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Preliminary Study on the Antisnake Venom Activity of Alcoholic Root Extract of *Clerodendrum viscosum* (Vent.) in Naja naja Venom

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Abstract – The antisnake venom activity of *Clerodendrum viscosum* Vent. (Fam. Verbenaceae), a plant traditionally used in India for the treatment of snake bite was evaluated by *in vitro* and *in vivo* methods. While *in vitro* studies were performed using human blood, *in vivo* studies were carried out using mice administered three different i.p doses of the extract, 5 min before the administration of *Naja naja* snake venom. The results of the *in vitro* studies showed that the extract probably interacts with but does not stabilize membrane protein. In the *in vivo* studies, the extract showed significant antisnake venom activity, which may be attributed to its possible interference with the acetylcholine receptor sites. Hence the present investigation justifies the traditional use of *Clerodendrum viscosum* (*C. viscosum*) as antisnake venom.

Keywords – *Clerodendrum viscosum*, *Naja naja*, snake venom, verbenaceae, alcoholic extract

Introduction

Snakebite, a rural and occupational hazard which mainly affects farmers, land workers and fishermen is a major health hazard in India (Chatterjee, 1965). Incidents of snake bite leading to death are common in many tropical countries during or after the rainy season because of increased human settlement in the natural habitats of snakes. Early administration of adequate amounts of horse antisnake venom serum (polyvalent or the better specific) followed by supportive treatment is the best way of treating envenomation. Although antiserum is an effective antidote, it quite often produces severe allergic reactions and side effects. Antiserum requires refrigeration; it is expensive and often not available in rural areas. All these hazards and drawbacks necessitate the search for antidotes without these defects. Thus the study of herbal antidotes against snake venom is of great importance. In folk medicine a plethora of plants are claimed to be antidotes for snake bites. These plants or their extracts, sometimes in combination are used as antidotes for snake venom by rural populations in many parts of the world. Compared to the numerous folklore claims the pharmacological and clinical investigations done on the same are meagre (Houghton *et al.*, 1993).

Clerodendrum viscosum (Fam. Verbenaceae) is a large gregarious tawny-villous shrub found throughout India as undergrowth in forests up to 1800 m and as a weed along the roadsides and wastelands (Warrier *et al.*, 1996). The plant has been used as antiseptic, anti-inflammatory, antipyretic, vermifuge, expectorant and in the treatment of tumors, leprosy, and skin diseases. The aerial part of the plant contains sterols; the root contains lupeol, β-sitosterol, and steroidal glycosides; the leaf contains a diterpene clerodin; the seed oil contains fatty acids and the flower contains glycoside acetoside, fumaric acid esters of caffeic acid, lupeol, β-sitosterol, β-sitosterol glucoside, cleridine and hentriacontane (Yoganarasimhan, 2000). Traditionally though *C. viscosum* has been used in the treatment of snake bites and scorpion sting (Nadkarni, 1954), there is no documentation of research work carried out with regard to this activity. Hence, the present study is an attempt to evaluate the antisnake venom activity of *C. viscosum*.

Experimental

Animals – Swiss albino mice were acclimatized in an experimental room having temp 23 ± 2 ℃, controlled humidity conditions and 12 : 12 hr light and dark cycle. Animals were caged in polypropylene cages with a maximum of two animals in each cage. The mice were fed with standard food pellets and water *ad libitum*. The study was conducted after obtaining ethical committee clearance from the Institutional Animal Ethics Committee, KMC, Manipal (IAEC/

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KMC/25/2001-2002).

Plant material – The roots of the plant material *C. viscosum* were collected from Shirva, Udupi, Karnataka, India during the month of April 2002. The plant was authenticated by Dr.Gopalakrishna Bhat, Department of Botany, Poorna Prajna College, Udupi, Karnataka, India. A voucher specimen (PP 515) has been deposited in the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal, India. The roots were shade dried and powdered.

Preparation of alcoholic extract – About 1 kg of the root powder was taken in a soxhlet extractor and extracted with ethanol for 6 hours. The solvent was recovered by distillation *in vacuo* and the residue (yield 42 g) was stored in dessicator and used for subsequent experiments. For animal studies, the dried extract was suspended in 2% gum acacia solution.

Preliminary phytochemical screening – The alcoholic extract was subjected to preliminary phytochemical analysis using standard methods (Kokate, 1994; Harborne, 1998).

Acute toxicity studies – Albino mice of either sex, starved for 3 - 4 hours were divided into four groups (n = 6) and orally fed with the alcoholic extract in increasing dose levels of 100, 500, 1000 and 3000 mg/kg body weight (Ghosh, 1984). The rats were observed continuously for 2 hr for behavioral, neurological and autonomic profiles and after 24 hr and 72 hr for any lethality (Turner, 1965).

Snake venom – Lyophilized *Naja naja* snake venom was collected from CSIR, Centre for Biochemicals, New Delhi and preserved at 4 ℃ until further use. The snake venom was dissolved in 0.9% w/v saline and centrifuged. The supernatant was used for the study. The venom concentration was expressed in terms of dry weight (mg/ml) of the stock venom.

***In vitro* antisnake venom activity** – Antisnake venom activity of *C. viscosum* was assessed through inhibition of *in vitro* Human Red Blood Corpuscles (HRBC) lysis. The hyposaline induced haemolysis was evaluated *in vitro* by the method of Roelofsen *et al.*, 1971 and Balu, 1995. This method was modified in the present study by venom induced haemolysis. Blood was collected from healthy human volunteers by vein puncture. Heparin was used as an anticoagulant. The collected blood was washed three times with saline. The preparation of cell suspension was carried out as described by Murugesh *et al.*, 1981. Venom of *Naja naja* was dissolved in physiological saline solution to make a stock solution of 100 μg/ml. To different tubes filled with 1 ml of venom (100 μg/ml), 1 ml phosphate buffer pH 7.4 and 1 ml of 1% HRBC, varying concentrations of alcoholic extract of *C. viscosum* (20, 40, 60, 80 and 100 μg/ml) in saline were added. Control had the same composition but was free of extract. The mixtures were incubated at 37 ℃ for 30 min and then centrifuged at 1000 rpm for 3 min. The absorbance of the supernatant was measured at 540 nm using a spectrophotometer. The percent of haemolysis was calculated by the following equation,

$$\% \text{ haemolysis} = \left[\frac{A_t - A_c}{A_t}\right] \times 100$$

At – absorbance of test (with extract) in venom solution.

Ac – absorbance of control (without extract).

***In vivo* snake venom activity** – The antisnake venom activity of alcoholic extract of *C. viscosum* was determined by using the LD50 of *Naja naja* venom (Alam *et al.*, 1994) in mice. The venom dissolved in 0.9% saline was administered intraperitoneally into mice. The effectiveness of the alcoholic extract in modifying the lethal effect of the test dose of venom was investigated by administering i. p doses of different concentration to three different groups comprising of six animals each, 5 min before the administration of snake venom. The number of animals which died within 24 hrs was recorded. The experiment was performed in duplicate and each value is the mean of two such determinations ± S.E.

Statistical analysis – Data were statistically evaluated by using one way Student 't' test using 7.5 version of SPSS computer software. The values were considered significant when $p < 0.05$.

Results and Discussion

The present study was undertaken to evaluate the antisnake venom activity of the alcoholic root extract of *C. viscosum*. Preliminary phytochemical screening revealed the presence of steroids, phenolic compounds, carbohydrates and fatty acids.

Results of the *in vivo* antisnake venom activity of *C. viscosum* are presented in Fig. 1. Administration of alcoholic extract of *C. viscosum* at three dose levels 250, 500 and 750 mg/kg progressively reduced the mortality in mice. Significant protection ($p < 0.05$) was observed at dose levels of 500 and 750 mg/kg body weight. Death from *Naja naja* results mainly due to the neurotoxicity caused by the curare like action of cobra toxin on respiratory failure (Warrel, 1987). Cobra neurotoxin acts at peripheral neuromuscular junction either posts synaptically by binding competitively at the acetyl choline receptor or presynaptically by preventing the release of acetyl choline transmitter from the nerve terminals (Gurumukh, 1999). The probable mechanism of prevention of neurotoxic effect by *C. viscosum*

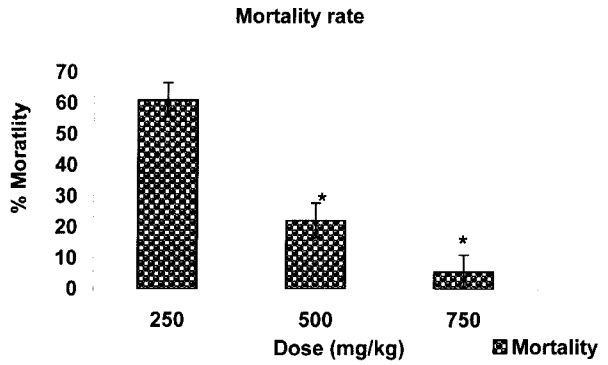

Fig. 1. *In vivo* antisnake venom activity of *C. viscosum.*
Each value represents mean ± S.E., n = 6; *Represents statistical significance between the groups ($p < 0.05$); analyzed by Student 't' test

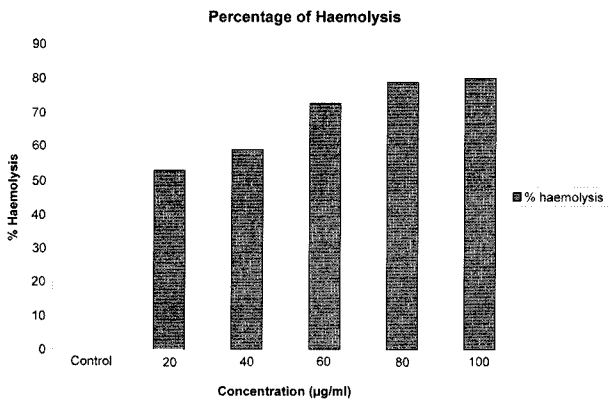

Fig. 2. *Invitro* HRBC membrane stabilization properties of the extract of *C. viscosum.*
Each value represents results of triplicate experiment. The figure represents concentration vs percentage haemolysis

may be by interfering with the acetyl choline receptor sites i.e., by antagonizing the actions of neurotoxic substances in the venom at the acetyl choline receptor sites (Haruna *et al.*, 1955).

In earlier studies, it has been shown that snakebite causes haemolysis, which is one of the contributing factors of snake venom. Most snake venoms contain phospholipase and haemolysin (Nandy, A., 1995; Rosenberg, 1979), which act on membrane associated phospholipids to liberate lysolecithin. Lysolecithin acts on the membrane of HRBC causing haemolysis (Maeno *et al.*, 1962). In our investigation, on *in vitro* HRBC membrane stabilization properties of the extract of *C. viscosum* (Fig. 2), the alcoholic extract at concentrations ranging from 20-100 µg/ml failed to inhibit haemolysis induced by *Naja naja* venom, but instead accelerated the process of haemolysis to a great extent.

Protection against venom induced haemolysis is thought to be caused by the stabilization of proteins in the membrane of HRBC (Abe *et al.*, 1991). Haque *et al.*, 2000 have reported the cytotoxic activity of *C. viscosum.* Cytotoxic drugs are known to accelerate haemolysis. A possible explanation for the acceleration of haemolysis by this plant may be attributed to its cytotoxic activity (Motta, 1971). Hence it may be suggested that *C. viscosum* extract may interact with but does not stabilize the membrane proteins. The results of *in vivo* studies have shown that the alcoholic extract of *C. viscosum* possesses significant antisnake venom activity and could have a promising role in the treatment of *Naja naja* snake bite. Further studies on the isolation of responsible active constituents and its mechanism of action have to be carried out.

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