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Inhibitory Studies of *Tamarindus indica* Seed Extract and Fractions on Hematological Activities of *Bitis arietans* Venom

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Authors' contributions

This work was carried out in collaboration among all authors. Author BIB designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author POY managed the analyses of the study. Author FTA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Envenomation is a serious public health and occupational hazard in tropical and subtropical countries. Antivenom serum treatment has been related to extreme hypersensitivity, high cost, and inaccessibility. Consequently, therapeutic plants have been investigated to give an alternative treatment. The aim of this study was to evaluate the ability of the methanolic extract and fractions of *Tamarindus indica* seeds to neutralize the hemolytic, anticoagulant and hemorrhagic activities of *Bitis arietans* venom. The powdered form of *Tamarindus indica* seed was extracted using methanol. The methanol extract was further fractionated using butanol solvent. The aqueous fraction of the methanolic extract inhibited hemolysis caused by crude venom by 95.5%, while methanolic extract significantly reduced the anticoagulant time of crude venom to 10.2%. The *in vivo* hemorrhagic activity of the crude venom of *Bitis arietans* was neutralized by both the crude methanolic extract and the subsequent two fractions of *Tamarindus indica* seed. However, the crude methanolic extract was found to be the most active by reducing the hemorrhage diameter caused by crude venom to 39%. The findings of this present research work demonstrated a very

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good antivenom properties of the seeds of *Tamarindus indica,* which suggests that it could be a potential source of effective, and safe compounds with inhibitory activities against *Bitis arietans* envenomation.

Keywords: Antivenom; Bitis arietans; Tamarindus indica; hemolytic; anticoagulant; hemorrhagic.

ABBREVIATIONS

ml : Milliliter
mM : Millimolar
mg : Milligram
PLA_2 : Phospholipase A_2
CP : Citrated plasma
SVMP : Snake Venom Metalloprotease
RBC : Red Blood Cell
Aqu : Aqueous fraction
Butanol : Butanol fraction
Meth : Methanol extract
V : Venom
MLD : Mean Lesion Diameter
WHO : World Health Organization

1. INTRODUCTION

Snakebite is an important public health and occupational hazard often faced by farmers, farm laborers, hunters and nomads of tropical and subtropical countries present in Asia, Africa and South America [1]. Envenomation by venomous snake is a common acute life-threatening and time-limiting medical emergency. Globally, approximately 5.4 million snakebites occur each year, resulting in 1.8 to 2.7 million cases of envenomings, with mortality ranging from 81 410 to 137 880 deaths and around three times as many amputations and other permanent disabilities each year [2,3]. The extent of snakebite globally, resulted the World Health Organization (WHO) declaration of snakebite as a neglected tropical disease in 2009. However, it was removed in 2013, and re-declared as category A on the 9th of June 2017 [4].

In Nigeria alone an estimate of 497 per 100,000 populations are bitten annually by venomous snakes. Nigeria is therefore reported to have one fifth of all West African region cases of snakebites [5]. However, an accurate measure of snakebites remains elusive as most victims approach traditional healers for aid and treatment. Snakebite is a major challenge that affects farmers, nomads and rural dwellers of all ages. Inadequate facilities at the primary health center (PHC), ignorance of conventional treatment of snakebite by medical doctors; further delays inappropriate treatment of victims leads to increased morbidity and mortality [6]. Treatment with antivenom serum remains the only specific therapy for snakebite envenomation. However, it is heterologous and therefore liable to cause adverse reactions, such as early anaphylactic, pyrogenic and delayed reactions [7]. Medicinal plants are rich sources of natural inhibitors that tend to reduce the pharmacological activities of various snake venom without resulting to any side effects [8].

Bitis arietans commonly known as Puff adder is a venomous snake thought to be the most common and widespread snake in Africa, occurring throughout the continent except in some forested regions and extreme deserts such as the Sahara. *Echis ocellatus* is responsible for most snakebite incidence in Nigeria. *Bitis arietans* is third in terms of medical importance [9]. This is due to a combination of factors, including its wide distribution, common occurrence, large size, potent venom that is produced in large amounts, long fangs, and sitting quietly when approached. The venom has cytotoxic effects and is one of the most toxic of any vipers based on LD_{50} [10]. Proteomic analyses has shown that hemorrghagins and hemolysins such as phospholipases A_2, metalloproteases, serine proteases, disintegrins, L-amino acid oxidase, Kunitz inhibitors, cystatins and C-type lectins are present in *Bitis arietans* venom [11].

Tamarindus indica of the Fabaceae, subfamily Caesalpinioideae, is an important food in the tropics. It is a multipurpose tree of which almost every part finds at least some use either nutritional or medicinal [12]. Asian countries such as India and Myanmar have used *Tamarindus indica* for the treatment of snakebite and can be found in their traditional medicine book [13,14]. Therefore it is necessary to carry out scientific studies that demonstrate the use of the extract of these seeds in the treatment of snake bites. It has been noted that several anti snake venom activities are known to contain phenolic compounds [15].

Tamarind seeds are reported to contain phenolic antioxidants, procyanidins, epicatechin, taxifolin

and eriodictyol. This plant is readily available; therefore this will resolve the problems of accessibility, cost, and storage of antivenom immunotherapy treatment encountered by patients. Hence, the proper utilization of the antivenom properties of *T. indica* seed by researchers, could help provide an alternative treatment that would curb the challenges of envenomation in rural areas.

The aim of this study was to determine the inhibitory effect of methanolic extract and fractions of *Tamarindus indica* seed on hemolytic, haemorrhagic and anticoagulant activities of crude venom of *Bitis arietans.*

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All solvents and All other chemicals: Sodium dihydrogen phosphate, Hydrogen disodium phosphate, sodium chloride, calcium chloride glycerol, Sodium hydroxide, sodium citrate were all purchased from Sigma Chemical Co. St. Louis, England and were of analytical grade.

2.2 Venom Collection

Two adult *Bitis arietans* were sourced from Fitika village, Giwa Local Government of Kaduna state, and allowed to acclimatize at the Herpetarium, Department of Veterinary Pharmacology and Toxicology, Ahmadu Bello University Zaria. The venom from the *Bitis arietans* was milked as described by Markfarlane [16].

2.3 Experimental Animals

Male and female adult albino mice (20-30 g) of Swiss strain, were used for the research. The mice were procured from animal house of the Department of Pharmacology and Therapeutics Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria.

2.4 Preparation of Plant Extract

Tamarindus indica seed was obtained from Sabon-gari, Zaria, Kaduna State and then taken for identification at the Herbarium unit, with voucher number 602 at Department of Biological Sciences, Ahmadu Bello University Zaria. The dried seeds were crushed into fine powder.

Dried powdered seeds of *Tamarindus indica* (1.2 kg) was extracted for 75 hours using n-hexane, ethyl acetate, methanol solvent (2.5 L) successively by maceration following the procedure described by Sukito and Tachibana [17].

2.5 Fractionation of Extract of *Tamarindus indica* Seed

The methanol seed extract (138.25 g) was dissolved in water (750 ml) and placed in a separating funnel and then 1.25 L of butanol was added mixed thoroughly and allowed to stand for 24 hours. The butanol fraction was collected carefully subsequently the aqueous fraction. They were both dried using water bath at 40°c and then weighed. This procedure was carried out as described by Abubakar [18].

2.6 Hemolytic Assay of Crude Venom of *Bitis arietans*

The method described by Gomes and Pallabi [19] was adopted using bovine erythrocytes to determine hemolytic activity. Twenty milliliters (20 ml) of bovine blood was collected from Zango abattoir, Zaria, Kaduna State, using sodium citrate as anticoagulant. A Thermo scientific sorvall primo R benchtop centrifuge was used to centrifuge blood sample at 646 x g for 10 min and the plasma discarded. Five milliliters (5 ml) of normal saline was mixed with the packed cell layer and centrifuged at 646 x g for 10 min and the supernatant discarded. This procedure was repeated 10 times to obtain plasma-free packed cells. Venom solution (5 mg/ml) was mixed with 1 ml of 1% cell suspension in saline and the mixture was incubated at 37°C for 1 hr. the reaction was stopped by adding 3 ml of chilled phosphate buffer saline. Then centrifuged at 449 x g for 10 mins and absorbance was measured at 540 nm using a Spectronic M508 Spectrophotometer. For inhibitory studies 1ml venom solution, with 1 ml of 1% v/v cell suspension in saline was incubated with 1ml of different concentrations of plant extract fractions at 37°C for 1 hr. The reaction was also been stopped by adding 1ml of chilled phosphate buffer then centrifuged at 449 x g 10 min and absorbance was measured at 540 nm. The control sample consist of 1% cell suspension treated with 3 ml chilled water, was considered as 100% hemolysis. This assay was repeated three times in triplicates.

% Inhibition of hemolysis = 100 x [OD1-OD2/OD1] [20].

Where:

OD1 = Optical density of hypotonic saline solution alone
OD2 = Optical density of test sample.

2.7 Anticoagulant Activity Assay of Crude Venom of *Bitis arietans*

Bovine blood sample of 20 ml was collected in 3.8% sodium citrate, centrifuged at 701 x g and 4°C for 2 hours to obtain platelet-poor plasma. Coagulant activity was measured by the method of Condrea et al. [21] using Bovine citrate plasma (2.0 mg/ml). Fifty microliters of $CaCl_2$ (25 mM) was added then, 50 uL of venom was added to 0.2 ml of citrated plasma solution and clotting time was recorded at 37°C. For inhibitory studies, 0.2 ml of citrated plasma solution was added to 1ml of $CaCl_2$ (25 mM), then the venom alongside different concentrations of plant extract fraction. The mixture was incubated at 37°C for 2 hours and the clotting time was recorded [22]. This assay was carried out three times in triplicates.

2.8 Haemorrhagic Assay of Crude Venom of *Bitis arietans*

The method described by Omori-Satoh et al. [23] was employed. *Bitis arietans* venom (5 mg/ml) was used where 0.2 ml was injected sub-cutaneously into the backs of mice. The mice were sacrificed by cervical fracture after 60 min, and then skinned and the haemorrhagic foci was measured in mm. For inhibitory studies, the venom was injected alongside with different concentrations of plant extract and fractions into mice. The mice were sacrificed after 2 hours and the haemorrhagic foci was measured in mm as described by [23,24].

Thirty (30) mice were assembled and divided into 5 groups of 6 mice each. (Male and female mice were evenly distributed in each group)

Group 1: Untreated control was treated with normal saline.

Group 2: Treated control was treated with Crude venom alone.

Group 3: Treated with aqueous fraction and Crude Venom

Group 4: Treated with n-butanol fraction and Crude Venom

Group 5: Treated with methanolic extract and Crude Venom

2.9 Statistical Analysis

Experimental results were expressed as means ± SD. The data was analyzed by an analysis of variance (at $p<0.05$) and the means separated by Duncan's multiple range tests. All statistical analysis was performed using coupled Microsoft Excel Software and; Statistical Package for Social Sciences (SPSS) version 20.0.

3. RESULTS

3.1 *In vitro* Studies Activity of Solvent Fractions of *Tamarindus indica* Seed Extracts on Crude Venom of *Bitis arietans*

The hemolysis caused by distilled water was considered as 100% hemolysis of the RBC, which served as control. The hemolytic activity on RBC by the crude venom of *Bitis arietans* as shown in (Fig. 1) revealed a significantly high value of 65.8%±0.26. At the highest concentration of 10 $mgml^{-1}$ the crude methanolic extract, butanolic fraction and aqueous fraction of *T. indica* seed incubated with 5$mgml^{-1}$ of crude *B. arietans* venom significantly (at $p<0.05$) reduced hemolysis of RBC to 8.1%±2.51, 11.7%±2.84 and 4.5%±2.62 respectively. At the least concentration of 2.5 $mgml^{-1}$ the methanolic extract, butanolic fraction and aqueous fraction reduced hemolysis to 55.8%±1.85, 73.9%±4.56 and 59.4%±1.11 respectively, suggesting that the methanolic extract, aqueous fraction and butanolic fraction *T. indica* seed protected the red blood cells from being lysed by toxins such as PLA_2 present in *Bitis aritans* venom.

The anticoagulant activity was carried out and the coagulation time was monitored for 2 hours. The crude venom of *B. arietans* did not yield clot formation after 2 hours of incubation, and this was considered 100% anticoagulation time as shown in (Fig. 2). Aqueous fraction, methanolic extract and butanolic fraction significantly reduced the anticoagulant time to 16.70±1.88%, 10.21±0.62% and 47.53±1.61% respectively at 10 $mgml^{-1}$ concentration. While at the least concentration (2.5 $mgml^{-1}$), aqueous fraction, the methanolic extract and butanolic fraction also significantly reduced the anticoagulant time to 50.21±3.26%, 50.08±2.24% and 63.44±6.04% respectively, but not as compared to the highest concentration.

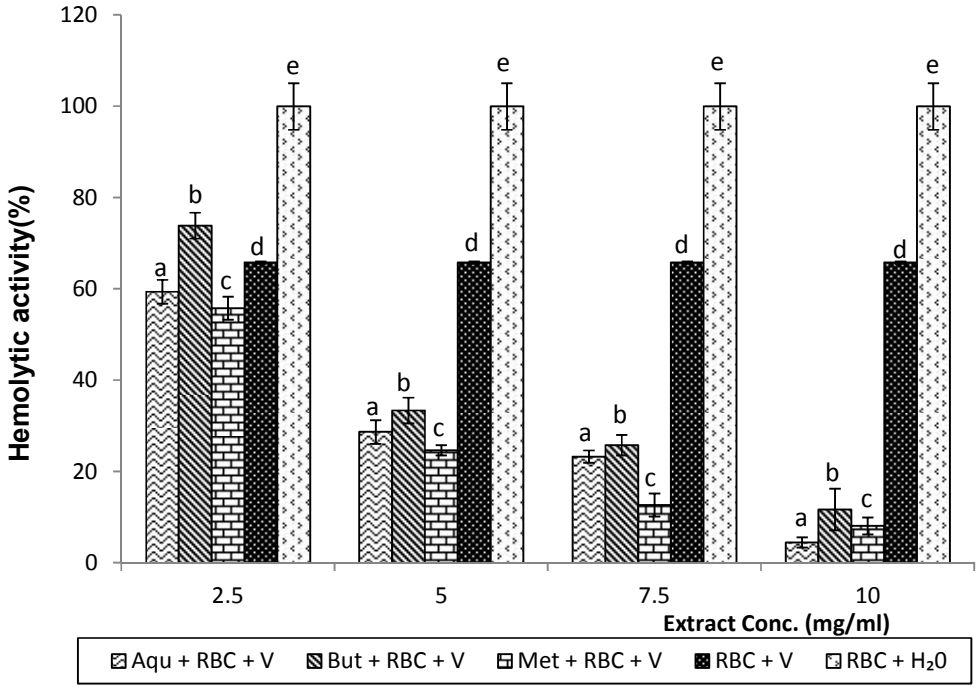

Fig. 1. Effect of *T. indica* seed extracts on the hemolytic properties of *B. arietans* venom
Bars Represent the Mean ±SD: with different superscripts are significantly different at P < 0.05 by Duncan's Multiple Range test

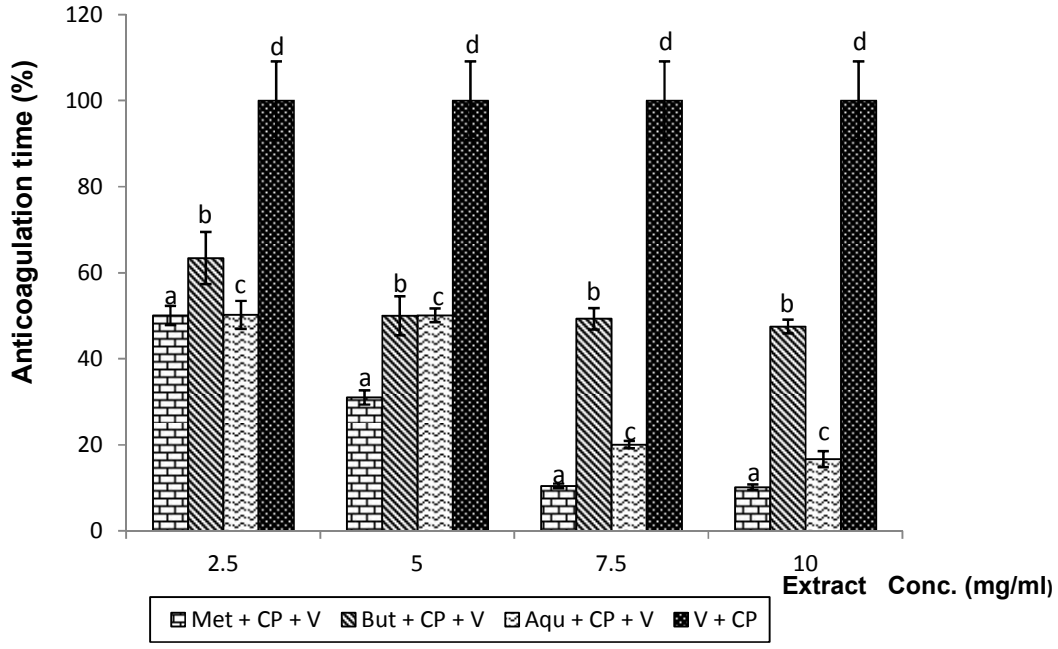

Fig. 2. Effect of *T. indica* seed extracts on the anticoagulant properties of *B. arietans* venom
Bars Represent the Mean ±SD: with different superscripts are significantly different at P < 0.05 by Duncan's Multiple Range test

3.2 *In vivo* Activity of Solvent Fractions of *Tamarindus indica* Seed Extracts on Crude Venom of *Bitis arietans*

The crude *B. arietans* venom resulted to an MLD of 32±3 mm, which was considered 100% hemorrhage. The methanolic extract, butanolic fraction, and aqueous fraction also significantly (at $p < 0.05$) reduce the diameter of lesion at high concentration to 39.1%±0.17, 40±0.1, and 42.9±0.2 respectively as shown in (Fig. 3). This is to suggest that the crude methanolic extract, butanolic fraction and aqueous fraction of *T. indica* seed were able to reduce degradation of the capillary vessel in mice induced by PLA_2 isoform and crude venom of *B arietans.*

4. DISCUSSION

The antihemolytic activity implies that fractions of *T. indica,* especially aqueous consist of bioactive compounds that interfere with the mechanism, in which haemolysins such as PLA_2 responsible for hemolysis present in the venom of *Bitis arietans,* interacts with phospholipids on cell membrane that results to hydrolysis of intact membrane releasing free fatty acids and lysopholipids [25,26,27].

Medicinal plants have been recorded to deactivate proteins and peptides because of diverse secondary metabolite like polyphenols, terpenoids, and flavonoids which have been reported to neutralize snake venom [28,29,30,31]. Similar results were also shown by the methanolic extract of *Indigofera pulchra* and *Aristolochia abida* plants against hemolysis caused by *Naja nigricollis* venom [18]. This work conforms to findings of Ushanandini et al. [32], who reported that crude ethanolic extracts of *T. indica* were found to neutralize the hemolytic activity of *Russelli* viper.

Anticoagulation and spontaneous bleeding are some of the hematological effects of viper envenomation in victims. These research study, revealed that treatment with the methanol crude extract and aqueous fraction neutralized the

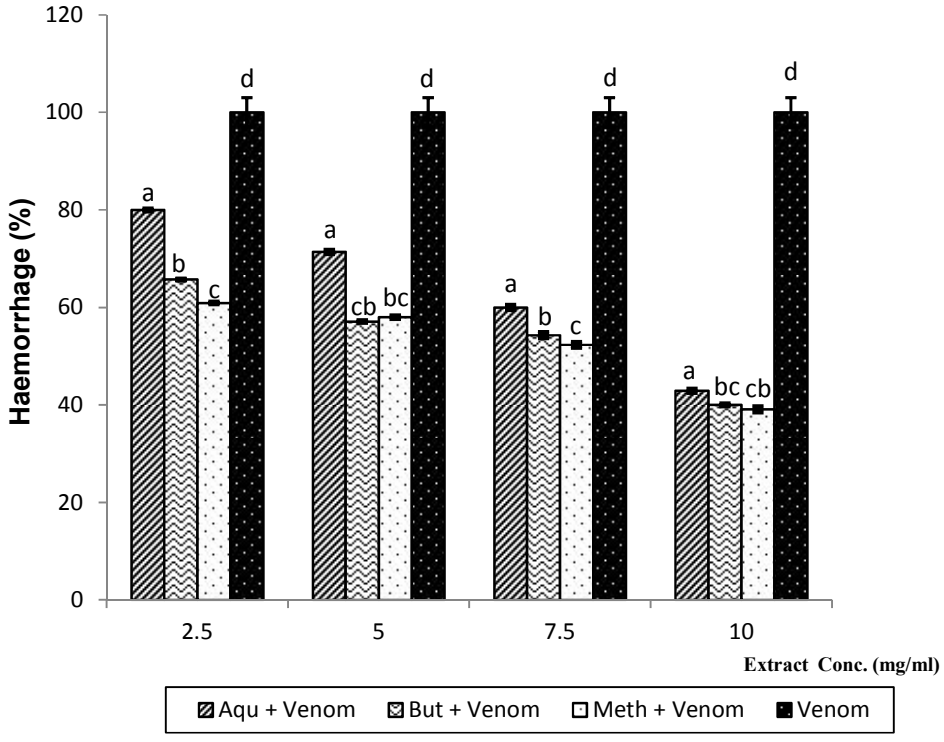

Fig. 3. Effect of *T. indica* seed extracts on the haemorrhagic properties of *B. arietans* venom

Bars Represent the Mean ±SD: with different superscripts are significantly different at P < 0.05 by Duncan's Multiple Range test

anticoagulant effect of crude venom of *B. arietans* in a concentration dependent manner, where an increase in extract concentration reflected a significant reduction in anticoagulant activity of *B. arietans* venom. These findings suggest that the aqueous fraction constitute of bioactive compounds that inhibits haemolysins and haemorrhagic toxins. PLA_2 is known to prevent the formation of the extrinsic tenase complex (TF, FVIIa and Ca^{2+}) and the activation of FX to FXa required for the formation of prothrombinase complex, a vital component of blood coagulation [33,34]. It is worth noting that the activation of FX and prothrombin are both totally dependent on the presence of phospholipid membrane [35,36,37]. Aside from PLA_2 in the venom of *B. arietans*, Zn^{2+} metalloproteinase and other nonenzymatic toxins have been shown to degrade Aα and Bβ chains of fibrinogen and act upon the coagulation factors and complexes [38,1,39]. It is possible that bioactive compound present in aqueous fraction and crude methanolic extract of *T. indica* antagonize the anticoagulant activities of Zn^{2+} metalloproteinase and other toxins in an unknown mechanism thereby preventing the hydrolysis of Aα and Bβ chains of fibrinogen. This work further authenticates the findings of Ushannidini et al. [32] who reported that the ethanolic extracts of *T. indica* was able to antagonize the defibrinogenating activity of *V. russelli* venom at a concentration of 25 ug. Other studies have shown that the aqueous extract of *J. gossypiifolia* at high concentration was able to inhibit fibrinogenolytic enzymes from *B. jararaca* by preventing the degradation of Aα and Bβ chains of fibrinogen [8].

Severe haemorrhage is a major challenge associated with envenomation of *Bitis arietans*. This study revealed that the crude venom of *B. arietans* induced a significantly high mean diameter of lesion in mice. This can be ascribed to the cumulative action of haemorrhagins such as, PLA_2, Zn^{2+} metalloprotease and nonenzymatic toxins present in *B. arietans* venom. However, irrespective of the cumulative effect of crude *B. arietans* venom, the methanolic extract and fractions of *T. indica* seed was still able to reduce hemorrhage. The reduction of haemorrhage by *T. indica* suggests that, it contains bioactive compounds that directly inhibits the major haemorrhagic toxins which are PLA_2 and Zn^{2+} metalloproteinase thus, preventing the degradation of the extracellular membrane components of the capillary vessels. It is possible that these bioactive compounds interacts with divalent ion such as Ca^{2+} and Zn^{2+} which are essential cofactors of PLA_2 and SVMP respectively, thereby leading to the inhibition of their enzymatic activity.

This findings, agrees with the report by Esmeraldino et al. [40] who showed that the butanolic fraction of *C. urucurana* plant was able to inhibit haemorrhage of *B. jararaca* venom by 97%, which was ascribed to catechin, a basic unit of condensed tannins. Similar findings by Pithayannkul et al. [41] showed that the butanolic extract of *Eclipta* plant was able to neutralize the lethal and haemorrhagic effect of Malaysia pit viper venom and speculated that it could be due to the hydrogen interaction between the phenolic hydroxyls and the carbonyl group in the peptide bonds of the venom protein.

5. CONCLUSION

Viper envenomation is a major life-threatening event among healthy individuals in tropical and subtropical countries. In viper venoms, phospholipase A_2 and proteases are extensively studied hydrolytic enzymes due to their relatively high abundance and participation in a wide range of pharmacological and toxic effects.

The methanolic extract, aqueous fraction and butanolic fraction of *T indica* seed was able to neutralize the pharmacological activities of *B. arietans* which included hemolytic, anticoagulant and haemorrhagic effects which are key systemic and local effect in *B. arietans* envenomation. This indicates that methanolic extracts of *T. indica* seed consist of compounds that might interfere with the combined effects of the enzymes and toxins present in *B. arietans* venom. The findings of this present research work demonstrated a very good antivenom properties of the seeds of *Tamarindus indica,* which suggests that it could be a potential source of effective, safe and cost efficient antivenom agent for treatment of *Bitis arietans* envenomation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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