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Evaluation of Anti-Venom Potential of *Areca catechu* Seed Extract on *Bungarus caeruleus* Venom

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Abstract: *Areca catechu* seeds and their extract/s are currently used to treat various ailments and infections including snakebites. The purpose of this investigation was to assess the inhibiting/neutralizing effect of ethyl acetate and aqueous ethanolic seed extracts of *A. catechu* on *Bungarus caeruleus* (krait) venom. The enzyme activities and their inhibition were evaluated using standard procedures (in vitro). In vivo studies were conducted using chick embryos and murine models. The extracts inhibited hyaluronidase and phospholipase A_2 activities. Protease activity was neutralized by the aqueous ethanolic extract only. The IC_{50} value of aqueous ethanolic extract for hyaluronidase was 0.001 g/mL, while that for the ethyl acetate extract for phospholipase A_2 was 0.006 g/mL. In addition, both the extracts neutralized the indirect hemolysis and fibrinogenolytic activity induced by *B. caeruleus* venom. The LD_{50} for the chick embryos was 4.9 µg/egg. The 50 and 100 µg aqueous ethanolic extracts neutralized the LD_{50} and the challenging dose ($3LD_{50}$) of venom effectively in the chick embryo model. The LD_{50} of *B. caeruleus* venom in mice was 0.1927 µg/kg; the extract extended the survival time of the mice from 25 min to 30 and 35 min in 1:10 and 1:20 ((*w*/*w*) venom:extract) ratios, respectively. The extract also neutralized myotoxic activity. The *A. catechu* seed extract showed promising inhibitory properties against *B. caeruleus* venom. In this regard, academia and industries should work collaboratively to develop and formulate a cost-effective first-aid drug.

Keywords: *Areca catechu*; *Bungarus caeruleus*; fibrinogenolytic activity; haemolytic activities; lethal toxicity; chick embryo

1. Introduction

A snakebite is a hazardous occupational and environmental injury. It is a neglected tropical injury, and is also dangerous in other zones [1,2]. In India, 216 species of snakes

are found; among them, 58 are venomous, and only 4 of these species of snakes are responsible for maximum-dose bites and mortality. These four species are the krait, the Indian Cobra, *D. russeli*, and *Echis carinatus.* [3]. Among the big four, *B. caeruleus* venom is highly neurotoxic and very rapid in action, and its producer is considered to be the most dangerous venomous snake species in India [4]. With no/little pain initially, a *B. caeruleus* bite causes severe abdominal pain, heaviness, blurred vision, partial ptosis, external ophthalmoplegia, and death, which usually occurs due to respiratory failure [5,6]. The venom is a mixture of enzymes that includes small peptides, metal ion amines, and carbohydrates, which exert neurotoxic and cytotoxic effects on the snake's prey [3]. Venom enzymes such as hyaluronidase, protease, and phospholipase are responsible for degrading the connecting materials in the attacked cells, thereby increasing the diffusion of the toxins from the venom. This may lead to cell damage (nucleic acids' liberation and cell membrane permeability) [7,8].

Snakebites are treated using anti-venom derived from immunized horse sera. However, such treatments cause several adverse effects such as anaphylactic shock, pyrogen reaction, and serum sickness [9]. Anti-venom can neutralize the free circulating venom components but has no effect on the damage already induced by the venom [10]. This has led biologists to investigate alternative treatments for snake envenomation. In this perspective, medicinal plants and their extracts could be potential anti-venom sources, which would be cheaper, non-toxic, and easily available under normal storage conditions. Alam [11] reported that people across the globe rely on traditional medicine for basic health issues. The knowledge of the use of medicinal plants as medicine is passed down the generations verbally. Soares et al. [12] reported that 850 species of higher plants from 138 families are used to treat snakebites. Various plants across the globe possess anti-venom properties [7,13–15].

A. catechu seeds have been widely used as disease-curing agents, especially in South Asian regions. Over 59 compounds have been isolated and authenticated, which include alkaloids, flavonoids, tannins, terpenoid fatty acids, and triterpenes [16]. Indeed, this plant's seeds have shown antifungal, antioxidant, hypolipidemic, anti-diabetic, antibacterial, anti-malarial, antiviral, anti-migraine, wound-healing, anti-ulcer, antihypertensive, antidepressant, anti-allergic, cytoprotective, anthelmintic, aphrodisiac, hepatoprotective, and anti-cancer properties [17–20]. The curative properties of *A. catechu* have been attributed to phytochemicals such as polyphenols; the interaction of these phytochemicals with venom enzymes or toxins leads to the neutralization of their activity and/or toxic effects [6,21]. The plant extracts inhibit the venom activity by protein binding or neutralizing the enzyme. Many of the phytocompounds identified have an affinity towards toxic constituents, cations, and biomolecules such as the enzymes present in the venom [5,11,21]. The available polyvalent anti-serum binds to the remaining toxin and thereby prevents further side effects but does not reverse the effects already caused by the venom. To overcome this drawback, an easily available alternative treatment for snakebites must be uncovered. Thus, in the present investigation, the ability of *A. catechu* seeds to neutralize *Bungarus caeruleus* (krait) venom was assessed.

2. Materials and Methods

2.1. Materials

2.1.1. Plant Material

The seeds of *A. catechu* were collected in February 2018 from Macchenahalli (Bengaluru rural district, India). The collected samples were authenticated and deposited at National Ayurveda Dietetics Research Institute, Bangalore, India (Ref.RRCBI-mus114).

2.1.2. Venom

The venom sample of *B. caeruleus* procured from Irula Snake Catcher's Co-operative Society (Chennai, India) was stored at 4 °C and maintained in the Toxicology Lab., Dayananda

Sagar University, bearing the license number 01/2017-18. The estimation of protein concentration was conducted as per Lowry et al. [22].

2.1.3. Chemicals

Fibrinogen and SDS were procured from Sigma-Aldrich (Aldrich, St. Louis, MO, USA) and all other chemicals from HiMedia (Mumbai, India).

2.2. Animals

Adult male Swiss Albino mice with an average body weight of 25–30 g were used for in vivo studies. They were procured from Sri Raghavendra enterprise and housed in cages at room temperature (28–32 °C) under a light period of 16–18 h daily and were fed with standard commercial feed. The procedure for handling of animals was followed in accordance with the ordinance of the Institutional Animal Ethics Committee (IAEC/NCP/91/2015). The six-day-old eggs were purchased from Lakshmi hatcheries, Bangalore.

2.3. Human and Animal Rights

The animals were handled according to the rules and regulations of the ethical committee. Blood samples used for experiments were collected from healthy volunteers with their consent. Human experiments using either venom or plant samples were not conducted.

2.4. Phytochemical Analysis of Seed Extracts

The rinsed seeds were thoroughly dried and ground. A total of 40 g of seed flour was soaked in aqueous ethanol solvent overnight followed by extraction with Soxhlet extractor. The extract was concentrated using a rotary vacuum evaporator. The residue was dried at 37 °C in incubator, weighed, and stored at room temperature conditions. The ethyl acetate extract was derived in the same manner. Tests for phenols, steroids, tannins, glycosides, and terpenoids were conducted [23].

2.5. In Vitro Enzyme Assay and Neutralization Studies

Hyaluronidase activity of *B. caeruleus* venom was estimated turbidimetrically following the method given by Pukrittayakamee et al. [24]. The activity of the phospholipase A_2 in the krait venom was estimated by the procedure suggested by Marinetti et al. [25]. The procedure of Greenberg [26] was considered to quantify the protease activity of *B. caeruleus* venom utilizing casein as a substrate. For neutralization and inhibition investigations, the venom was pre-incubated with ethyl acetate and aqueous alcohol extracts of 100 µg concentration at 37 °C for 30 min.

2.6. Pharmacological Assays

For indirect hemolytic assay, PLA_2 activity of krait venom was quantified using the method of Gutierrez et al. [27]. The RBC hemolysis took place on agarose gel plate containing egg yolk and erythrocyte. For fibrinogenolytic assay, Ouyang and Teng's [28] procedure was followed. Inhibition studies were carried out using venom pre-incubated with the extracts.

2.7. Acute Toxicological In Vitro Studies Using Chick Embryo Model

2.7.1. Acute Toxicity Determination of Seed Extract

Six-day-old eggs were first analyzed by candlelight procedure to assess their fertility. Acute toxicity of the *A. catechu* seed extract was checked utilizing in vitro chick embryo model [29,30]. Aqueous ethanolic seed extract of *A. catechu* was dissolved in saline to obtain a concentration of 100 µg in 0.2 mL saline. A hole was made in the eggs (n = 6) at the apex of the air cell without disturbing the embryo. The contents were transferred into the egg through the hole using insulin syringe. A total of 0.2 mL of seed extract and 0.2 mL of saline were used to inject test and control groups, respectively. After overnight incubation at 65% relative humidity and 37 °C, candling procedure was utilized to check eggs' survival status.

2.7.2. Lethal Toxicity (LD_{50}) Quantification

LD_{50} ranges were determined by injecting various concentrations (2–10 µg) of *B. caeruleus* venom in 0.2 mL saline and by incubating them at 37 °C for 24 h. The survivability of the eggs was determined by candling method followed by the opening of the eggs' apexes. Meier and Theakston's [31] procedure was followed to record LD_{50}.

2.7.3. Lethal Toxicity Neutralization by the Chick Embryo Model

For neutralization, 3 LD_{50} of the *B. caeruleus* venom was considered as the challenging dose. The challenging dose of *B. caeruleus* venom and 50 µg/0.2 mL seed extract were incubated together at 37 °C for 30 min. The pre-incubated mixture and challenging dose were separately injected into eggs (n = 6). Control eggs were administered with saline. Embryos were checked every hour and at 5h the survivors were noticed. The same procedure was repeated with 100 µg/0.2 mL seed extract.

2.8. Acute Toxicological In Vivo Studies Using Murine Model

2.8.1. Acute Oral Toxicity Investigation

The acute oral toxicity of the seed extract was determined as per Vineetha et al. [32]. The mice were evaluated for changes in behavior, gross toxicity signs, and mortality every day for 14 days. Body weights were also recorded at time zero (prior to administration), on day 7, and on the 14th day.

2.8.2. Lethal Toxicity Neutralization and Evaluation

The lethal toxicity was evaluated as per the technique proposed by Theakston and Reid [33]. For the determination of median lethal dose (LD_{50}), different doses of krait venom in 200 µL of saline were administered to the mice by intraperitoneal route. After 24 h, deaths were counted and the Meier and Theakston [31] procedure was utilized to quantify lethal toxicity. Mice used for lethal toxicity neutralization studies were divided into four groups. Control mice were injected with saline alone (Group I). For inhibition study, 3 LD_{50} of venom was selected as challenging dose. Group II mice were injected with 3LD_{50} of venom. Groups III and IV received venom pre-incubated for 30 min at 37 °C with 2 concentrations of seed extracts (krait venom: seed extract corresponded to 1:10 and 1:20 (*w*/*w*), respectively). The survival times were recorded.

2.8.3. Neutralization of Myotoxicity

Histopathological studies were conducted to analyze the neutralization potential of plant extracts against venom-induced myotoxicity. The mice were injected with 50 µL (5 µg) of the samples in saline by intramuscular route. After 180 min, histological studies of thigh muscle tissue were conducted by dissecting it and fixing it in Bouin's reagent overnight. Later, a mixture of alcohol/chloroform was utilized to dehydrate the samples for 24 h. After staining, sections were observed with the help of microscope. For inhibition investigations, venom was pre-incubated aqueous ethanolic seed extract.

2.9. Statistical Analysis

All the measurements were taken 3 times. The independent student's *t*-test analysis was adopted and was performed using SPSS software 20.

3. Results

3.1. Analysis of Phytochemicals from A. catechu Seeds

Two solvents were utilized to extract the phytochemicals from the *A. catechu* seeds. The percent yield of the extract (*w*/*w*) was found to be 4.113 and 7.484 g for the ethyl acetate and aqueous ethanolic extracts, respectively. The assessment of the phytocompounds showed the presence of phenols, steroids, tannins, glycosides, and terpenoids in both the aqueous ethanolic and ethyl acetate seed extracts.

3.2. In Vitro Enzyme Assay and Neutralization Studies

The protein concentration of the 0.2% venom was 260 µg/mL. The specific activities of PLA_2, hyaluronidase, and protease of *B. caeruleus* venom were 2.10 ± 1.44 U/mg, 1.13 × 10^{-5} ± 0.33 TRU/min/mg, and 1.5 × 10^{-6} ± 0.015 µmol/h/mg, respectively. The anti-venom activity of *A. catechu* seeds against *B. caerules* venom was determined by in vitro and ex vivo investigations. The enzyme activity was calculated by percentage by considering the krait venom activity alone as 100%. The enzyme inhibition studies revealed that both the extracts at 100 µg/mL completely inhibited the hyaluronidase activity, whereas at 50 µg/mL, hyaluronidase inhibitions of 80 and 100% were obtained for ethyl acetate and aqueous ethanol extract, respectively. The phospholipase inhibition studies revealed that both the extracts inhibited the enzyme. However, protease neutralization was only observed in the aqueous ethanolic extract at a 0.002 g/mL concentration. The IC_{50} values of the aqueous ethanolic extract were lower than those of the ethyl acetate extract, which were 0.001 g/mL and 0.006 g/mL for hyaluronidase and phospholipase A_2, respectively.

3.3. Inhibition of Pharmacological Activity

The Aα of the fibrinogen was hydrolyzed by 0.1% krait venom. At a 1:10 ratio of krait venom to seed extract (*w*/*w*), fibrinogen degradation was completely neutralized (Figure 1). The indirect hemolytic assay for PLA_2 action was studied. A total of 1 µg krait venom was noted as the dose for the minimum hemolytic halo (MHH), which produced an 11 mm clear zone in the hemolytic gel plate. The aqueous ethanolic extract totally deactivated hemolytic action at 1:100 (*w*/*w*) venom: extract (Figure 2). However, a reduction in diameter by 7 mm was observed in the ethyl acetate extract at the same concentration (Figure 2).

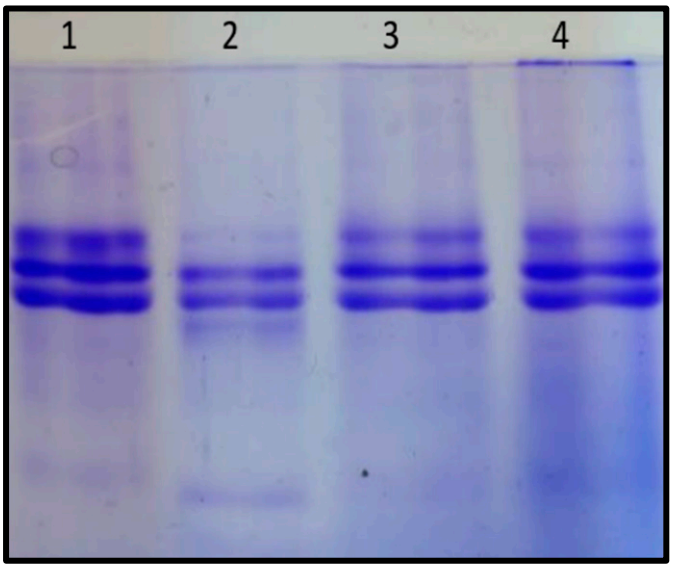

Figure 1. Neutralization of fibrinogenolytic action of *B. caeruleus* venom by *A. catechu* seed extracts. Lane 1—fibrinogen control (without the venom), Lane 2—venom incubated with fibrinogen alone, Lane 3—ethyl acetate extract + fibrinogen + venom, and Lane 4—aqueous ethanolic extract + fibrinogen + venom.

3.4. Acute Toxicological Studies Using Chick Embryo Model

The toxicological studies were carried out on a six-day-old chick embryo possessing a vascularized yolk sac as well as a primitive embryonic heart. The aqueous ethanolic seed extract (100 µg/0.2 mL) was checked for toxic effects. The chick embryo grew normally and thus the seed extract was not toxic (Figure 3). The LD_{50} value for *B. caeruleus* venom was determined to be 4.9 µg/egg. For the neutralization assessments, a $3LD_{50}$ of venom was selected as the challenging dose. The plant extract exhibited promising inhibition

by extending the survivability of the eggs. When the challenging dose of venom was injected first followed by the extract, three embryos survived up to 2 h and the remaining embryos for 4 h. However, all the embryos survived when the challenging dose of venom was pre-incubated with the seed extract of either 1:10 or 1:20 krait venom: seed extract (*w*/*v*). Thus, the experiment indicates the anti-venom activity of this seed extract against *B. caeruleus* venom (Figure 4; Table 1).

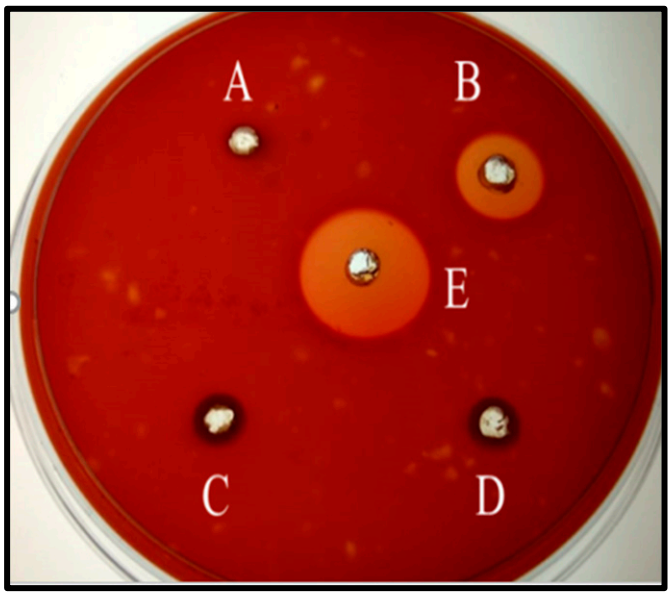

Figure 2. Neutralization of indirect hemolytic activity of *B. caeruleus* venom by *A. catechu* seed extracts. Well A: ethyl acetate extract (EA), well B: EA +Venom, well C: aqueous ethanolic extract (AE), well D: AE +Venom, and Well E: Venom.

Figure 3. Acute toxicity studies of *A. catechu* using six-day-old chick embryo.

3.5. Acute Toxicological Studies Using Murine Model

3.5.1. Acute Oral Toxicity of the *A. catechu* Extracts

Ethical permission (DSCP/DST/IAEC/06/16-17) and IAEC/NCP/92/2015 acting as ethical committee approval were granted before the investigation. In this investigation, no toxic effects, abnormal behavior, and/or pharmacological symptoms were noticed in the

mice dosed with 100 µg/kg of *A. catechu* aqueous alcohol during the assessment period. In addition, all the mice grew normally and gained weight normally during the study period of 14 days.

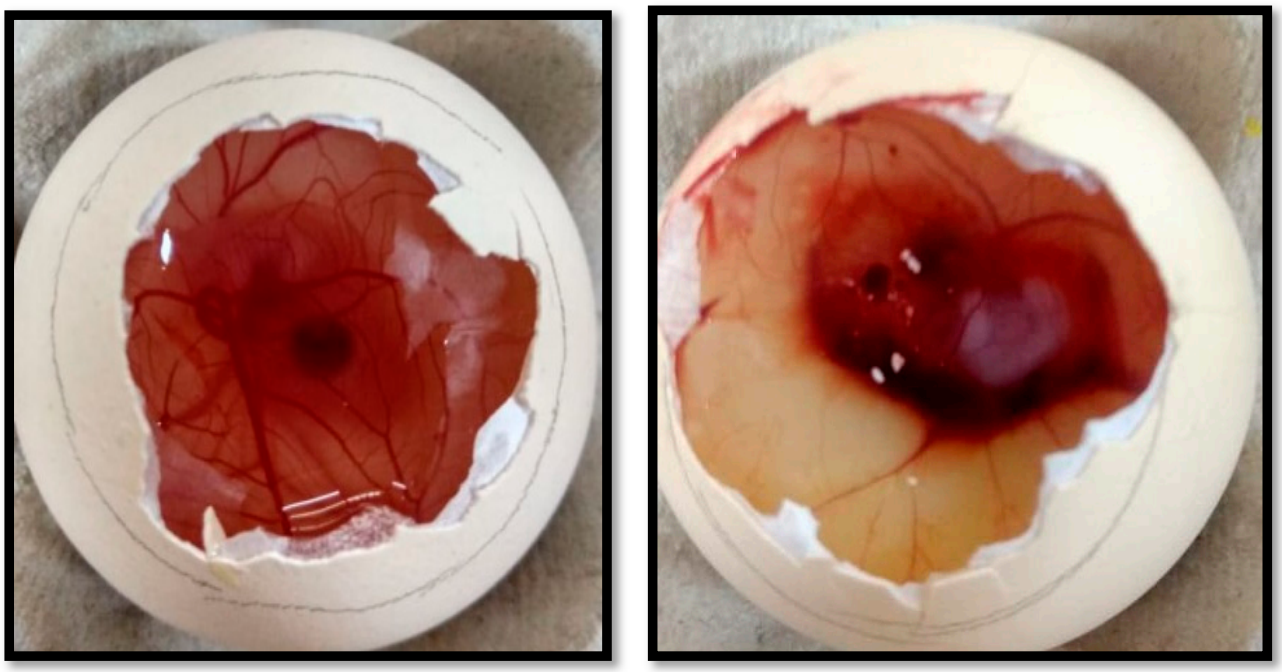

Figure 4. Anti-venom potential of *A. catechu* aqueous ethanol extract against *B. caeruleus* venom using chick embryo model. (**Left**)—normal egg with clear vasculature. (**Right**)—immobile dead egg without clear vasculature.

Table 1. Lethal toxicity (LD_{50}) determination of *B. caeruleus* venom in chick embryo model with saline, venom, and venom: extract (1:10; 1:20 *w*/*w*).

Venom/Extract Concentration	Time of Death
Saline (Group 1)	24 h
Venom alone (3 × LD_{50}) (Group 2)	2–4 h
Venom: extract (1:10 *w*/*w*) (Group 3)	24 h
Venom: extract (1:20 *w*/*w*) (Group 4)	24 h

3.5.2. Lethality Analysis and Neutralization

The lethal toxicity studies were conducted using murine models. To determine the LD_{50} value, various amounts of *B. caeruleus* venom were injected intraperitoneally as represented in Table 2. Various concentrations of *B. caeruleus* venom, i.e., 2, 4, 6, 8, and 10 µg, were injected to estimate the lethal dose, which was calculated as 0.1927 µg/kg body weight of the mice. Table 3 shows the neutralization of the challenging dose of krait venom (3 LD_{50}) by *A. catechu* aqueous alcohol extract in the murine models. The survival period of mice was 24h when saline was utilized alone for the injection, whereas the mice survived for 25 min when they were administered the extract pre-incubated with krait venom alone (3LD_{50}). However, 30 and 35 min were the survival times when the venom was pre-incubated differently with 1:10 and 1:20 ratios (*w*/*w*, venom: aqueous alcohol seed extract), respectively.

Table 2. Lethal toxicity (LD_{50}) quantification of *B. caeruleus* venom in murine models.

Group	Venom in µg per /25–27 g of Murine Body Weight	Mice Dead Out of 5	Mice Survived
Saline	-	0	100%
Group I	2 µg	1	80%
Group II	4 µg	2	60%
Group III	6 µg	2	60%
Group IV	8 µg	3	40%
Group V	10 µg	5	0%

Table 3. Deactivation of challenging dose of *B. caeruleus* venom (3 LD_{50}) by *A. catechu* aqueous alcohol extract in murine models.

Venom/Extract Concentration	Time of Death
Saline (Group 1)	24 h
Venom alone (3 × LD_{50}) (Group 2)	25 min
Venom: extract (1:10 *w*/*w*) (Group 3)	30 min
Venom: extract (1:20 *w*/*w*) (Group 4)	35 min

3.5.3. Neutralization of Myotoxicity

For the determination of the myotoxicity of the venom, 5 µg of krait venom was injected intramuscularly. Three hours after the venom administration using diethyl ether, the mice were euthanized and their muscle sections were excised and stained. Moderate edema was seen in between the muscle fibers in the microscopic examination. Minimal edema was observed when the aqueous ethanolic seed extract was injected (Figure 5).

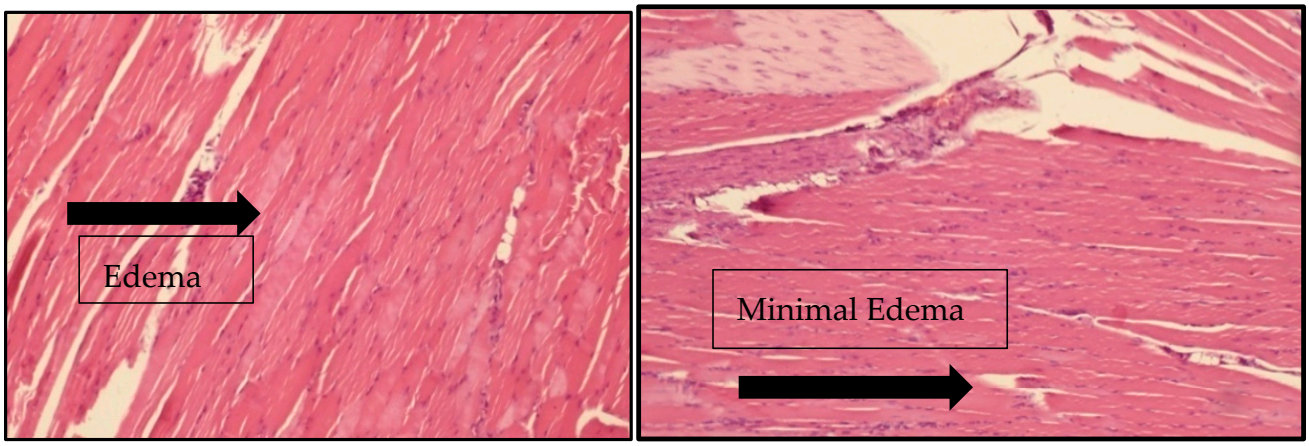

Figure 5. Neutralization of *B. caeruleus* venom's myotoxicity via aqueous ethanolic extracts of *A. catechu* plant.

4. Discussion

Snake bites are among the major health issues in India, causing deaths especially in rural areas [3]. The victims of snakebites have been treated with plant extracts for a very long time; even now, the search for medicinal plants that can cost-effectively neutralize the effects of snake venom continues. In this study, the activities of protease, hyaluronidase, and phospholipase A_2, which are important constituents of *B. caeruleus* venom, were evaluated. These enzymes are responsible for early envenomation reactions, such as tissue degradation, inflammation, etc., resulting in the release of other toxic substances present in

the venom. In these conditions, the anti-venom compounds may fail to halt and deactivate local tissue damage [7,34].

The anti-venom potential of *A. catechu* plant was evaluated, and neutralization studies were conducted with the seed extracts of different concentrations. It was observed that both ethyl acetate and the aqueous ethanolic extracts neutralized hyaluronidase and PLA_2 activities. However, the protease activity was inhibited only by the aqueous ethanol extract. At low concentrations, both extracts of *A. catechu* inhibited the hyaluronidase action. This inhibition can stop the release of other toxic substances and thus prevent the negative effects of the krait venom. The aqueous ethanol seed extract was active against the venom enzymes in vitro. Similarly, the *A. tetracantha* leaf extract inhibited the effect of toxic enzymes such as the hyaluronidase, phosphomonoesterase, and acetylcholinesterase from krait and *V. russelli* venoms [35,36]. Likewise, the leaf extracts of *C. serratum* exhibited promising inhibition against the venom enzymes of *Daboia russelii* and the krait [37]. The inhibition of krait venom by *A. catechu* seeds may be ascribed to the phytochemicals such as the phenols, steroids, tannins, glycosides, and terpenoids present within them. Baggai et al. [38] suggested an interaction between bioactive substances and calcium ions, which are a vital cofactor of PLA_2.

The fibrinogenolytic action of *B. caeruleus* venom was determined on 12% agarose gel, where the venom degraded the Aα band of the fibrinogen. The fibrinogenolytic activity caused by the venom was attributed to various types of proteases present in the venom. The 1:10 (*w*/*w*) ratio (venom: seed extract) was observed as an effective concentration. Similarly, the fibrinogenolytic action of the krait venom was totally prevented by the bioactive compounds in the *A. tetracantha* extracts [36]. This investigation was authenticated by the findings of Baggai et al. [38], where the phytosubstances available in both aqueous and crude methanolic extracts of *Tamarindusindica* seeds stopped the hydrolysis of fibrinogen's Aα and Bβ chains. *Clerodendrum serratum* extracts also exhibited promising inhibition of *B. caeruleus* and *Daboia russelii* venom-induced fibrinogenolytic activity [37]. In contrast, the degradation of the human fibrinogen Bβ chain was stopped by the phytocompounds present in the seeds of *Tamarindus indica* [8]. Thus, the *A. catechu* seeds exhibited promising inhibition of *B. caeruleus* venom-induced fibrinogenolytic activity.

The indirect hemolytic activity was studied by investigating the phospholipid hydrolysis of the krait venom by PLA_2. The 1 µg sample of krait venom formed an 11 mm hemolytic halo and the aqueous ethanolic extract totally neutralized hemolytic action at 1:100 (*w*/*w*, krait venom: seed extract). However, a reduction in diameter (7 mm) was observed in the ethyl acetate extract at the same concentration. Previous studies have showed that 15 µg of cobra venom and 10 µg of *B. caeruleus* venom produced an MHH of 11 mm, which was neutralized by 0.15 mg and 0.18 mg of *Mucuna pruriens* extract, respectively, on agarose–sheep–erythrocyte gel plates [39]. *Leucas aspera* extract also deactivated all other actions caused by the *N. naja* venom, but the activity of PLA_2 was not stopped totally [40]. The aqueous fraction of the methanolic extract from the *Tamarindus indica* seeds stopped the hemolysis and hemorrhagic action caused by the crude venom of *Bitis arietans* [38]. The inhibition of hemorrhaging resulting from *B. jararaca* venom was attributed to the catechin of tannins present in the butanolic *C. urucurana* extract [41]. However, the neutralization of the hemorrhagic effect of Malaysian pit viper venom by the butanolic *Eclipta* plant extract was ascribed to the formation of hydrogen bonds between the hydroxyl groups of the phenols and carbonyl functional groups of the peptide bonds present in the proteins/enzymes of the venom [42]. Ushanandini et al. [8] also reported a neutralization of indirect hemolysis resulting from *V. russelli* venom via the seed extract of *Tamarindus indica*. Thus, the bioactive phytochemicals of *A. catechu* seeds inhibit hydrolytic enzymes at a low dose along with other hemorrhagic toxic substances responsible for venom's negative effects.

A. catechu aqueous ethanol extracts were found to be nontoxic in both models. Sari et al. [43] reported similar results with the same plant but in rats. Likewise, the ethyl acetate extract from the *A. tetracantha* leaf stopped the LD50 of venom in both mice

and embryonic models [36]. The neutralization of toxicity was performed by selecting a threefold higher concentration of venom (3 × LD_{50}, challenging dose). The chick embryos injected with pre- incubated venom survived for 24 h, similar to that of saline. This result indicates the neutralizing potential of the seed extract. The lethality of the venom of the saw-scaled viper and krait was neutralized by a cocktail prepared by mixing four plant extracts [44]. Current studies prefer the use of chick embryo models for venom studies, wherein the LD_{50} value is evaluated for each batch. The major advantages associated with the chick embryo model is that it requires less venom to calculate the LD_{50} values and ethical approval is not needed.

Myotoxicity was induced by two different types of PLA_2 from the venom: one type constitutes the neurotoxic PLA_2 and the other category constitutes non-neurotoxic types of PLA_2. The higher level of PLA_2 from the krait venom shows a higher degree of myotoxic action linked with the venom. This was importantly neutralized by the seed extract under study. Janardhan et al. [36] and Ushanandini et al. [8] reported similar neutralization of the myotoxic properties of krait and *V. russelli* venom by *A. tetracantha* leaf and *Tamarindus indica* seed extracts, respectively. The myotoxic effects caused by the Russell's viper and krait venom were also stopped with an herbal cocktail prepared by utilizing four medicinal plants [44].

5. Conclusions

The toxic enzyme inhibition of *B. caeruleus* venom and pharmacological actions exhibited by the *A. catechu* seed extract were observed. This can be ascribed to the phytosubstances present in the *A. catechu* seeds that may act as enzymatic inhibitors/modulators and/or activators. Further, the purification and characterization of the *A. catechu* extracts would confirm whether a single phytocompound or a synergistic action of phytocompounds is responsible for the anti-venom aspects. Academicians must work synergistically with commercial companies to develop a new first-aid medicine from *A. catechu* seeds to combat snake bites.

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References

1. Warrell, D.A. Snake bite. *Lancet* **2010**, *375*, 77–88. [CrossRef]
2. Snakeman, W.Z. *The Story of a Naturalist*; Penguin Books Ltd.: London, UK, 1990; p. 192.
3. Gupta, Y.K.; Peshin, S.S. Snake bite in India: Current scenario of an old problem. *J. Clin. Toxicol.* **2014**, *4*, 182. [CrossRef]
4. Alam, M.I. Inhibition of toxic effects of viper and cobra venom by Indian medicinal plants. *Pharmacol. Pharm.* **2014**, *5*, 828–837. [CrossRef]
5. Girish, K.S.; Mohanakumari, H.P.; Nagaraju, S.; Vishwanath, B.S.; Kemparaju, K. Hyaluronidase and protease activities from Indian snake venoms: Neutralization by *Mimosa pudica* root extract. *Fitoterapia* **2004**, *75*, 378–380. [CrossRef] [PubMed]

6. Yingprasertchai, S.; Bunyasrisawat, S.; Ratanabanangkoon, K. Hyaluronidase inhibitors (sodium cromoglycate and sodium auro-thiomalate) reduce the local tissue damage and prolong the survival time of mice injected with *Naja kaouthia* and *Calloselasma rhodostoma* venoms. *Toxicon* **2003**, *42*, 635–646. [CrossRef]
7. Melo, P.A.; Ownby, C.L. Ability of wedelolactone, heparin, and para-bromophenacyl bromide to antagonize the myotoxic effects of two crotaline venoms and their PLA2 myotoxins. *Toxicon* **1999**, *37*, 199–215. [CrossRef]
8. Ushanandini, S.; Nagaraju, S.; Harish, K.; Vedavathi, M.; Machiah, D.K.; Kemparaju, K.; Vishwanath, B.S.; Gowda, T.V.; Girish, K.S. The anti-snake venom properties of *Tamarindus indica* (Leguminosae) seed extract. *Phytother. Res.* **2006**, *20*, 851–858. [CrossRef]
9. Cannon, R.; Ruha, A.M.; Kashani, J. Acute hypersensitivity reactions associated with administration of crotalidae polyvalent immune Fab antivenom. *Ann. Emerg. Med.* **2008**, *51*, 407–411. [CrossRef]
10. Theakston, R.D.; Phillips, R.E.; Warrell, D.A.; Galagedera, Y.; Abeysekera, D.T.; Dissanayaka, P.; de Silva, A.; Aloysius, D.J. Envenoming by the common krait (*Bungaruscaeruleus*) and Sri Lankan cobra (*Najanajanaja*): Efficacy and complications of therapy with Haffkineantivenom. *Trans. R. Soc. Trop. Med. Hyg.* **1990**, *84*, 301–308. [CrossRef]
11. Alam, M.I.; Gomes, A. Snake venom neutralization by Indian medicinal plants (*Vitexnegundo* and *Emblicaofficinalis*) root extracts. *J. Ethnopharmacol.* **2003**, *86*, 75–80. [CrossRef]
12. Soares, A.M.; Ticli, F.K.; Marcussi, S.; Lourenço, M.V.; Januário, A.H.; Sampaio, S.V.; Giglio, J.R.; Lomonte, B.; Pereira, P.S. Medicinal plants with inhibitory properties against snake venoms. *Curr. Med. Chem.* **2005**, *12*, 2625–2641. [CrossRef] [PubMed]
13. Makhija, I.K.; Khamar, D. Anti-snake venom properties of medicinal plants. *Der. Pharm. Lett.* **2010**, *2*, 399–411.
14. Geetha, R.M. Medicinal plants viz a viz indigenous knowledge among the tribals of Pachamalai hills. *Indian J. Tradit. Knowl.* **2010**, *9*, 209–215.
15. Shwetha, V.; Veen, S.M.; Zameer, G.F.; Niyonzima, N.F.; More, S.S. In vitro neutralization of *Najanaja* venom enzymes by folk medicinal plant extracts. *J. Biol. Act. Prod. Nat.* **2019**, *9*, 278–288. [CrossRef]
16. Meenatchisundaram, S.; Prajish, G.; Parameswari, S.T.; Michael, A. Studies on anti-venom activity of *Andrographispaniculata* and *Aristolochiaindica* plant extracts against *Echiscarinatus* venom. *Internet J. Toxicol.* **2008**, *6*, 1–7. [CrossRef]
17. Umdor, M. Indigenous practice on protection of *Areca catechu* Linn. Seedlings—A case study in Meghalaya. *Indian J. Tradit. Knowl.* **2004**, *3*, 253–255.
18. Amudhan, M.S.; Begum, V.H.; Hebbar, K.B. A review on phytochemical and pharmacological potential of *Areca catechu* L. seed. *Int. J. Pharm. Sci. Res.* **2012**, *3*, 4151–4157. [CrossRef]
19. Badami, S.; Rai, S.R.; Suresh, B. In-vitro antioxidant properties of Indian traditional paan and its ingredients. *Indian J. Tradit. Knowl.* **2004**, *3*, 187–191.
20. Bhat, S.K.; Sarpangala, M.; Ashwin, D. Antilipidemic activity of arecanut, *Areca catechu* L.: A valuable herbal medicine. *Int. J. Herb. Med.* **2017**, *5*, 35–38.
21. Alcaraz, M.J.; Hoult, J.R. Actions of flavonoids and the novel anti-inflammatory flavone, hypolaetin-8-glucoside, on prostaglandin biosynthesis and inactivation. *Biochem. Pharmacol.* **1985**, *15*, 2477–2482. [CrossRef]
22. Lowry, O.H.; Rosenberg, W.J.; Farr, A.L.; Randell, R.J. Quantitation of protein using FolinCiocalteu reagent. *J. Biol. Chem.* **1951**, *193*, 265–275. [CrossRef]
23. Harborne, J.B. Methods of Plant Analysis. In *Phytochemical Methods*; Chapman and Hall: London, UK, 1973; p. 132.
24. Pukrittayakamee, S.; Warrell, D.A.; Desakorn, V.; McMichael, A.J.; White, N.J.; Bunnag, D. The hyaluronidase activities of some southeast Asian snake venoms. *Toxicon* **1988**, *26*, 629–637. [CrossRef]
25. Marinetti, G.V. The action of phospholipase A on lipoproteins. *Biochim. Biophys. Acta* **1965**, *98*, 554–565. [CrossRef]
26. Greenberg, D.M. Plant Proteolytic Enzymes. In *Methods in Enzymology*; Colowick, S.P., Kalpan, N.O., Eds.; Academic Press Inc.: New York, NY, USA, 1955; pp. 54–64.
27. Gutierrez, J.; Avila, C.; Rojas, E.; Cerdas, L. An alternative in vitro method for testing the potency of the polyvalent antivenom produced in Costa Rica. *Toxicon* **1988**, *26*, 411–413. [CrossRef]
28. Ouyang, C.; Teng, C.M. Fibrinogenolytic enzymes of *Trimeresurusmucrosquamatus* venom. *Biochim. Biophys. Acta* **1976**, *420*, 298–308. [CrossRef]
29. Sells, P.G.; Ioannou, P.; Theakston, R.D. A humane alternative to the measurement of the lethal effects (LD50) of non-neurotoxic venoms using hens' eggs. *Toxicon* **1998**, *36*, 985–991. [CrossRef]
30. van der Valk, T.; van der Meijden, A. Toxicity of scorpion venom in chick embryo and meal worm assay depending on the use of the soluble fraction versus the whole venom. *Toxicon* **2014**, *88*, 38–43. [CrossRef]
31. Meier, J.; Theakston, R.D. Approximate LD50 determination of snake venoms using eight to ten experimental animals. *Toxicon* **1986**, *24*, 395–401. [CrossRef]
32. Vineetha, M.S.; Bhavya, J.; Mirjakar, K.M.; More, S.S. In vitro evaluation of active phytochemicals from *Tabernaemontanaalternifolia* (Roxb) root against the *Najanaja* and *Echiscarinatus* Indian snake venom. *J. Biol. Act. Prod. Nat.* **2014**, *4*, 286–294. [CrossRef]
33. Theakston, R.D.; Reid, H.A. Development of simple standard assay procedures for the characterization of snake venoms. *Bull. World Health Organ.* **1983**, *61*, 949–956.
34. Krishnan, S.A.; Dileepkumar, R.; Nair, A.S.; Oommen, O.V. Studies on neutralizing effect of *Ophiorrhizamungos* root extract against *Daboiarusselii* venom. *J. Ethnopharmacol.* **2014**, *151*, 543–547. [CrossRef] [PubMed]

35. Janardhan, B.; Shrikanth, V.M.; Mirajkar, K.K.; More, S.S. In vitro screening and evaluation of anti-venom phytochemicals from *Azimatetracantha* Lam. leaves against *Bungaruscaeruleus* and *Viperarusselli*. *J. Venom. Anim. Toxins Incl. Trop. Dis.* **2014**, *20*, 12. [CrossRef] [PubMed]
36. Janardhan, B.; Shrikanth, V.M.; More, V.S.; Melappa, G.; Zameer, F.; More, S.S. *Bungaruscaeruleus* venom neutralization activity of *Azimatetracantha* Lam. Extract. *Heliyon* **2019**, *5*, e02163. [CrossRef] [PubMed]
37. Vasudev, S.; More, S.S.; Annappa, G.S.; More, V.S. Anti-snake venom potential of *Clerodendrumserratum* extracts on *Bungaruscaeruleus* and *Daboiarusselii* venom. *Bangladesh J. Pharmacol.* **2018**, *13*, 187–191. [CrossRef]
38. Baggai, B.I.; Yusuf, P.O.; Alloh, F.T. Inhibitory studies of *Tamarindusindica* seed extract and fractions on hematological activities of *Bitisarietans* venom. *J. Adv. Biol. Biotechnol.* **2020**, *23*, 48–57. [CrossRef]
39. Meenatchisundaram, S.; Michael, A. Antitoxin activity of *Mucunapruriens* aqueous extracts against *Cobra* and krait venom by *in vivo* and *in vitro* methods. *Int. J. Pharm. Tech. Res.* **2010**, *2*, 870–874.
40. Gopi, K.; Renu, K.; Jayaraman, G. Inhibition of *Najanaja* venom enzymes by the methanolic extract of *Leucasaspera* and its chemical profile by GC–MS. *Toxicol. Rep.* **2014**, *1*, 667–673. [CrossRef]
41. Esmeraldino, L.E.; Souza, A.M.; Sampaio, S.V. Evaluation of the effect of aqueous extract of *Croton urucurana* Baillon (Euphorbiaceae) on the hemorrhagic activity induced by the venom of *Bothropsjararaca*, using new techniques to quantify hemorrhagic activity in rat skin. *Phytomedicine* **2005**, *12*, 570–576. [CrossRef]
42. Pithayanukul, P.; Laovachirasuwan, S.; Bavovada, R.; Pakmanee, N.; Suttisri, R. Anti-venom potential of butanolic extract of *Ecliptaprostrata* against Malayan pit viper venom. *J. Ethnopharmacol.* **2004**, *90*, 347–352. [CrossRef]
43. Sari, L.M.; Suyatna, F.D.; Utami, S.; Chairul, C.; Subita, G.P.; Auerkauri, E.I. Acute oral toxicity study of *Areca catechu* Linn. aqueous extract in sprague-dawley rats. *Asian J. Pharm. Clin. Res.* **2014**, *7*, 20–22.
44. Vasudev, S.; More, V.S.; Ananthraju, K.S.; More, S.S. Potential of herbal cocktail of medicinal plant extracts against 'big four' snake venoms from India. *J. Ayurveda Integr. Med.* **2021**, *12*, 458–464. [CrossRef] [PubMed]