



Research Article

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IN VITRO AND IN VIVO NEUTRALIZING POTENTIAL OF *TERMINALIA ARJUNA* BARK EXTRACT AGAINST NAJA NAJA VENOM

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ABSTRACT

Envenoming's by snake bite to involve medical emergencies and its clinical management is by the administration of antivenom. As antivenom was reported to induce early or late adverse reactions against human beings, snake venom neutralizing potential for aqueous bark of *Terminalia arjuna* extract was tested for the present research by *in vitro* and *in vivo* methods against *Naja naja* venom. *In vitro* assessment of venom toxicity and neutralization assays was carried out. The bark extracts from *Terminalia arjuna* were used to evaluate the *in vitro* neutralization tests like acetyl cholinesterase, protease, direct haemolysis assay, phospholipase activity and procoagulant activity. The *in vivo* assessment of venom lethality (LD₅₀) of *Naja naja* venom was found to be 0.301 µg. *Terminalia arjuna* bark extract was effectively neutralized the venom lethality and effective dose (ED₅₀) was found to be 10.47 mg/ 3LD₅₀ of *Naja naja* venom. *Terminalia arjuna* bark extract was found effective against neutralizing all the toxic effects induced by the venom. The study concludes that further investigations are needed for identification and purification of the active components involved in the neutralization of the snake venom.

Keywords: *Terminalia arjuna*, *Naja naja*, Acetyl cholinesterase, Phospholipase A2, Protease

INTRODUCTION

Snakes are the most misunderstood and generally disliked animals in the world. They are global, marine, or amphibious in nature and found in thick forest as well as near human habitation like in homes, private grounds, stockpile rooms and in many cracks and crevices. Usually more than 3000 poisonous and non-poisonous species of snakes are found in the world which are grasping carnivores in nature with wide range of prey species^{1, 2}. It is difficult to calculate actual frequency of snakebites because in rural areas it is not properly recognized. There are around 1.2 million and 5.5 million snakebites occur worldwide each year, with 421,000-1,841,000 envenomation's and 20,000 - 94,000 death³. The four-main species of deadly snakes omnipresent in India known as "Big four" are considered conscientious for serious envenomation around the country. The frequent poisonous snakes found in India are *Naja naja*, *Bungarus Caeruleus*, *Daboia russelli* and *Echis Carinatus*⁴. The venom of *Naja naja* and *Bungarus Caeruleus* are neurotoxic, they affect the victim's central nervous system and cause heart failure. The venoms of *Daboia russelli* and *Echis Carinatus* are histotoxic and haemorrhagic, therefore they exaggerate haemorrhagic manifestation that includes nosebleeds and cardiac manifestations such as myocarditis and cardiac failure. The foremost pathophysiological problems associated with snake bite usually start with the widespread side effects like nausea, vomiting, headache, diarrhoea, abdominal pain, fall of blood pressure etc., followed with late systemic effects, like neuromuscular blockage, respiratory, haemorrhage etc. occasionally, snake envenomation is associated with local side effects like ache inflammation and necrosis⁵. The most effective and accepted therapy for snakebite patients are immediate administration of specific or polyvalent antivenom following

envenomation. The first anti venom (called an 'anti-ophidic serum') was developed by Albert Calmette, a French scientist of the Pasteur Institute in 1895. But it has several drawbacks in actual clinical practice like species specificity, difficulty in availability, affordability and ideal storage conditions⁶. The treatment of snake bites is as variable as the bite and its symptoms. Antiserum contains purified, enzyme-refined and concentrated heterologous immunoglobins⁷. Antivenom immunotherapy leads to various side effects such as anaphylactic shock, pyrogen reaction and serum sickness. Most of these symptoms may due to high concentration of non-immunoglobulin proteins present in commercially available antivenom. In many cases the mortality of victim happens due to the wrong choice of antiserum because of misidentified snake species by the treating physicians which results in severe life-threatening envenoming to the victim⁸.

Hence, in almost all parts of world, numerous plant species are used as folk medicine to treat snake bite instead of using antivenom due to the above said limitations. In India, the rural areas are mostly affected by snake envenomation. About 54 million indigenous people constantly practice herbal medicine for diverse types of diseases and disorders including the treatment of snakebites. They counteract snake venom activity by their manual methods like applying herbal extracts on affected skin, chewing and eating the leaves and barks of plants and injecting or drinking the herbal extracts directly⁹. The phytochemicals isolated from plants are not only used in traditional treatment but also has an immense importance as raw materials for preparation of modern medicine¹⁰.

The plant constituents were identified to play a major role in neutralizing the effects of any types of snake venoms. The snake

bite management was accomplished using herbal plants either using a single herbal ingredient or by using combination of herbal plants. According to Kuntal Das (2009)¹¹, the recent scientific investigations have confirmed the efficacy of many herbal preparations for snake bites. The most effective herbs were also reported as relatively non-toxic and have substantial documented efficacy. Among them some herbs are *Aristolochia* species, *Cissus assamica*, *Echinacea* species, *Guiera senegalensis*¹², *Hemidesmus indicus*, *Parkia biglobosa*¹³, *Securidaca longipedunculata*, *Thea sinensis*, *Tamarindus indica*, *Trianosperma tayuya*, *Withania somnifera*¹⁴.

In the present research, *Terminalia arjuna* bark was screened for its antivenom properties. *Terminalia arjuna* (Wight & Arn.) is an important medicinal plant, belongs to the family Combretaceae. *Terminalia arjuna* is distributed over India, Burma and Sri-Lanka¹⁵. It mainly grows along the banks of the river and streams. Bark powder boiled with water and inhaled to cure headache and to kill worms in the teeth¹⁶. The powdered bark is useful for the treatment of heart troubles, juice is used as antacid and fruits are found useful as tonic. Bark is used in the treatment of the snakebite and scorpion sting. It is also useful as expectorant, aphrodisiac, tonic and diuretic¹⁷. Although the plant has been a widely used as folklore medicine with reportedly high diuretic and anti-inflammatory properties, the antivenom potential remains still uncharacterized. Hence in this research, the neutralization efficacy of this plant root extract against *Naja naja* snake venom was determined both under *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Snake venom

The freeze-dried snake venom powders of *Naja naja* were obtained from Irula's Snake Catchers Industrial Co-operative Society Limited Chennai and was stored at 4^o C. Stock solution was prepared by dissolving 1mg of lyophilized venom in 1ml of physiological saline (1mg/ml).

Collection and authentication of plant material

Terminalia arjuna bark was collected from Anakkal region, Malampuzha, Palakkad district, Kerala after questionnaire with tribal people and from vaidyas in and around Palakkad district. It was authenticated by Dr. Althaf Ahamed Kabeer, Scientist 'D', Botanical Survey of India Southern Regional Centre, Coimbatore. (Herbarium voucher specimen number 1161)

Preparation of Extracts

About 20g of powdered sample of the herb was extracted by soaking in 180 ml of distilled water in a beaker, stirred for about 6 min and left over night. Thereafter, the solution was filtered using filter paper (What man No. 1) and the extracts were evaporated to dryness under reduced pressure below 40°C. The plant extracts were expressed in terms of dry weight.

Acute Oral Toxicity

Acute oral toxicity of all the selected plant extracts was performed as per OECD guidelines 423. A limit test of 2000 mg/kg body weight of the extracts was administered. Briefly, two thousand milligrams of the test substance per kilogram of body weight was administered to 3 healthy mice by oral gavages. The animals were observed for mortality, signs of gross toxicity, and behavioural changes at least once daily for 14 days. Body weights were recorded prior to administration and again on

Days 7 and 14 (day of termination). Necropsies were performed on all animals at terminal sacrifice. (Ethics committee approval number: JSSCP/IAEC/PH.D/PH.COLOGY/02/2014-15)

In vitro Assessment of Venom Toxicity and Neutralization assays

Acetylcholinesterase activity

Acetyl cholinesterase inhibition assay was carried out according to the modified method¹⁸ 200 µl of venom (1 mg/ml) was pre-incubated (1 h) with different concentrations of plant extract 200,250, 300 µl and supernatant was added to the assay mixture which consists of 100 µl of 75mM acetylcholine iodate 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

Proteolytic activity

Proteolytic activity was determined according to the method¹⁹ using 2% casein as substrate in 0.02M Tris-HCl buffer (pH 8.5). Venom 200µl (1mg/ml) and different dilutions of plant extract 200µl, 250µl, 300µl were pre-incubated with 1ml of substrate for 2h at 37 °C. The undigested casein was precipitated by the addition of 1.5ml of 0.44M trichloroacetic acid (TCA) and centrifuged. The digested casein in the supernatant was determined using Folin ciocalteu's reagent. Venom without plant extract was considered as control or 100% activity.

Direct Hemolysis Assay

The hemolytic action of *Naja naja* venom and plant extracts was studied *in vitro* by using RBC. Briefly, 5ml of citrated blood was centrifuged for 10minutes at 900rpm. The supernatant was poured off and the pellet was washed twice with physiological salt solution. 5ml of physiological saline and 0.5ml of RBC mixture served as a control. 5ml of distilled water with 0.5ml of washed RBC was used for 100% hemolysis. 5ml of venom/extract and 0.5ml of washed RBC served as experimental sample. The tubes were put in a thermostat for 1hr at 37°C and centrifuged at 2000rpm for 20mts. The supernatant fluid was poured off to separate tubes to measure the optical density using spectrophotometer at a wave length of 540nm against water.

Indirect Hemolysis Assay (PLA₂ activity)

Phospholipase A₂ activity was measured using an indirect hemolytic assay on agarose-erythrocyte-egg yolk gel plate by the method described by²⁰. Increasing concentrations of *Naja naja* venom (µg) was added to 3mm wells in agarose gels (0.8% in PBS, pH 8.1) containing 1.2% sheep erythrocytes, 1.2% egg yolk as a source of lecithin and 10mM CaCl₂. Slides were incubated at 37°C overnight and the diameters of the haemolytic halos were measured. Control wells contained 15µl of saline. The minimum indirect haemolytic dose (MIHD) corresponds to a concentration of venom, which produced haemolytic halo of 11mm diameter. The efficacy of *Terminalia arjuna* bark extracts in neutralizing the phospholipase activity was estimated by mixing constant amount of venom (µg) with different amount of plant extracts (µl) and incubated for 30 minutes at 37°C. Then, aliquots of 10 µl of to the mixtures were added to wells in agarose-egg yolk-sheep erythrocyte gels. Control samples contain venom without plant extract. Plates were incubated at 37°C for 20 hours. Neutralization expressed as the ratio mg plant extract/mg venom able to reduce by 50% the diameter of the haemolytic halo when compared to the effect induced by venom alone.

Procoagulant Activity

The procoagulant activity were done according to the method²¹. Various amounts of venom dissolved in 100 μ l 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 concentration, which induced clotting of plasma within 60 seconds. Plasma incubated with PBS alone served as control. In neutralization assays constant amount of venom was mixed with various dilutions of plant extract. 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 were recorded. In control tubes plasma was incubated with either venom alone or plant extract. Neutralization was expressed as effective dose (ED₅₀), defined as the ratio μ l antivenom (plant extract)/mg venom at which the clotting time increased three times when compared with clotting time of plasma incubated with two MCD of venom alone.

In vivo assessment of venom toxicity and anti-venom effect of plant extracts lethal toxicity

Various doses of venom in 0.2 ml of physiological saline were injected into the tail vein of mice, using groups of 3-5 mice for each venom dose. The LD₅₀ was calculated with confidence limit of 50% probability by the analysis of deaths occurring within 24 h of venom injection²².

The anti-lethal potentials for plant extract were determined against 2LD₅₀ of *Naja naja* venom. Various amount of plant extracts (μ l) were mixed with 2LD₅₀ of venom sample and incubated at 37°C for 30 minutes and then injected intravenously into mice. 3–5 mice were used at each antivenom dose. Control mice received same amount of venom without antivenom (plant extracts). The median Effective Dose (ED₅₀) calculated from the number of deaths within 24h of injection of the venom/antivenom mixture. ED₅₀ was expressed as μ l antivenom/mouse and calculated by probit analysis²³.

Statistical Analysis

Statistical evaluation was performed using XL stat 2008 and SPSS 10 Software. P<0.005 was considered statistically significant

RESULTS

In vitro Assessment of Venom Toxicity and Neutralization assays

All the *in vitro* assessment of venom toxicity and neutralization assays showed remarkable results. The inhibitory effect of *Terminalia arjuna* bark extracts from acetyl cholinesterase activity of venom was determined *in vitro* and the results were presented in (Figure 1). Noteworthy results from other tests like protease activity (Figure 2), direct haemolysis, phospholipase activity, and procoagulant activity were evident during the

analysis. Interestingly, *Terminalia arjuna* bark extracts also showed good neutralization on the toxicity of venom.

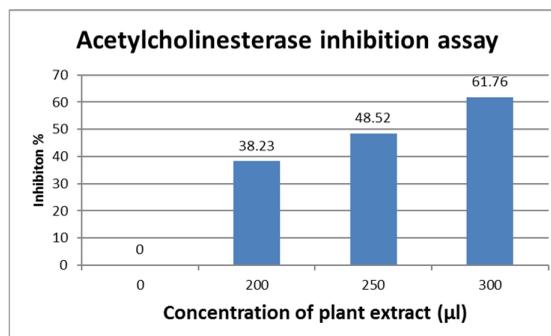


Figure 1: *In vitro* Assessment of Neutralization assay: Acetylcholinesterase activity

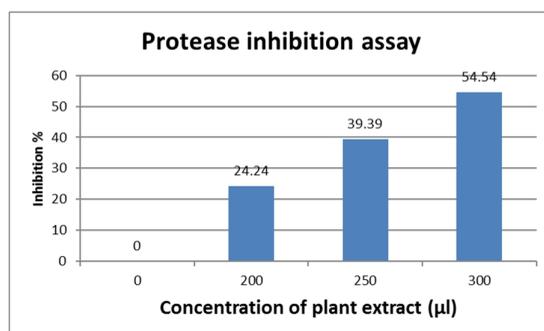


Figure 2: *In vitro* Assessment of Venom Toxicity and Neutralization assays

Protease activity

In vivo assessment of venom toxicity and anti-venom effect of plant extracts lethal toxicity

In vivo assessment of venom lethality (LD₅₀) of *Naja naja* venom was assessed and calculated by Miller and Tainter method. The tests of determining venom lethality (LD₅₀) and antivenom neutralizing capacity (ED₅₀) are currently the only validated means of assessing venom toxicity and antivenom neutralizing potency by both manufacturers and regulatory authorities worldwide. *In vivo* assessment of venom toxicity (LD₅₀) of the venom was assessed by LD₅₀ range-finding test and the median lethal dose (LD₅₀) assays using mice (18–20 g). LD₅₀ of *Naja naja* venom was calculated and found to be 0.301 μ g/g. (Table 1 and Figure 3) Venom-neutralizing potency test (ED₅₀) using *Terminalia arjuna* bark extract was carried out by pre-incubating constant amount of venom (2LD₅₀) with various dilutions of the plant extracts prior to injection. Calculation of ED₅₀ of *Terminalia arjuna* bark against 2LD₅₀ of venom was done by Miller and Tainter method and found to be 10.47 mg /2LD₅₀ venom. (Table 2 and Figure 4)

Table 1: Calculation of LD₅₀ of *Naja naja* venom in mice receiving various doses of *Naja naja* venom by Miller and Tainter method (n=5).

Dose (µg/g)	Adjusted (Dose×100)	Log dose	Death/Total	Dead %	Corrected formula %	Probit values
0.025	2.5	0.4	0/5	0	5	3.36
0.05	5	0.7	1/5	0	20	4.16
0.1	10	1	1/5	20	20	4.16
0.25	25	1.4	2/5	40	40	4.75
0.5	50	1.7	3/5	60	60	5.25
1.0	100	2.0	4/5	80	80	5.84
2.5	250	2.4	4/5	80	80	5.84
5.0	500	2.7	5/5	100	95	6.64

Corrected formula: For the 0% dead: $100(0.25/n) = 100(0.25/5) = 5$
 For the 100% dead: $100[(n-0.25)/n] = 100[(5-0.25)/5] = 95$, n is the number of animals in the group

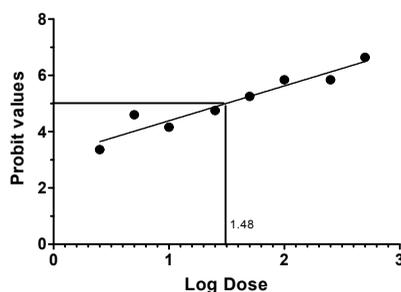


Figure 3: Calculation of lethal dose LD₅₀ of *Naja naja* venom

LD₅₀ of *Naja naja*
 = antilog (log dose)/100
 = antilog 1.48/100
 = 30.19/100
 = 0.301 µg/g

Table 2: Calculation of ED₅₀ of *Terminalia arjuna* against *Naja naja* venom in mice by Miller and Tainter method (n=5).

Dose (mg/100µL)	Adjusted (Dose×100)	Log dose	Survival/Total	Dead %	Corrected formula %	Probit values
1	100	2	0/5	0	5	3.36
2.5	250	2.4	0/5	0	5	3.36
5	500	2.7	1/5	20	20	4.16
10	1000	3	2/5	40	40	4.75
20	2000	3.3	3/5	60	60	5.25
40	4000	3.6	5/5	100	95	6.64

Corrected formula: For the 0% dead: $100(0.25/n) = 100(0.25/5) = 5$
 For the 100% dead: $100[(n-0.25)/n] = 100[(5-0.25)/5] = 95$, n is the number of animals in the group

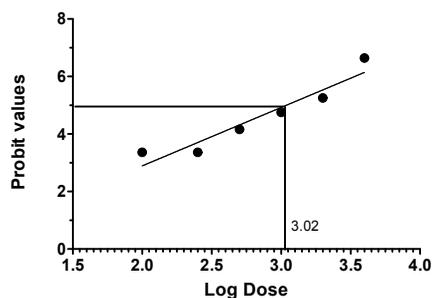


Figure 4: ED₅₀ of *Terminalia arjuna* against *Naja naja* venom

= antilog (log dose)/100
 = antilog 3.02/100
 = 1047/100
 = 10.47 mg

DISCUSSION

Snakebite is always considered to be as a major health hazard which leads to high mortality rate world-wide. The true global incidence of snake bites and associated mortality is difficult to estimate. Anti-snake venom remains the specific antidote to snake venom poisoning with different limitations on its usage. It consumes lots of time for the development and expensive. Since it contains horse immunoglobulins, it leads to complement mediated side effects, like serum sickness and anaphylactic shock. Due to these limitations of anti-venoms, since last 20 years more scientific attention on using plants and medically significant herbs against different snakebites were given importance²⁴. More interestingly, many Indian medicinal plants were recommended for the treatment of snakebites world-wide. In one of the previous study *Terminalia arjuna* bark ash is used in the treatment of the snakebite and scorpion sting²⁵. In another work, *Terminalia arjuna* bark is used in the treatment of the snakebite²⁶. Antivenom potential for *Terminalia arjuna* bark extract against *Naja naja* venom was investigated in the present study by both *in vitro* and *in vivo* experiments. During this experiment, maximum acetyl cholinesterase inhibition was recorded as 61.76% for a known concentration of 300µl plant extract (Figure 1). To assess the *in vitro* antagonism of protease, the venom degrading the substrate (casein) into peptide precipitation was observed at 600 nm. Maximum protease inhibition of 54.54% was observed for 300 µl concentration of aqueous plant extract (Figure 2). It was justified that the increased concentration of plant extract could increase the inhibition of protease activity of venom. Direct hemolysis of *Naja naja* venom produced 91.66% hemolysis. *Terminalia arjuna* bark extract neutralized the hemolysis of RBC's produced by the venom up to 42%. In phospholipase activity (PLA₂) 10 µg of *Naja naja* venom was able to produce 11mm diameter haemolytic halo, which is considered to be 1Unit. *Terminalia arjuna* bark extract can capable of inhibiting PLA₂ dependent hemolysis of sheep RBC's induced by *Naja naja* venom in a dose dependent manner. In procoagulant activity 100 µg of *Naja naja* venom was found to clot human citrate plasma in 60 seconds. In the neutralization assay, the absence of clot formation shows the neutralizing ability of plant extract. High concentration of venom caused rapid clotting that required very high concentration of plant extract to neutralize. *In vivo* assessment of venom lethality (LD₅₀) and venom-neutralizing potency test (ED₅₀) using *Terminalia arjuna* bark extract was carried out in the present research. From the obtained results, it was found that *Terminalia arjuna* bark extract could able to neutralize the toxin of venom. LD₅₀ of *Naja naja* venom was calculated and found to be 0.301 µg/g. ED₅₀ of *Terminalia arjuna* bark against 2LD₅₀ of venom was done by Miller and Tainter method and found to be 10.47 mg /2LD₅₀ venom. In Acute Oral Toxicity, all animals survived and appeared active and healthy throughout the study. There were no signs of gross toxicity, adverse pharmacological effects or abnormal behaviour. Gross necropsy findings at terminal sacrifice were unremarkable. Based on the above findings, the LD₅₀ of *Terminalia arjuna* bark extract was >2000 mg/kg.

The acute oral toxicity of plant extract also observed and it was found that there was no any toxic effect on any mice due to the intake of crude plant extract. The result from this preliminary study indicates that *Terminalia arjuna* bark extract possess some compounds which can neutralizes the toxins present in *Naja naja* venom.

In the present study, protease and phospholipase activities were completely inhibited in a dose dependent manner. This could be

due to the strong anti-venom activity of the phytosterol (stigmasterol) present in the extract.

Hence, the presence of these multiple bioactive (anti-snake venom) compounds in the extract could have contributed to its efficient antivenom activity in the present study. Based on the finding of the present study, it is demonstrated that aqueous extracts from *Terminalia arjuna* bark possess antivenom activity against *Naja naja* venom enzymes and supports their traditional use of herbal medicine against snakebites.

CONCLUSION

The *in vitro* enzymatic analysis reveals that the *Terminalia arjuna* bark extract could inhibit most of the toxic enzymes of the *Naja naja*. The result from this preliminary study indicates that *Terminalia arjuna* bark extract possess significant compounds neutralizes the toxins present in *Naja naja* venom. Further investigations are needed for identification and purification of the active components involved in the neutralization of the snake venom.

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