Protein Characterization of Javan Cobra (*Naja sputatrix*) Venom Following Sun Exposure and Photo-Oxidation Treatment

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Abstract. Snake venom has always been known for its toxicity that can cause fatality, however, it is also one of the important biological resources to be used for disease treatment. In Indonesia, snake venom previously expose under the sun has been used for alternative treatment of some diseases such as dengue fever, atherosclerosis, cancer, and diabetes. There has been very little scientific evidence on the use of snake venom of Indonesia origin as well as its protein characteristic. Thus, the objective of this research is to characterize the protein content and the specific activity of the venom of Javan Cobra (*N.sputatrix*) when treated with sun exposure in comparison with photo-oxidation by ultraviolet. Qualitative analysis of protein contents was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The L-amino acid oxidase activity (LAAO) and the phospholipase A_2 (PLA_2) activities were determined using spectrophotometry. The venom's protein was separated into 5 main protein bands with molecular weight ranging from 14 to 108 kDa. A time course study showed that the venom lost 91% of its LAAO activity and 96% of PLA_2 activity after 6 hours of sun exposure. UV photo-oxidation carried out for 3 hours decreased 91% of LAAO activity, and almost diminished all of PLA_2 activity (99.8%). These findings suggest that the exposure of *N. sputatrix* venom under the sun and UV photo-oxidation decreased its toxicity as shown by the significant reduction of the enzymes activity, but did not affect the protein's integrity. Therefore, these approaches produced *N.sputatrix* venom with less toxicity but still withheld other characters of intact proteins.

1. Introduction

Snake venom has always been known for its toxicity that is potential for lethality, however, it is also one of the important biological resources to be used for disease treatment. The first use of snake venom in modern medicine has been reported in 1970 and has been approved by the US *Food and Drug Assosiation* (FDA) in 2004 as medicinal source for treatment of high blood pressure- and cardiovascular-related diseases [1]. Its applications ranging for treatment of heart disease, diabetes, cancer, as well as *Human Immunodeficiency Virus* (HIV) infection [2]. In China and India, snake venom has been used for treatment of stroke, ischemia, and haemorraghia [3]. Oral administration of snake venom had been showed to increase immune response [4]. It has been reported also that photo-oxidized venom was able to immunized rats against snake venom toxin [5]. Protein isolated from snake venom such as phospholipase A2 (PLA_2), L-amino acid oxidase (LAAO) and other proteins have been showed in vitro as potential cure for cancer through various mechanisms such

as necrosis and apoptosis [6,7]. Snake venom also exhibited coagulation and fibrinolytic effects associated with hemostasis [2].

Several approaches could be used to reduce the toxic activity of the snake venom [5]. In India, photo-oxidation by ultraviolet was used to decrease the toxicity of snake venom, so the remaining effect is therapeutic activity [8,9]. The components of snake venom and its effects are varied and depending on the species, geographical location, climate, niche, sex, and age. Vyas et al. reported that the amounts of toxin being produced was decreased as the snake gets older [6]. In Indonesia, snake venom previously expose under the sun has been used for treatment of some diseases such as dengue fever, atherosclerosis, cancer, and diabetes. Particularly, javan cobra (*N. sputatrix*)'s venom pre-treated with sun exposure has been used for oral treatment of human cancer. Javan cobra, also known as *ular sendok*, is an Elapidae commonly found in Indonesia. There has been very limited information on the characteristics of the venom of this species of snake, especially the one that has been traditionally 'detoxified'. Thus, the objective of this research is to characterize the protein content and the specific activity of the venom of *N.sputatrix* when treated with sun exposure in comparison with photo-oxidation by ultraviolet. The venom's characterization includes: total protein and protein molecular weight determination, LAAO and PLA_2 enzymes activities, and anticoagulation activity. This research will provide preliminary scientific evidence on the application of snake venom as alternative therapy for infectious as well as non-infectious diseases.

2. Materials and Methods

The venom of *N.sputatrix* was obtained from The Indonesian Cobra Show (ICS), Bogor. Buffer solutions used in the experiments were as follows: Tris-HCl pH 7.4, Tris-HCl pH 6.8 and Tris-HCl pH 8.8. The standards solutions used in the experiments were *Bovine Serum Albumin* (BSA), L-leucin. and palmitic acid. The reagents and substrates used in enzymatic assays were Bradford reagent, ninhydrin, phenol-red, egg-yolk, and bovine whole blood. Supplies for running the SDS PAGE were acrylamide, bis-acrylamide, Sodium Dodecyl Sulphate (SDS), amonium persulphate (APS), tetramethyl-ethylenediamine (Temed), bromphenol blue, glicerol, mercaptoethanol, *Comassie Brilliant Blue*, methanol, glacial acetic acid, glicyine, dan unstained low molecular weight protein marker. All SDS PAGE materials were purchased from Thermo Scientific, USA. All chemicals for solutions such as NaOH, concentrated HCl, blue methylene, activated charcoal, DMSO, ethanol, sodium citrate, and $CaCl_2$.all were purchased from Merck, Darmstad, Germany.

2.1. Experimental Design

The experimentals approach of this research was to characterize several parameters of *N. sputatrix* venom activities when untreated or treated with either sun exposure or UV-photooxidation. Those parameters were: total protein content, protein molecular weight qualitative analysis, specific activity of LAAO and PLA_2 enzymes, and coagulation/anticoagulation activity of the venom.

2.2. Samples Preparation

Snake venom was milked from N. Sputatrix using a glass drinking cup, after which the collected venom was transported in a cool box (4-8°C) and kept at -20^{o}C until used for experiment. Sun-exposed venom was prepared by placing the sample in a closed petri dish directly under the sun during the daylight. The sun exposure was carried out for: 2, 4, and 6 hours. The UV photooxidation was done following the modified method of Gawade et al.[9]. Briefly, snake venom sample was diluted (1:2) with Tris-HCl buffer pH 7.4 and 2 ml of 0.05 M methylene blue solution was added. This mixture was poured in a petri dish and was incubated in a 37^{o}C waterbath under the UV lamp (Philips 25 Watt, germicidal 254 nm) at distance of 10 cm. The UV-photooxidation was carried out for 3 hours. Following the incubation, the UV-treated sample was transfered into a beaker glass and 0.5 gram of activated charcoal was added and mixed for 15 minutes. The mixture was filtered and the filtrate was kept in a vial at -20^{o}C until experiment.

2.3. Qualitative Protein Analysis with SDS PAGE

Qualitative analysis of protein contents was determined using SDS PAGE with 13% polyacrylamide. Diluted samples of snake venom (2%,v/v) were added with mercaptoethanol-containing reducing sample buffer. The sample mixtures was heated in boiling water for 15 minutes. After cooled down, all samples were loaded into wells (10µl each) in the polyacrylamide. Protein molecular weight marker was loaded in a 5-µl volume.The SDS PAGE was run for 1 hour 30 minutes at 80 volts. The gel was stained with solution of Coomassie brilliant blue for 12 hours. Destaining of the gel was done to clear up the remaining stain by soaking the gel in boiling water until all stains in the background was gone and all markers clearly stained. The electrophoregram was analyzed using PhotocapMw application (Vilber Lourmat, versi 10.0)

2.4. Determination of Total Protein Content

Total protein concentration was determined quantitatively using Bradford method [10] Samples of snake venom in aliquotes of 50 µL were diluted to 100 mL with destilated H_2O. The BSA standard solutions at concentration of: 0,40, 80, 120, 160, and 200 µg/mL and diluted samples (100 µL) were mixed thoroughly with Bradford reagents and incubated at room temperature for 5 minutes. The absorbancy was measured using spectrophotometer at 615 nm wave-length.

2.5. Assay of L-Amino Acid Oxidase (LAAO) Acitivity

The LAAO activity was determined by ninhydrin test [11] The activity of the enzyme was determined by measuring the remaining amino acid substrate left after certain time of incubations. Samples of snake venom in 0.1M Tris-HCl Buffer pH 7.4 at concentration of 10 mg/mL (0.1 mL) were added to 4.9 mL of L-leucin solution in similar buffer (800 µg/mL) and the mixtures were incubated at 37^{o}C for periods of: 0, 2.5, 5, and 10 minutes. The reactions were stopped by heating the mixtures in waterbath at 100 ^{o}C for 10 minutes. Subsequently , 4 mL of this mixtures was taken for amino acid concentration analysis. Amino acid standard curve was made using 4 ml each of L-leucine standard solutions at concentrations of: 0, 500, 600, 700, 800, 900, and 1000 µg/mL. All samples and standard solutions were added with 1 ml of ninhydrin reagen. All reaction tubes were sealed with aluminium foil and the mixtures were shaken well, after which all were put in 100^{o}C waterbath for 15 minutes. After cooled down, all samples were measured their absorbance at 570 using spectrophotometer. All measurements were in triplicates.

2.6. Assay of Phospholipase A_2 (PLA_2) Activity

The PLA_2 activity was determined according to the modified method of de AraÚjo and Radvanyi [12]. The activity of the PLA_2 was determined by measuring the rate of product increment which was fatty acid that reduced the pH of the solution. The pH of the substrate, the fatty acid standard, and the phenol red indicator were all 7.4 at the beginning of the reaction. The phospholipid solution (2 ml) was incubated with the venom sample (1 mg/ml) at 37^{o}C for 0, 20, 40, and 60 detik. The reations were stopped by incubating the mixtures in boiling water (water bath) for 10 minutes. The fatty acid contents in the reaction mixtures and standard solutions (3 ml each) were measured by addition of 0.5 ml of 0.1% phenol red. The fatty acid standard curve was made by using palmitic acid standard at concentrations of : 0, 30, 60, 90, 120, and 150 µg/mL. The absorbances were read using spectrophotometer at 540 nm. All assays were performed in triplicates.

2.7. Assay of Coagulant and Anticoagulant Activity [13]

The coagulant effect of N. Sputatrix venom was determined by recording the clotting times after addition of the venom to citrate-containing bovine plasma according to the method of Theakston & Reid [14]. Addition of 250 mM $CaCl_2$ alone to aliquots of plasma was used as a positive control. Bovine plasma was obtained from the whole blood by addition of 3.2% sodium citrate anticoagulant to the whole blood (1:9, v/v). The plasma was separated from the blood cell by centrifugation in low-speed centrifuge (Hettich Universal) at 3000 rpm. The supernatant was the plasma which contained

fibrinogen and other inactivated coagulation factors. The plasma aliquots (3mL) to be assayed were prepared in reaction tubes and kept in 37°C waterbath. The venom at concentration of 1 mg/ml (0.2 mL) was mixed with the plasma and the recording of the clotting times was immediately begun. The anticoagulant activity was assayed using modified method of Suarez et al. [13] by incubating the citrated bovine plasma with the mixture of venom and 250 mM $CaCl_2$ and the clotting times recorded.

3. Results and Discussions

3.1. Protein Molecular Weight Analysis

Figure 1 shows that using SDS PAGE the venom's protein was separated into 5 main protein bands. PhotocapMw Application showed that the molecular weight of those bands were ranging from 15.5 to 107.9 kDa (Table 1). These observations are consistent with the ones reported by Suarez *et al*. (2016). The five observed protein bands were closely matched with phosphodiesterase, hyaluronidase, LAAO, prostatin-like protein, and PLA_2 reported in their works.There was no different between control venom and pre-treated venom samples. This observation indicates that the protein structure remained intact in the venom treated with either sun exposure or UV-photooxidation.

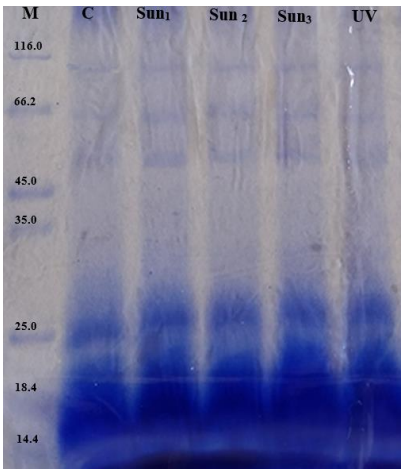

Figure 1. Electrophoregram of SDS PAGE of *N. sputatrix* venom. Lanes: C=control, untreated venom, Sun 1-3 = sun exposed (2,4,6hrs), UV=UV photo-oxidized (3 hrs)

Table 1. Molecular weight of *N. sputatrix* venom protein.

Molecular Weight (kDa)				
Control	Sun Exposure (2 hrs)	Sun exposure (4 hrs)	Sun exposure (6 hrs)	Photo-oxidation
102.9	102.9	106.1	102.9	107.8
64.9	64.5	65.9	65.2	63.8
53.9	54.3	55.1	55.5	54.7
25.3	25.7	26.2	26.6	27.2
14.9	15.5	15.7	15.8	15.6

3.2. Total Protein Content

Data in Table 2 shows that the protein content of the untreated venom was 20.46 % (w/v). The sun exposure for 6 hours increased protein content by 38.7%, which probably due to the concentrating effect of the heat. On the other hand, UV photo-oxidation for 3 hours decreased protein content by

22%. This reduction may be due to adsorption by activated charcoal. The data on protein content showed that the venom of *N.sputatrix* is a protein rich substance which account for approximately 90% of its total dry weight [15]. This protein content was used later to calculate the specific activity of the venom.

Table 2. Total protein content.

Samples Treatments	Protein content (% w/v)
Control	20.46 ± 0.43
Sun exposure, 2 hours	23.35 ± 0.16
Sun exposure, 4 hours	28.10 ± 0.16
Sun exposure, 6 hours	28.38 ± 0.16
Photo-oxidation	15.89 ± 0.00

3.3. L-Amino Acid Oxidase (LAAO) Activity

The LAAO specific activity of the control (untreated) venom of *N. sputatrix* was 0,229 µmol/min./mg (Figure 2). LAAOs (EC1.4.3.2) are hemorrhagic toxins present in snake venoms which are protein responsible for the yellowish color of the venom. The enzyme catalyzes oxidative deamination of L-amino acid to form ammonia, hydrogen peroxide, and ketoacid and commonly found in many kinds of snake venom. [16]. This enzyme has been demonstrated to alter platelet function [17]. A time course study on the effect of length of sun exposure showed that there was a significant decrease in LAAO enzyme activity during the first 2 hours of exposure and the enzyme lost 91% of its activity by 6 hours. Similar findings was observed for samples pre-treated with UV photo-oxidation for 3 hours which cause 91% reduction in the enzyme activity.

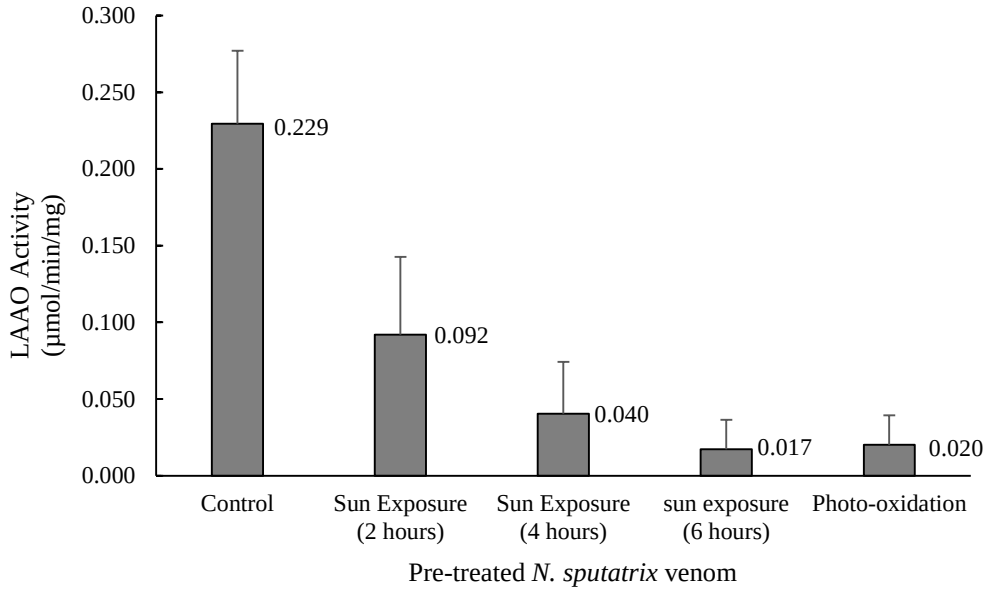

Figure 2. LAAO specific activity of pre-treated *N. sputatrix venom.*

3.4. Phospholipase A_2 (PLA_2) Activity

The specific activity of PLA_2 from *N. sputatrix* control (untrated) sample was 1.672 µmol/min./mg. Similar with LAAO, 96% of PLA_2 activity was lost after 6 hours of sun exposure (Figure 3). UV photooxidation carried out for 3 hours almost diminished all of PLA_2 activity (99.8%). Previously,

Sallau *et al*. [18] reported that PLA_2 activity of *Echis ocellatus* was as much as 2.39 µmol/min.t/mg. This enzyme is the most abundant enzymes found in snake venom and is responsible for the phospholipid degradation which eventualy damaged the cell membrane. This activity results in the venom's toxicity, particularly when it is entering the blood circulation. The PLA_2 catalyzes the hydrolysis of the fatty acid by attacking the ester bond at the *sn*-2 of the phospholipid molecule thus liberating free fatty acid and lysophospholipid [18]. Thus, the decrease in the activity of PLA_2 as well as that of LAAO following sun exposure and UV photo-oxidation indicates that the venom had lost its toxicity due to the treatments. Our observations are consistent with the works of Tejasen & Ottolenghi [19] which reported the reduction of snake venom toxicity following UV photo-oxidation as shown by the decrease in PLA_2 and phosphodiesterase activities.

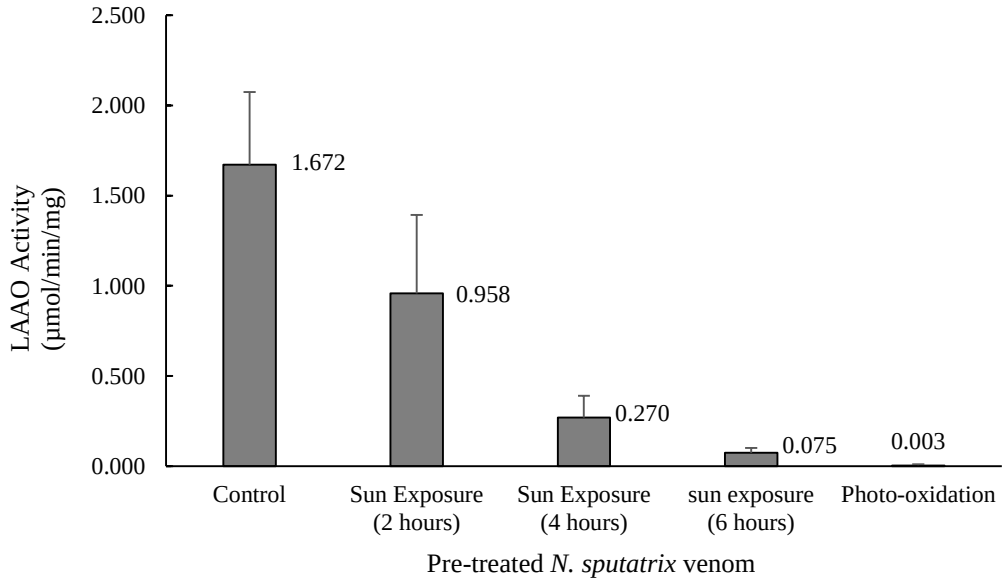

Figure 3. PLA_2 specific actifity of pre-treated *N. sputatrix* venom.

3.5. Coagulant and Anti-Coagulant Activity

As shown in Table 3, the positive control for coagulant activity was observed by addition of $CaCl_2$ solution into the citrate-containing plasma which will induce the plasma to clot. The calcium added into the citrated plasma stimulated the mechanisme of clotting. When the citrated plasma was mixed with *N. sputatrix* venom alone, no clotting was observed, indicating that the venom does not posses coagulant activity. This lack of coagulant activity were still exhibited regardless of the sun exposure or the UV-photo-oxidation pre-treatment of the venom, which suggest that the *N. Sputatrix* venom does not contain enzymes that clot fibrinogen, unlike the venoms of several snakes previously reported by Pirkle et al. [20]. The lack of coagulant activity of the venom of the Colombian coral snake *Micrurus dumerilii* has been reported recently [13]. On the other hand, when tested for anticoagulant activity, the untreated *N. sputatrix* venom exhibited positive result. The anticoagulant activity was also observed with the venom exposed to the sun for 2- and 4- hours. Interestingly, the 6 hour-sun exposed and photo-oxidized venoms were no longer demonstrated the anticoagulant activities. Zingali [21] reported several snake venom proteins which possessed anticoagulant activity, among others were PLA_2 and LAAO. The PLA_2s affect blood clotting through the hydrolysis of procoagulant phospholipid or by binding to them [22, 23]. The anticoagulant activity of the untreated venom shown in our present data is correlated with the highest specific activity showed in the LAAO and PLA_2 assay. This anticoagulant activities were no longer presence when the activity of both enzymes were diminished following 6-hour sun exposure or UV photo-oxidation.

Table 3. Plasma clotting time with the addition of pre-treated *N. sputatrix* venom.

Samples	Clotting time
Plasma +$CaCl_2$	Clots observed after 193 ± 7 sec.; coagulant effect
Plasma +Untreated venom	None (after 20 minutes observation); no coagulant effect
Plasma +Sun exposed (2 hrs) venom	None (idem)
Plasma +Sun exposed (4 hrs) venom	None (idem)
Plasma +Sun exposed (6 hrs) venom	None (idem)
Plasma +Photo-oxidized venom	None (idem)
Plasma +Untreated venom + $CaCl_2$	None (idem), anticoagulant effect
Plasma + Sun exposed (2 hrs) + $CaCl_2$	None (idem), anticoagulant effect
Plasma+Sun exposed (4 hrs) + $CaCl_2$	None (idem), anticoagulant effect
Plasma+Sun exposed (6 hrs) + $CaCl_2$	Clots observed after 307 ± 13 sec. ; no anticoagulation effect
Plasma+$CaCl_2$+Photo-oxidized venom	Clots observed after 227 ± 22 sec. ; no anticoagulant effect

These observations suggest that prolong (6 hours) sun-exposure and the UVphoto-oxidation were able to inactivate the LAAO and PLA_2 which are consistent with the venom detoxification [Tejasen &Ottolenghi]. The inactivation of those enzymes resulted in the loss of anticoagulant activity of *the N. sputatrix* venom. Nevertheless, the proteins contained in the venom remain intact under the prolong sun exposure as well as UV photo-oxidation. Taken together, these findings suggest that sun exposure and UVphoto-oxidation significantly decreased the activity of LAAO and PLA_2.of the *N.sputatrix* venom, but both treatments did not affect the physical characteristic of the venom's protein content. Further studies on the immunogenicity of the treated venom will provide insight to the traditional healing property of the snake venoms for various diseases.

References

[1] Mohney G 2013 Toxic medicine: How venom can heal [internet] (March 22, 2016) http://abcnews.go.com/Health/toxic-medicine-venom-lead-medical-breakthroughs/story?id=18558299
[2] Fatima LD and Fatah C 2014 *J Clin Toxicol* **4(2)** 1-9
[3] Koh DCI, Armugam A, Jeyaseelan K 2006 *Cell Mol Life Sci* **63** 3030-3041
[4] Anindyajati G, Budiono B, Joyosumarto R 2009 *Jurnal Matematika, Sains, dan Teknologi* **10(2)** 75-80
[5] Kocholaty W 1966 *Toxicon* **3** 175-186
[6] Vyas VK, Brahmbhatt K, Bhatt H, Parmar U *Asian Pac J Trop Biomed* **3(2)** 156-162
[7] Lee ML, Fung SY, Chung I, Pailoor J, Cheah SH, Tan NH 2014 *Int J Med Sci* **11** 593-601
[8] Mukund H, Gawade SP 2011 *J Venom Anim Toxins incl Trop Dis* **17(4)** 422-429
[9] Gawade SP, Tamboli MA, Thorat SS 2016 *Indian J Pharmaceutical Ed Res* **50** 123-129
[10] Bradford MM 1976 *Analytical Biochem.* **72** 248-254
[11] Yu Z, Wang Y, Zhou N, Zhao M, Qiu J, Lin J 2014 *Appl. Biochem. Biotechnol.* **174** 13-27
[12] Araújo AL, Radvanyi F 1987 *Toxicon* **25** 1181-1188
[13] Suarez PR, Nunez V, Fernandez J, Lomonte B *J Proteomics* **16** 2-52
[14] Theakston RDG, Reid HA 1983 *Bull World Health Org* **61** 949-956
[15] Aarti C and Kusro A 2013 *Int. J. Pure App. Biosci.* **1(6)** 24-29
[16] More SS, Kiran KM, Veena SM, Gadag JR 2010 *J Venom Anim Toxins incl Trop Dis* **16(1)** 60-75
[17] Sakurai Y, Takatsuka H, Yoshioka A, Matsui T, Suzuki M, Titani K, Fujimura Y 2001 *Toxicon* **39** 1827-1833

[18] Sallau AB, Ibrahim MA, Saluhu A, Patrick FU 2008 *African J Biochem Res* **2(4)** 98-101
[19] Tejasen P, Ottolenghi A 1970 *Toxicon* **8** 225-233
[20] Pirkle H, Theodor I, Miyada D, Dimmons G 1986 *J Biol. Chem***. 261** 8830-8835
[21] Zingali RB 2007 *Toxin Reviews* **26** 25-46
[22] Markland FS 1998 *Toxicon* **39** 371-375
[23] Mounier CM, Bon C, Kini RM 2001 *Haemostasis* **31** 279-287