.3 Inhibition of lethal venom effect

2.3.1 Intramuscular administration

The lethal dose (LD_{100}) of *Naja kaouthia* venom (0.1 ml) was injected intramuscularly to mice followed plant solutions (0.1 ml) in another thigh. Male Swiss albino mice weighed 18-20 g were used. The lethal dose of *N. kaouthia* venom was prepared in 0.9% saline solution at a final concentration of 8 μg/mouse and fixed volume of 0.1 ml. The mixed-plants, *T.redioides* 1.5 g and *A. catechu* 0.5 g, was dissolved in 50 ml distilled water and stirred 5 min then filtered pass Wathman filter no. 2. The filtrate 0.02 ml (*T. reidioides* : *A. catechu* = 0.6:0.2 mg/mouse) was mixed with 0.9% saline to the final volume of 0.1 ml. The solution of *T. reidioides* alone and areca nut alone were prepared by crude powder of plant (*T. reidioides* 1.5 g, *A. catechu* 0.5 g) dissolved with 50 ml distilled water and 0.02 ml (*T. reidioides* 0.6 mg/mouse, A. catechu 0.2 mg/mouse) of filtrate was mixed with 0.9% saline to the final volume of 0.1 ml. The plant solution was injected intramuscularly in the right thigh of mice after immediately injected lethal dose (8 μg/mouse) of *N.kaouthia* venom. The survival time was observed 24 hr. Mice in the control group was injected with 0.9% saline instead of plant solutions. Six mice were used in each group.

2.3.2 Oral administration

A. Water extracts

1) *Filtrate of plant solutions*

a) Feeding plant extract immediately after snake venom injection

Animals were starved 5 hr before the injection of snake venom. Male Swiss albino mice weighed 18-20 g were used. The lethal dose of *Naja kaouthia* venom was prepared in 0.9% saline solution at a final concentration of 6 μg/mouse and 0.1 ml fixed volume. The *N. kaouthia* venom was injected intramuscularly in left thigh of mice. The mixed plant solution (*T.reidioides* 0.15 g+ *A. catechu* 0.05g) was dissolved in 50 ml distilled water and stirred 5 min. The solution was filtered. The 0.2 ml filtrate was fed to mice immediately after snake venom injection. The 0.2 ml filtrate (*T.reidioides:A.catechu*) were 0.6:0.2 mg/mouse. The single plant extracts also used. The powder of *T. reidioides* 0.15

g and powder of *A. catechu* 0.05 g were dissolved with 50 ml distilled water and filtered. The 0.2 ml filtrate was fed immediately after snake venom injection. The dose of *T.reidioides* filtrate (0.2 ml) was 0.6 mg/mouse and *A.catechu* was 0.2 mg/mouse. The survival time was observed 24 hr. Control group was fed with distilled water instead of plant solution. Six mice were used for each group.

b) Feeding plant extract immediately before snake venom injection

This experiment was done as described in (a), but the filtrate of plant extract was fed before snake venom injection. The animals were starved 5 hr. The 0.2 ml filtrate of plant extract was fed and then injected *N. kaouthia* venom intramuscularly. Food was filled after snake venom injection of. The survival time was observed 24 hr. Control group was fed distilled water instead of plant solution. Four mice were used for each group.

c) Feeding plant extract 1 hr before snake venom injection

The plant extract was prepared as experiment (a). Animals were starved 4 hr before fed with plant extract. One hour after feeding, animals were injected *N. kaouthia* venom intramuscularly. Food was filled after snake venom injection. The survival time was observed 24 hr. Control group was fed distilled water instead of plant solution. Six mice were used for each group.

d) Feeding plant extract 2 hr before snake venom injection

The plant extract was prepared as experiment (a). Animals were starved 3 hr before fed with plant extract. After feeding 2 hr, animals were injected *N. kaouthia* venom intramuscularly. Food was filled after snake venom injection. The survival time was observed 24 hr. Control group was fed distilled water instead of plant solution. Six mice were used for each group.

f) Feeding plant extract 3 hr before snake venom injection

The plant extract was prepared as experiment (a). Animals were starved 2 hr before fed with plant extract. After feeding 3 hr, animals were injected *N.*

kaouthia venom intramuscularly. Food was filled after snake venom injection. The survival time was observed 24 hr. Control group was fed distilled water instead of plant solution. Five mice were used for each group.

2) *Plant solution with powder*

a) Feeding plant solution with powder immediately after injection of snake venom

The mixed plant solution (*T.reidioides* o.15 g+ *A. catechu* 0.05g) was dissolved in 50 ml distilled water and stirred 5 min (not filtered). The single plants also used. *T. reidioides* alone 0.15 g or *A.catechu* alone 0.05 g was dissolved with 50 ml distilled water. The solution was stirred 5 min. The dose of plant extract, mixed-plant (*T.reidioides* : *A. catechu*) was 0.6 :0.2 mg/mouse, *T. reidioides* alone was 0.6 mg/mouse and *A.catechu* alone was 0.2 mg/mouse. Animals were starved for 5 hr, then 0.2 ml plant solution with powder was fed and injected lethal dose of *Naja kaouthia* venom (6 μg/mouse). The lethal dose of *Naja kaouthia* venom was prepared in sterile normal saline solution at a final concentration of 6 μg/mouse and fixed volume of 0.1 ml. The survival time was observed 24 hr. Control group was fed distilled water. Four mice were used in each group.

b) Feeding plant solution with powder 1 hr before injection of snake venom

The mixed plant solution (*T.reidioides* o.15 g+ *A. catechu* 0.05g) was dissolved in 50 ml distilled water and stirred 5 min (not filtered). Group 1 animals were starved 4 hr. The mix-plant solution was fed 0.2 ml after starvation 4 hr. One hour after feeding of plant solution, the *N. kaouthia* venom (6 μg/mouse) was injected intramuscularly to left thigh of mice. Group 2 animals were starved 4 hr. The mixed-plant solution was fed 0.2 ml after starvation 4 hr and was repeated at 30 min after the first feeding. Animals were injected venom 30 min after the second feeding. Food was filled after snake venom injection. The percent survival was observed 24 hr. Control group was fed distilled water once

4 hr after starvation. The *N. kaouthia* venom was injected after feeding 1 hr. Sixteen mice were used in each group.

B. Ethanol extract

1) Residue suspended with distiled water

a) Feeding ethanol extract immediately after snake venom injection

The dried residue of ethanol extract was suspended with distilled water. The mixed-residue was suspended to final concentration 0.6:0.2 mg/mouse of crude plants. Male Swiss albino mice weighed 18-20 g were used. Animals were starved 5 hr. The lethal dose of *Naja kaouthia* venom was prepared in 0.9% saline solution at a final concentration of 6 μg/mouse and fixed volume of 0.1 ml. The *N. kaouthia* venom was injected intramuscularly to left thigh of mice. The 0.2 ml solution was fed immediately after snake venom injection. Control group was fed with 0.2 ml distilled water. The survival time was observed 24 hr. Four mice were used in each group.

b) Feeding ethanol extract immediately before snake venom injection

Liked experiment (a), but the ethanol extract was fed immediately before snake venom injection.

c) Feeding ethanol extract 1 hr before snake venom injection

The dried mixed-residue of ethanol extract was suspended with distilled water. In contrast to experiment a) and b), the mixed-residue was suspended to final concentration of 1.2:0.4 mg/mouse of crude plants in 0.2 ml distilled water. The 0.2 ml suspension was fed to 4 h starved mice and then 1 hr later *N.kaouthia* venom at dose 6 μg/mouse in 0.1 ml 0.9% saline was injected intramuscularly to left thigh mice. Food was filled after snake venom injection. Control group was fed 0.2 ml distilled water. The survival time was observed 24 hr. Four mice were used in each group.

2) Residue suspended with alcohol

a) Feeding plant extract 1 hr before snake venom injection

The dried residues of ethanol extract were suspended with ethanol 28°. The mixed-residue was suspended to final concentration 0.6:0.2 mg/mouse of crude plants. The residue of *T.reidioides* alone was suspended to final concentration 0.6 mg/mouse and *A.catechu* alone was suspended to final concentration 0.2 mg/mouse. Male Swiss albino mice weighed 18-20 g were used. Animals were starved 4 hr. The 0.2 ml solutions were fed. The lethal dose of *Naja kaouthia* venom was prepared in 0.9% saline solution at a final concentration of 6 μg/mouse and fixed volume of 0.1 ml. The *N. kaouthia* venom was injected intramuscularly to left thigh of mice after feeding of ethanol extract 1 hr. Control group was fed with 0.2 ml ethanol 28°. The survival time was observed 24 hr. Four mice were used in each group.

2.4 Inhibition of myotoxicity effect

Inhibition of myotoxic effect of venom was measured by quantitation of plasma creatine phosphokinase (CPK) activity as described previously (Mukherjee and Maity, 1998a). The mixed-plants powder (*T.reidioides* 0.15 g+ *A. catechu* 0.05g) was dissolved in 50 ml distilled water and stirred 5 min (not filtered). Animals were starved for 4 hr before feeding of 0.2 ml plant solution. The sublethal dose of *Naja kaouthia* venom was prepared in 0.9% saline solution at a final concentration of 4 μg/mouse and fixed volume 0.1 of ml. The venom solution was injected intramuscularly to mice 1 hr after feeding of plant solution. Control mice were injected with venom alone. Mice were sacrificed by ether inhalation 4 hr after injection. A blood sample was collected from inferior vena cava and centrifuged at 3000 rpm for 10 min. Plasma was collected. Plasma was assayed for creatine phosphokinase (CPK) activity by Professional Laboratory. Six mice were used in each group.

CHAPTER IV

RESULTS

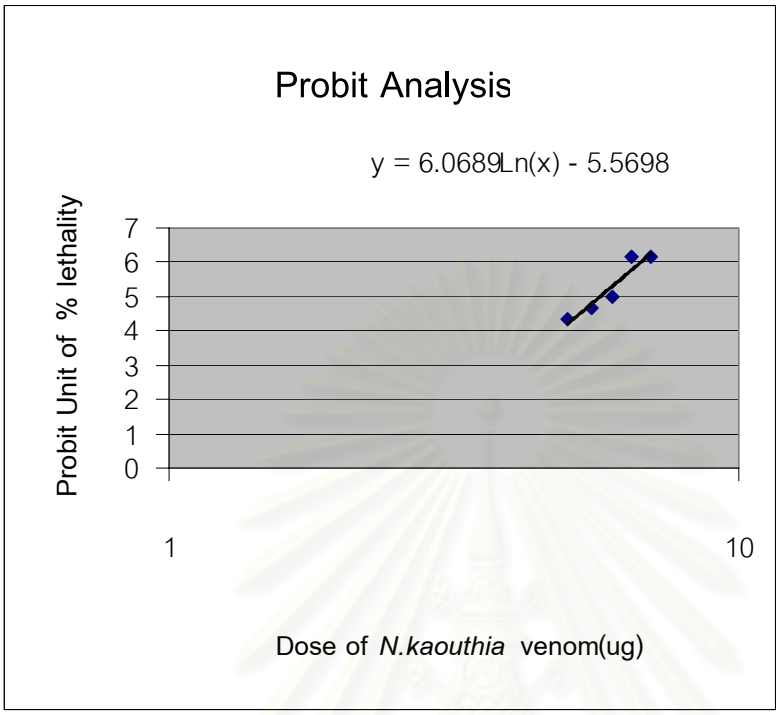
1. Median lethal dose (LD_{50}) of *Naja kaouthia* venom

Median lethal dose (LD_{50}) of *Naja kaouthia* venom was assayed by injecting different doses of venom in 0.1 ml physiological saline intramuscularly to left thigh of Swiss albino mice of both sexes weighed between 18-20 g. Lethality data of *Naja kaouthia* venom was shown in Table 4. Median lethal dose (LD_{50}) was calculated by probit analysis. The median lethal dose (LD_{50}) of *Naja kaouthia* venom obtained from this study was 5.71 μg/mouse.

Table 4. % Death of mice receiving various doses of *Naja kaouthia* venom

Dose (μg/mouse)	Dead animal/total	% Death
5.0	2/8	25
5.5	3/8	37.5
6.0	4/8	50
6.5	7/8	87.5
7.0	7/8	87.5

Figure 4. Probit analysis



LD_{50} was calculated from y = 6.0689 Ln (x) – 5.5698

LD_{50} is probit unit = 5, so at LD_{50} y = 5

5 = 6.0689 Ln (x) – 5.5698

Ln (x) = 10.5698/6.0689

x = 5.7067

2. Neutralization of lethality

The lethal dose (LD_{100}) of *Naja kaouthia* was pre-incubated with different doses of water extract of mixed-plants (*T. reidioides*: *A. catechu*) and single plants at 37° C for 1 hr. The results of neutralization were shown in table 5.

N. kaouthia venom at dose 8 mg/mouse produced 100% death of mice. The water extracts of *A. catechu* alone and mixed-plants showed neutralization of *N. kaouthia* venom. They protected death of mice from *N. kaouthia* venom. The extract of *A.catechu* alone at dose 0.2 mg/mouse could protect mice from the lethal dose *N. kaouthia* venom 100%. The survival time of mixed-plant at dose 2.4:0.8 mg/mouse and

A.catechu alone significantly different from control at 95% confidence. However, the survival time of mixed-plant solutions at dose 0.6:0.2 and 1.2:0.4 mg/mouse were not significantly different from control, they increased percent survival from 0% to 66.67% (four mice survived from six mice). The extract of *T. reidioides* alone did not neutralized *N. kaouthia* venom. Mice in this group died 100% and survival time was not significantly different from control mice.

Table 5. The percent survival of mice injected with pre-incubated plant extracts and *N. kaouthia* venom

Groups	Survival animal/Total	% Survival	Survival time (min): Mean±SE
Control	0/6	0	106 ± 6.76
Mix (0.6:0.2 mg/mouse)	4/6	66.67	1003 ± 276.26
Mix (1.2:0.4 mg/mouse)	4/6	66.67	1038 ± 254.92
Mix (2.4:0.8 mg/mouse)	4/6	66.67	1145 ± 192.34 *
T. redioides 0.6 mg/mouse	0/6	0	88 ± 6.44
A.catechu 0.2 mg/mouse	6/6	100	1440 ± 0.00 *

* Significantly different from control, $p < 0.05$ (One way ANOVA ;Tamhane' s T2)

Data of survival time was calculated from all mice (died and survived mice).

For survived mice the survival time was calculated from 24 hr (1440 min).

3. Inhibition of lethality

3.1 Intravenous administration

The survival time of *T.reidioides* and *A.catechu* extracts either mixed-plant (0.6:0.2 mg/mouse) or each single plants (*T.reidioides* 0.6 mg/mouse, *A.catechu* 0.2 mg/mouse) was not significantly ($p<0.05$) different from control mice as showed in Table 6.

Table 6. Inhibition of plant extracts by intramuscular administration

Group	Survival animal/Total	Survival time (min): Mean ± S.E.
Control	0/6	56 ± 4.89
T. reioides +*A. catechu* (0.6:0.2 mg/mouse)	0/6	109 ± 19.48
T. reioides (0.6 mg/mouse)	0/6	66 ± 3.64
A. catechu (0.2 mg/mouse)	0/6	61 ± 5.43

3.2 Oral administration

A. Water extracts

1) *Filtration of plant solutions*

a) Feeding plant extract immediately after snake venom injection

The *Naja kaouthia* venom at dose 6μg/mouse produced 100% death of mice. The water extract of *T.reidioides* and *A. catechu*, mixed-plant or each single plant, could not significantly inhibit the lethal activity of *N. kaouthia* venom at 95 % confidence when feed immediately after injection snake venom. This experiment was simulated the real usage by folk healer, but filtered plant extract before use for easy feeding. Data of survival time showed in Table7.

Table 7. Survival time of mice feeding water extract immediately after injection of *N.kaouthia* venom

Group	Survival animal/Total	Survival time (min): Mean ± SE
Control	0/6	79 ± 4.94
T.reidioides + *A.catechu* (0.6:0.2 mg/mouse)	0/6	109 ± 19.05
T.reidioides (0.6 mg/mouse)	0/6	83 ± 11.86
A.catechu (0.2 mg/mouse)	0/6	75 ± 8.24

b) Feeding plant extract immediately before snake venom injection

Liked experiment a), *Naja kaouthia* venom at dose 6μg/mouse produced 100% death of mice. The filtrate of water extract of *T. reidioides* and *A. catechu*, mixed-plant or each single plants could not significantly inhibit the lethal activity of *N. kaouthia* venom at 95 % confidence. Data of survival time was shown in Table 8.

Table 8. Survival time of mice feeding water extract immediately before injection of *N.kaouthia* venom

Group	Survival animal/Total	Survival time (min): Mean ± SE
Control	0/4	86 ± 20.88
T.reioides + *A.catechu* (0.6:0.2 mg/mouse)	0/4	119 ± 2.49
T.reioides (0.6 mg/mouse)	0/4	106 ± 2.90
A.catechu (0.2 mg/mouse)	0/4	98 ± 5.60

c) Feeding plant extract 1 hr before snake venom injection

Like experiments a) and b), filtrate of plant extracts either single or mixed-plants could not significantly inhibit the lethal activity of *N.kaouthia* venom at 95 % confidence when fed 1 hr before injection of snake venom. Data was shown in Table 9.

Table 9. Survival time of mice feeding water extract 1 hr before injection of *N.kaouthia* venom

Groups	Survival animal/Total	Survival time (min): Mean ± SE
Control	0/6	79 ± 10.03
T.reidioides + *A.catechu* (0.6:0.2 mg/mouse)	0/6	170 ± 44.02
T.reidioides (0.6 mg/mouse)	0/6	106 ± 9.75
A.catechu (0.2 mg/mouse)	0/6	106 ± 22.55

d) Feeding plant extract 2 hr before snake venom injection

Filtrate of plant extracts either mixed or each single plant could not significantly inhibit the lethal activity of *N.kaouthia* venom at 95 % confidence. Data of survival time showed in Table 10.

Table 10. Survival time of mice feeding water extract 2 hr before injection of *N.kaouthia* venom

Group	Survival animal/Total	Survival time (min): Mean ± SE
Control	0/6	159 ± 24.26
T.reidioides + *A.catechu* (0.6:0.2 mg/mouse)	0/6	183 ± 58.49
T.reidioides (0.6 mg/mouse)	0/6	165 ± 14.89
A.catechu (0.2 mg/mouse)	0/6	225 ± 27.34

f) Feeding plant extract 3 hr before snake venom injection

Filtrate of plant extracts either mixed or each single plants could not significantly inhibit the lethal activity of *N.kaouthia* venom at 95 % confidence. Data of survival time showed in Table 11.

Table 11. Survival time of mice feeding water extract 3 hr before injection of *N.kaouthia* venom

Group	Survival animal/Total	Survival time (min): Mean ± SE
Control	0/5	164 ± 37.82
T.reidioides + *A.catechu* (0.6:0.2 mg/mouse)	0/5	148 ± 10.68
T.reidioides (0.6 mg/mouse)	0/5	141 ± 0.65
A.catechu (0.2 mg/mouse)	0/5	147 ± 18.84

2) *Plant solution with powder*

a) Feeding plant solution with powder immediately after injection of snake venom

Like part 1), the *Naja kaouthia* venom at dose 6μg/mouse produced 100% death of mice. The water extract of *T.reidioides* and *A. catechu*, mixed-plant or each single plant could not significantly inhibit the lethal activity of *N. kaouthia* venom at 95 % confidence. Data was shown in Table 12.

Table 12. Survival time of mice feeding plant solution with powder immediately after injection of snake venom

Group	Survival animal/Total	Survival time (min): Mean ± SE
Control	0/4	98 ± 25.37
T.reidioides + *A.catechu* (0.6:0.2 mg/mouse)	0/4	78 ± 9.55
T.reidioides (0.6 mg/mouse)	0/4	120 ± 26.03
A.catechu (0.2 mg/mouse)	0/4	75 ± 11.35

b) Feeding plant solution with powder 1 hr before injection of snake venom

Water extract of mixed-plant (*Trigonostemon reidioides* : *Areca catechu*) increased percent survival of mice when feeding 1 hr before injection of *N.kaouthia* venom. The dose of *N.kaouthia* venom at 6 μg/mouse did not produce 100% death. The water extract of *T.reidioides*: *A.catechu* at dose 0.6:0.2 mg/mouse increased percent survival from 6.25% to 18.75 % and at dose 1.2:0.4 mg/mouse increased percent survival to 31.25 %. Data was shown in Table 13.

Table 13. Percent survival of mice feeding plant solution with powder 1 hr before injection of snake venom

Group	Survival mice/total	% Survival	Survival time(min): Mean±SE
Control	1/16	6.25	172 ± 84.68
T.reidioides + *A.catechu* (0.6:0.2 mg/mouse)	3/16	18.75	401 ± 130.35
T.reidioides + *A.catechu* (1.2:0.4 mg/mouse)	5/16	31.25	531 ± 158.38

Data of survival time was calculated from all mice (died and survived mice).
For survived mice the survival time was calculated from 24 hr (1440 min).

B. Ethanol extract

1) Residue suspended with distilled water

a) Feeding ethanol extract immediately after snake venom injection

Result showed that *N.kaouthia* venom at dose 6 μg/mouse produced 100% death of mice. The survival time of mice feeding suspension of ethanol extract at dose 0.6:0.2 mg/mouse not significantly differed control mice at 95% confidence. Data showed in Table 14.

Table 14. Survival time of mice feeding ethanol extract (residue suspended with distilled water) immediately after snake venom injection

Group	Survival animal/Total	Survival time (min.): Mean ± SE
Control	0/4	65 ± 3.68
T.reidioides + *A.catechu* (0.6:0.2 mg/mouse)	0/4	93.75 ± 27.71

b) Feeding ethanol extract immediately before snake venom injection

Survival time of mice feeding suspension of ethanol extract at dose 0.6:0.2 mg/mouse immediately before snake venom injection showed no significant difference with control mice at 95% confidence. Data showed in Table 15.

Table 15. Survival time of mice feeding solution of ethanol extract (residue suspended distilled water) immediately before snake venom injection

Group	Survival animal/Total	Survival time (min.): Mean ± SE
Control	0/4	90 ± 10.51
T.reidioides + *A.catechu* (0.6:0.2 mg/mouse)	0/4	74 ± 12.68

c) Feeding ethanol extract 1 hr before snake venom injection

The result showed no significant difference in survival time of mice feeding ethanol extract compared to control mice at 95% confidence. Data showed in Table 16.

Table 16. Survival time of mice feeding ethanol extract (residue suspended with distilled water) 1 hr before snake venom injection

Group	Survival animal/Total	Survival time (min): Mean ± SE
Control	0/4	90 ± 11.03
T.reidioides + *A.catechu* (1.2:0.4 mg/mouse)	0/4	133 ± 28.88

2) Residue suspended with alcohol

a) Feeding plant extract 1 hr before snake venom injection

The *Naja kaouthia* venom at dose 6μg/mouse produced 100% death of mice. Results were showed in Table 17. The survival time of mice in sample groups was not significantly different from control group at 95 % confidence.

Table 17. Survival time of mice feeding ethanol extract (residue dissolved in ethanol 28°) 1 hr before snake venom injection

Group	Survival animal/Total	Survival time (min.): Mean ± SE
Control	0/4	130 ± 15.39
T.reidioides + *A.catechu* (0.6:0.2 mg/mouse)	0/4	135 ± 38.64
T.reidioides (0.6 mg/mouse)	0/4	193 ± 77.69
A.catechu (0.2 mg/mouse)	0/4	90 ± 9.12

4. Inhibition of myotoxicity effect

Injection of *N.kaouthia* venom induced myonecrosis as measured by serum CPK activity which increased from 102 ± 2.63 units/litre in normal mice (untreated control) to 2632 ± 498.64 units/litre. The water extract of *Trigonostemon reidioides* and *Areca catechu* at dose 0.6:0.2 mg/mouse feeding with powder of plant two times significantly decreased the CPK activity about 77%. However, The water extract of *T. reidioides* and *A. catechu* at dose 0.6:0.2 mg/mouse feeding with powder one time decreased CPK activity with no significant difference from control. Data was shown in Table 19.

Table 18. Inhibition of Myotoxicity by water extracts

Groups	CPK (units/L): Mean±SE	% Inhibition
Normal mice	102 ± 2.63	-
Control mice	2632 ± 498.64	0
T.reidioides + *A.catechu* (0.6:0.2 mg/mouse) feeding one time	1729 ± 607.36	34.31
T.reidioides + *A.catechu* (0.6:0.2 mg/mouse) feeding two time	585 ± 139.38*	77.77

* Significant different from control, $p<0.05$ (One way ANOVA; Tamhane's T2)

N = 6

CHAPTER V
DISCUSSION AND CONCLUSIONS

Venomous snakebite is still an important public health problem in Thailand. Approximately 7,000 cases of snakebites are reported annually. The most dangerous snake causing life-threatening envenomation is the Thai monocellate cobra, *Naja kaouthia*. It is one of the common poisonous snakes found throughout Thailand. Their toxins are composed of neurotoxin, cardiotoxin, enzymes and proteins (Aksaranugraha, Penchart and Pipatanakul, 1983; Lee, 1971; Sivamogsthem and Tejasen, 1973; Lai, Wen and Lee, 1972). The victims may die from respiratory muscle paralysis which is the major cause of death (Aksaranugraha et al., 1983; Sivamogsthem and Tejasen, 1973; Chulee Mitrakul et al., 1984). Minor cause includes sepsis and neumonia (Payom Buranasin, 1993). Cobra antivenom and assisted ventilation can save life in many cases of neurotoxic sign. However, antivenom carried a risk of severe adverse reactions (Warrell, 1999; Gilon et al, 1989; Chippaux and Goyffon, 1998) and other problems such as difficulty to manage and usage, variety of dose and high cost (Sornchai Looareesuwan et al., 1988; Chippaux and Goyffon, 1998). Furthermore, antivenom sometimes does not provide enough protection against snake envenomation, especially local poisoning (Sutherland, 1977; Corregan et al., 1987; Gilon et al., 1989; Warrell, 1989; Leon et al., 2000).

The use of plants against the effects of snakebite has long been recognized, even in modern times, only for the last 20 years it has merited to closer scientific attention (Nakagawa et al., 1982; Selvanayagam et al., 1995; Pereira et al., 1994). Several reviews are published (Mors, 1991; Martz, 1992; Houghton and Osibogum, 1993; Selvanayagam et al., 1994). While quite a number of reports from different geographical areas, mention plants reputed to neutralize the action of snake venom, only a few attribute such activity to certain chemical compounds identified in them (Pereira et al., 1994), and even less are concerned with a possible mechanism of action.

In Thailand, many plants were recognized against snake envenomation (มาลี บรรจบ และคณะ ,2543; สมหมาย กระจ่างลิขิต และคณะ, 2525; พระเทพวิมลโมลี, 2522). The

preparation of Lot Thanong Daeng (*Trigonostemon reidioides* Criab.) and nut seed (*Areca catechu* Linn.) was used by folk healer in Surin Province to treat people who were bitten by venomous snakes for many years ago. Because of the problems concerning the failure of antivenom, the doctor at Kabchoeng Hospital has been using this preparation for treatment of snakebite in hospital for more than 10 year. Although, this preparation is effective to treat patients with snakebite and not having any death, there is still little scientifically proved for its action.

The water extract of nut seed (*Areca catechu* Linn.) significantly neutralized lethal dose of *Naja kaouthia* venom when pre-incubated plant extract and snake venom before injected intramuscularly to mice. All mice in this group survived, whereas all control mice died. In the same way, extracts of mixed-plants (*T.reidioides* and *A.catechu*) also neutralized lethal dose of *N. kaouthia* venom. Four mice survived from total six mice. When increasing dose of mixed-plant extract survival time of mice also increased. On the other hand, the extract of *T.reidioides* alone did not neutralize *N.kaouthia* venom, all mice in this group died and survival time did not differ from control mice. Therefore, the neutralization of *N.kaouthia* venom may be the effect of *A.catechu* only. However, effect of *A.catechu* presented in mixed-plant extract had the lesser activity than extract from *A.catechu* alone. This result may be related to pH of the extracts. Extract of *A.catechu* had higher than extract mixed with *T.reidioides*. When increased dose of mixed-plant extracts the capacity of neutralization of snake venom was also increased, so it seems to be dose dependent. This result similar to previous studies. Pakatip Ruenraroengsak (2002) reported that aqueous-ethanol (50%) extract of *Areca catechu* Linn. seed could inhibit lethal activity of *N.kaouthia* venom and *Doboia russelli siamensis* venom and also inhibit acetylcholinesterase activity of *N.kaouthia* venom. The venoms were neutralized by precipitation of venom proteins. The effective dose (ED_{50}) of tannin content against lethal activity varied depending on the content of condensed tannin in the extract. The seed of *A.catechu* contained high content of polyphenols (tannin) both hydrolysable and condensed tannins. Gallic acid and tannic acid were found as the tannin contents of hydrolysable tannins whereas dimmer, trimer and tetramer of procyanidin were found as the condensed tannin in *A.catechu*. It is also known that polyphenols in plant materials may inhibit lethal activity by precipitating

enzyme proteins and the degree of inhibition is proportional to the polyphenols concentration.

Filtrate from water extract of *T.reidioides* and *A.catechu* could not inhibit lethal activity of *N.kaouthia* venom in mice when administered by intramuscular injection immediately after envenomation or by oral route at varying time (1, 2, 3 hr and immediately before envenomation or immediately after envenomation). However, water extract that did not filter before used (feeding extract with powder of mixed-plants) increased percentage of survival animal especially when administered orally 1 hr before envenomation. Inhibition of lethal activity of the extract is dose dependent. In addition, this water also inhibited myotoxicity in mice when administered orally 1 hr prior to envenomation. It significantly decreased creatinine phosphokinase (CPK) activity in serum of mice from 2632 $\pm$ 498.64 units/L to 585 $\pm$ 139.38 units/L at dose of *T.reidioides* 0.6 mg/mouse: *A.catechu* 0.2 mg/mouse when administered orally two times 1 hr prior to envenomation. Although, at the same dose when feeding one time, CPK activity did not significantly differ from control mice, but it decreased to 1729 $\pm$ 607.36 which was nearly half of the control mice, the nonsignificance may due to the variation in response of animals in this group.

From the above results, it showed that active compounds of *T.reidioides* and *A.catechu* preparation may be both the soluble and non-soluble fraction of mixed-plant extract. As showed in previous studies, the chemical constituents of *T.reidioides* are high content of steroids including β-*sitosterol*, *stigmasterol* and *campesterol* and the mixture of steroid in other form including *steroid palmitate* (β-sitosteryl palmitate, stigmasteryl palmitate, campesteryl palmitate and cholesteryl palmitate) and *steroid glycoside* (β-sitosteryl-3-O-glucopyranoside, stigmasteryl-3-O-glucopyranoside and campesteryl-3-O-glucopyranoside) which are not soluble in water (Theerawut Wangamnauyporn, 1998; วิภา เชิดชูสกุลชัย, 2530). Sitosterol and stigmasterol were reported to have anti-snake venom activity. They were able to neutralize lethal dose of South American rattlesnake venom (Mors et al, 1989) and inhibit myotoxicity of crotalid venoms (Melo et al., 1994). In addition, sitosterol increased percent survival of mice when administered orally 1 h prior to envenomation of *Bothrops jararaca* snake (Pereira et al., 1994).

Mahanta and Mukherjee (2001) used *Mimosa pudica* root extract against *Naja kaouthia* venom measuring lethal activity, myotoxicity and toxic enzymes. The result showed that aqueous extract displayed a significant inhibitory effect on lethality, myotoxicity and toxic enzymes (protease, phospholipase A_2 and acetylcholinesterase) when pre-incubated with venom before injection intramuscularly to mice. Whereas alcoholic extract failed to inhibit lethal activity and myotoxicity and was less effective in inhibition of toxic enzymes compared with aqueous extract. This result is quite similar to our study using the preparation of *T.reidioides* and *A.catechu.* Ethanol extract of *T.reidioides* and *A.catechu* either in mixed or single plant both re-suspended in water and ethanol 28° could not inhibit lethal activity of *N.kaouthia* venom at dose 6 μg/mouse when administered orally at varying time. The chemical constituents of *Mimosa pudica* roots contained ascorbic acid, crocetin, D-gluronic acid, palmitic and steric acid, mimosine, D-xyrose, linoleic acid, linolenic acid and β-sitosterol. The latter compounds, *β-sitosterol* is also found in the root of *T.reidioides*.

Other chemical constituents of *T.reidioides* root included a mixture of long chain acid (C_{16}-C_{35}), acetyl aleuritolic acid, Trigonostemone (1,1,7-trimethyl-3,6,9-trimethoxy-2-phenanthrenone), 5-hydroxy-6,7-dimethoxycoumarin, 5,7-dihydroxy-6-methoxy coumarin, a mixture of long chain amide (C_{44}-C_{48}), and water soluble fraction constituents as sugars (glucose, fructose, arabinose and rhamnose), amino acids (glycine, alanine, isoleucine, and γ-aminobutyric acid) and chloride salts (Wangamnauyporn, 1998; วิภา เชิดชูสกุลชัย, 2530) which may be potentiated the effectiveness especially coumarin, that once reported to have anti-snake venom activity (Pereira et al., 1994; Mors et al., 2000).

Inconclusion, the preparation of *T.reidioides* and *A.catechu* could inhibit lethality and myotoxicity of *N.kaouthia* venom when dissolved in water and administered the solution with powder of plants. The important active components may be polyphenols from *A.catechu* that could neutralize snake venom and the steroids from *T.reidioides*. Due to the fact that the steroid components are not soluble in water, so the water extract of mixed-plants should be administered together with powder. This preparation are more safely and effectively than other preparations studied.

This present investigation will support scientifically the use of preparation from *T.reidioides* and *A.catechu* for treating snakebite patients in the same previous way described by folk healer and doctor at Kabchoeng Hospital, Surin Province.

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