Received: February 9, 2006
Accepted: May 11, 2006
Abstract published online: June 26, 2006
Full paper published online: November 30, 2006

J. Venom. Anim. Toxins incl. Trop. Dis.
V.12, n.4, p.632-652, 2006.
Original paper.
ISSN 1678-9199.

EVALUATION OF THE EFFECTS OF PHOTOOXIDIZED *Echis carinatus* VENOM ON LEARNING, MEMORY AND STRESS

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ABSTRACT: Snake venoms are a mixture of complex proteins, which have many physical and pharmacological properties. Photochemical detoxification has been suggested to generate photooxidized *Echis carinatus* venom product (POECVP). Antigenically-active photooxidized species of *Echis carinatus* venom could be obtained by exposing the venom to ultraviolet radiation (UVR) in the presence of methylene blue. The aim of the present study was to evaluate the effects of POECVP on learning, memory and stress in rats. Detoxification of the photooxidized venom was evident since the POECVP-treated group had longer survival time than the group of mice treated with *Echis carinatus* venom product (ECVP) following intraperitoneal and intracerebral injections. Photooxidized *Echis carinatus* venom product showed antidepressant activity by prolonging sleep onset and shortening the duration of pentobarbitone-induced hypnosis in mice. In single and chronic dose studies with rats, we observed that POECVP significantly decreased the time needed to reach food in T-maze, shortened transfer latency in elevated plus-maze, and decreased immobility time in forced swim test. We concluded that although there is a possibility of employing POECVP in the treatment of depressive and chronic degenerative illnesses as a nonherbal and nonsynthetic alternative for patients not responding to the available therapy, further investigation is still needed.

KEY WORDS: learning and memory, photooxidized *Echis carinatus* venom product, T-maze, forced swim test, elevated plus-maze.

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INTRODUCTION

Venom is defined as a highly potent, biologically-active mixture of chemical constituents produced or secreted by the venom gland and injected into the prey to paralyze and digest it or for defense. Snake venom is a mixture of complex proteins with many physical and pharmacological properties such as neurotoxins used in the treatment of brain injury, stroke or Alzheimer's disease. It has been a powerful tool to elucidate biological processes of vital importance (18). Several proteins isolated from snake venoms have been useful as pharmaceutical and diagnostic agents or as preparative tools and surrogate markers for hematology, neurobiology and complement research (20, 27).

Photochemical reactions have been extensively used to produce photooxidized venom products from protein molecules and nucleic acids of the venom (8, 12, 15, 25, 26). Photooxidation can be carried out under gamma, visible and ultraviolet radiations using methylene blue as a sensitizer to generate antigenically-active or detoxified species, which can also be used in the production of antivenom (3). Studies have demonstrated that irradiating snake venoms with UVR for different time extents in the presence of methylene blue can lead to significant changes (9). Photochemical detoxification has been suggested as an alternative approach to investigate the pharmacological significance of snake venom proteins when their main toxophoric regions are altered. Radiation methods were initially used in the detoxification of venom proteins to shorten the immunization schedule for antivenom production (24).

Echis carinatus venom has shown prominent analgesic, anticonvulsant, phospholipase A_2, and fibrinogen clotting activities, besides skeletal muscle mild relaxant action; whereas its photooxidized product has shown algesia and antidepressant activity in experimental animal models. The antidepressant action of POECVP has been evidenced in mice injected with pentobarbitone. Photooxidized *Echis carinatus* venom product has also shown to retain the central nervous system (CNS) stimulant actions such as circling movements following intracerebral injections (10).

Many potential areas of research have used snake venom as medicine. However, no attempts have been made to investigate the effects of acute and chronic administration of POECVP on the cognition, dementia and stress in animal models as an alternative mechanism to generate lead compounds or surrogate markers for

the therapy of neurodegenerative diseases. Thus, the aim of the present study was to evaluate the effect of POECVP on rat behavior using T-maze, elevated plus-maze and forced swim test.

MATERIALS AND METHODS

Animals

Male mice (25-30 g) were used for toxicity studies and male rats (180-240 g) were used for behavioral studies. Animals were kept under standard light/dark cycle with standard commercial food and tap water *ad libitum.*

Venom

Echis carinatus venom was obtained from Haffkine Institute, Mumbai, India.

Antivenom

Polyvalent land snake antivenom was also supplied by Haffkine Institute, Mumbai, India.

Echis carinatus venom solution (ECV)

Twenty-one milligrams of *Echis carinatus* venom were dissolved in 0.05 M phosphate buffer (1.5 g Na_2HPO_4 and 1.36 g KH_2PO_4 in 400 ml double distilled water; pH 6.8 was adjusted using 0.1N NaOH) to a final concentration of 2.1 mg/ml.

Methylene blue solution (MB)

To prepare a 0.003% w/v solution, 3 mg of methylene blue was dissolved in 100 ml of phosphate buffer (pH 6.8).

Activated charcoal (1% w/v)

Charcoal (100 mg) was weighed and transferred to a 10ml volumetric flask; then, a few milliliters of phosphate buffer were added, shaken gently and the volume was completed to 10 ml with phosphate buffer (pH 6.8).

Photooxidation method

Reaction mixture (containing 2 ml ECV and 2 ml MB in phosphate buffer) was kept on magnetic stirrer, exposed to UV light (tubular ultraviolet 15W, G15 T8 Phillips,

Holland, UV output: 4.8 W, 49 μW/cm^2) at a distance of about 10 cm and was gently stirred for 90 minutes at 37±1°C (10). Then, 200 μl of 1% activated charcoal was added; mixture was stirred for more 5 minutes without exposure to UV light and filtered through 0.2μ filter using a syringe filtration unit (Sartorius).

Similarly, control mixture contained 2 ml ECV and 2 ml MB in phosphate buffer. It was stirred for 5 minutes on the magnetic stirrer and then filtered through 0.2μ filter using a syringe filtration unit, without exposure to UV light nor addition of activated charcoal. Absorbances of photooxidized venom product (POECVP) and control (ECVP) were measured separately at 200-400 nm using Shimadzu spectrophotometer.

Toxicity studies

Determination of minimum lethal dose: To determine the lethal dose of a venom, intracerebral (IC) and subcutaneous (SC) injections have been used (7).

In our study, three groups of three mice each received IC injections into the brain at the angle of 90°. The first group was injected with 100 μg/kg phosphate buffer (control); the second group was injected with 200 μg/kg ECVP; and the third group received 400 μg/kg POECVP.

Other three groups of three mice each received injections in the peritoneal cavity (IP). The first group received 1 mg/kg phosphate buffer (control); the second group was injected with 2 mg/kg ECVP; and the third group received 4 mg/kg body weight of POECVP.

The sequence of symptoms and time of death were recorded. Student's t-test was used to calculate the minimum lethal dose.

Antigenicity test: Antigencity of POECVP was determined using the immunogel diffusion method (13), in which antigens and antibodies migrate through agarose gel and react, resulting in precipitin lines.

Antidepressant activity: The method described by Balazs and Grice was closely followed (1). Pentobarbital sodium (35 mg/kg; IP) was used as a standard hypnotic agent. Thirty minutes prior to the start of the experiment, one group of three mice was injected with normal saline (control) and another group of three mice (test), with

POECVP (100 µg/25 g; IP). After 30 minutes, both groups were injected with pentobarbital sodium. Onset and duration of sleep were recorded.

Behavioral studies

Learning and memory in T-maze: T-maze was used to assess spatial working memory of rats (6, 19). It was made up of perspex and consisted of a stem, a starting box (86.5 cm X 8 cm), and two arms (left and right arm dimensions: 62 cm X 18 cm), which were the goal areas. Food pellets were kept at the end of both arms. It was placed at 87.5 cm from the ground.

Three groups of six rats each were used for the experiment. Group 1 (control) received normal saline; Group 2 (standard) received Piracetam (150 mg/kg body weight); and Group 3 (test) received POECVP (2.8 mg/kg body weight) on the 2nd day (single dose studies). For chronic dose studies, control group received normal saline; standard group received Piracetam (75 mg/kg body weight); and test group received POECVP (1.4mg/kg body weight).

For three consecutive days, starting at 10:00 A.M., animals were tested in the T-maze. Before starting a new trail, animals were allowed to stay for some time in their home cages. On the 1st day, each rat completed 15 trails to become familiarized with the maze and the food. Fifty percent of the animals were induced to prefer the left arm and the remaining, the right arm of the maze. Then, animals were deprived of food for 24 hours. On the 2nd day, 30 minutes before the experiment, each group was treated as described above. Rats were trained until they attained nine correct arm choices out of 10 consecutive trails. On the 3rd day, each animal was subjected to three trails. The number of correct responses and the time needed to reach food (TRF) in each trail were recorded (22). The percentage of correct responses (%CR) was calculated.

On the 13th and 14th days (chronic dose studies), the experiment procedures started 30 minutes after treatment. On the 15th day, TRF in each trial and %CR were calculated.

Transfer latency in elevated plus-maze: Elevated plus-maze was employed to measure transfer latency (16, 17). Three groups of six rats each were used. Group 1 (control) received normal saline; Group 2 (standard) received Piracetam (150 mg/kg body weight); and Group 3 (test) received POECVP (2.8 mg/kg body weight) for

single dose studies. For chronic dose studies, Group 1 received normal saline; Group 2 received Piracetam (75 mg/kg body weight); and Group 3 received POECVP (1.4 mg/kg body weight) at 14 days.

On the 1st day, rats were individually placed at the end of one arm facing away from the central platform. Rats were allowed to freely explore the maze for 20 seconds. The time rats needed to find and enter with all four paws in one of the closed arm was recorded and defined as transfer latency (TL). On the 2nd day, 30 minutes prior to the start of the experiment, animals were treated as described above.

Twenty-four hours later, rats were again placed at the end of one of the open arms, facing away from the central platform, and TL was recorded. After each rat trial, the apparatus was cleaned and dried. Transfer latencies measured on the 1st day and on the 2nd day after treatment served as parameters for acquisition and retrieval, respectively (23).

On the 14th day (chronic dose studies), the experiment procedures started 30 minutes after treatment and the TL recorded was used as a parameter for acquisition. Transfer latency obtained on the 15th day was used as a parameter for retrieval.

Forced swim test: The test was performed using the method of Porsolt *et al*. (4, 5, 11, 21). Experiments were carried out between 10:00 A.M. and 12:00 P.M. and three groups of six rats each were used. Group 1 (control) received normal saline; Group 2 (standard) received Imipramine (4 mg/kg body weight); and Group 3 (test) received POECVP (2.8 mg/kg body weight) on the 2nd day (single dose studies). For chronic dose studies, Group 1 (control) received normal saline; Group 2 (standard) received Imipramine (2 mg/kg body weight); and Group 3 (test) received POECVP (1.4 mg/kg body weight) at 14 days.

On the 1st day, each rat was allowed to swim individually for about 15 minutes in a water tank (height: 40 cm, diameter: 24 cm) containing 15 cm water at 25±2°C. Animals were removed and wiped with a dry towel, warmed under 100W bulb for 15 minutes and returned to their home cage after each trial.

On the 2nd day, 30 minutes prior to the test each group of animals was treated as described above and allowed to swim in the similar environmental conditions for 6 minutes. Onset and total time of immobility were recorded in the last 5 minutes (test session). An animal was considered immobile when it ceased struggling and

remained floating motionless in the water, making only those movements necessary to keep the head above the water. After the experiment, animals were removed and wiped with a dry towel, warmed under 100W bulb for 15 minutes and returned to the home cage.

At 14 days (chronic dose studies), animals were treated 30 minutes prior to the start of the experiment, which was carried out as described for the 2nd day.

Statistical analysis

Mean and standard error mean were calculated for all parameters studied. Comparisons between groups were made using one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test; $p<0.05$ was considered to be significant.

RESULTS

Photooxidation of venom proteins

Photooxidation of venom proteins was previously standardized by exposing the reaction mixture to UV light for 0, 15, 30, 60, 90, and 120 minutes. Antigenicity was clearly visible at 90 minutes; therefore, the reaction time of 90 minutes at 37°C was used to generate antigenically-active POECVP. Absorbance results of 6 control (ECVP) and 6 photooxidized (POECVP) samples were subjected to unpaired Student's t-test and were statistically significant (Table 1).

Spectrum Analysis: UV spectrum of ECVP was biphasic with maximum optical density (OD max) of 1.089 AU at 279.0 nm and 3.612 AU at 226.0 nm. For UV spectrum of POECVP, maximum wavelength (λ max) shifted from 279.0 nm to 279.5 nm and for that of ECVP, λ max shifted from 226.0 nm to 213.5 nm. Absorbance increased from 1.089 to 1.482 at 279.5 nm for POECVP.

First order (ΔT = 1) derivative UV spectrum of ECVP showed 5 peaks and that of POECVP showed 8 peaks. The peak at 217.0 nm observed for POECVP was not observed for ECVP. The absorbance of the downward peak at 236.5 nm decreased from 0.281 AU to 0.296 AU.

Second order (ΔT = 2) derivative UV spectrum of POECVP was resolved into 14 peaks and that of ECVP into 12 peaks. The peak at 239.5 nm, characteristic of ECVP, shifted towards right in POECVP at 240.0 nm and its absorbance decreased from 0.044 AU to 0.034 AU.

Toxicity studies: Intracerebral injection of ECVP at the dose of 400 µg/kg body weight into mice showed characteristic CNS stimulant manifestations such as circling movements and hyperexcitation to external stimulus. The average time of death of all animals in this group was 100 minutes.

Intracerebral injection of 400 µg/kg POECVP presented similar symptoms: circling movements and continuous run. Mice showed hyperexcitation to external stimulus, which slowly decreased with time. All the animals injected with POECVP survived.

Intraperitoneal injection of ECVP at the dose of 4 mg/kg body weight into mice showed continuous writhing symptoms, such as stretching of hind legs, immobility, increased bowel movements, cataleptic behavior and gasp. Animals died due to respiratory arrest at an average of 190 minutes.

Intraperitoneal injection of POECVP showed less severe writhing symptoms and all animals survived.

Antigenicity test: Prominent precipitin lines were observed with ECVP, and their intensity decreased with time of exposure to UVR. Photooxidized *Echis carinatus* venom product (POECVP), which was produced by exposing the venom to UV radiations for 90 minutes, showed precipitin lines on immunogel diffusion indicating that the venom antigenicity was retained.

Antidepressant activity: Pentobarbital sodium prolonged sleep onset by 1.023 fold, and time of sleep decreased by 0.94 fold in the POECVP-treated group compared with control.

Behavioral studies

Learning and memory in T-maze:

1) Single dose studies

On the 2nd day (acquisition process), TRF decreased in Groups 2 and 3 by 32.98% and 31.36%, respectively, after treatment (Table 2 and Figure 1). On the 3rd day (retrieval process), TRF decreased by 52.0% and 37.83% in Groups 2 and 3, respectively, when compared with control group (Table 2 and Figure 2). Results of learning process and memory retention were statistically significant [p = 0.0172]. During the retrieval process, the %CR increased by 21.31% in Group 2 and by

13.34% in Group 3, when compared with control group [77.73%] (Table 2 and Figure 3).

2) Chronic dose studies

In comparison with control group, TRF on the 2nd day (acquisition process) decreased in Groups 2 and 3 by 62.22% and 56.94%, respectively (Table 3 and Figure 4). During the retrieval process, results were statistically significant for both groups [p = 0.024]; TRF decreased by 39.35% and 22.59% in Groups 2 and 3, respectively, when compared with control (Table 3 and Figure 5). The percentage of correct responses in Groups 2 and 3 increased by 41.57% and 16.64%, respectively, when compared with control group [66.7%] (Table 3 and Figure 6).

Transfer Latency in elevated plus-maze:

1) Single dose studies

On the 1st day, TL decreased by 38.98% and 29.62% in Groups 2 and 3, respectively, in comparison with control [29.5 ± 3.18] (Table 4 and Figure 7). On the 2nd day, TL decreased by 29.49% and 23.48% in Groups 2 and 3, respectively (Table 4 and Figure 8).

2) Chronic dose studies

On the 14th day, TL decreased by 37% and 36.34% in Groups 2 and 3, respectively, in comparison with control (Table 5 and Figure 9). On the 15th day, TL decreased by 23.33% and 42% in Groups 2 and 3, respectively (Table 5 and Figure 10).

Forced swim test:

1) Single dose studies

In comparison with control group [108.0 ± 3.537], immobility time decreased by 22.15% in Group 2 and by 28.01% in Group 3. These results were statistically significant [p=0.021] (Table 6 and Figure 11).

2) Chronic dose studies

Immobility time significantly decreased [p<0.0001] by 22.23% and 23.68% in Groups 2 and 3, respectively, when compared with control group (Table 7 and Figure 12).

Table 1: Evaluation of photooxidation of venom proteins using UV absorption spectrum after exposure to UV light for 90 minutes (Mean ± SEM).

Parameters	ECVP	POECVP
λmax (nm)	278 ± 0.38*	279.8 ± 0.54*
Absorbance (AU)	1.132 ± 0.02*	1.434 ± 0.04*

*p = 0.0022
ECVP = *Echis carinatus* venom product (control)
POECVP = Photooxidized *Echis cartinatus* venom product (test)

Table 2: Effect of treatment with Piracetam and photooxidized *Echis carinatus* venom product on learning and memory using T-maze in single dose studies (Mean ± SEM).

Parameters	Group 1	Group 2	Group 3
Familiarization, 1st day [sec]	20.86 ± 4.31	18.73 ±4.14	15.65 ± 2.56
Acquisition, 2nd day [sec]	12.37 ± 1.27	8.29 ± 0.946*	8.49 ± 0.649*
Retrieval, 3rd day [sec]	20.72 ± 2.44	9.927 ± 1.68*	12.88 ± 3.16
Percentage of correct responses (%CR)	77.73	94.4	88.88

* = Significant
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Piracetam (standard)
Group 3 = Six rats injected with Photooxidized *Echis carinatus* venom product (test)

Table 3: Effect of treatment with Piracetam and photooxidized *Echis carinatus* venom product on learning and memory using T-maze in chronic dose studies (Mean ± SEM).

Parameters	Group 1	Group 2	Group 3
Familiarization, 1st day [sec]	22.87 ± 5.71	15.42 ± 1.86	24.82 ± 1.96
Acquisition, 2nd day [sec]	27.27 ± 2.34	10.33 ± 1.223*	11.74 ± 1.781**
Retrieval, 3rd day [sec]	16.24 ± 2.246	9.767 ± 1.078**	12.57 ± 2.217**
Percentage of correct responses (%CR)	66.7	94.4	77.8

* = Significant
** = Highly significant
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Piracetam (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

Table 4: Effect of treatment with Piracetam and photooxidized *Echis carinatus* venom product on transfer latency in single dose studies (Mean ± SEM).

Parameters	Group 1	Group 2	Group 3
1st day [sec]	29.5 ± 3.178	18.0 ± 1.803 *	20.08 ± 3.414
2nd day [sec]	21.67 ± 1.754	15.25 ± 1.131 *	16.58 ± 2.196

* = Significant
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Piracetam (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

Table 5: Effect of treatment with Piracetam and photooxidized *Echis carinatus* venom product on transfer latency in chronic dose studies (Mean ± SEM).

Parameters	Group 1	Group 2	Group 3
1st day	57.17 ± 3.283	36.0 ± 2.497 **	43.83 ± 5.079 *
2nd day	34.92 ± 2.275	22.33 ± 2.348 **	20.25 ± 2.819 **

* = Significant
** = Highly significant
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Piracetam (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

Table 6: Effect of treatment with Imipramine and photooxidized *Echis carinatus* venom product using forced swim test in single dose studies (Mean ± SEM).

Parameter	Group 1	Group 2	Group 3
Immobility time [sec]	107.9 ± 3.537	84.0 ± 6.627 **	77.67 ± 4.780 **

** = Highly significant
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Imipramine (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

Table 7: Effect of treatment with Imipramine and photooxidized *Echis carinatus* venom product using forced swim test in chronic dose studies (Mean ± SEM).

Parameter	Group 1	Group 2	Group 3
Immobility time [sec]	100.9 ± 2.951	78.67 ± 2.654 **	77.0 ± 3.464 **

** = Highly significant
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Imipramine (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

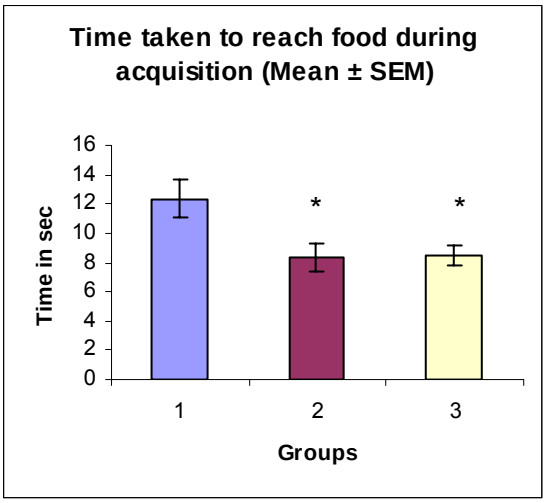

Figure 1: Effect of Piracetam and photooxidized *Echis carinatus* venom product on acquisition in single dose studies. Data are expressed as mean plus standard error mean.
* = Significant
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Piracetam (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

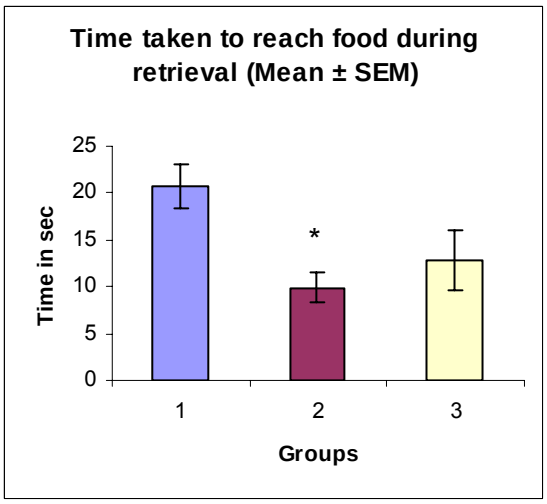

Figure 2: Effect of Piracetam and photooxidized *Echis carinatus* venom product on retrieval in single dose studies. Data are expressed as mean plus standard error mean.
* = Significant
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Piracetam (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

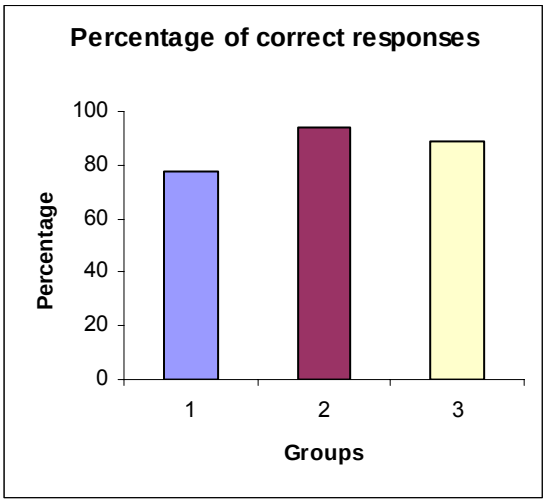

Figure 3: Effect of Piracetam and photooxidized *Echis carinatus* venom product on the percentage of correct responses in single dose studies.
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Piracetam (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

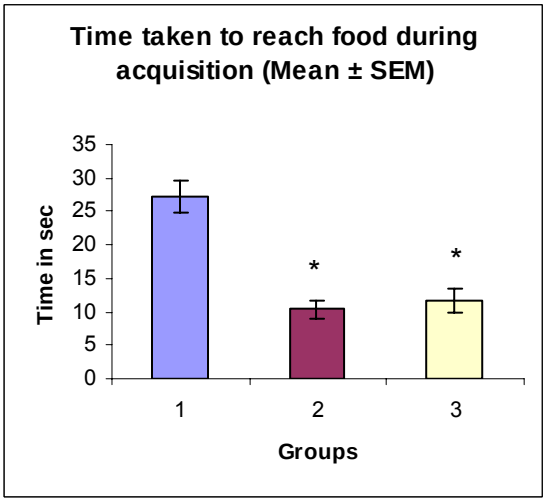

Figure 4: Effect of Piracetam and photooxidized *Echis carinatus* venom product on acquisition in chronic dose studies. Data are expressed as mean plus standard error mean.
* = Significant
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Piracetam (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

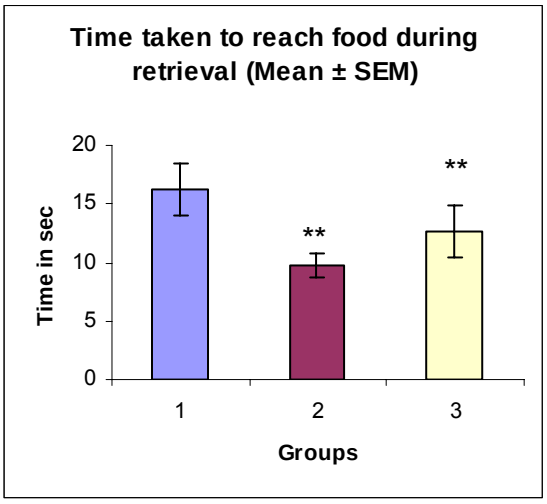

Figure 5: Effect of Piracetam and photooxidized *Echis carinatus* venom product on retrieval in chronic dose studies. Data are expressed as mean plus standard error mean.
** = Highly significant
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Piracetam (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

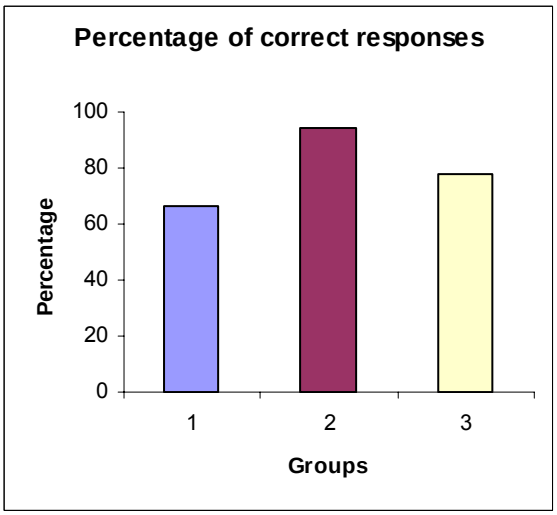

Figure 6: Effect of Piracetam and photooxidized *Echis carinatus* venom product on the percentage of correct responses in chronic dose studies.
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Piracetam (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

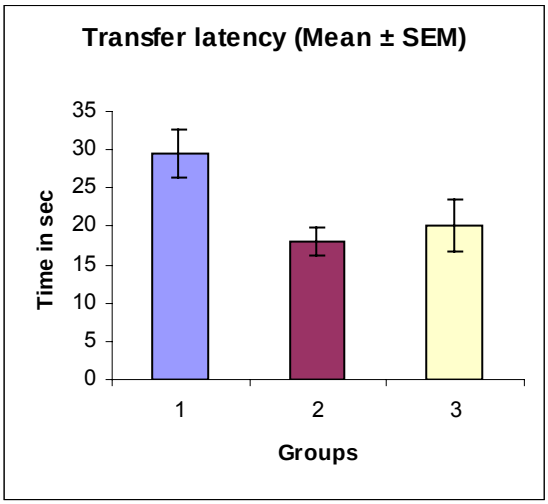

Figure 7: Effect of Piracetam and photooxidized *Echis carinatus* venom product on transfer latency during the 1st day in single dose studies. Data are expressed as mean plus standard error mean.
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Piracetam (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

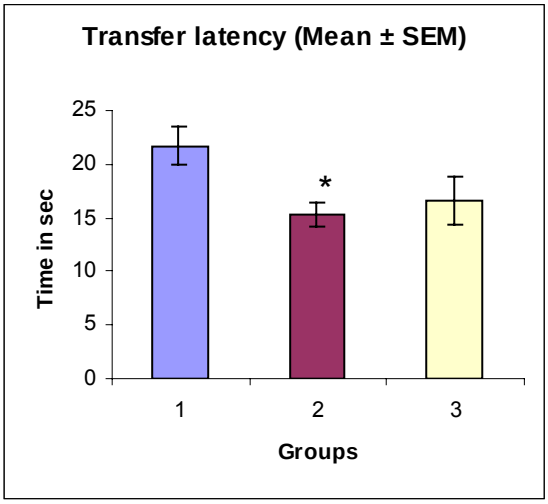

Figure 8: Effect of Piracetam and photooxidized *Echis carinatus* venom product on transfer latency during the 2nd day in single dose studies. Data are expressed as mean plus standard error mean.
* = Significant
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Piracetam (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

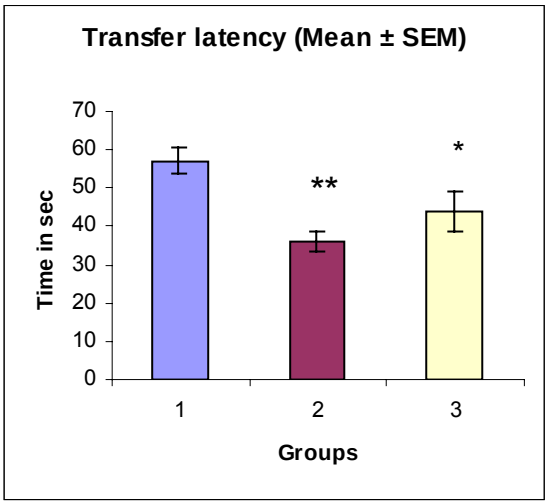

Figure 9: Effect of Piracetam and photooxidized *Echis carinatus* venom product on transfer latency during the 14th day in chronic dose studies. Data are expressed as mean plus standard error mean.
* = Significant
** = Highly significant
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Piracetam (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

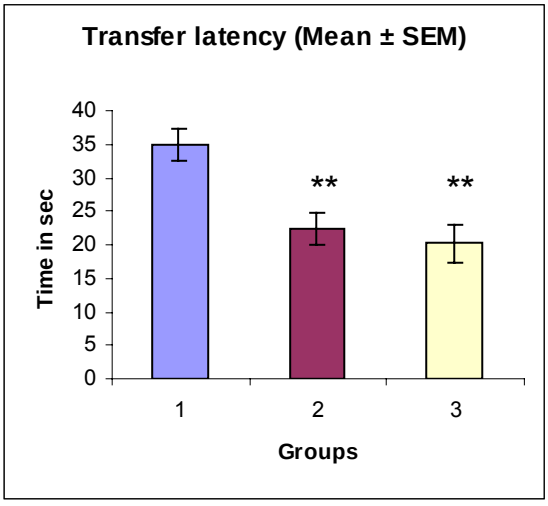

Figure 10: Effect of Piracetam and photooxidized *Echis carinatus* venom product on transfer latency during the 15th day in chronic dose studies. Data are expressed as mean plus standard error mean.
** = Highly significant
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Piracetam (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

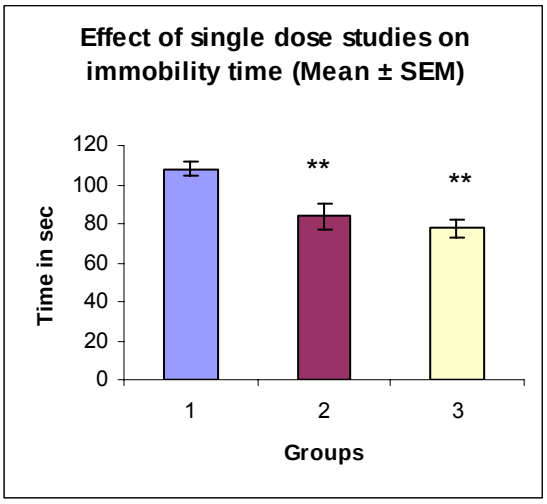

Figure 11: Effect of Imipramine and photooxidized *Echis carinatus* venom product on immobility time during forced swim test in single dose studies. Data are expressed as mean plus standard error mean.
** = Highly significant
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Imipramine (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

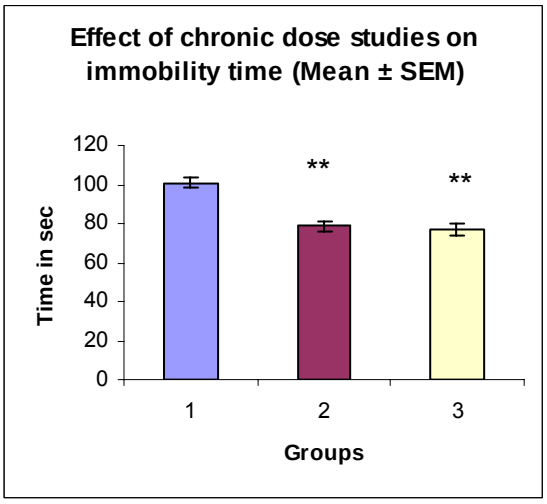

Figure 12: Effect of Imipramine and photooxidized *Echis carinatus* venom product on immobility time during forced swim test in chronic dose studies. Data are expressed as mean plus standard error mean.
** = Highly significant
Group 1 = Six rats injected with normal saline (control)
Group 2 = Six rats injected with Imipramine (standard)
Group 3 = Six rats injected with photooxidized *Echis carinatus* venom product (test)

DISCUSSION

Photochemical detoxification has been suggested as an alternative approach to investigate the pharmacological significance of snake venom proteins when their toxophoric regions are altered (8). Photochemical reactions between light-absorbing molecules and proteins in the presence or absence of the sensitizer methylene blue have been extensively used to investigate alterations in the biological activity of venoms (2, 14).

In the present study, a reaction mixture containing venom proteins (P), methylene blue (MB) and phosphate buffer (PB) was exposed to UVR. Electrons were transferred from the UV-sensitized MB to P. By photodynamic action, stable excited species (S) of MB produced reduced substrates (SH_2) and, in the presence of atmospheric oxygen, generated photooxidized products (POP). Therefore, S + P in MB medium = reduced form of the substrate (SH_2) + atmospheric oxygen→ POP.

To generate photooxidized *Naja naja siamensis* venom protein species, UVR radiations in the presence of methylene blue have been used. To generate a photooxidized *Echis carinatus* venom product, we used a similar method with selective modifications to suit our laboratory conditions (9).

Echis carinatus venom mainly affects the cardiovascular and central nervous systems and tissues. Neurotoxins from *Echis carinatus* venom are responsible for many pharmacological alterations (9).

In the present work, an antigenically-active species of *Echis carinatus* venom was generated by using UVR radiation for 90 minutes at 37 ± 1°C in the presence of methylene blue.

The POECVP mixture showed an alteration in the absorbance when compared with that of ECVP and which is probably due to unfolding of proteins in the presence of dye. Flat spectrum with increased absorbance was observed for POECVP from 238.5 to 372.5 nm, which may be due to the transitions generated in the side chain of aromatic amino acids.

Precipitin lines were observed when POECVP was subjected to immunogel diffusion, indicating that antigenicity was certainly retained after exposing ECVP to UVR for 90 minutes.

The selective changes in absorbance due to photooxidation were further resolved using 1^{st} and 2^{nd} order derivative spectrum analysis, at $\Delta T=1$ and $\Delta T=2$. The 1^{st} order derivative spectrum of ECVP showed 5 peaks whereas that of POECVP was

resolved into 8 peaks, suggesting transitional changes in the side chain of aromatic amino acids. There were about 14 peaks resolved in the POECVP spectrum and 12 peaks in the 2nd order derivative spectrum of ECVP at ΔT=1 between 200 and 294 nm. The peak at 240 nm decreased from 0.044 AU to 0.034 AU in the 2nd derivative UV spectrum at ΔT=1, which may also be due to selective alterations in the side chain of the aromatic amino acids tryptophan or tyrosine.

The POECVP generated at 90-minutes UVR-exposure showed indirect CNS stimulant/antidepressant properties on pentobarbitone-induced hypnosis in mice, similarly to the POECVP generated at 120-minutes UVR-exposure. Pentobarbitone-induced hypnosis in mice is known to be due to prolonged activation of ionophoric receptors such as GABA A with consequent increases in the chloride conductance.

Besides intracerebral injections, detoxification of POECVP was studied using the intraperitonial route, which showed to be convenient for the chronic administration of POECVP to rats. Abdominal complications and writhing induced by ECVP were not observed with POECVP. Significant antidepressant effects of POECVP were evidenced by the shortening of the immobility time on Porsalt forced-swim test in both single and chronic dose treatments. Indirect antidepressant activity induced by POECVP was further evaluated for the involvement of nootropic properties using T-maze and elevated plus-maze. Photooxidized *Echis carinatus* venom product significantly shortened the time rats needed to reach food in T-maze and the transfer latency in elevated plus-maze following single and chronic dose studies.

From the present findings, we can conclude that there is a possibility of using POECVP in the treatment of depressive and chronic degenerative illnesses as a nonherbal and nonsynthetic alternative for patients not responding to the available therapy; however, further studies are still needed.

ACKNOWLEDGEMENTS

The present work is part of M. Pharm-Pharmacology dissertation presented by Chandra Mohan Reddy to Rajiv Gandhi University of Health Sciences, Bangalore, 2005. The authors are indebted to B. G. Shivananda, Principal of Al Ameen College of Pharmacy, for support and help.

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