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Research Article

Anti-Snake Venom Activity of the Leaves and Stem Bark Extract of *Alstonia Venenata* R.Br. By *In Vitro* and *In Vivo* Methods in Swiss Albino Mice

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Abstract: Objective: The main objective of this study is to evaluate the *in vitro* and *in vivo* Anti-snake venom activity in the leaves and stem bark extract of *Alstonia venenata* R.Br. **Method:** Powdered leaves and stem bark materials were extracted with ethanol using soxhlet apparatus. The dried extracts were subjected to preliminary phytochemical analysis and the extracts were evaluated for acute oral toxicity by OECD guideline No 425. By using the MLD of venom the neutralization activity was found out. Acetylcholinesterase activity was performed to determine the percentage of inhibition of acetylcholinesterase by the different dilutions of plant extracts. Phospholipase A_2 activity was measured using indirect hemolytic assay to determine the reduction of hemolytic halo by the plant extract when comparing with the venom. Fibrinolytic activity was performed to determine the reduction of fibrinolytic halo by the plant extract when comparing with that of venom. In the both activity the MIHD and MFC was determined. *In vivo* neutralization activity was performed in mice (n=06). The mice were administered with ethanolic extracts of *Alstonia venenata* R.Br. (200 & 400 mg/kg) through oral 1h prior to administration of 1 to 2 folds of MLD of venom by intraperitoneal route; all the animals were observed for mortality for 24h. **Results:** Preliminary phytochemical examinations showed the presence of Alkaloids, Flavonoids, Tannins, Acids, Triterpenoids, Proteins and Amino acids, Sterols and Carbohydrates. The acute oral toxicity study results showed that the extract was found to be safe up to 2000mg/kg. The ethanolic extract of dried leaves and stem bark of *Alstonia venenata* R.Br. (Family: Apocynaceae) was tested for their protective or Anti-venom activity against *Naja naja* venom induced toxic actions by *in vitro* and *in vivo* methods. The 200 and 400mg/kg of EEAV significantly neutralizes the lethality (up to 2 folds) induced by the *Naja naja* venom in *in vitro* and *in vivo* methods.**Conclusion:** The results obtained from the study indicates that the leaves and stem bark of *Alstonia venenata* R.Br. possess promising Anti-snake venom activity. The activity might be due to the presence of the phytoconstituents including Alkaloids, Flavonoids, Tannins, Protein and Amino acids, Sterols, Triterpenoids and Carbohydrates in the extract. Further studies are required to identify the active principle responsible for the Anti-snake venom activity.
Keywords: Anti-snake venom activity, *Naja naja* venom, *Alstonia venenata* R.Br.

1. INTRODUCTION

Plants, including many now used as culinary herbs and spices, have been used as medicines, not necessarily effectively, from prehistoric times. A medicinal plant is a plant that is used with the intention of maintaining health, to be administered for a specific condition, or both, whether in modern medicine or in traditional medicine. Primitive men observed and appreciated the great diversity of plants available to them. Plants provide food, clothing, shelter and medicine. Much of the traditional use of plants seems to be developed through observations of wild animals, and by area to its knowledge base. They methodically collected information on herbs and developed well defined herbal pharmacopoeias (www.naturopathic.org; www.herbpalace.com).

Snake venom has been the cause of innumerable deaths worldwide. It is a global problem, especially in subtropical and tropical countries like India. It has been estimated that 5 million people are bitten by venomous snakes annually around the world,

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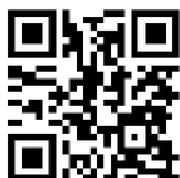

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thereby resulting in about 100,000 fatalities (Chippaux, J. P. 1998; Sharma, S. K). However, these largely hospital based figures are likely to be underestimates, as the majority of snake bite victims seek traditional treatment and may die at home unrecorded. The signs, symptoms and prognosis in poisonous snakebite cases depend upon the species and the type and quantity of venom injected. Bites by venomous snakes exact a great toll in terms of human morbidity and mortality in India. World-wide, most bites are inflicted on the feet and ankles of agricultural workers, hunter gatherers, fishermen and fish farmers and professional handlers of snakes (for food, skins, etc.). Seasonal variations in snakebite incidence reflect rainfall and other climatic cycles but also changes in agricultural activity, such as the paddy harvest in South East Asia. Venomous snakebite without clinically evident envenoming ('dry bites') occurs in about 50% of all venomous snake bites, ranging from 80% in the case of Australian brown snake (Pseudonaja) bites to less than 10% with sawscaled viper (Echis) bites. Case fatality of snake bite before the advent of Anti-venom therapy was reported to be high (more than 50%) in the case of bites by species such as the tropical rattlesnake and Australian elapids (Taipan, death adder, tiger snake). With Anti-venom and modern ancillary treatments it can be reduced to less than 5% (Theakston, R. D. G., & Reid, H. A. 1983).

The immediate treatment of Anti-venom is the best treatment over Snake bites. Horse polyvalent Anti-venom is the only specific treatment for snake venom poisoning. Therapeutic Anti-venom against snake was first produced by Albert Calmette in 1894 (Calmette, A. 1894). Since then Anti-venom have saved the life of countless snake bite victims. Anti-venom are usually hyper immune sera collected from animals which bind and inactivate venom components. Antiserum development in animals is time consuming, expensive and requires ideal storage condition. Anti-venom currently available are not only expensive, but do not effectively neutralize venom induced haemorrhage, myonecrosis and nephrotoxicity. Some of the Anti-venom cause allergic reaction in patients (Grant, J. *et al*., 200; Gutiérrez, J. M. *et al*., 1980; Ferreira, M. L. *et al*., 1992). Plants have reportedly been used locally to treat diverse cases of snakebites but many of the studies lack systematic scientific procedures, which are necessary for the development of an Anti-venom agent from plants.

The methanolic root extracts of *Vitex negundo* and *Embilica officinalis* were tested for Anti-snake venom activity (Alam, M. I., & Gomes, A. 2003). *Hemidesmus indicus* root extracts (Alam, M. I. *et al*., 1996), the butanolic extract of *Eclipta prostrata* plant (Pithayanukul, P. *et al*., 200) were tested for their Anti-venom activity. The root extract of Indian sarsaparilla *Hemidesmus indicus* also neutralized *Naja kaouthia* venom induced lethality, cardiotoxicity, neurotoxicity and respiratory changes in experimental animals (Chatterjee, I. *et al*., 2005). It may be concluded that evidence are now available to establish the scientific background of the traditional use of plants against snakebite. The Anti-snake venom plants contain more than one compound (secondary metabolites) that is responsible for venom neutralization. Thus medicinal plants with Anti-venom activity could be considered as an effective alternative to mammalian antibody production for the treatment of snake bite envenomation.

Alstonia venenata R.Br.(Apocynacea) is the rare species which is a shrub having distribution in Maharashtra, Karnataka and Kerala. In Kerala it is mostly found in Malappuram, Palakkad, Thrissur and Wayanad. *Alstonia venenata* R.Br. is also known as Analivegam in Malayalam, Sinnapalai in Tamil, Vishagni in Sanskrit and Adda sarpa in Kannada. The various extracts of this plant are proved effective for the treatment of Ascaris, blood impurity and cough. They are mainly used as Anthelmintic, Antibacterial, Blood purifier, Stimulant, Aphrodisiac and Tonic. Mainly the leaves and stem bark of *Alstonia venenata* R.Br. are used in for the snake bite (Abdul Malick, V.M., & Hedge, K. 2018; Bhole, R. P., & Bhavsar, V. A. 2017; Vadivelan, R. 2017). The present study was aimed to examine the snake venom neutralization potential of ethanolic extract of leaves and stem bark of *Alstonia venenata* R.Br. to scientifically validate the traditional claim.

2. MATERIALS AND METHODS

2.1 Collection Of Plant Materials

The fresh leaves ad stem bark of *Alstonia venenata* R.Br. were collected from Narikkuni, Kozhikode district and from Kasargod district, Kerala in the month of October 2018 and were authenticated (Specimen No. 148236) by Dr. A.K. Pradeep, Assistant Professor, Department of Botany, University of Calicut, Kerala, India.

2.2 Preparation Of Plant Extracts

The collected leaves and stem bark materials were shade dried and coarsely powdered by using a mechanical grinder and was subjected to successive solvent extraction with ethanol by continuous hot percolation method using Soxhlet apparatus (Abdul Malick, V.M., & Hedge, K. 2018). The crude samples were subjected to qualitative chemical test for the detection of various plant constituents like Alkaloids, Terpenoids, Tannins, Glycosides, Flavonoids, Saponins, Amino acids, Carbohydrates and Phenolic compounds (Kokate, C.K. 2005).

2.3 Experimental Animals

Albino mice of Swiss strain (25 to 30g), 8 to 12 weeks old were used for the experiments. Animals were housed individually in polypropylene cages, maintained under standard conditions (12h light and

12h dark cycle;25±3^{0}C). The animals were fed with standard pellet diet manufactured by Amrut laboratory, Animal Feed company, Sangli, Maharashtra and water *ad libitum*. The study was approved by Institutional Animal Ethics Committee (IAEC): Reference No: DAMCOP/IAEC/052.

2.4 Venom

The *Naja naja* venom was obtained from Snake park (order no. S4/2900/2017/AVC), Department of Agadathantra of Govt. Ayurveda college, Thiruvanathapuram, Kerala, India and was preserved in a refrigerator at 4^{0}C. Before use the venom was dissolved in phosphate buffer solution.

2.5 Polyvalent Snake Anti-Sera

Snake venom anti-sera I.P manufactured by Vins Bio-products Limited, Telengana, and it was purchased from Alfa Medical Distributor, Calicut, Kerala, India

2.6 Acute Oral Toxicity Study

The acute oral toxicity study was carried out as per OECD 425 guideline. The limit test for acute toxicity was carried out at 2000mg/kg oral dose. Nulliparous and non-pregnant female mice were used for the study and observed continuously for 24h for behavioural, neurological and autonomic profiles and after a period of 24h and 72h, for any lethality, morbidity state or death (Vadivelan, R. 2017).

2.7 IN VITRO STUDY

2.7.1 Acetylcholinesterase Activity

Different dilutions of plant extracts were prepared. Along with these diluted plant extracts, 200µg of venom was added in the concentration of 1 mg/ml. this mixture was incubated for 1 hour at 37^{0}C. On the other hand, assay mixture was prepared (100µl of 75mM acetylcholine iodide in 1ml of phosphate buffer) and the final P^H should be in 6.8. After incubation of the plant-venom mixture, the supernatant was added to the assay mixture and incubated for 15 minutes at room temperature in dark condition. Venom and assay mixture without plant extract was used as the control. The absorbance at 412nm was measured and the acetylcholinesterase inhibition was calculated.

2.7.2 INDIRECT HEMOLYSIS ASSAY

2.7.2.1 Determination Of Mihd

Phospholipase activity was measured using an indirect hemolytic assay on agarose- erythrocyte egg yolk gel plate method. Various doses of venom was added to 3mm wells in agarose gels (0.8% in PBS,P^H 8.1) containing 1.2% sheep erythrocytes, 1.25 egg yolk as source of lecithin and 10mM $CaCl_2$. Plates were incubated overnight at 370^{0}C and the diameters of hemolytic halos were measured. Control wells containing 10µl saline used to compare the results. The minimum indirect hemolytic dose (MIHD) corresponds to the dose of venom that produced a hemolytic halo of 11mm diameter was measured.

2.7.2.2 Neutralization Assay

The neutralization assay was carried out by mixing the MIHD of venom with various amounts of plant extracts and incubating at 370^{0}C for 30 minutes. Then aliquots of 10µl of these mixtures were added to wells in agarose – egg yolk sheep erythrocytes gels. Plates were incubated at 370^{0}C for 20h. Results were compared with the control samples which contained venom without plant extracts.

2.7.3 INHIBITION OF FIBRINOLYTIC ACTIVITY

2.7.3.1 Determination of MFC

The minimum fibrinolytic concentration (MFC) was defined as the concentration of venom that induced a fibrinolytic halo of 10mm diameter.

2.7.3.2 Neutralization Assay

Neutralization assay were performed by incubating the MFC of venom with various amount of plant extracts at 370^{0}C for 1h. After incubation, 10µl of the mixture was applied to wells in the plaque. After 18 hours of incubation at 370^{0}C, the diameters of fibrinolytic halos were measured. Neutralization was expressed as the effective dose (ED) which is the ratio of mg of plant extract to the mg of venom that caused 50% reduction of the hemolytic halo was compared to the effect of venom alone (Mani, M. *et al*., 2018).

2.8 IN VIVO STUDY

Neutralization of lethal effect *in vivo* neutralization activity of *Alstonia venenata* R.Br against lethality induced by *Naja naja* venom.

2.8.1 Neutralization Of Lethal Effect

The plant extracts administered to animals 1h before challenging with MLD (1-3 folds) dose of venom to animals. *In-vivo* neutralization was performed in 5 groups (n=4) of mice. The mice were administered with ethanolic extracts of *Alstonia venenata* R.Br. (200mg/kg and,400mg/kg) through oral 1h prior to administration of 1-3 folds of MLD of venom by intraperitoneal route. All the animals were observed for mortality for 24h (Mukherjee, A. K., & Maity, C. R.2002; Mukherjee, A. K. *et al*., 2000; Theakston, R. D. G., & Reid, H. A. 1983; Calmette, A. 1894).

2.9 Statistical Analysis

The data will be expressed as mean ± standard error of the mean (n=6) and are analyzed by one way analysis of variance (ANOVA) using InStat v 2.02 software (GraphPad software Inc, La Jolla, CA. Trial version 6).

3. RESULTS

200g of dried powdered leaves and stem bark mixtures of *Alstonia venenata* R.Br yield 20.25g of

crude drug which was about 10.38%w/w. The phytochemical studies of ethanolic extract of *Alstonia venenata* R.Br. showed the presence of phytochemical constituents such as Flavonoids, Alkaoids, Tannins, Glycosides, Terpenoids, Proteins and Amino acids, Steroids, Saponins, Phenols and Carbohydrates.

3.1 Acute Oral Toxicity Study

Oral administration of ethanolic extract of *Alstonia venenata* R.Br. did not produce any toxicity up to the dose of 2000mg/kg. All the animals were found to be normal. The ethanolic extract of *Alstonia venenata* R.Br... was found to be safe up to the dose of 2000mg/kg.

3.2 *IN VITRO* PHARMACOLOGICAL SCREENING

3.2.1 Acetylcholinesterase Activity

Table.No.1: Inhibition of acetylcholinesterase activity by the leaves and stem bark extract of *Alstonia venenata* R.Br.

S. no	Concentration (µg/ml)	% Inhibition	IC_{50} (µg/ml)
1	100	25.36 ± 0.08	208.8
2	150	36.30 ± 0.11	
3	200	48.56 ± 0.09	
4	250	57.26 ± 0.13	

The percentage inhibitions are expressed as mean ±SEM.

Acetylcholinesterase inhibition

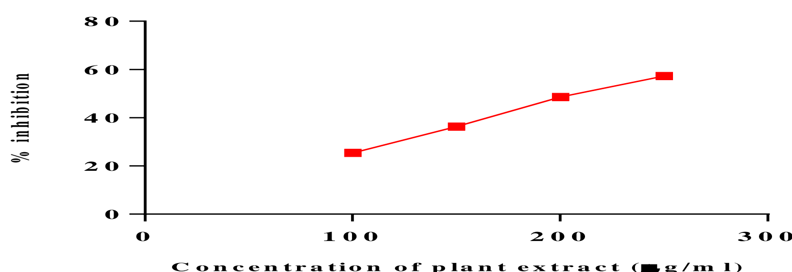

Fig.No. 1: The effect of EEAV on inhibition of acetylcholinesterase activity.

3.2.2 Indirect Hemolysis Assay

TableNo.2: Reduction of hemolytic halo produced by the *Naja naja* venom by ethanolic extract of *Alstonia venenata* R.Br.

S. No	Venom dose that gives 11mm zone	Concentration (µg/ml)	Hemolytic zone produced by plant extract (mm)
1	200µg	100	11
2		150	9
3		200	7
4		250	6

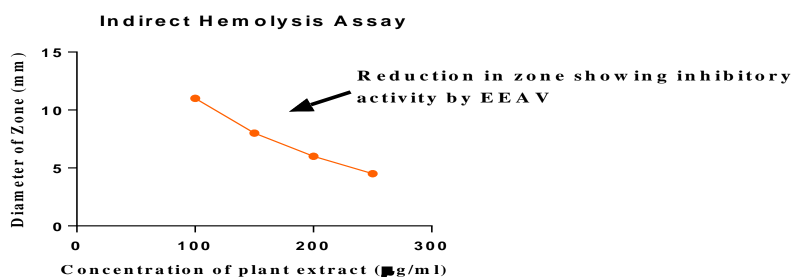

Fig.No.2 : Effect of Concentration of EEAV on diameter of Indirect hemolysis assay.

3.2.3 Inhibition of Fibrinolytic Activity

Table.No.3: Reduction of fibrinolytic halo produced by the *Naja naja* venom by leaves and stem bark extract of *Alstonia venenata* R.Br.

S. No	Venom dose that gives 10mm Zone	Concentration (µg/ml)	Fibrinolytic halo produced by the plant extract (mm)
1	200µg	100	10
2		150	9
3		200	7
4		250	6

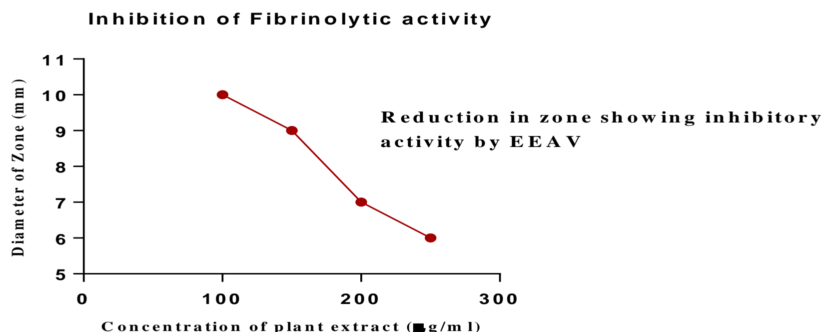

Fig.No.3: Effect of Concentration of EEAV on diameter on inhibition of fibrinolytic activity.

3.3 *IN VIVO* STUDY

3.3.1 *in Vivo* Neutralization Activity of *Alstonia Venenata* R.Br. against Lethality Induced By *Naja Naja* Venom.

Table.No.4: *In vivo* neutralization effect of *Alstonia venenata* R.Br. extract in mice administered with LD_{50} (63.1µg/kg) of *Naja naja* venom.

S. No	Groups	*Naja naja* venom LD_{50} (1 fold)			
		Mortality	% Survival	Corrected %	Probit*
1	Control (*Naja naja* venom)	06/06	0	4.16	3.6
2	*Naja naja* venom + standard anti-venom	00/06	100	99.95	8.85
3	*Naja naja* venom + EEAV 200mg/kg	01/06	75	75	6.32
4	*Naja naja* venom + EEAV 400mg/kg	00/04	100	99.95	8.85

Corrected % formula; for 0% dead: 100 (0.25/n), for 100% dead: 100 (n-0.25/n).
*Miller LC, Tainter ML. Estimation of LD_{50} and its error by means of log- probit graph paper.

Table No:5 *In-vivo* neutralization effect of *Alstonia venenata* R.Br. extract in mice administered with 2 LD_{50} (126.2µg/kg) of *Naja naja* venom.

S. No	Groups	*Naja naja* venom 2 LD_{50} (2 fold)			
		Mortality	% Survival	Corrected %	Probit*
1	Control (*Naja naja* venom)	06/06	0	4.16	3.6
2	*Naja naja* venom + standard anti-venom	00/06	100	99.95	8.85
3	*Naja naja* venom + EEAV 200mg/kg	02/04	50	50	4.68
4	*Naja naja* venom + EEAV 400mg/kg	01/04	75	75	6.32

Corrected % formula ; for 0% dead: 100 (0.25/n), for 100% dead: 100 (n-0.25/n)
*Miller LC, Tainter ML. Estimation of LD_{50} and its error by means of log- probit graph paper.

4. DISCUSSION

Snake bite or snake envenoming is a major public health issue in the rural tropics in world as it causes injury or death. In India 35,000 to 50,000 people die every year due to snake envenomation. Anti-venom immunotherapy is the only effective treatment against snake venom poisoning. Anti-venom are usually hyper immune sera collected from the animals which bind and inactivate the venom component. Products of animal serum can produce adverse side effects such as anaphylatic reactions and serum sickness. Because of the unavailability of the anti-venoms, the people in the tropic areas mostly uses the various plant extracts for treating the snake bite. More than 700 plants have been reported as used in folk medicine in the world for snake bites. Many plants are recommended in Indian traditional medicine to treat snake bites, some of which have been examined for their activity like, *Pluchea indica*, *Vitex negundo* and *Embelica officinalis* etc., for the neutralization of snake venom.

Alstonia venenata R.Br. is the rare species which is a shrub having distribution in Maharashtra, Karnataka and Kerala. In Kerala it is mostly found in Malappuram, Palakkad, Thrissur and Wayanad. *Alstonia venenata* R.Br. is also known as Analivegam in Malayalam, Sinnapalai in Tamil, Vishagni in Sanskrit and Adda sarpa in Kannada. The various extracts of this plant are proved effective for the treatment of Ascaris, Blood impurity and Cough. They are mainly used as Anthelmintic, Antibacterial, blood purifier, Stimulant, Aphrodisiac and Tonic. Mainly the leaves and stem bark of *Alstonia venenata* R.Br. are used in for the snake bite.

In this present investigation the ethanolic extracts of *Alstonia venenata* R.Br. leaves and stem bark extracts of 200 & 400mg/kg were tested for its neutralization capacity of the *Naja naja* venom induced lethality. The snake venom contains more than 20 different constituents, mainly proteins, including toxic enzymes and polypeptide toxins which produces the toxic reactions on the body after snake envenomation. The *Naja naja* venom contains several pharmacologically active component like cobra venom factor, cardiotoxin and myotoxin. Some of the local pathological effects of envenomation such as myonecrosis and muscular degeneration, lethality etc., are due to the presence of protease in snake venom. As plant contains Alkaloids, Flavonoids as well as Tannins and Glycosides which have the ability to bind the proteins which produces toxic effects. The LD_{50} of *Naja naja* venom was calculated by Miller and Tainter method and the Minimum Lethal Dose of *Naja naja* venom was found to be 63.1µg/kg. Inhibition of acetylcholinesterase activity was performed to determine the percentage of inhibition produced by the plant extract against the *Naja naja* venom. It was found that the plant extract was effective in the inhibition of acetylcholineserase activity. Maximum inhibition was found in the concentration of 250µg/ml. The percentage of inhibition was found to be 57.26%. The plant extract inhibited the enzyme acetylcholinesterase in a dose dependent manner. The IC_{50} value of different concentration of plant extract was found to be 208.8µg/ml. The inhibition of acetylcholinesterase by the EEAV may be due to the presence of phytoconstituents such as Alkaloids which is one of the cholinergic inhibitor.

The indirect hemolysis assay was performed to determine the inhibition of phospholipase A_2 by the EEAV. The Minimum Indirect Hemolytic Dose (MIHD) of *Naja naja* venom that produced hemolytic halo of 11mm diameter is determined. It was found that a dose of 200µg/ml of *Naja naja* venom gaves the 11mm diameter of hemolytic halo. This hemolytic zone produced by the *Naja naja* venom was reduced by the EEAV in a dose dependent manner. The maximum reduction of hemolytic halo was observed in the dose of 250µg/ml of EEAV. It gaves a hemolytic zone of 6mm of diameter by the plant extract. This reduction of hemolytic halo produced by the *Naja naja* venom by the EEAV is may be due to the presence of phytoconstituents like acids, steroids, glycoproteins etc., Phospholipase A_2 present in the *Naja naja* venom hydrolyses the ester bond at Sn-2 position of phospholipids producing free fatty acids and lysophospholipids. This may results to myotoxicity, odema formation, anticoagulant effects etc., The hydrolyses of phospholipid A_2 was inhibited by the phytoconstituents present in the EEAV.

In inhibition of fibrinolytic activity the EEAV showed effective reduction of fibrinolytic halo produced by the *Naja naja* venom. The Minimum Fibrinolytic Concentration (MFC) produced by the *Naja naja* venom was found to be 200µg/ml. The presence of phytochemicals such as Terpenoids and tannins may be the reason of the reduction of fibrinolytic halo produced by the *Naja naja* venom.

The ethanolic extract of *Alstonia venenata* R.Br. 200 & 400mg/kg significantly inhibits the lethality induced by the *Naja naja* venom. The Pentacyclic triterpenes (free or as glycosides) are widely found in the plant extract of *Alstonia venenata* R.Br. may be responsible of Anti-venom activity as it is mentioned that Triterpenes found widely in Anti- snake venom plants and provide nearly 20% protection against snake venom. It also contains Glycosides, Tannins, Flavonoids which is abundant in the plants phytoconstituents might have neutralized the toxic enzymes of the *Naja naja* venom. Similar reports on plant *Alstonia scholaris* containing Pentacyclic triterpenes which had the ability to neutralizes the cobra venom toxic reactions. For the development of plant-derived therapeutic antagonist against snakebite for the

community in need further investigation of the plant; the isolation and investigation of active chemical constituent needed. It is noteworthy that these studies were performed using the crude extracts and thus an increase in the extent of activity can be expected with the use of highly purified extracts or isolated compounds.

5. CONCLUSION

This works reveals the scientific validation for the usage of *Alstonia venenata* R.Br. leaves and stem bark as an Anti-venom drug. I hope these findings may clarify the supposed efficacy of the use of herbal drug as an anti-snake venom agent. Further study is required to conclude the mechanism of action isolation, purification and structural elucidation of active compounds to develop a new chemical antidote for snake envenomations.

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