Photooxidized Vespa orientalis venom Improves Memory and Learning Activities in Rats

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ABSTRACT
The work was aimed to study the effect of photooxidized Vespa orientalis venom on memory and learning activity on rats in presence of scopolamine and ondansetron using T maze, Elevated plus maze and passive avoidance. UV radiation exposure of Vespa orientalis venom (VOV) for 15, 30, 45 and 60 min intervals in presence to methylene blue to detoxify venom and studied by change in UV spectrum. Antigenicity study and in-in vivo and in-vitro neutralization study of Photooxidized Vespa orientalis venom (PVOV) against immunoglobulin from hyperimmunized rabbit was performed. The memory and learning activity of PVOV in presence and absence of scopolamine and ondansetron was studied. Forty five minute UV Radiation exposed VOV showed shift in λmax and increase absorbance indicated alternations in venom protein concentration, this in supported when PVOV showed loss of toxicity and decrease in mortality time in neutralization study in mice. Administration of PVOV for 28 days produced a notable improvement in spatial and long memory in rats when subjected in several tasks. When PVOV administered with scopolamine and ondansetron, all the parameters of spatial and long term memory tasks were significantly reduced inferred that PVOV acted by modulating either muscarinic or serotonergic receptors. However, other possibility of low-molecular weight protein and peptides or enzymes, which might also act by serotonergic / cholinergic system that affect CNS action. We concluded that although there is a possibility of employing PVOV in the treatment of depressive and chronic degenerative illnesses as a nonherbal and nonsynthetic alternative for patients not responding to the available therapy.

Keywords: Vespa orientalis venom (VOV), Photooxidized Vespa orientalis venom (PVOV), T-Maze, Elevated plus maze, Scopolamine, Ondansetron.

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INTRODUCTION
Hymenoptera venoms are complex mixtures of biochemically and pharmacologically active components such as biogenic amines, peptides and proteins. Vespa orientalis venom contain many deferent biological components acetylcholine, serotonin, adrenaline, nor-adrenaline, dopamine, kinins, and high molecular weight compounds such as phospholipase-A; hyaluronidase; histidine decarboxylase; acid, alkaline, and neutral DNase; poly and disaccharidase; and several polycationic peptide and protein that act together to produce biological effects. Photooxidation is one of the method carried out to detoxify venom exposing gamma, visible and ultraviolet radiations (UVR) to generate antigenically-active detoxified venom products. Detoxified snake venom has been used treat dengue fever, atherosclerosis, cancer, and diabetes. Photooxidized Echis carinatus venom demonstrated antidepressant and central nervous system stimulant activities, and enhancement of memory and learning in rats. Envenomation with hornets caused specific central nervous system effects in laboratory animals due to cholinesterase like compounds present in the wasp venom. Numerous studies have been carried out to test the properties of wasp venom as a pharmacological agent. However, no attempts have been made to investigate the effects of Photooxidized Vespa orientalis venom (POV) on cognition and dementia and effect in presence of various antagonists like scopolamine and ondansetron in animal models as an alternative mechanism that may create lead compounds for the treatment of neurodegenerative diseases. Thus, the present work aimed to evaluate the psychopharmacological effects of POV using suitable animal models for memory and learning.

MATERIAL AND METHODS
Animals
Swiss albino mice (20 -25 g) for toxicity study, Wistar rats (180-220 g) for psychopharmacological studies and albino rabbits (2-2.5 kg) were used in hyperimmunization 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 on Experiments on Animals (CPCSEA), Government of India, under recommended temperature and relative humidity.

Drugs and chemicals
Piracetam was received as a gift sample from UCB India Private Ltd. (India), Freund’s complete and incomplete adjuvants 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 were collected in the month of November to March from a village Belavanaki, India in the whole period of work (Seasonal collection) and were authenticated by the entomologist Dr. Chandrashekar, 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. The lyophilized venom subsequently referred to as Vespa orientalis venom (VOV). The venom concentration was expressed in terms of mg/ml.

Determination of minimum lethal dose
Groups of three mice each were injected (via intraperitoneal and intravenous routes) with VOV dose range from 12-20 mg/kg and 10-15 mg/ kg. Control group animals were treated with phosphate buffer only. Following with administration of VOV, the signs of toxicity and behavioral changes were observed for 1, 2, 4, 24 and 72 h.

Photooxidation of Vespa orientalis venom
Standard photochemical method with suitable modification was followed to generate photooxidized venom products. Briefly, the reaction mixture containing 2 ml of VOV (25 mg in 2 ml of 0.05M phosphate buffer at pH 6.8) and 2 ml 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, 49μW/cm²) at about 10 cm distance and gently stirred in different time intervals (15, 30, 45, and 60 minutes) at 37°C. Then, 200µl 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) exposed different intervals were filtered through a 0.2µm 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 200-400 nm using UV spectrophotometer.

Hyperimmunization of rabbit 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 1 mg of VOV in 5 ml of phosphate buffer mixed with Freund’s adjuvant were inoculated intradermally at 16 sites along the animals’ spine. Two weeks later, animals were boosted with 1.5 mg VOV 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 appearance of precipitin lines.

Purification of immunoglobulins
The isolation of antisera from rabbit was carried out by ion-exchange method using diethyl aminoethyl...
cellulose (DEAE). The purified immunoglobulins were and tested for antigenicity against PVOV. 

**Antigenicity Test**
The antigenicity test of PVOV submitted to different UV time exposure was carried out using immunogel diffusion method, in which antigens and antibodies migrate through an agarose gel and react resulted precipitin lines. 

**In vitro venom neutralization Studies with Immunoglobulins**
A constant amount of VOV (1 MLD) was allowed to react with 1% and 0.1% dilutions of isolated immunoglobulin and the mixtures were incubated at 37°C for 30 minutes. The protection or death (if any), was determined after intraperitoneal injection 0.2 ml into mice (n = 3) and observed for seven days. 

**In-vivo detoxification Test in Mice**
The detoxification of PVOV exposed to UV light for 45 minutes (which preserved antigenicity as tested out by immunogel diffusion method) was intraperitoneal injected into a group of mice (n = 3) at a dose based on body weight (0.1 ml/25 g or 5 mg/ml) and observed up to seven days. 

**Total protein content**
Total protein concentration of VOV and PVOV was determined quantitatively by Bradford method using Bovine serum Albumin as a standard. 

**Memory and learning activity using T-maze**
Eight groups of six Wistar rats of either sex weighing between 180-200 g were used for the experiment. Group 1 normal control animals received vehicle for 28 days; Group 2 animals received 1.5 mg/kg VOV alone for 28 days; Group 3 animals received 1.5 mg/kg PVOV alone for 28 days; Group 4 received first 21 day 1.5 mg/kg PVOV following by scopolamine for (0.5 mg/kg) up to 28 days; Group 5 received first 21 day 1.5 mg/kg PVOV following by Scopolamine for (1 mg/kg) up to 28 days; Group 6 received first 21 day 1.5 mg/kg PVOV following by Ondansetron (50 mg/kg) up to 28 days; Group 7 received first 21 day 1.5 mg/kg PVOV following by Ondansetron (100 mg/kg) up to 28 days and Group 8 piracetam (150 mg/kg) up to 28 days. Training of the animals was carried out for three consecutive days. On the first day, each rat placed on the maze completed 15 trials to become familiarized with the maze and access food. Then, animals were deprived of food for 24 hours. On the second day, 60 minutes before experiment, each group was treated as described above. Rats were trained until they attained nine correct arm choices out of 10 consecutive trials. The number of correct responses and time needed to reach the food (TRF) in each trial were recorded. The percentage of correct responses (%CR) was calculated. 

**Transfer latency in elevated plus maze**
In elevated plus maze (EPM) rats were placed individually at end of the open arm facing away from the central platform. The time taken to enter any closed arm with all four paws was taken as transfer latency (TL). On the first day, rats were allowed to explore the maze for five minutes and TL was recorded (acquisition) and on the second day, 60 minutes prior to experiment, animals were treated as described above and the retention of the learned task was examined. TL was recorded on completion of the fourth week of treatment.

**Step down passive avoidance**
The method suggested by Camacho et al, 1996 was followed. Briefly, on the first day, each rat was placed on the shock free zone, when it stepped down and placed all four paws on the grid floor, an electric shock was delivered (1 mA for 5 seconds). Rats were given 3 to 5 trials until the time spent in the shock free zone was 120 seconds, the step down latency (SDL) and time spent in the shock zone (TSS) were recorded (familiarization). Twenty four hours later, acquisition phase was recorded again. Animals were treated as described above. All Animals were given three trials and parameters such as SDL, step down error (SDE) and time spent in shock zone were recorded. Retention was tested on the third day and all the parameters were recorded again. The drugs were administered next day of familiarization and one hour before the acquisition phase on 28th day and recorded. 

**RESULTS**

**Minimum lethal dose of venom**
About 5.212 g of lyophilized VOV was obtained from 3000 Vespa orientalis with an average of 1.5 mg VOV/sac. Toxicity by intraperitoneal and intravenous routes in mice showed characteristic symptoms such as, dyspnoea, akinesia, diarrhoea, hematuria, stupor, loss righting reflex, hyper excitation to external stimuli and death at 15 mg/kg and 12 mg/kg by i.p and i.v route respectively. The average time of death ware 1.57 ± 0.2 & 1.25 ± 0.1 h by i.p and i.v route, hence the dose of 15 mg/kg i.p was considered as 1 MLD of VOV and chosen for Photooxidation studies to generate antigenically active detoxified PVOV.
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Photooxidation of venom

Photooxidation of VOV in presence methylene blue caused changes in UV optical absorbance value and λmax shift at various exposure time. 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 λmax shifted from 265 nm to 255.5 nm. The absorbance increased from 1.034 to 1.424 at 265.5 nm and venom products obtained after UV exposure were abbreviated as PVOV (Photooxidized Vespa orientalis venom).

Antigenicity Test

Immunogel diffusion was performed using antiserum to all four photooxidized venoms to test retention of antigenicity after Photooxidation at different time interval. The figure 1 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 exposure showing reduced intensity and number of precipitin lines (Fig. 1D).

Table 1: Effect of Photooxidized Vespa orientalis venom (PVOV) step down latency in rats using passive avoidance paradigm.

| Treatment       | SDL      | SDE     | TSS       | SDL      | SDE     | TSS       |
|-----------------|----------|---------|-----------|----------|---------|-----------|
| Normal control  | 140.5 ± 1| 4.4     | 40.5 ± 0.8| 120.7 ± 0.8| 3.8     | 32.5 ± 0.5|
| VOV             | 115.1 ± 0.5| 3.5     | 55.2 ± 0.1| 102.2 ± 0.4 | 3.4     | 41.2 ± 0.6 |
| PVOV            | 162.6 ± 0.3| 1.6     | 25.2 ± 0.2 | 165.1 ± 0.8 | 1.4     | 23.4 ± 0.9 |
| PVOV + Scop (0.5) | 155.2 ± 0.2 | 1.7     | 60.3 ± 0.5 | 163.1 ± 0.3 | 1.5     | 244.4 ± 0.5 |
| PVOV + Scop (1) | 110.2 ± 0.3 | 4.2     | 61.2 ± 0.5 | 122.6 ± 0.5 | 3.5     | 53.7 ± 0.7 |
| PVOV + Ond (50) | 112.4 ± 0.2 | 3.9     | 59.2 ± 0.5 | 119.1 ± 0.5 | 3.6     | 51.8 ± 0.4 |
| PVOV + Ond (100) | 113.6 ± 0.2 | 5.1     | 62.2 ± 0.6 | 122.8 ± 0.6 | 3.4     | 49.8 ± 0.8 |
| Piracetam       | 167.9 ± 0.4 | 4.9     | 22.5 ± 0.4 | 170.5 ± 1.2 | 1.4     | 21.7 ± 0.6 |

Statistical analysis: a = Control Vs VOV & b= VOV Vs PVOV by Paired t- test; c= PVOV Vs all groups by one way ANOVA and Dunnet’s multiple comparison test, ***P<0.001; **P<0.01; * P<0.05; ns= non-significant.
The results of protein content of crude venom VOV was 68.5% (w/v) upon UV photo-oxidation at various time interval, a reduction in protein content was observed. There was a 8.61%, 11.8%, 17.8 and 22.3% protein content was lost when VOV was exposed at 15, 30, 45 and 60 min respectively indicated that alteration in protein concentration.

Neutralization and detoxification Studies of Purified Immunoglobulins
It was observed that 1% of immunoglobulins neutralized the 1 MLD of VOV in mice indicated that the 1% antivenin was sufficient to protect mice toxicity. In vivo detoxification studies, the mice exhibited continuous restlessness, respiratory abnormality, immobility, urination, defecation, dyspnoea, and all animals died within 2.45 ± 0.3 hour. Based on antigenicity and toxicity tests, 45 minute UV-exposed venom product was considered photooxidized Vespa orientalis venom (PVOV), and it was used to study psychopharmacological activities.

Memory and learning using T-maze
Administration of VOV to group of animals for 28 days has significantly increased (P<0.001) by 59.5% for reach food (transfer latency) and decreased the % Correct responses by 23% compared to control animals during retrieval phase. These effects were countered significantly (P<0.001) by 1.5mg/kg PVOV (45 min) administration for 28 days with decrease in transfer latency by 66.8% and increase in correct response by 28.4% compared VOV administered group animals. When PVOV was administered with scopolamine a dose dependant change in transfer latency & % CR was observed with significant (P<0.05) increase in latency by 64 and 76.6% and correct of correct responses were reduced by 69.4 and 73% respectively compared to PVOV. Whereas PVOV with ondansetron 50 mg/kg and 100 mg/kg, there was significant (P<0.001) increase by 50.7 and 28% in time latency compared to control and number of correct choices were increase by 75.4 and 86.2% compared to PVOV alone. Piracetam was used as reference standard. The details of results are shown in Figure 2.

Transfer latency in Elevated plus maze
During acquisition study, VOV administration to rats for 28 days caused significant (P<0.5) increase in transfer latency by 57.8 % compared to normal animals, whereas, this effect was significantly (P<0.5) reversed by treatment with 1.5 mg/kg PVOV for 28 days by 64.4% compared to VOV. PVOV administration along with scopolamine at 0.5 and 1 mg/kg for 28 days significantly (P<0.001) increase time latency by 15 and 38.6% and with PVOV with Ondansetron at 50 and 100 mg/Kg increased significantly (P<0.001) by 36.5 and 29.8% compared to PVOV alone. In Retrieval phase, the transfer latency of VOV was significantly (P<0.05) increased by 86.6% compared to normal animals whereas, this effect was significantly (P<0.5) increased when administered with PVOV by 59.1% when compared VOV group animals. When PVOV was combined with two doses of scopolamine and Ondansetron, there was a significant (P<0.001) increase in latency time by 55.7 & 65.1% and 55 & 56.3% when compared with PVOV group. Piracetam was used as reference standard produced a significant decrease of 3.3% change in transfer latency compared to PVOV. The details are depicted in Figure 3.

Step down passive avoidance test
During acquisition phase of study, VOV treated animals showed significant (P<0.05) increase in SDL by 81.9% compared to control animals, in turn the PVOV group animals showed significant decrease (P<0.001) in SDL by 41.2% compared to VOV treated animals. When PVOV was combined with two doses of scopolamine and Ondansetron, the step down latency was significantly (P<0.001) increased by 45.3 & 32.2% and 30.3 & 30.1 % when compared with PVOV group, where as piracetam treated animals showed significant (P<0.05) decrease by 3.2% compared to PVOV. Similarly there was an increase in step down errors and significant (P<0.001) increase in time spent in shock zone by animals when PVOV was administered with scopolamine and ondansetron compared PVOV alone. During retrieval phase, the step down latency of was significantly (P<0.001) increased by 15.3% with VOV compared to control, whereas, the PVOV group animals showed significant decrease (P<0.001) in SDL by 61.5% compared to VOV treated animals, similarly piracetam decrease significantly (P<0.001) by 3.2% when compared PVOV group. When PVOV was administered with scopolamine and ondansetron, there was a dose dependant significant (P<0.001) increase in SDE and TSS in comparison with PVOV alone group. The details of results are shown in the table 1.

DISCUSSION
Vespa venom is still widely studied for their component activity and applications in toxicology related fields. This venom contains high molecular mass proteins, biological active amines (acetylcholine, histamine, serotonin and catecholamines) and small peptides. Many bioactive peptides (kinins, mastoparans and chemotactic peptides) have been isolated and characterized from various species of vespid venoms due these components, most of venom cause complications. In the present study, the minimum lethal dose of VOV was found to be 15 mg/kg and 12 mg/kg by i.p and i.v route in mice, which is higher than the reported dose of 2.5 mg/kg probably due presence non-protein material might have increased the MLD. We have selected 1/10th of MLD of VOV for Photooxidation studies to generate antigenically active detoxified Vespa orientalis venom products (PVOV) and to study effect on memory and learning activity in presence and absence of scopolamine and ondansetron. The UV-Photooxidation of venom proteins in presence methylene blue have been widely used to detoxify and to study the biological activity. UV exposure of...
VOV caused shift in the ODmax in PVOV absorption profile compared to VOV. An increased absorbance was reciprocated with loss of venom protein in photooxidized venom. Comparative absorbance changes in reaction mixture of VOV and PVOV revealed the loss of protein in PVOV probably due to unfolding of proteins in the presence of sensitizer dye. Similar alterations were also observed in photooxidized venom product of *Naja siamensis* and *Echis carinatus*, which support our hypothesis. In immunogel diffusion test, Inmunoglobulins isolated from rabbit sera showed prominent precipitin lines with 45 min exposure of venom indicated the retention of antigenicity, where as other Photooxidized venom products produced either reduced intensity or number of precipitin line, hence we have selected 45 min exposure PVOV for *in-vivo* and *in-vitro* neutralization study of venom. When isolated immunoglobulins were tested against 1 MLD VOV, it was found that 1% diluted antivenom was sufficient to neutralize 1 MLD and protected mice from death and even when PVOV when injected in mice the mortality time was prolonged compared to VOV indicated that decreased toxicity with retention of antigenicity. From the results of above study, we have chosen 1.5 mg/kg (1/10% of 1MLD) of PVOV as therapeutic dose to rats in memory and learning model. *Vespa orientalis* venom is complex mixture of many enzymatic and non-enzymatic proteins. Nearly 4% of soluble proteins of *Vespa orientalis* are known to contain acetylcholine-like substances. In the pure venom and venom sac extract of *Vespa orientalis* provoked CNS anticholinesterase-like activity in various animals, and may open the blood-brain barrier and cause central mediated changes in auditory and somatosensory activities in cats. and pharmacological and toxicological effects of toxins of snake and wasps thought to mediated through central acetylcholine receptor and ion channels. These facts encouraged us explore study of effect of Photodetoxified Vespa orientalis venom along administered with Scopolamine and Ondansetron to check mechanism of memory enhancement. PVOV generated at 45 min UVR exposure showed improvement both spatial and long term memory in rats when subjected in T maze, EPM and conditioned avoidance paradigm. Administration of PVOV shortened the time required to reach the food in T-maze and transfer latency in EPM, while a significant improvement was observed in step down passive avoidance test, which indicates a clear cut improvement in learning and memory function in all the models. These experimental results indicated that PVOV components might have acted directly or indirectly upon repeated administration and some water soluble components of venom responsible for boosting of blood flow in central cortex. Scopolamine, an anticholinergic agent acts as a competitive inhibitor at post ganglionic muscarinic M1 receptors site of parasympathetic system. The improvement was significantly suppressed when scopolamine was administered indicated that activity of PVOV could be mediated through cholinergic mechanism which was antagonized by scopolamine and ondansetron. However, we cannot rule out the role of other low-molecular weight protein and peptides or enzymes, which may act by serotonergic / cholinergic system that affect CNS action.

We provide first reported evidence that photooxidized Vespa orientalis venom with UV radiation for 45 min undergo detoxification having improvement in both spatial and long term memory. There is a possibility of using PVOV in the treatment of degenerative illnesses such Dementia, Alzheimer’s disease as a nonherbal and nonsynthetic alternative for patients not responding to the available therapy; The results supports that possible mode of action could be through M1 receptor or 5HT-3 modulation or antiacetylchonesterase action.

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