



## STUDIES ON EFFICACY OF MEDICINAL PLANTS AGAINST THE LETHALITY OF *NAJA NAJA* SNAKE ENVENOMATION

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### ABSTRACT

Chloroform and aqueous extracts of the selected medicinal plant were evaluated for qualitative phytochemical analysis and inhibiting property of (toxic) lethal factors like PLA<sub>2</sub>, coagulant activity, proteolytic activity and acetylcholine esterase activity of venom. Many of the extracts under this study exhibited significant inhibitory properties of venom components. (Aqueous and chloroform extracts of *Jatropha gossypifolia* effectively inhibited the PLA<sub>2</sub> activity of *Naja naja* up to 87 and 85%). Proteolytic activity induced by venom of *Naja naja* was inhibited up to 63% and 57 % by the aqueous extracts of *Antidesma buniis* and *Sida acuta*. Serum coagulation of the venom was inhibited by *Commelina bengalensis*, *Antidesma buniis* and many others while Inhibition of acetylcholine esterase was exhibited by chloroform extracts of *Antidesma buniis* (67%) and aqueous extract of *Jatropha gossypifolia* (72%) Some of the plants showed promising antivenom potential by inhibiting various *in vitro* activities of venom. The extracts of *Antidesma buniis* and *Calyopteris floribunda* showed good inhibition of venom pathological activities. Further investigation on the identification and characterization of the compounds responsible for the antivenom properties has to be done which can revolutionize the treatment of snake bite.

**Key words:** *Naja naja*, Phospholipase A<sub>2</sub>, Caseinolytic assay, anticoagulant activity

### INTRODUCTION

Snake bite is considered as major occupational problem causing both disabilities and mortalities. Snakes belong to ophidians, a class of cold blooded vertebrates of which a few produce poisonous venom - a modified form of saliva, produced by Jacobson's glands situated above the upper jawbone and below the eyes<sup>1</sup>. The venom is helpful in capturing prey and defense mechanism. This venom paralyses the victim by different mechanisms ultimately leading to death.

Snake venom is most complex mixture of protein and non protein molecules. The non-protein components of venom are very few like anticonvulsant non-protein cardiotoxin<sup>2</sup>. Of the 25 enzymes found in the proteinaceous mixture of the venoms 6 to 12 occur in all venoms<sup>3</sup>. They include phospholipase A<sub>2</sub>, L-amino acid oxidase, phosphodiesterase, proteases including serine and metalloproteinases, 5'-nucleotidase, phosphomonoesterase, deoxyribonuclease, ribonuclease, adenosinetriphosphatase, hyaluronidase, NAD-nucleosidase, arylamidase, phosphatases (acid as well as alkaline), esterases, acetylcholinesterase and transaminase<sup>4</sup>.

The high mortality of people prone to snake bite are from rural and tribal areas because of insufficient medication. The first antivenom was developed by Albert Calmette (from immunized horse) i.e. against *Naja naja* from the Pasteur institute in 1895. Till date this is only method available to develop Anti snake venom which faces some limitations like anaphylactic shocks, immediate hypersensitive reactions, require ideal storage conditions and it sometimes fails to stop the local damage like necrosis of tissue<sup>5</sup>.

Because of developing adverse effects by serum treatment, men turned to ethnopharmacognosy. Although there are several hundreds of plants reported with antivenom activity in folks and ethno medicine, only a few were investigated both phytochemically and pharmacologically,

*Naja naja* snake of *elapidae* family poses a serious threat and dangerous public health problem in tropical and subtropical countries like India<sup>6</sup>. *Naja naja* (Indian cobra) is associated with high mortality of victims in India as it produces systemic poisoning by rapid action of neurotoxin causing respiratory paralysis and death<sup>7,8,9</sup>.

Knowels was the first scientist to investigate on treating the snake envenomation with the plants extracts reported in traditional medicine but failed to report their efficacy either due to sub lethal dose or non-lethal dose of venom. Later Mhaskar and Caius screened 314 plants and 184 combinations against venom lethality.

Natural products are integral part of the ancient medicine systems in Chinese, Ayurvedic and Egyptian<sup>10</sup>. Over the years they have assumed a very central stage in modern civilization as natural source of chemotherapy as well as amongst scientist in search for alternative sources of drugs.

Indian Subcontinent has a wide collection of medicinal plants helpful in the treatment of snake envenomation. But lack of scientific evidence and evaluation we are not able to use those medicinal properties. In present study we studied characterization of medicinal properties of plants in specifically the anti-edema, and anti-hemolytic activity was carried out against the Snake venom of *Naja naja*. The plants are selected on literature basis of traditional medicine and tribal medicine

articles and antivenom properties are studied on different solvent extracts of the plant.

## MATERIALS AND METHODS

**Snake venom:** Lyophilized form of *Naja naja* snake venom is purchased from Irula snake park, Chennai, and was stored at -20°C. The venom was dissolved in saline just before use.

**Plant selection & extract preparation:** Plants are selected based on the reports of traditional medicine. Plants were identified with the help of Taxonomist P.Sathyanarayana Raju of Acharya Nagarjuna University, Guntur, Andhrapradesh, India. Leaf material was collected from the plant, shade dried and ground to fine powder packed in soxhlet and compounds are extracted by using chloroform and water of varying polarity. The extract was concentrated through rotary evaporator and the obtained powder was stored at 4°C.

### Phytochemical analysis

Qualitative phytochemical screening of the phytochemicals present in the chloroform and aqueous extracts of the selected plants were carried by according to Harborne, 1973<sup>11</sup>; Trease and Evans 1983<sup>12</sup>. Different classes of phytochemicals like alkaloids, phenolic compounds, flavonoids, saponins, tannins, were identified using standard procedures

### Phospholipase A<sub>2</sub> activity

The MHD minimum hemolytic dose of the venom is calculated by incubating venom with different doses of plant extract and the quantity of venom which is able to lyse 70 % of RBC is considered as MHD. First the 200µg of the plant crude extract was incubated with 50µg of venom i.e. MHD for 30 min at 37°C. Assay was carried out by indirect hemolytic assay<sup>13,14</sup> by preparing fresh red blood cells in PBS buffer. To this 1% of egg albumin was added and erythrocyte-egg yolk suspension was made. Later the prepared erythrocyte- egg yolk mixture is added as substrate to venom-plant extract mixture and incubated for 30min at 37°C. The reaction is stopped by adding double the volume of ice cold PBS buffer and centrifuged at 7000rpm for 10 min. PLA<sub>2</sub> activity is determined by calculating the hemolysis spectrophotometrically at 540nm. The results are mean ±SD of three experiments

$$\% \text{ inhibition of PLA}_2 \text{ activity by plant extracts} = 100 - 100 \times \frac{\text{OD of test sample at 540 nm}}{\text{OD of control sample at 540 nm}}$$

### Caseinolytic assay

Proteolytic activity was determined by calculating the digestion of casein according to Laing<sup>15</sup>etal. A solution of 1% of casein

was dissolved in 0.02M Tris-HCL buffer of pH 8.0 containing 150mM NaCl which was used as substrate. 200 µg of the venom was incubated with 200 µg of the different plant extracts for 30 min at 37°C. Later incubated with the substrate for 2hr at 37°C. Reaction was stopped by adding 0.5 ml of 10%TCA. The digested protein in the supernatant (1 ml) was determined using Folin Ciocalteu's reagent. One unit of activity is defined as the amount of enzyme required to cause an increase in O.D by 0.01 at 660 nm/min.

### Procoagulant activity

The minimum coagulant dose is defined as the amount venom required to coagulate the citrated blood plasma in 60 sec. For *Naja naja* the MCD was calculated by incubating the citrated blood plasma with varying concentrations of venom and the minimum concentration able to coagulate the blood plasma was recorded. was assayed according to the method described by akston and Reid<sup>16</sup>. Various amounts of venom dissolved in 100µl PBS (pH 7.2) was added to human citrated plasma at 37°C. Coagulation time was recorded and the minimum coagulant dose (MCD) was determined as the venom dose, which induced clotting of plasma within 60 seconds. Plasma incubated with PBS alone served as control. In neutralization assays constant amount of venom (MCD) was mixed with various dilutions of plant extracts. The mixtures were incubated for 30 minutes at 37°C. Then 0.1ml of mixture was added to 0.3ml of citrated plasma and the clotting times recorded. In control tubes plasma was incubated with either venom alone or plant extracts alone. The extract is defined as effective if it is able to inhibit the clotting time for 6 minutes when incubated with minimum clotting dose.

### Acetylcholinesterase activity

Inhibition of Acetyl cholinesterase in the venom was carried out according to the modified method of Ellman<sup>etal.</sup>,<sup>17</sup> 200 µg of venom (1 mg/ml) was preincubated (1 h) with 800µg of the extract and supernatant was added to the assay mixture which consists of 100 µl of 75 mM acetylcholine iodide in 1 ml of phosphate buffer. The activity was measured by taking the absorbance at 412 nm. Venom without plant extract was considered as control or 100% activity.

## RESULTS

Successive extraction of the phytochemicals was carried out by chloroform and water as solvents. Extraction with chloroform yielded green colored extract while with that of water yielded dark brown colored dried compound. The concentrated and dried extracts were subjected to qualitative phytochemical analysis to investigate the various classes of phytochemicals present in it and the results were shown in the table 1 & 2 below

**Table 1: Qualitative phytochemical analysis of chloroform extracts**

	Alkaloids	Flavanoids	Phenols	Tannins	Terpenoids	Saponins
<i>Antidesma bunius</i>	+	—	—	+	—	—
<i>Bixa oreallana</i>	—	+	—	—	+	—
<i>Biophytum sensitivum</i>	+	+	+	—	—	+
<i>Calyopteris floribunda</i>	—	—	—	+	—	+
<i>Sida acuta</i>	+	—	+	—	—	—
<i>Boerhavia diffusa</i>	—	+	—	—	+	—
<i>Solidago virgaurea</i>	—	—	—	—	—	+
<i>Commelina benghalensis</i>	+	+	—	+	—	—
<i>Jatropha gossypifolia</i>	+	+	+	+	+	+

Table 2: Qualitative phytochemical analysis of aqueous extracts

	Alkaloids	Flavanoids	Phenols	Tannins	Terpenoids	Saponins
<i>Antidesma bunius</i>	+	—	+	+	+	+
<i>Bixa oreallana</i>	—	+	+	—	+	—
<i>Biophytum sensitivum</i>	+	+	+	—	+	+
<i>Calyopteris floribunda</i>	—	—	—	+	—	+
<i>Sida acuta</i>	+	—	+	—	+	—
<i>Boerhavia diffusa</i>	—	+	—	+	+	—
<i>Solidago virgaurea</i>	+	—	+	—	+	+
<i>Commelina benghalensis</i>	+	—	—	+	—	—
<i>Jatropha gossypifolia</i>	—	+	—	+	+	+

**Inhibition of PLA<sub>2</sub> activity**

The hemolytic assay clearly demonstrates that the venom of *Naja naja* have PLA<sub>2</sub> dependent hemolysis. Inhibition of PLA<sub>2</sub> was assessed by observing the rate of hemolysis occurred during the Incubation period. Overall inhibition of PLA<sub>2</sub> activity

is well noted in the aqueous extracts than the chloroform extracts of the respective plants. The aqueous extract and chloroform extracts of *Jatropha gossypifolia* showed 87.5% and 85.9% of PLA<sub>2</sub> inhibition followed by aqueous extract of *Commelina benghalensis* (59%).

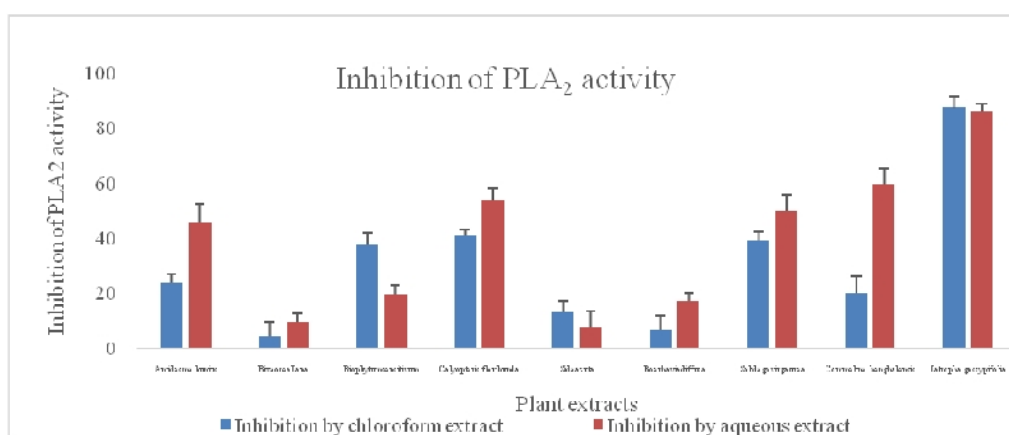


Figure 1

**Caseinolytic assay**

The inhibition of venom proteolysis was well observed with the aqueous extracts of *Antidesma bunius* and *Sida acuta* around 63% and 57%, Chloroform extracts were not efficient in inhibiting the acetylcholine esterase activity.

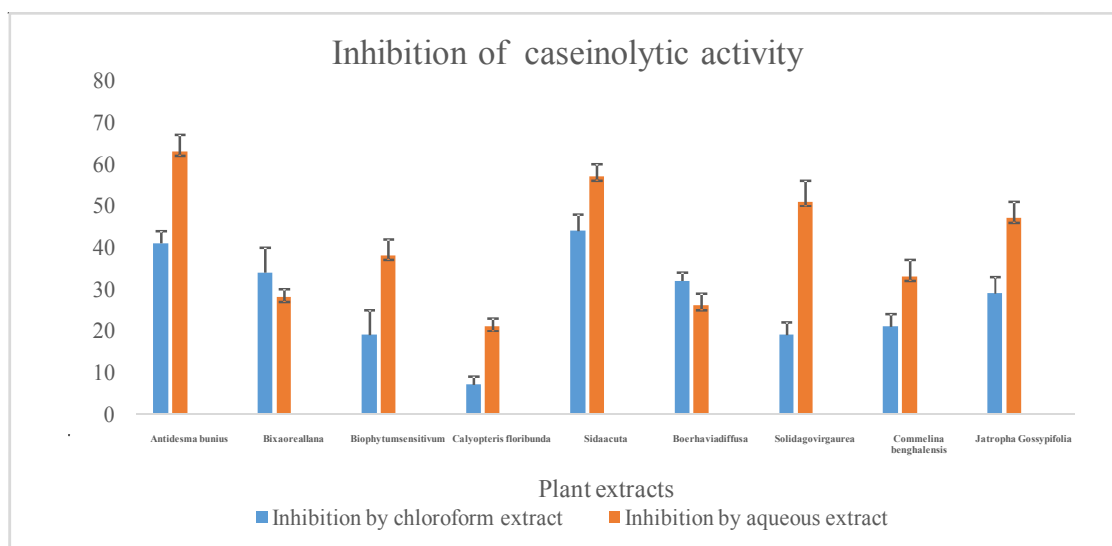


Figure 2

### Procoagulant assay

By incubating different concentrations of venom with human citrated plasma 28µg of the venom is recorded as the (MCD). After incubating the MCD with 800µg of different plant extracts separately chloroform extracts of *Calopterys floribunda*, *Antidesma buniis* and aqueous extracts of *Commelina benghalensis*, *Antidesma buniis*, *Biophytum sensitivum*, *Boerhavia diffusa* showed inhibition of the coagulation even

after 60 sec of the addition of the venom incubated with the plant extract.

### Acetylcholinesterase activity

Results of the work evidenced that chloroform extract of *Antidesma buniis* showed maximum inhibition (67%) of acetylcholine esterase of *Naja naja* and *Bixa oreallana* showed least inhibition. Among the aqueous extracts *Jatropha gossypifolia* showed high inhibition of 72%.

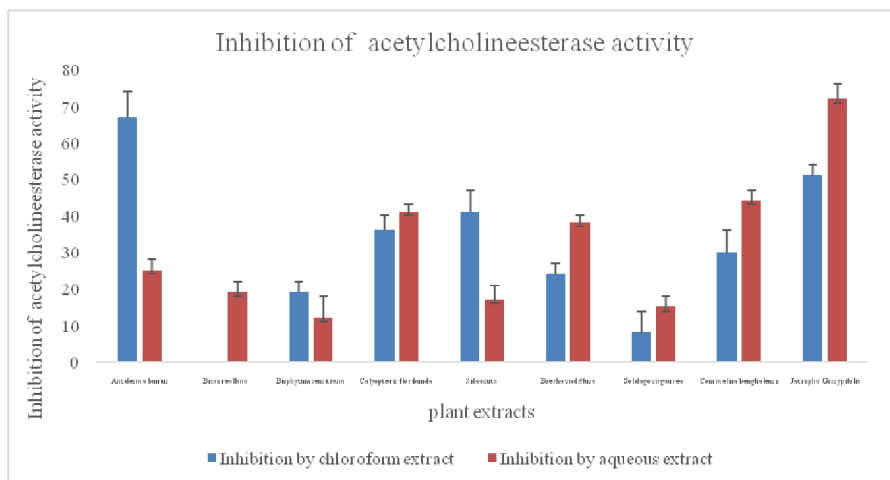


Figure 3

### DISCUSSION

Snake bite is frequent medical emergency encountered in many rural areas of India. Venom is a mixture of proteins, lipids, steroids, amino polysaccharides, amines and other compounds which damage various functional systems of the body at the same time. The management of snake bite is a critical problem due to scarcity of Antivenom and adverse reactions ranging from pruritus, urticaria to potential fatal anaphylaxis shocks that develop during treatment<sup>18</sup>. The commercially available polyvalent antivenom neutralizes the systemic effects of venom lethality but fails to nullify the local tissue damage like necrosis which results in permanent sequelae<sup>19</sup>. Development of therapeutic agents which can solve the above issues could have significant social and economic impact on developing countries

The Cobra bite envenomation are generally characterized by neurotoxicity leading to flaccid paralysis, hypertension and death by respiratory failure<sup>20</sup>.

Alam and Gomes suggested the administration of herbs and commercial polyvalent antiserum can give the better protection for snake bite<sup>21</sup>. Here we evaluated the efficacy of various plants reported in Folks and traditional medicine. The secondary metabolites like alkaloids, flavanoids, lipids of plants are responsible for antivenom property<sup>22, 23</sup>. Preliminary Phytochemical analysis revealed the presence of alkaloids, flavanoids, and phenols among the chloroform extracts of various plants; alkaloids flavanoids, phenols, tannins, terpenoids and saponins in the aqueous plant extracts.

*Elapid* venoms have higher concentrations of acetylcholine esterase that exerts effect on nervous system<sup>24</sup>, proteolytic enzymes which play key role in digestive reactions,

Phospholipase A and B that degrade lipids to free fatty acids causing lysis and apoptosis of the cell. PLA<sub>2</sub> is important lethal factor of venom with various pharmacological activities like neurotoxicity, hemolytic activity, myotoxicity, anti-coagulant and antiplatelet activities<sup>25,26</sup>

Local necrosis is frequently seen in cobra bite victims which is difficult to treat. Neurotoxins, Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and cardiotoxins are the major group of enzymes involved in toxicity and pharmacology of cobra bite<sup>27,28</sup>. Phospholipases A<sub>2</sub> class of enzymes are wide spread in snake venom playing major role in immobilization and killing of prey/ victim<sup>29,30</sup>

So present work is focused on identification of medicinal plants inhibiting various pharmacological activities like acetylcholine esterase, PLA<sub>2</sub> proteolytic enzymes and coagulant factors of *Naja naja*.

The PLA<sub>2</sub> enzyme in the snake venom converts lecithin in egg yolk to lysolecithin which in turn causes the lysis of the RBC. Inhibition of PLA<sub>2</sub> by *Jatropha gossypifolia* and *Antidesma buniis* can help in reducing the necrosis of local tissue which otherwise can be irreversible damaged because commercially available polyvalent antiserum is ineffective in nullifying the local tissue damage. Meenatcisundaram and Sindhu<sup>31</sup> demonstrated that root extracts of *Acorus calamus* and *Withania somnifera* were capable of inhibiting PLA<sub>2</sub>-dependent hemolysis induced by *Echiscarinatus* venom in a dose-dependent manner. Blood cell lysis by phospholipase A<sub>2</sub> was clearly inhibited by the extracts of *Jatropha gossypifolia* (89%). The factors responsible for PLA<sub>2</sub> inhibition can act as potent factors in treating snake envenomation. Proteolysis enzyme inhibition by *Antidesma buniis* and *Calopterys floribunda* was observed.

The coagulant factors in the venom of *Naja naja* were inhibited by the chloroform extracts of *Calyopteris floribunda*, *Antidesma buniis* and aqueous extracts of *Commelina bengalensis*, *Biophytum sensitivum* where no coagulation of citrated blood plasma is observed in 6 minutes after the addition of venom incubated with the extract.

Acetyl choline esterase is important neurotoxic factor of *Naja naja*. The inhibition of acetyl choline esterase by *Antidesma buniis* and *Jatropha gossypifolia* was seen. The data obtained from the Invitro studies demonstrate the efficacy vegetal extracts from the selected of the plants in treating snake envenomation of *Naja naja*. Significant protection was observed with the extracts of *Jatropha gossypifolia*, *Biophytum sensitivum*, *Calyptras floribunda* and *Antidesma buniis*. The Inhibitory Property and efficacy of these plants should be further confirmed by isolation and Purification of bioactive principle and *INVIVO* studies using animal model.

The presence of compounds responsible for PLA<sub>2</sub>inhibition, coagulation inhibition, acetyl choline esterase and proteolytic inhibition gives us the chance to search for natural therapeutic agents for snake envenomation. These findings encourage in identification of the potent herbal antagonists in treating snake envenomation.

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