Synopsis
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TITLE OF THESIS EXPERIMENTAL EVALUATION OF ANTISNAKE VENOM ACTIVITY OF INDIAN MEDICINAL PLANTS

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INDIA.
Snakebite is a serious health problem worldwide (Russells et al, 1975; Sawai 1992) and specially in the tropics (Swaroop and Grab, 1954). Approximately 15,000-25,000 people die in the Indian subcontinent alone (Porges, 1953; Hati, 1979). The major venomous snakes found in India are the Indian Cobra (Naja naja sp), Russell’s viper (Vipera russellii), Saw scaled viper (Echis carinatus), Common krait (Bungarus caeruleus) and Banded Krait (Bungarus fasciatus).

Snake envenomation causes immediate burning sensation and pain at the site of bite followed by numbness. There is local pain with radiation and tenderness followed by edema. Due to increase in capillary permeability there may be loss of blood and plasma volume into the extravascular space. This accumulation of fluid in the interstitial space is responsible for edema. Tingling and numbness over the tongue, mouth and scalp and paraesthesias around the wound occur mostly in viper bites (Reddy, 1980). Regional lymphadenopathy has also been reported (Trevett et al, 1994). Secondary infection including tetanus and gas gangrene may also result (Sathyanathan, 1993). In case of neurotoxic venoms like cobra envenomation, there is selective d-tubocurarine like neuro-muscular blockade, which results in flaccid paralysis of muscles (Paul, 1993) though cobra venom is 15-40 times more potent than tubocurarine. Ptosis is the earliest (Philip, 1994) neuroparalytic manifestation followed closely by ophthalmoplegia. Paralysis might gradually progress involving muscles of palate, jaw, tongue, larynx, neck and muscles of deglutition (Paul, 1993). Generally muscles innervated by cranial nerves are affected earlier. Muscles of chest are involved relatively late with diaphragm being the most resistant. This accounts for the respiratory paralysis, which is often terminal. In case of Vipera russelli which has procoagulant activating factors V and X, might cause defibrinogenation by activating endogenous fibrinolytic system (Budzynski, 1984 and Kitchens, 1983). Besides direct effects on the coagulation cascade, venoms also can cause defects in platelet function (Mirtschin, 1991). Bleeding may occur from multiple sites including gums (Warrell, 1996). Death may occur due to cerebral haemorrhage in some cases (Mirtschin, 1991).

In 1894, Albert Calmette developed the antiserum, the only specific treatment available for snake envenomation, till date. The antiserum is given
i.v. drip in normal saline. Antiserum though considered the most viable antidote has numerous drawbacks. It does not provide enough protection against venom induced haemorrhage, necrosis, nephrotoxicity and often develops hypersensitivity reactions (Sutherland, 1977; Corrigan et al., 1978; Stahel et al., 1985). Antiserum development in animals is very time consuming and it requires ideal storage conditions which are not necessarily met with. The greatest disadvantage in a developing country like India is the high cost of the antiserum specially for the rural people.

To overcome these drawbacks, studies have been conducted to find an alternative treatment. One of the most important resource of India is its medicinal plants. Many Indian medicinal plants are mentioned in the literature, which are used to treat snakebite victims specially in rural areas (Chopra et al., 1956; Biswas and Ghosh, 1977). Very few scientific reports on the active constituents from plants are there, which inhibit snake venom action (Nazimuddin, 1978; Sudarsanan et al, 1975; Leung 1980; Morton 1981; Duke 1985; Mors 1991; Martz, 1992) From this laboratory, an active compound 2-hydroxy-4-methoxy benzoic acid was isolated from root extract of *Hemidesmus indicus* R.Br, which effectively neutralized *Vipera russellii* venom induced pathophysiological changes (Alam et al., 1994).

The present investigation aimed to study the antisnake venom properties of a few selected Indian medicinal plants. Eighteen medicinal plants were selected on the basis of literature survey and geographic availability of these plants in this region (Indian subcontinent). These plants were studied against snake venom (viper and cobra) induced lethal action in experimental animals. Venom neutralization studies were carried out against viper venom and cobra venom only. Out of the eighteen plant materials, ten were found to effectively neutralize venom induced lethal action. The observation clearly validates the claims of traditional literature and medicine men, that Nature has provided us with the antidote of snake venom in the form of herbs. There are very few scientific evidence of these claims and the present investigation aimed at providing information on selected Indian medicinal plants having antisnake venom activity.

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The first step was to do an exhaustive search for selecting the medicinal plants. It was carried out through literature survey and local tours to snake infested districts of West Bengal, like Midnapur, 24 Parganas, Bankura, Birbhum and Hooghly. Information was gathered from the local healers, ojhas and villagers and plants in bulk were collected from them. These were then sent to a team of taxonomists, Prof. N. Paria, University of Calcutta and Dr. A. Mukherjee, Burdwan University, India for identification of the collected plant sample. The selected plants were purchased from M/s United chemicals and Allied Products, Calcutta, India. The plant materials were stabilized by drying the material at ambient temperature in a shady place. The dried plant materials were then powdered and taken for extract preparation. Extract preparation was carried out in Soxhlet apparatus by passing petroleum-ether for defatting followed by a high polar solvent (methanol/ethanol). The extracts were dried and stored in vacuo at room temperature till further use.

The dried plant extracts were dissolved in 0.9% saline and tested for snake venom neutralization in experimental animals. Solubility of each extract were noted. The plant extracts were highly soluble in water and methanol and less soluble in chloroform. The yield of the extracts obtained, varied from plant to plant (4-8%). It was expressed in terms of dry weight. The toxicity of the plant extracts were tested and snake venom neutralization studies were carried out with their non toxic dose. Out of the eighteen plant materials selected for testing, ten plants were found to be active in neutralizing snake venom induced lethal action in experimental animals. They are:

- Seed extract of *Croton tiglium* Linn, *Dolichandrone spathacea* (L.f) k. Schum and *Strychnos nux vomica* Linn.
- Aerial parts of *Andrographis paniculata* and
- Flower extract of *Crotalaria juncea*.

In the present investigation, out of the ten plant materials, three of them, the root extracts of *Pluchea indica*, *Hemidesmus indicus* and seed of *Strychnos*...
*nux vomica* were found to possess highest snake venom neutralizing capacity against viper and cobra venom induced lethal action. These three extracts were therefore taken for a detailed study.

**Studies with *Pluchea indica* root extract**

*Pluchea indica* is a very common shrub found in the eastern region of India. Visit to the snake infested areas in West Bengal, India revealed that the villagers plant *Pluchea indica* around their huts to keep the snakes away. Local healers and medicine men also prescribe the plant root to treat snake bite. Methanolic root extract of *Pluchea indica* was taken for snake venom neutralization studies. In the present study, the methanolic root extract of *Pluchea indica* was found to induce a fall in body temperature, prolong pentobarbitone induced sleeping time as well as reduce mice paw edema. In the venom neutralization studies, the methanolic root extract was found to neutralize viper venom induced lethal, haemorrhagic and defibrinogenating action and cobra venom induced lethal, cardiotoxic and neurotoxic activity. This observation suggested that methanolic root extract of *Pluchea indica* was an extremely potent antagonist against both viper and cobra venom.

Search for the active constituent responsible for snake venom neutralization was undertaken. The methanolic root extract was fractionated over silica gel chromatography. The fractions were eluted using solvent systems petroleum-ether, chloroform and methanol in increasing proportions. Pure compound was eluted by petroleum-ether: chloroform (40 : 60, v/v) and it was designated as PINF1. Another active fraction was eluted by chloroform. This fraction was further rechromatographed and pure compound was eluted by petroleum-ether: chloroform 70 : 30, v/v). The homogeneity of the compounds were tested by thin layer chromatography. Crystallization of the pure compounds were carried out. PINF1 was found to be soluble in alcohol/methanol but insoluble in water. Spectral studies of PINF1 reveal that it is a mixture of straight chain compounds and PINF2 reveal that it is a mixture of β-sitosterol and stigmasterol. Both PINF1 and PINF2 were taken for a detailed venom neutralization study, mechanism of venom inhibition and antiserum action potentiation study in experimental animals.
Spectral study of PINF1 indicated that it was a mixture of triterpenoids. Due to the very low yield and also difficulty in fractionating the compound into a single compound, further structure analysis could not be undertaken. Snake venom neutralization studies were carried out with PINF1. It was found that PINF1 did not produce any lethal effect up to a dose of 25mg/kg body weight. There was no change in the body temperature and pentobarbital induced sleeping time in mice, indicating it to be a safe and neutral compound. The venom neutralization studies showed that PINF1 was active against viper venom induced lethal (5 LD₅₀), haemorrhagic (5 MHD), defibrinogenating (2 MDD), edema (2 MED) and PLA₂ (4 units) enzyme activity. It was also active against cobra venom induced lethal (4.8 LD₅₀), cardiotoxicity (2 MCTD), neurotoxicity (1 MNTD) and PLA₂ (6 units) enzyme activity in experimental models, suggesting PINF1 as a potential snake venom antidote. The second active compound PINF2 was also similarly taken for detailed study. PINF2 was found to significantly decrease body temperature of male albino mice. Snake venom neutralization studies were carried out in experimental animals against both viper and cobra venom induced action. PINF2 was found to effectively neutralize viper venom induced lethal (6.2 LD₅₀) haemorrhagic (5 MHD), defibrinogenating (1 MDD), edema (3 MED) and PLA₂ (6 units) enzyme activity and cobra venom induced lethal (4.8 LD₅₀), cardiotoxicity (3 MCTD) and neurotoxicity (2 MNTD), respiration (2 MAD) and PLA₂ (8 units) enzyme activity, studied in experimental models. Studies of the mechanism of venom inhibition showed that both PINF1 and PINF2 prevented snake venom induced activity by inhibiting changes in serum phosphatase and transaminase levels. They also inhibited changes in superoxide dismutase and lipid peroxidation due to snake venom envenomation, in experimental animals.

**Studies with *Hemidesmus indicus* root extract**

Literature study and visit to local villages suggested that this plant root was extensively used for snake bite treatment. Earlier, a pure compound 2-hydroxy 4-methoxy benzoic acid was isolated and purified from the root extract of *Hemidesmus indicus* which effectively neutralized viper venom induced action (Alam *et al.*, 1994). Methanolic root extract of *Hemidesmus*
Indicus was taken for snake venom neutralization studies. The methanolic root extract was highly soluble in water and less soluble in chloroform. The extract was found to be non lethal upto 1.0 gm/kg of experimental mice. The non lethal dose was taken for all the snake venom neutralizing study in experimental animals. The methanolic root extract was found to significantly neutralize viper venom induced lethal (16.6 LD_{50}), haemorrhagic (10 MHD) and defibrinogenating (5 MDD) action and cobra venom induced lethal (4.8 LD_{50}), cardiotoxic (2 MCTD) and neurotoxic (2 MNTD) action. The root extract was therefore taken for isolation and purification of active constituents, which can neutralize snake venom induced action in experimental animals.

The methanolic root extract was fractionated by column chromatography. Each fraction eluted was dried and tested for venom neutralization in male albino mice. One fraction eluted with chloroform: methanol 95:05, v/v was found to possess antisnake venom capacity. TLC pattern showed that it was not a single compound. Therefore further purification was carried out by rechromatography. Two active constituents were isolated. The first compound was obtained by eluting with chloroform: methanol 95.5:0.05, v/v. TLC showed that it had a single spot. Spectral analysis of the compound indicated the compound is a mixture of triterpenoids. Detailed structure analysis was not carried out. It was therefore taken for neutralization studies in the form of a mixture. The fraction was designated as HINF1.

HINF1 was found to antagonize both viper and cobra venom induced action in both in vitro and in vivo studies in experimental animals. HINF1 did not produce any change in body temperature and pentobarbitone induced sleeping time in male albino mice. HINF1 significantly neutralized *Vipera russellii* induced lethal action (2.08 LD_{50}), haemorrhagic (2.5 MHD), defibrinogenating (1 MDD), edema (1 MED) and PLA_{2} (6 units) activity in male albino mice. HINF1 also significantly neutralized *Naja kaouthia* induced lethal action (3.2 LD_{50}) in male albino mice and cardiotoxicity (1 MCTD). It did not neutralize venom induced neurotoxicity and respiratory and PLA_{2} activity. Mechanism of venom inhibition by HINF1 was partially determined. It was found to neutralize the venom induced free radical formation as evaluated by thiobarbituric acid product (malonyldialdehyde) formation. It also inhibited venom induced SOD changes.
A second active constituent was isolated by rechromatography of the above fraction. It was eluted with chloroform : methanol 50 : 50 v/v. This component was designated as HINF2. TLC pattern indicated that the compound was a pure one. Spectral analysis of the compound confirmed it to be Lupeol acetate. This is the first report of antisnake venom activity of lupeol acetate isolated from plants. HINF2 produced a significant fall in body temperature in male albino mice and also significantly increased pentobarbitone induced sleeping time. HINF2 (lupeol acetate) was found to be a potent snake venom neutralizing factor. Viper and cobra venom induced action was antagonized by HINF2. HINF2 significantly neutralized *Vipera russellii* venom induced lethal (4.5 LD$_{50}$), haemorrhagic (5 MHD), defibrinogenating (2 MDD), edema (3 MED) and PLA$_{2}$ activity (8 units) in male albino mice. HINF2 also neutralized *Naja kaouthia* induced lethal action (3.8 LD$_{50}$), cardiotoxicity (1 MCTD), respiratory activity (1 MAD) and PLA$_{2}$ activity (4 units). Neurotoxicity induced by the venom was not neutralized. Mechanism of venom inhibition was partially evaluated. It was found to be similar to the mechanism of venom inhibition of HINF1.

**Studies with *Strychnos nux vomica* seed extract**

*Strychnos nux vomica* seed is well known for its medicinal value. There has been no earlier report of anti snake venom activity of the seed extract. However local tours and information gathered from local medicine men revealed that *Strychnos nux vomica* seed extract has been used to treat some of the snake bite victims. This investigation, for the first time reports on the antisnake venom activity of *Strychnos nux vomica* seed. Ethanolic seed extract of *Strychnos nux vomica* was prepared. The extract did not produce any lethal effect upto 5mg/kg, i.v. upto 24 hours of observation in male albino mice. The *Strychnos nux vomica* seed extract significantly neutralized *Vipera russellii* induced lethal action (4.1 LD$_{50}$), haemorrhagic action (3 MHD) and defibrinogenating activity (2 MDD) in male albino mice. It also significantly antagonized *Naja kaouthia* venom induced lethality (2.9 LD$_{50}$) in male albino mice, cardiotoxicity (2 MCTD) in isolated guineapig auricle and neurotoxicity (2 MNTD) in isolated rat phrenic nerve diaphragm. The seed extract was taken for isolation and purification of the active constituents responsible for snake venom antagonism.
Ethanolic extract of *Strychnos nux vomica* seed was applied to TLC plates of silica gel (GF<sub>254</sub>). Numerous bands were obtained when the plates were run in the solvent system 0.1(N) HCl : Isopropanol 3 : 7, v/v. Each band were eluted in methanol and tested for antisnake venom activity in male albino mice. The active fraction obtained at R<sub>f</sub> 0.6 was further fractionated on TLC II and each band eluted were again taken for neutralization studies. Active compound was found at R<sub>f</sub> 0.5. The band eluted in methanol was filtered and passed through silica gel column chromatography. The pure fraction was eluted with ethyl acetate : water : methanol 80 : 19 : 1, v/v. TLC pattern revealed a single spot at R<sub>f</sub> 0.4 in benzene : ethyl acetate 80 : 20, v/v solvent system. The active compound was designated as SNVNF. Yield of SNVNF was 0.04%. Structural identification could not be carried out due to less yield. Venom neutralization studies were carried out with SNVNF. The active compound SNVNF, effectively neutralized *Vipera russellii* induced lethal action (3.75 LD<sub>50</sub>), haemorrhagic (2.5 MHD), defibrinogenation (1.5 MDD), edema (1MED) and PLA<sub>2</sub> (2 unit) enzyme activity in male albino mice. SNVNF effectively neutralized *Naja kaouthia* induced lethal action (1.94 LD<sub>50</sub>) in male albino mice and cardiotoxicity (1 MCTD) and neurotoxicity (1 MNTD). It did not neutralize venom induced respiratory activity.

**Studies on antiserum action potentiation**

The methanolic root extracts of *Pluchea indica* and *Hemidesmus indicus* and the seed extract of *Strychnos nux vomica* were found to significantly potentiate the action of commercially available snake venom antiserum against both viper and cobra venom induced lethal action in male albino mice. The active constituents isolated from these extracts were then taken for antiserum potentiation studies.

The two compounds isolated and purified from root extract of *Pluchea indica*, PINF1 and PINF2 were taken to study antiserum action potentiation against venom induced lethal action. PINF1 was found to give significant protection upto 5 LD<sub>50</sub> of *Vipera russellii* venom induced lethal action. In antiserum action potentiation study, PINF1 gave significant protection upto 6.6 LD<sub>50</sub> of venom induced lethal action thereby indicating antiserum action.
potentiation of 330% with respect to protection given by antiserum alone. PINF2 also gave significant protection upto 6.2 LD₅₀ of Vipera russellii venom induced lethal action. It gave significant protection upto 10.4 LD₅₀ of venom induced lethal action when administered along with antiserum, thereby indicating antiserum action potentiation of 520% with respect to protection given by antiserum alone. Similarly, PINF1 gave significant protection upto 4.8 LD₅₀ of Naja kaouthia venom induced lethal action. In antiserum action potentiation study, PINF2 gave significant protection upto 6.4 LD₅₀ of venom induced lethal action thereby indicating antiserum action potentiation of 320% with respect to protection given by antiserum alone. Naja kaouthia venom induced lethal action was inhibited upto 4.8 LD₅₀ by PINF2. In antiserum action potentiation study, PINF2 gave significant protection upto 7.1 LD₅₀ of venom induced lethal action thereby indicating antiserum action potentiation of 355% with respect to protection given by antiserum alone.

HINF1 and HINF2 are the two compounds isolated and purified from the root extract of Hemidesmus indicus. These two compounds were also taken for studying antiserum action potentiation against venom induced lethal action in male albino mice. HINF1 gave significant protection upto 2.08 LD₅₀ of Vipera russellii venom induced lethal action. In antiserum action potentiation study, HINF1 gave significant protection upto 4.1 LD₅₀ of venom induced lethal action thereby indicating antiserum action potentiation of 205% with respect to protection given by antiserum alone. Similarly, HINF2 was found to give significant protection upto 4.5 LD₅₀ of Vipera russellii venom induced lethal action. In antiserum action potentiation study, it gave significant protection upto 8.3 LD₅₀ of venom induced lethal action thereby indicating antiserum action potentiation of 415% with respect to protection given by antiserum alone. HINF1 was found to give significant protection upto 3.2 LD₅₀ of Naja kaouthia venom induced lethal action. It gave significant protection upto 3.8 LD₅₀ of the same venom induced lethal action thereby indicating antiserum action potentiation of 190% with respect to protection given by antiserum alone. Similarly, HINF2 gave significant protection upto 3.8 LD₅₀ of Naja kaouthia venom induced lethal action.
action. In antiserum action potentiation study, it gave significant protection upto 4.8 \( LD_{50} \) of venom induced lethal action thereby indicating antiserum action potentiation of 240\% with respect to protection given by antiserum alone.

An active constituent SNVNF was isolated and purified from the seed extract of \textit{Strychnos nux vomica}. SNVNF was taken for studying antiserum action potentiation in male albino mice. SNVNF was found to give significant protection upto 3.75 \( LD_{50} \) of \textit{Vipera russellii} venom induced lethal action. In antiserum action potentiation study, significant protection upto 5 \( LD_{50} \) of venom induced lethal action was found, thereby indicating antiserum action potentiation of 250\% with respect to protection given by antiserum alone. Similarly, SNVNF antagonized upto 1.94 \( LD_{50} \) of \textit{Naja kaouthia} venom induced lethal action. SNVNF gave significant protection upto 3.8 \( LD_{50} \) of venom induced lethal action when given along with antiserum. This observation indicated antiserum action potentiation of 190\% with respect to protection given by antiserum alone.

\textbf{The major findings of the present investigation}

- This study validates the claim of medicine men and traditional literature that certain Indian medicinal plants, can neutralize cobra and viper venom induced activities in experimental animals. Ten Indian medicinal plants have been identified to possess antisenake venom activity. Three plants with highest neutralizing capacity were selected for further studies.

- Active constituents have been isolated and purified and structure of the compound elucidated from three plant materials. These three plants are \textit{Pluchea indica} root, \textit{Hemidesmus indicus} root and seeds of \textit{Strychnos nux vomica}.

- Two active compounds have been isolated and purified from the root extract of \textit{Pluchea indica}. One was identified as a mixture of \( \beta \) sitosterol and stigmasterol and the other as a mixture of long chain compounds. From the root extract of \textit{Hemidesmus indicus}, two compounds were isolated and purified. One of them had been identified as Lupeol acetate. The other compound is a mixture of triterpenoids.
The pure compounds isolated and purified from the three plants mentioned above were found to significantly neutralize both viper and cobra venom induced action in experimental animals.

The mechanism of venom inhibition was partially studied. The active constituents were found to significantly inhibit venom induced free radical formation and changes in serum transaminase and phosphatase levels.

The pure compounds were found to significantly potentiate antiserum action against venom induced lethal action in experimental animals.

Conclusion

The present investigation confirms that there are Indian medicinal plants which possess snake venom neutralizing capacity. Active constituents isolated from three plants *Pluchea indica*, *Hemidesmus indicus* and *Strychnos nux vomica* were capable of antagonizing snake venom induced action both in the presence and absence of snake venom antiserum. Further studies in the direction of finding new active compounds and their detail study is warranted before clinical trials. The informations from this study may open newer concepts in the management of snakebite victims at least in the rural areas of our country.