Studies on antivenom activity of Andrographis paniculata and Aristolochia indica plant extracts against Echis carinatus venom

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Citation

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Abstract

Andrographis paniculata and Aristolochia indica plant extracts were tested for antivenom activity against Echis carinatus venom. Both plant extracts were effectively neutralized the Echis carinatus venom induced lethal activity. About 0.19mg of Andrographis paniculata and 0.17mg of Aristolochia indica plant extracts were able to completely neutralize the lethal activity of $2LD_2$ of Echis carinatus venom. Various pharmacological activities like haemorrhagic, coagulant, edema and phospholipase activities were studied and these pharmacological activities were significantly neutralized by both plant extracts The above observations confirmed that Andrographis paniculata and Aristolochia indica plant extracts possess potent snake venom neutralizing capacity and could be used for therapeutic purposes in case of snakebite envenomations.

INTRODUCTION

Snake envenoming is a major public health issue in the rural tropics with large numbers of envenoming and deaths. In India there are about 216 different species are found, of which 53 species are reported to be poisonous. The common poisonous snakes found in India are Cobra (Naja naja), Krait (Bangarus Caeruleus), Russell's viper (Daboia russelli) and Saw Scaled Viper (Echis Carinatus) (Bawaskar, 2004). About 35,000 to 50,000 people die of snakebite every year in India. Echis carinatus (Saw-scaled viper) occurs in both India and Pakistan, where it is responsible for a large number of snakebite case, reaching 95% of envenomations in the state of Jammu (Chippaux, 1998). Antivenom immunotherapy is the only specific treatment against snake venom envenomation. Antivenoms are usually hyper immune sera collected from animals which bind and inactivate venom components. There are various side effects of antivenom such as anaphylactic shock, pyrogen reaction and serum sickness. Most of these symptoms may be due to the action of high concentrations of nonimmunoglobulin proteins present in commercially available hyper immune antivenom (Maya Devi et al, 2002). Antiserum development in animal is time consuming, expensive and requires ideal storage condition. Over the years many attempts have been made for the development of snake venom antagonists especially from plants sources. Extracts from plants have bee used among traditional healers, especially in tropical areas where here are plentiful sources, as therapy for snakebite for a long time (Saka Daduang et al., 2005). Several medicinal plants, which appear in old drug recipes or which have been passed on by oral tradition, are believed to be snakebite antidotes. In modern science, there have been many attempts to study these plants to clarify their effectiveness (Houghton et al., 1993 and Otero et al., 2000). India has a rich tradition of the usage of medicinal plants. Many Indian medicinal plants are recommended for the treatment of snakebite (Alam et al., 2003). Some of these plant extracts has shown antivenom property on land snakes and their possible mechanism of action have been suggested. In almost any part of the world, where venomous snakes occur, numerous plant species are used as folk medicine to treat snakebite. The present investigation explored the Echis carinatus venom neutralizing activity of Andrographis paniculata and Aristolochia indica plant extracts by in vivo and in vitro methods.

MATERIALS AND METHODS

VENOM AND EXPERIMENTAL ANIMALS

The free-dried snake venom powder of Echis carinatus was obtained from Irula's Snake Catchers Industrial Co-operative

Society Limited, Chennai and was stored at 4ºC. Male inbreed Swiss albino mice 18-20 gm were used for the studies of venom toxicity and in the experiments of venom neutralization. Institutional Animal Ethics Committee clearance at Institute of vector control and Zoonooses, Hosur, was obtained to conduct the experiment.

MEDICINAL PLANS

Andrographis paniculata and Aristolochia indica plants were obtained from RVS Ayurveda College, Sulur.

PREPARATION OF EXTRACTS

The whole plant material was dried in shade and the air-dried plant was ground and extracted first by refluxing with petroleum ether (50–60 °C, for 72 h) and then with methanol (60–80 °C for 72 h). The methanol extract was concentrated in vaccuo and kept in a desiccator at room temperature for further use. Before use, it was dissolved in saline and centrifuged at 2000 rpm for 10 min at room temperature. The supernatant was used for further investigation and kept at 4 °C. The plant extracts were expressed in terms of dry weight.

TESTS FOR ANTI-SNAKE VENOM ACTIVITIES

LETHAL TOXICITY

The median lethal dose (LD_{50}) of Echis carinatus venom was determined according to the method developed by Theakston and Reid 1983. Various doses of venom in 0.2 ml of physiological saline was injected into the tail vein of mice (18-20gms), using groups of 3-5 mice at each venom dose. The LD_{50} was calculated with the confidence limit at 50% probability by the analysis of deaths occurring within 24 h of venom injection. The anti-lethal potentials of Andrographis paniculata and Aristolochia indica plant extracts were determined against $2LD_{50}$ of Echis carinatus venom. Various amount of Plant extracts (µl) were mixed with $2LD_{50}$ of venom sample and incubated at 37ºC for 30 minutes and then injected intravenously into mice. 3 – 5 mice were used at each antivenom dose. Control mice received same amount of venom without antivenom (Plant extracts). The median Effective Dose (ED_{50}) calculated from the number of deaths within 24h of injection of the venom/antivenom mixture. The ED_{50} was expressed as µl antivenom/mouse and calculated by probit analysis.

EDEMA- FORMING ACTIVITY

The Minimum edema-forming dose (MED) of Echis carinatus venom was determined by the method of Lomonte et al. (1993) and Camey et al. (2002). Group of four mice were injected subcutaneously in the right footpad with various amounts of venom (0.25µg - 10µg) dissolved in 50µl of Phosphate – Buffered Saline (PBS) pH 7.2. The left footpad received 50µl of PBS alone (control). Edema was calculated as percentage of increase in the thickness of the right foot injected with venom compared to the left foot. The thickness of each footpad was measured every 30 min after venom injection with a low-pressure spring caliper (Rojas et al., 2005). Minimum edema-forming dose (MED) was the venom dose that induced 30% edema within 6 hours of venom injection when compared to control. The ability of both plant extracts in neutralizing the Edema- forming activity were carried out by pre-incubating the constant amount of venom and various dilutions Andrographis paniculata and Aristolochia indica plant extracts and incubated for 30 minutes at 37ºC. Then, groups of four mice (18 - 20g) were injected subcutaneously in the right footpad with 50µl of the mixtures, containing venom/plant extracts, whereas the left footpad received 50µl of PBS alone. Control mice were injected with venom in the right footpad and 50µl of PBS in the left footpad. 1 hour after injection edema was evaluated as described by Yamakawa et al., 1976. Edema was expressed as the percentage increase in thickness of the right footpad compared to the right footpad of the control mice.

HAEMORRHAGIC ACTIVITY

The minimum haemorrhagic dose (MHD) of Echis carinatus venom was determined by the method described by Theakston and Reid, 1983. The minimum haemorrhagic dose was defined as the least amount of venom which when injected intradermaly (i.d.) into mice results in a haemorrhagic lesion of 10mm diameter in 24 hours. Neutralization of the haemorrhagic activity was estimated by mixing a fixed amount of venom with different amounts plant extracts. The Plant extract–venom mixture was incubated at 37°C for 1 h and 0.1 ml of the mixture was injected intradermaly into mice. The haemorrhagic lesion was estimated after 24 h.

PHOSPHOLIPASE ACTIVITY

Phospholipase A2 activity was measured using an indirect hemolytic assay on agarose–erythrocyte–egg yolk gel plate by the methods described by Gutierrez et al., 1988. Increasing doses of Echis carinatus venom (µg) was added to 3mm wells in agarose gels (0.8% in PBS, pH 8.1) containing 1.2% sheep erythrocytes, 1.2% egg yolk as a source of

lecithin and 10mM $Cacl_2$. Slides were incubated at 37˚C overnight and the diameters of the hemolytic halos were measured. Control wells contained 15l of saline. The minimum indirect hemolytic dose (MIHD) corresponds to a dosage of venom, which produced a hemolytic halo of 11mm diameter. The efficacy of antivenom (Plant extracts) in neutralizing the phospholipase activity was carried out by mixing constant amount of venom (µg) with different amount of plant extracts (µl) and incubated for 30 minutes at 37C. Then, aliquots of 10l of the mixtures were added to wells in agarose-egg yolk-sheep erythrocyte gels. Control samples contain venom without Plant extracts. Plates were incubated at 37C for 20 hours. Neutralization expressed as the ratio mg antibodies/mg venom able to reduce by 50% the diameter of the hemolytic halo when compared to the effect induced by venom alone.

PROCOAGULANT ACTIVITY

The procoagulant activity was done according to the method described by Theakston and Reid, 1983 modified by Laing et al., 1992. Various amounts of venom dissolved in 100µl PBS (pH 7.2) was added to human citrated plasma at 37C. Coagulation time was recorded and the Minimum Coagulant Dose (MCD) was determined as the venom dose, which induced clotting of plasma within 60 seconds. Plasma incubated with PBS alone served as control. In neutralization assays Constant amount of venom was mixed with various dilutions of plant extracts. The mixtures were incubated for 30 minutes at 37C. Then 0.1ml of mixture was added to 0.3ml of citrated plasma and the clotting times recorded. In control tubes plasma was incubated with either venom alone or plant extracts alone. Neutralization was expressed as effective dose (ED), defined as the ratio l antivenom (plant extracts)/mg venom at which the clotting time increased three times when compared with clotting time of plasma incubated with two MCD of venom alone.

STATISTICAL ANALYSIS

Statistical evaluation was performed using XL stat 2007 and SPSS 10 Softwares. P< 0.005 was considered statistically significant.

RESULTS

The antivenom potential of Andrographis paniculata and Aristolochia indica plant extracts were tested against Echis carinatus venom by in vivo and in vitro methods. The lethal toxicity (LD_{50}) of Echis carinatus venom was assessed using 18g, Balb/c strain mice. About 12g of Echis carinnatus venom was found to be LD_{50} for 18g of mice. The neutralization of lethality was done by mixing constant amount of venom with various dilutions of Andrographis paniculata and Aristolochia indica Plant extracts and incubated at 37°C for 30 minutes prior to injection. We found that 0.19mg of Andrographis paniculata and 0.17mg of Aristolochia indica plant extracts were able to completely neutralize the lethal activity of $2LD_{50}$ of Echis carinatus venom (Table 1). When comparing to Andrographis paniculata , Aristolochia indica effectively neutralize the lethal toxicity (Fig 1).

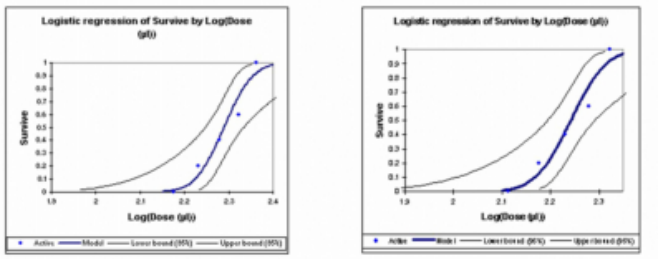
Figure 1

Table 1: Neutralization of venom induced lethality by and Plant extracts

Plant Extracts	Dose of *Echis carinatus* venom (µg)	Neutralization of venom by Plant extracts (ED_{50} in mg)
Andrographis paniculata	24 ($2LD_{50}$)	0.19 mg
Aristolochia indica	24 ($2LD_{50}$)	0.17 mg

Figure 2

Figure 1: Dose Response curve for Neutralization of Lethality by a) and



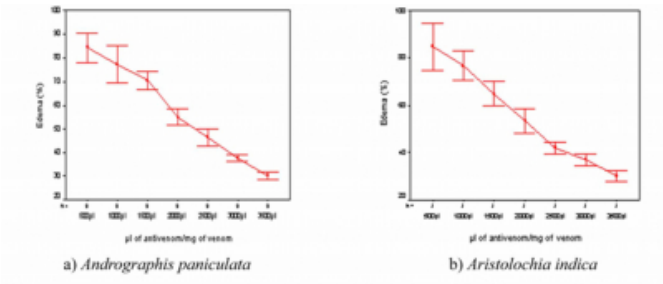
a) *Andrographis paniculata* b) *Aristolochia indica*

Fig 1 - Dose response curve for Neutralization of Lethality by a) *Andrographis paniculata* and b) *Aristolochia indica* plant extracts against *Echis carinatus* venom in experiments involving preincubation of venom ($2LD_{50}$) and various concentrations of antivenoms (Plant extracts). The median effective dose for $2LD_{50}$ of *Echis carinatus* venom was 194.67µl (176.753-215.702) for *Andrographis paniculata* and 174.54µl (156.53-195.69) for *Aristolochia indica* plant extracts. P < 0.005

In edema forming activity, the mice immunized with Echis carinatus venoms showed increase in footpad thickness. About 7g of Saw-scaled viper venom induced edema formation within 3h which is considered as 100% activity. The edema was reduced up to 30% when 3.5mg of plant extracts /mg venom was given. There was no further reduction in the percentage of edema even when there was an increase in antivenom dose (Fig 2).

Figure 3

Figure 2: Neutralization of edema induced by Echis carrinatus venom by a) and



a) *Andrographis paniculata* b) *Aristolochia indica*

Fig 2 - Neutralization of Edema induced by *Echis carinatus* venom by *Andrographis paniculata* and *Aristolochia indica* plant extracts in experiments with pre-incubation. Various mixtures of venom and plant extracts were incubated and tested in the foot pad assay. Edema was assessed 1 hour after injection and expressed as percentage. Edema induced in control mice (venom alone) was considered as 100% activity. Results presented as mean ± SE (N=3). P<0.005 at all antivenoms/venom ratios.

In the case of hemorrhagic activity, Saw-scaled viper venom produced visible hemorrhagic spot. About 8g of venom produced a hemorrhagic spot of 10mm diameter (MHD). Both Plant extracts were able to neutralize the hemorrhage induced by the venom. In phospholipase activity (PLA2), Echis carinatus venom able to produce hemolytic haloes in agarose-sheep erythrocytes gels. About 10g of Saw scaled viper venom produced 11mm diameter hemolytic halo, which is considered to be 1U (U/10g). This shows that Echis carinatus venoms have the enzymes (PLA2) that has the ability to lyse sheep RBC's. Andrographis paniculata and Aristolochia indica Plant extracts were capable of inhibiting PLA2 dependent hemolysis of sheep RBC's induced by Echis carinatus venom in a dose dependent manner (Fig 3). We found that that 0.17mg of Andrographis paniculata and 0.14mg of Aristolochia indica plant extracts were able to completely inhibit PLA2 dependent hemolysis of sheep RBC's induced by Echis carinatus venom

Figure 4

Figure 3: Inhibition of Phospholipase activity by a) and

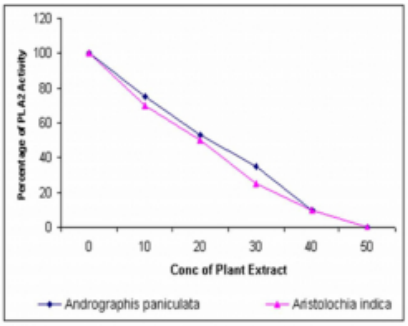


Fig 3 - Inhibition of Phospholipase activity by plant extracts. *Echis Carinatus* venom was pre-incubated with various concentrations of plant extracts for 1hr at 37 °C. Plant extracts were capable of inhibiting PLA_2 activity of sheep RBCs induced by *Echis Carinatus* venom in dose dependent manner. The PLA_2 activity in the absence of plant extacts was considered as 100%. Values represent mean of triplicate values.

The minimum coagulant dose (MCD) was determined as the venom dose inducing clotting of plasma in 60s. About 120g of Saw-scaled viper venom clotted human citrated plasma within 60s. In the neutralization assay, the absence of clot formation shows the neutralizing ability of both Plant extracts. We found that that 2.1mg of Andrographis paniculata and 1.8mg of Aristolochia indica plant extracts were able to completely neutralize coagulant activity. High dose of venom caused rapid clotting that required very high dose of antivenom to neutralize.

DISCUSSION

Snakebite is a common medical emergency encountered in the tropics and estimated 35,000 to 50,000 people die of snakebite every year in India (Sharma et al., 2004). Saw scale vipers or Echis carinatus are commonly found in the semi-arid deserts of Western Rajasthan with predominant nocturnal habitus. Snake venom is a highly complex mixture of a variety of biological substances including 90% of water, 20-25 enzymes, and a large number of low molecular weight peptides. Envenomation by Echis carinatus is associated with a mortality rate of 10-20%, if effective treatment is not initiated early. The major cause of mortality is due to increased bleeding tendency caused by the venom. Venom of viper is a mixture of multipleenzymes and low molecular weight peptides, some of which are responsible for the bleedingmanifestations. Multiple mechanisms have beensuggested for the bleeding occuring afterenvenomation. These include disseminatedintravascular coagulation (DIC) due to spontaneous activation of factor V and factor Xby pro-coagulants present in the venom. It has also been suggested that the small dose of venom,as typically injected

in humans, leads to continuousactivation of fibrinogen, producing a fragile fibrinmore susceptible to lysis than ordinary fibrin,leading to bleeding manifestations. Vascular endothelial damage caused by the haemorrhagin present in the venom also contributes to bleeding manifestations. (Manoj Lakhotia et al., 2002). Antivenom is the specific antidote for snakebite envenomation. Antivenom against snakes bites are lacking in the rural areas of coastal region. Antiserum; being the only therapeutic agent, its development from animal source is time consuming and expensive. Although, use of plants against the effects of snakes bite has been long recognized, more scientific attention has been given since last 20 years (Santosh et al., 2004). Many Indian medicinal plants are recommended for the treatment of snakebites (Alam et al., 2003). In our present study we check the antivenom potential of Andrographis paniculata and Aristolochia indica plant extracts against Echis carinatus venom. It is essential to understand the pharmacological action of snake venom in order to device a rational treatment for snakebite. An important initial step in finding antivenom potential of plant extracts is the pre-clinical testing using in vivo and in vitro methods to assess the neutralizing potential of the antivenom (plant extracts) against a range of different venom effects. The neutralization ability of snake antivenoms is still assessed by the traditional in vivo lethality assay (minimum effective dose ED_{50}), comparable to those used for bacterial antitoxins, usually performed in mice (WHO, 1981). Thus various pharmacological activities like lethality, edema forming activity, hemorrhagic activity, phospholipase activity (PLA2), procoagulant activity caused by Echis carinatus venom were carried out. Neutralization of these pharmacological effects was carried out using Andrographis paniculata and Aristolochia indica plant extracts. Neutralization studies can be performed by incubating of venom and plant extracts prior to testing (pre-incubation method). The results showed that the both plant extracts were capable of neutralizing the lethality induced by the venom. The Echis carinatus venom showed the presence of PLA2 enzymes by means of producing hemolytic haloes in indirect hemolytic assays. Both plant extracts were capable of inhibiting PLA2 dependent hemolysis of sheep RBCs in a dose dependent manner. The medicinal plants Thea sinensis Linn and Cordia verbenacea were effectively neutralized the phospholipase A2 activity induced by snake venoms (Yao-Ching Hung et al., 2004 and Ticli et al., 2005). Edema-forming activity was assessed for Echis carinatus venom and both plant extracts were found to be effective in neutralization of edema induced by venoms. There was a significant decrease in the edema (footpad thickness) when there was an increase in the antivenom (plant extract) dose. Procoagulant activity induced by Echis carinatus venom was studied using human citrated plasma and Andrographis paniculata and Aristolochia indica plant extracts were found to be effective in the neutralization of procoagulant activity. The present experimental results indicate that Andrographis paniculata and Aristolochia indica plant extracts were effective in neutralizing the main toxic and enzymatic effects of Echis carinatus venom. The antivenom properties of both plant extracts were potent enough to neutralize the lethality and various pharmacological activities of venom. The result from this preliminary study indicates that both plant extracts could be used for therapy in patients with snakebite envenomation. Further investigations are needed for identification and purification of the active components involved in the neutralization of the snake venom.

References

r-0. Alam, M.I., and Gomes. A. 2003. Snake venom neutralization by Indian medicinal plants (Vitex negundo and Emblica officinalis) root extracts. Journal of Ethnopharmacology. 86: 75–80

r-1. Bawaskar, H.S., 2004. Snake venoms and antivenoms: critical supply issues. Journal of the Association of Physicians of India 52, 11–13.

r-2. Chippaux, J.P. 1998. Snake-bites: appraisal of the global situation. Bull WHO.
76: 515 - 524.

r-3. Ermila Rojas., Lil Quesada, Viviana Arce, Bruno Lomonte, Gustavo Rojas and Jose Maria Gutierrez. 2005. Neutralization of four Peruvian Bothrops sp. snake venoms by polyvalent antivenoms produced in Peru and Costa Rica: preclinical assessment. Acta Tropica. 93: 85 – 95.

r-4. Gutierrez, J.M., C.Avila, E.Rojas, L.Cerdas. 1988. An alternative in vitro method for testing the potency of the polyvalent antivenom produced in Costa Rica. Toxicon. 26: 411– 413.

r-5. Houghton. P.J., I.M. Osibogun. 1993. Flowering plants used against snakebite, J. Ethnopharmacol. 39 : 1–29.

r-6. Kyoko.U.Camey., David T. Velarde, Eladio F. Sanchez. 2002. Pharmacological characterization and neutralization of the venom used in the production of Bothropic antivenom in Brazil. Toxicon. 40: 501 – 509.

r-7. Laing, G.D., Theakston, R.D.G., Leite, R.P., Dias Da Silva, W.D., Warrell, D.A., BIASG, 1992. Comparison of the potency of three Brazilian Bothrops antivenoms using in-vivo rodent and in-vitro assays. Toxicon 30 (10), 1219–1225.

r-8. Lomonte, B., Tarkowski, A., Hanson, H.A., 1993. Host response to Bothrops asper snake venom: analysis of edema formation, inflammatory cells, and cytokine release in mouse model. Inflammation 17, 95–105.

r-9. Manoj Lakhotia, Dinesh Kothari, Dharma Ram Choudhary, Sanjeev Sharma, Pravesh Jain. 2002. A Case of Saw Scale Viper Snakebite Presenting as Pleuro-pericardial Haemorrhage. JIACM. 3(4): 392-4.

r-10. Maya Devi, C., Vasantha Bai, Mary, Vijayan Lal, Arthur, Umashankar, P.R., Krishnan, Lissy K., 2002. An improved method for isolation of antiviper venom antibodies

from chicken egg yolk. Journal of Biochemical and Biophysical Methods 51, 129–138.
r-11. Otero.R.., R. Fonnegra, S.L. Jimenez, V. Nunez, N. Evans, S.P. Alzate, M.E. Garcia, M. Saldarriaga, G. Del Valle, R.G. Osorio, A. Diaz, R. Valderrama, A. Duque, H.N. Velez. 2000. Snakebites and ethnobotany in the northwest region of Colombia: I. Traditional use of plants. J. Ethnopharmacol. 71: 493–504.
r-12. Sakda Daduang, Nison Sattayasai, Jintana Sattayasai, Pattara Tophrom, Achra Thammathaworn, Arunrat Chaveerach, Monruedee Konkchaiyaphum.2005. Screening of plants containing Naja naja siamensis cobra venom inhibitory activity using modiWed ELISA technique. Analytical Biochemistry. 341: 316–325
r-13. Santosh, R., Fattepur and Shivaji P Gawade.2004. Preliminary Screening of Herbal Plant Extracts for Anti-venom activity against Common Sea Snake (Enhydrina schistosa)Poisoning. Pharmacognosy Magazine. 16: 56 – 60
r-14.
r-15. Sharma Sanjib K, Chappiux Francois, Arjha Nalhamb, Patrick A, Loutan Louis, Koirala Shekhar. 2004. Impact of snake bites and determinants of fatal outcomes in southeastern Nepal. Am J Trop Med Hyg.71(2):234—8.
r-16. Theakston, R.D.G., Reid, H.A., 1983. Development of simple standard assay procedures for the characterization of snake venoms. Bulletin of the World Health Organization 61, 949–956.
r-17. Ticli FK, Hage LI, Cambraia RS, Pereira PS, Magro AJ, Fontes MR, Stábeli RG, Giglio JR, França SC, Soares AM, Sampaio SV.2005. Rosmarinic acid, a new snake venom phospholipase A2 inhibitor from Cordia verbenacea (Boraginaceae): antiserum action potentiation and molecular interaction. Toxicon. 46(3):318-27.
r-18. World Health Organization.1981.Progress in characterization of venoms and standardization of antivenoms. WHO offset publication number.58, Geneva.
r-19. Yao-Ching Hung, Vasyl Sava, Meng-Yen Hong, G. Steven Huang. 2004. Inhibitory effects on phospholipase A2 and antivenin activity of melanin extracted from Thea sinensis Linn. Life Sciences. 74 : 2037–2047
r-20. Yamakawa M, Nozaky M, Hokama Z. 1976. Fractionation of sakishimahabu (Trimeresurus elegans) venom and lethal, hemorrhagic and edema-forming activities of the fractions. In: Ohsaka A, Hayashi K, Sawai Y, editors. Toxins: animal, plant and microbial. New York: Plenum Press; 1976. p. 97-109.

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