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Comparative Enzyme Activity of *Vespa Orientalis* Venom and its Photooxidized Venom Products

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Abstract

The *Vespa orientalis* venom, a secretary substance from arthropod wasps has attracted considerable interest as a potential source of pharmacological substances. Photo detoxified snake venom has been used for dementia, dengue fever, atherosclerosis, cancer, and diabetes. There was no scientific evidence on the photooxidized *Vespa orientalis* venom of Indian origin as well as its protein characteristic. Thus, the objective of this research work was to estimate the protein content before and after photooxidation of venom and compare the specific activity of enzymes. The protein content was determined by SDS PAGE, the specific activity of *Phospholipases, Hyaluronidase* enzyme, as well as the haemolytic and proteolytic activity of the venom determined by spectrophotometrically. The venom's protein was separated into many protein bands with a molecular weight ranging from 21 to 59.9kDa and showed retention of antigenicity. Photooxidation of venom caused 45 to 49% loss in Phospholipase-A and Phospholipase-B and a maximum loss in *hyaluronidase* activity was observed after 45 and 60min of exposure. A time course study showed that the venom lost 84-96% of hemolytic and a significant loss of proteolytic activity after 45-60 min of UVR. These findings suggest that the venom lost its toxicity as shown by the significant reduction of the activity of the enzyme. Therefore, UVR produced *Vespa orientalis* venom with less toxicity without affecting its immunogenicity.

Keywords: *Vespa orientalis*; Photooxidation; *Phospholipase; Hyaluronidase;* Proteolytic activity

Introduction

The family of Vespidae (Order; Hymenoptera) is a large, diverse, cosmopolitan family of wasps, including nearly all the known eusocial wasps and many solitary wasps. There are 23 valid species known from the world so far of which 16 species from the Indian subcontinent. *Vespa orientalis* (VO) belong to the genus *Vespa* frequently found in south India [1,2]. Wasp venoms are complex mixtures of biochemically and pharmacologically active components, such as biogenic amines, peptides and high molecular proteins such as *phospholipase* A and B, *hyaluronidase*, acid *phosphatase, protease*, and *nucleotidase*. The compositions of the various Vespidae venoms are rather similar each other. However, very few is known about the neotropical *Vespa orientalis* venom [3,4]. Three major venom proteins; Viz. *hyaluronidase*, *phospholipase*, and antigen 5 of *Vespa orientalis* venom having different biochemical functions with the property of inducing allergy have been isolated and characterized. The *phospholipase* appears to have dual enzymatic specificities of the A1 and B types [5]. It has shown to possess a high hydrolytic activity towards 1-acyllysophosphatidylcholine and caused an intensive hemolysis of washed human erythrocytes [6]. *Hyaluronidases* are glycoside *hydrolases* cleaving beta-1,4- glycosidic bonds between N-acetyl glucosamine and D-glucuronic acid of hyaluronic acid and thereby act as spreading factors in Hymenoptera and other animal venoms [7]. It is an important allergen in honeybees as well as yellow jacket venoms, in which it represents major allergens together with antigen 5 and phospholipase A [8,9].

Photochemical reactions have been extensively used to produce photooxidized venom products from protein molecules and nucleic acids of the venom [10-13]. Photooxidation can be carried out to detoxify venom by exposing gamma, visible and ultraviolet radiations to generate antigenically-active detoxified venom products [14]. In India, photo-oxidation by ultraviolet radiation was used to decrease the toxicity of snake venom, so the remaining effect is therapeutic activity [15]. In south east Asian countries, sun ray detoxified snake venom has been used for dengue fever, atherosclerosis, cancer, and diabetes [16]. Since, there is very limited information available on the characteristics of photo detoxified venom of *Vespa orientalis*, the objective of this research work was

to characterize the protein content and compare the specific activity of enzymes of the venom treated with UVR with that of native venom. This research will provide scientific evidence on the application of wasp venom as a possible natural nonherbal therapeutic alternative (NNTA) for the treatment of drug unresponsive illnesses under newer sub discipline of photodynamic and pharmaco-photodynamics.

Materials and Methods

Albino rabbits (2-2.5kg) were used in studies. The Institutional Animal Ethics Committee of PES College of Pharmacy, Bangalore, India, approved all animal experiments. All procedures were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, under recommended temperature and relative humidity.

Chemicals and Reagents

All the chemicals and reagents required for running SDS-PAGE were purchased from Thermo Scientific, MA, USA, Freund's complete and incomplete adjuvant were purchased from Genie Bioscience, Bangalore, (India), Diethylaminoethyl cellulose, Methylene blue (MB – Qualigens Fine Chem Ltd, Mumbai, India) and all other chemicals used in the study were of analytical grade.

Collection of *Vespa orientalis* Venom

Total of 3000 wasps was collected in the month of Nov to March from the village Belavanaki, India in the whole period of work (Seasonal collection) and was authenticated by the entomologist Dr. Chandrashekhar, University of Agricultural Sciences, Bangalore, India. A specimen wasp is maintained in the Department of Entomology. The method reported by Duvdevani, *et al.* with suitable modification used for milking of venom was followed [17]. Briefly, venom was milked from sting tip procedure entailed extruding the string with forceps and applied pressure on the abdomen, whereupon the small drop of venom that appeared on sting was collected with a micropipette. The content was pooled stored at -20 °C after lyophilization. The lyophilized venom subsequently referred to as *Vespa orientalis* venom (VOV). The venom concentration was expressed in terms of dry weight (mg/ml stock solution). In total, 5.312 g of lyophilized venom was obtained.

Photooxidation of *Vespa orientalis* Venom

The standard photochemical method with suitable modification was followed to generate photooxidized venom products [18-20]. Briefly, the reaction mixture containing 2ml of VOV (25mg in 2 ml of 0.05M phosphate buffer at pH 6.8) and 2ml of methylene blue (0.003% w/v in phosphate buffer) solution was kept on a magnetic stirrer in a photooxidation chamber exposed to UV light (tubular ultraviolet 15 W lamp, 615, T8, Philips, Holland, UV output 4.8 W, 49 μW/ cm^2) at about 10-cm distance and gently stirred in different time intervals (15, 30, 45, and 60 minutes) at 37 °C. Then, 200 μl of activated charcoal (1% w/v in phosphate buffer at pH 6.8) was added, the mixture was stirred five more minutes without light exposure. The photooxidized *Vespa orientalis* venom (PVOV) was filtered through a 0.2-μm filter using a syringe filtering unit (Minisart®, Sartorius, India). The absorbance of the control mixture (2 ml of VOV and MB without UV exposure maintained in similar conditions) was measured separately at 200-400nm using UV- spectrophotometer.

Production of Antibodies by Hyper iImmunization for Antigenicity Test

The purpose of the hyper immunization of rabbit and preparation of IgG was to test the detoxified PVOV for its antigenicity. Male rabbits were immunized with 5 mg of VOV in 5ml of phosphate buffer mixed with Freund's adjuvant at 16 sites along the animals' spine. Two weeks later, the animals were boosted with 7 mg VOV in 1 ml of phosphate buffer *i.p* route. The same procedure was repeated twice, three and four weeks later; then animals were bled and their sera were pooled. During the immunization schedule, serum was continuously applied on to the immunodiffusion gel for the appearance of precipitin lines [21]. The isolation of immunoglobulin from rabbit antisera was carried out by an ion-exchange method using diethylaminoethyl cellulose (DEAE). The purified immunoglobulin was tested for antigenicity against PVOV [22].

Antigenicity Test

The antigenicity test of PVOV submitted to different UV time exposure was carried out using immunogel diffusion method, in which antigens and sera (antibodies) migrate through an agarose gel and react resulted in precipitin lines [21].

Qualitative Protein Analysis with SDS PAGE

Qualitative analysis of protein contents was determined using SDS PAGE with 13% polyacrylamide. Diluted samples of VOV and PVOV (2%, v/v) were added with mercaptoethanol-containing reducing buffer. The mixtures were heated in boiling water for 15 minutes. After cooled down, 30μl each mixture and protein molecular weight marker were loaded into wells in the polyacrylamide. The SDS-PAGE was run at 150V, 25mA for 90min. The gel was stained with 0.1% Coomassie brilliant blue followed by destained with methanol: acetic acid and water for 2h.

Total Protein Content

Total protein concentration was determined quantitatively using Bradford method [23]. Samples of venom in aliquots of 50 µL were diluted to 100 ml with distillated water. 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 absorbency was measured using spectrophotometer at 615 nm wave-length.

Enzymatic Activities

Phospholipase activity: Phospholipase-A (PLA) and phospholipase B (PLB) activities were measured by titration of free fatty acids. The substrate, either 5 ml egg yolk homogenate (PLA) or 2 mg lyosophosphatidylcholine (PLB) was incubated with the venom in a final volume of 10 ml, containing 1.3×10^{-3}M sodium desoxycholate, 0.5% bovine serum albumin, and 3 mM $CaCl_2$. Enzyme activity at 40 °C was followed by automatic titration of free fatty acids at pH 8, with 0.01N NaOH. One unit of activity releases 1 µM fatty acid/min/mg protein.

Hyaluronidase activity: was assayed by the turbidity method of Tolksdorf, S [24]. The amount of hyaluronic acid digested was determined from a hyaluronic acid standard curve. Activity was expressed as milligrams hyaluronic acid digested per 10 min per milligram protein.

Direct haemolytic activity: Direct hemolytic activity of the VOV and PVOV toxins were assayed on washed red blood cells of the mouse. The red cells (1%) were incubated with the toxins in Tris-buffer (0.01 M, pH 7.4) saline (0.15 M NaCl) at 37 °C for 60 min as described elsewhere [25,26]. Hemolytic action was stopped by addition of cooled (4 °C) Tris-bufer saline. The degree of hemolysis was determined by measurement of the released hemoglobin at a wavelength of 540 nm. Results were expressed as Mean ± S.D of five experiments.

Assay of proteolytic activity: The proteolytic activity was assayed by using casein and denatured hemoglobin as substrates. Casein digestion: A casein suspension was incubated with 0.5 ml varying concentration of venom at 37 °C for 15 min. The reaction mixture was stopped by adding 0.1 ml of 0.44 M Trichloroacetic acid. After standing for 30 min, 2.5 ml of 0.4 M Sodium carbonate and 0.5 ml of Folin-ciocalteu reagent were added. The optical density was measured at 660nm. One unit of enzyme activity was defined as the amount of enzyme required to increase in absorbance of 0.01 per min at 660 nm at 37 °C. *Denatured hemoglobin*: 1g of hemoglobin in 40 ml water was denatured by heating for 30 min at 80 °C in pH 8.5 and then same assay procedure was carried out as described above by replacing casein with hemoglobin. Specific activity was expressed as units of activity/mg protein/min.

Results

About 5.212 g of lyophilized VOV was obtained from 3000 *Vespa orientalis* with an average of 1.5 mg VOV/sac. Photooxidation of VOV in presence methylene blue caused changes in absorbance value and λmax shift at various exposure time and products obtained are abbreviated as photooxidized *Vespa orientalis* venom (PVOV) in this article. The UV spectrum of VOV was biphasic with maximum optical density of 1.034 AU at 265 nm and 3.432 AU at 214 nm. In the UV spectrum of PVOV, the λ max shifted from 265 nm to 255.5 nm. The absorbance increased from 1.034 to 1.424 at 265.5nm.

Antigenicity Test

Immunogel diffusion was performed using antisera to each four photooxidized venoms to test retention of antigenicity after photooxidation at different time interval. The Figure 1A shows distinct precipitin lines visible with 30 and 45min PVOV, when incubated with Immunoglobulins from in central well, however the number of precipitin lines are less with 30 min PVOV (Figure 1B) than 45 min (Figure 1C), whereas, with PVOV 60 min exposure showing reduced intensity and number of precipitin lines (Figure 1D).

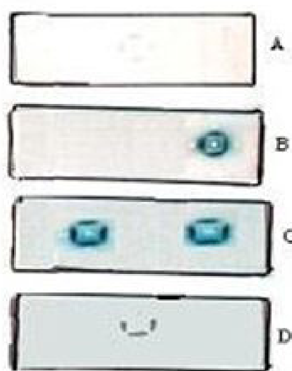

Figure 1: Immnunogel diffusion slide. The photooxidized *Vespa orientalis* venom at different time interval were incubated with immunoglobulins isolated from rabbits sera (central well). **(A)** 15min exposure, **(B)** 30min (right well), **(C)** 45min, (right and left well) and **(D)** 60min

Molecular Weight Analysis

The electrophoresis analysis of native venom and photooxidized with UV for 45min are shown in the Figure 2. The native venom revealed that 10 bands having molecular masses of 59.9, 57.2, 44.3, 42.2, 31.8, 31.9, 21.7 kD (Figure 2, lane 2). When the photooxidized venom was analyzed, the pattern was changed, the venom components were altered after Photooxidation, some of the proteins disappeared and streaking in the gel was appeared (Figure 2, lane 3)

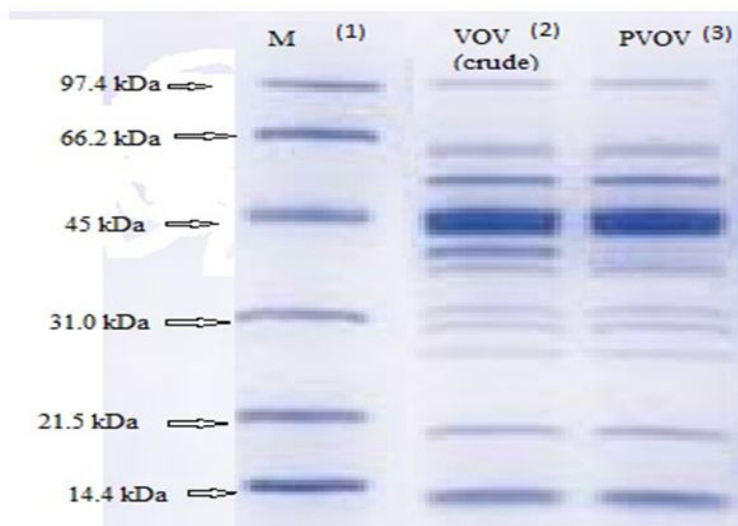

Figure 2: SDS-Polyacrylamide gel electrophoresis of native *Vespa orientalis* venom **(lane 2)**, Photooxidized *Vespa orientalis* **(lane 3)**, corresponding to the following (with molecular mass in Dalton): α-lactalbumine, 14.4; trypsin inhibtor, 20.1; carbonic anahydrase, 30.0; ovalbumin, 45.0; bovine serum albumin, 66.2; phosphorylase-b, 97.4

Total Protein Content

Data in Table 1 shows the protein content of crude venom VOV was 68.5% (w/v) upon UV photo-oxidation, a reduction in protein content was observed. At 45 and 60 min exposure, the protein content was reduced by 17.8% and 22.3% indicating that alteration in protein concentration.

Catalytic activity of PL (μmol/min/mg)			
Toxin	**Egg yolk**	**Lyosphaphatidyl-choline (w/v)**	**Protein content (%)**
Vespa orientalis venom	4204±22 (4)	1420±116 (4)	68.5±0.43
PVOV 15 min	3540±180 (4)	620±226 (4)	62.6±0.16
`PVOV 30 min	2850±156 (4)	930±109 (4)	60.4±0.16
PVOV 45 min	2250±127(4)	770±021 (4)	56.3±0.15
PVOV 60 min	2106 ±251(4)	1020±216 (4)	53.2±0.11

Table 1: Egg yolk/lyosphaphatidylcholine was incubated with the venom substrates, containing sodium desoxycholate, bovine serum albumin, and $CaCl_2$. Enzyme activity at 40 °C was followed by automatic titration of free fatty acids with NaOH. The changes in the total protein content after UV Photooxidation of *Vespa orientalis* venom estimated by Bradford method using Bovine serum albumin as standard. The number of experiments is given in parentheses. PVOV=Photooxidized *Vespa orientalis* venom

Phospholipase Activity

The specific activity of PLA from VOV was 4204μmol/min/mg, upon 15 min photooxidation specific activity decreased by 15%, similarly, as the duration of UV exposure increased, the time dependent loss of PL activity was observed. A decrease in PL activity was observed with PVOV (45min) and PVOV (60 min) by 46% and 49% compared to control venom. Similarly, 45% of PLB activity was lost after 45 min exposure PVOV compared to control, however, the activity was not lost much with 60 min exposed PVOV when compared to control venom (Table 1).

Hyaluronidase Activity

Figure 3 presents the hyaluronidase activities of VOV and PVOV on the basis of protein content, by a turbidimetric and a colorimetric assay using hyaluronic acid (HS) and chondroitin sulfate (CS) as substrate. Both testicular *Hase* and VOV possess considerable activity in depolymerizing both substrates. The rate of degradation CS was lower than that of HA and in this respect, the VOV resembles mammalian testicular *Hase*. There was a significant fall in depolymerizing CS and HA by PVOV (45 min and

60 min) exposure compared to control VOV.

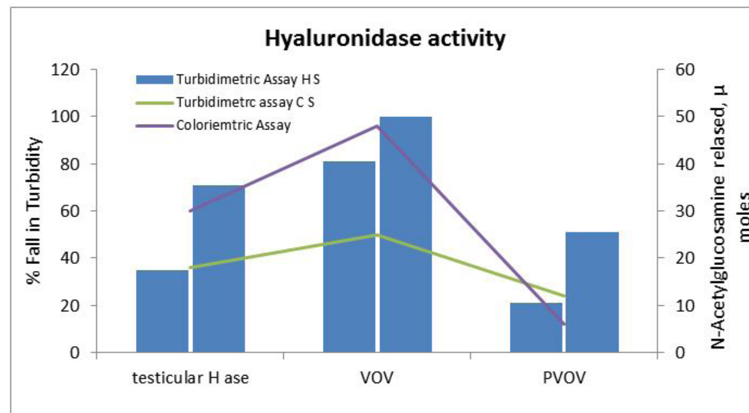

Figure 3: Hyaluronidase activity of Photooxidized *Vespa orientalis* venom. The Hyaluronidase activity (Hase) was assayed turbidimetrically incubating with Hyaluronic acid (HA) and chondroitin sulphate (C.S) and by colorimetric measurement of N-acetyl gucosamaine reacting material released from hyaluronic acid after 6-hr incubation at 37 °C. Activity is expressed on the basis of protein. (VOV=*Vespa orientalis* venom; PVOV=photooxidized venom *Vespa orientalis*)

Direct haemolytic Activity

Hemolysis of mice erythrocytes was examined for both VOV and PVOV, the results are represented in Table 2. Photooxidation of PVOV at 50μg/ml exposed for 45-60 min produced almost diminished hemolytic activity by 84-96% as compared to control VOV which showed concentration dependent potent direct hemolytic activity with red blood cells.

Venom/photooxidized	Venom concentration (μg/ml)	Haemolysis (%)
Vespa orientalis venom	10.0	65.3±4.5 (5)
Vespa orientalis venom	40.0	84.5±6.8 (5)
Vespa orientalis venom	60 .0	96.2±5.5 (5)
PVOV (15 min)	50.0	60.1 ±2.5 (5)
PVOV (30 min)	50.0	41.5±3.5 (5)
PVOV (45 min)	50.0	16.8±0.5 (5)
PVOV (60 min)	50.0	10.1±0.5 (5)

Table 2: Washed red blood cells of the mouse were incubated with the *Vespa orientalis* and its photooxidized venom in Tris-buffered saline for 60 min. Data shown is Mean±SEM with the numbers of experiments in parentheses.

Proteolytic activity by casein digestion

Figure 4 shows the relative proteolytic activity of VOV and PVOV on casein and denatured Hemoglobin at different time exposure. Forty five min and 60min UV exposed PVOV produced 42.5% & 49.6% decrease in casein digestion compared control venom at 200(μg/ml) indicating the time dependent loss of proteolytic property of venom.

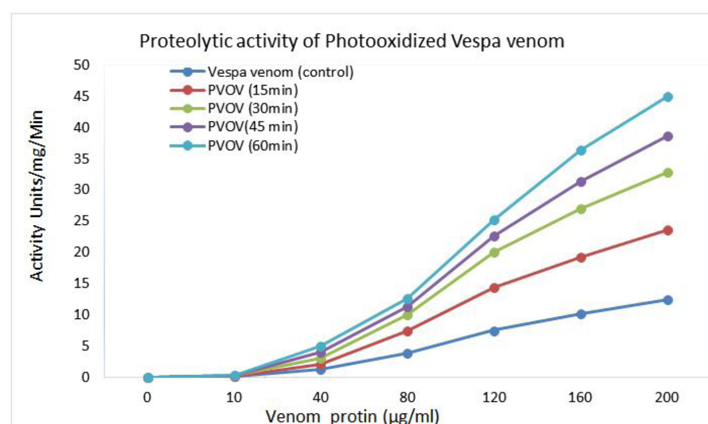

Figure 4: Proteolytic activity of *Vespa orientalis* and its photooxidized venom products at various time intervals was incubated with casein, Folin- ciocalteu reagent, trichloroacetic acid and sodium carbonate at 37 °C for 60 min.

Proteolytic activity on denatured Hb

The greatest inhibitions, obtained to the proteolytic activity induced by PVOV were observed with UV exposure time 45 and 60 min at lower concentrations similar to Vespa venom which was reduced the activity by 63% and 83% after incubation denatured Hb with venom and PVOV respectively.

Discussion

Photochemical detoxification reaction in the presence of sensitizer methylene blue made it possible to unravel the pharmacologic and therapeutic potential of some venom proteins, which are otherwise highly lethal weapons used by predators to paralyze their prey. Ultra violet exposed photo detoxification has been advocated as an alternative method to explore the pharmacological implication of snake venom proteins where in their toxophoric regions are altered [19]. The Photooxidation of light-absorbing molecule and protein in the presence methylene blue have been widely used to explore alterations in the biological activity of venoms [18,27]. In our study, a mixture of VOV and Methylene blue was exposed to UVR. The probable mechanism of photo-oxidation of venom protein by UV radiation in the presence of methylene blue can be explained as follows; exchange of energy depends on the frequency of radiation, which corresponds to a quantum of energy. When methylene blue sensitizer molecules absorb a quantum of energy (i.e. a photon), the sensitizer molecules are rapidly entered into the excited state from the ground state, before dropping to the lowest excited singlet state by internal conversion. Energy is also transferred from the lowest excited singlet state (S1) to a triplet-excited state (T1) by the process of inter system crossing. The excited electron of the sensitizer either returns to the vacant position on the ground state or enters into a vacant higher orbital of the acceptor. At the same time, an electron donor fills the vacant position at the ground state of the sensitizer. Thus, absorption of a photon and transfer of an electron to the excited state and subsequently into vacant higher orbital of the acceptor or to the ground state generates two new species, the Reduced acceptor (A-) and Oxidized donor (D+). In the photo-oxidation process of venom proteins, the sensitizer methylene blue molecule is exposed to the incident UV radiation in a phosphate buffer containing venom proteins. The sensitizer methylene blue is activated by photons from UV light and enters into an excited singlet state. In the presence of environmental oxygen, an electron transfer quenching reaction of an excited species of methylene blue adsorbed to venom proteins is augmented. Electrons are transferred from the sensitized methylene blue to the protein molecules [28].

Based on antigenicity test, 45-minute exposure venom product was selected as PVOV. The presence of antigenicity of PVOV (Figure 1C) is an indication of retention of the intact antigenic domain while the absence of toxicity revealed alteration of toxophoric groups of VOV. (Unpublished reports of Pacific University, India). In the present study, there was a shift in the ODmax in PVOV absorption profile compared to VOV. An increased absorbance was reciprocal with loss of venom protein in photooxidized venom. Changes in the absorbance of VOV and PVOV revealed the loss of protein in PVOV. In our study, a moderate loss of protein content was observed with 45 and 60 min UV exposure (Table 2) which justified the above facts. Similar alterations were observed in photooxidized venom product of *Naja siamensis and Echis carinatus*, which supports our reports [29,30]. This may possibly due to the unfolding of proteins and/or may be due to adsorption of proteins by activated charcoal. SDS PAGE analysis of the venom's protein was separated into many protein bands. Comparison with standard markers showed that the molecular weight of those bands was ranging from 14 to 59.4 kDa. These observations are consistent with the ones reported by Sophida, *et al*. [31]. The observed protein bands were closely matched with major toxins namely *PL-A, hyaluronidase, protease, phosphodiseterase* and antigen 5 reported in their works. The UVR of *Vespa orientalis* venom decreased Haemolytic, *Hyaluronidase*, caseinolytic activities and almost abolished the PLA and PLB activities with 45 and 60 min. *Phospholipase* enzyme is most abundant enzyme found in *Vespa orientalis* and responsible for phospholipid degradation and eventually damages cell membrane [32]. PLs catalyses the hydrolysis of fatty acid by attacking ester bond of phospholipid molecules, liberating free fatty acid and phospholipid, thus decrease in the PLA & B activity following UV photo-oxidation indicated that venom had lost its toxicity due to treatment [33]. Our observations are in consistent with works of Tejasen & Ottolenghi which reported the reduction of snake venom toxicity following UV photo-oxidation as shown by the decrease in PLA2 and phosphodiesterase activities [34]. Venom Hase, a "spreading factor", is well-known for its toxin-enhancing activity. Its activity may play a vital role in allergenicity and toxicity of venoms. Inhibition of *Hase* activity prevents local tissue damage, retards the venom toxin diffusion into the tissues and blood circulation, resulting in the delay of fatality [35]. In our report, there was a decrease in the depolymerising activity of Hyaluronic acid and chondroitin sulphate activity following UV photo-oxidation indicated that venom had lost its toxicity enhancing activity due to Photooxidation.

The PLA1 from *Vespa orientalis* exhibited direct hemolytic action against washed red blood cells despite being a hemolysin it is much less potent than the PLA1's from other vespid venoms such as the verutoxins from *V. verutina* and the lethal toxin from *V. basalis* [36]. As the duration of exposure time increased, the percentage hemolysis was decreased up to 90% at 60 min exposure. Our results are in parallel with the reports of Bennacef-Heffar, *et al*. 2003 which affirmed that a complete loss of PLA2 activity after irradiation of *Vipera lebitina* venom with 1and 2 kGy of gamma rays [14]. They reported that irradiated toxin was unable to bind to calcium and a 2kGy dose of gamma radiation cleaves approximately six sulphide bridges of toxin molecule rendering loss of toxicity of venom [14].

An exposure of *Vespa orientalis* venom to UV radiation decreased the caseinolytic and anticoagulant activity and reduced the PLA activity at 45 and 60 min exposure. The enzyme inactivation may be due to the destruction of the active site or to a complete

denaturation. For most enzymes, the loss of activity is associated with a change in the Michaelis-Menten constant. In our study, we have seen that exposure UVR for about 45 & 60 min, there was a significant loss of proteolytic activity. Similar loss activity has been reported elsewhere, which reported that irradiation of venom has the implication of loss of toxicity parallels the loss of enzymes which include PLA2, *protease*, and *Phosphodiesterase*.

Conclusion

In conclusion, the present results indicate that UVR of *Vespa orientalis* proteins can promote significant detoxification. Indeed, exposure of venom to 45 to 60 min may offer an effective method of reducing toxic effects of venom without affecting its immunogenicity. These results are promising for preparing the best toxoids and vaccines.

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