

Neutralization of the Lethal Effect of *Bitis arietans* Venom by *Euphorbia cotinifolia* Leaf Extract

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Abstract: Snake bite is an important health challenge in Northern Nigeria and especially in the North Central Zone. *Euphorbia cotinifolia* is one of the plants reportedly used in traditional medicine against snake bite. This study evaluates the neutralizing potential of *Euphorbia cotinifolia* leaf extract against *Bitis arietans*. Phytochemical screening of the leaf extracts was carried out and the LD₅₀ of the venom was determined through probit analysis. Two Protocols were used to evaluate the venom neutralizing ability of the extract at 250, 500, 1000 and 2500 mg/kg dose levels respectively. In the first protocol, the animals were challenged with venom and immediately treated with the extract. In the second protocol, animals were pre-treated with extract for five days and then challenged with the venom. The result of the phytochemical screening show that the extract contains alkaloids, tannins, terpenoids, flavonoids, carbohydrate, cardiac glycosides and saponins and LD₅₀ is greater than 5000 mg/kg. The venom neutralizing potential of the extract was not significant at (p<0.05) when the animals were challenged with the venom before the extract. When the animals were pretreated with the extract before being challenged with the venom before the extract. When the animals were pretreated with the extract before being challenged with the venom, the neutralizing potency was statistically significant at (p<0.05). However, the extract produced an increase in mean survival time and protection fold but could not protect the animals from death in both protocols. These suggest that the ethanolic extract is orally safe in laboratory animals and possesses significant antivenom potential against *Bitis arietans* venom thereby, authenticates its ethno medicinal use.

Key words: *Bitis arietans* venom, *Euphorbia cotinifolia* leaf, LD₅₀, phytochemicals.

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I. Introduction

Euphorbia cotinifolia is a broad leaf evergreen shrub that belongs to the family *Euphorbiaceae*. Its common name includes African nut tree (English), *Erin mado* (Yoruba) and *Waawan kurmi* (Hausa). *Euphorbia cotinifolia* is native to Mexico and South America treated as shrub, it reaches 10 to 15ft (3.0 to 4.6m) but can be grown as a tree reaching 30ft (9.1m). The purplish stems, when broken, exude a sap that is a skin irritant (Nelson *et al.*, 2007). *Euphorbia cotinifolia* is commonly grown as an ornamental plant in gardens and pots, due to its colorful and distinctive foliage. It prefers a site with well-drained soil and full sun. While relatively hardy, it does not react well to wind, salt, or frost (Welsch *et al.*, 2000). In a study by Ribeiro *et al.* (2015), all extract of *Euphorbia Cotinifolia* showed antibacterial, antifungal and antioxidant activities. *E. cotinifolia* is known to have biological, molluscidal, (Percia *et al.*, 2003) antioxidant, anti-cancer and anti-tumor properties (Hohmann *et al.*, 2003). Antibacterial activity of isopropyl alcohol extract of *E. cotinifolia* has also been evaluated by Rojas and his co-workers (Rojas *et al.*, 2008). Previous studies have reported that the dichloromethane extract of this plant showed mediocre antiviral activity with cytotoxic properties (Galvis *et al.*, 2002). The leaves of the plant contain Ingenol esters, (Herota *et al.*, 1980) which induces apoptosis (anticancer, and anti-HIV properties) as reported by Blanco-Molina *et al.* (2001). Furthermore, phytochemical analysis of the extract of *Euphorbia cotinifolia* showed the presence of flavanoids, terpenoids, tannins and steroids suggesting the use of these plants in traditional medicine for treatment of various diseases (Hohman *et al.*, 2003).

Snake bites is an injury caused by the bite of a snake. It often results in two puncture wounds from the animal's fangs. Sometimes poisoning from the bite may occur this may result in redness, swelling, and severe pain at the area which may take up to an hour to appear (WHO, 2015). Vomiting, trouble serving, tingling of the limbs and sweating may result (Gold *et al.*, 2002). Most bites are on the hands or arms (Gold *et al.*, 2002). Fear following a bite is common with symptoms of a racing heart and felling faint (Gold *et al.*, 2002). The venom may cause bleeding kidney failure, a severe allergic reaction, tissue death around the bite, or breathing problems (WHO, 2015). Bites may result in the loss of a limb or other chronic problems (Gold *et al.*, 2002) the outcome

depends on the type of snake, the area of the body bitten, the amount of venom injected, on the health conditions of the person (Marx, 2010) problems are often worse in children than adults (Peden *et al.*, 2008).

Bitis arietans is a venomous viper species found in savannah and grasslands from Morocco and Western Arabia throughout Africa except for the Sahara and rain forest region. It is responsible for causing the most snakebite fatalities in Africa owing to various reasons in highly populated regions and aggressively disposition (Mallow *et al.*, 2003). The species is commonly known as the puff adder (Mallow *et al.*, 2003), African puff adder or common puff adder. Due to the important health challenge posed by snake bite, problems with modern treatment have led the local populace to use numerous plant species to treat snake bite bites. Hence, this study was designed to evaluate the neutralization of lethal effect of *Bitis arietans* venom by *E. cotinifolia* leaf extract.

II. Materials And Methods

Chemicals and Reagents

Lyophilized viper venom (*Bitis arietans*), Spatula, distilled water, 70% Ethanol, Hydrochloric acid, Dragendoff's reagent, Ninhydrin reagent, Conc. H₂SO₄, Benzene, Dilute ammonia, 1% lead acetate, Graduated cylinder, Dilute sodium hydroxide, Sodium nitroprusside, solution, Pyridine, Chloroform, Acetic anhydride.

Collection of Plant materials

Fresh leaves of *Euphorbia cotinifolia* were collected in 2016 December from Jos, Plateau, Nigeria and used for the preparation of ethanol extract. A voucher specimen of the Plant has been deposited in the herbarium, Department of Botany, Federal College of Forestry, Jos, Nigeria. Mr. Azila, a senior taxonomist in the Department of Botany, Federal College of Forestry Jos, identified the plant.

Preparation and Extraction of *E. cotinifolia*

Thoroughly washed mature leaves were air-dried and then powder in a blender. 50 g of the powder leaves was exhaustively extracted with 70% ethanol in a Soxhlet extractor for 72 hours. All the extracts were concentrated to dryness on a water bath and weighed. It was preserved out from temperature in an air tight bottle until further use.

Phytochemical screening

Phytochemical analysis of ethanol extract was carried out for the detection of active secondary metabolite or different constituents such as tannins, alkaloids, flavonoids, terpenoids, steroids, carbohydrates, proteins and saponins using the methods employed by (Trease and Evans, 2002)

Experimental Animals

Wistar albino rats obtained from animal breeding house University of Jos weighing about 66 - 350 g was used for the study. They were housed in polypropylene cages, maintain under standard conditions of day and night, the animal was fed with standard rat pellet diet (Vital Feed Agro Industries Ltd, Nigeria) and water *ad libitum*.

Bitis arietans Venom Sample

The raw Venom of *Bitis arietans* was obtained from Prof. A.S Abubakar, Ahmadu Bello university Zaria, Nigeria and was preserved at 2-8°C before use, the venom was dissolved in distilled water.

Acute Toxicity (LD₅₀) of the Extract

Rat weighing (66 - 121 g) were used to determine the median lethal dose (LD₅₀). Different doses of the extract were administered intraperitoneal using Lorke's method (1983). In phase one, three groups of three animals each were used. Group one received 10 mg/kg body weight of rats; while group two received 100 mg/kg and group three received 1000 mg/kg. Phase two consisted of three groups with one animal per group, Group one received 1600 mg/kg, group two received 2900 mg/kg and group three received 5000 mg/kg. The control group was administered with distilled water.

Acute Toxicity of the Venom (LD₅₀)

The method reported by Turner was adopted for detection of LD₅₀ (Turner, 1965). The *Bitis arietans* snake venom was dissolved in distilled water and given to rats IP in graded doses and mortality was recorded for 24 hours. There are five groups of five animals each were used of the following doses: 0.2, 0.6, 1.8, 5.4, 16.2 mg/kg body weight.

Neutralization of *Bitis arietans* Venom by *E. cotinifolia* Leaf Extracts

This was carried out according to the procedure reported by Martz (1992) with little modifications. This modification involved multiplying the LD₅₀ by 3 instead of using the LD₉₉. Two protocols were employed, in the first protocol four groups of five animals each were used and 3 X LD₅₀ of the *Bitis arietans* venom was administered to individual animal across all groups intraperitoneal and immediate administration of the plant extract

Followed with different doses per group. Group one received venom only and dose 250, 1000, and 2500 mg/kg for group 2, 3 and 4 respectively, then the duration of survival and survived animals were recorded for the

period of 24 hours. In the second protocol, four groups of five animals each were used and pretreated for five days with different doses of plant extract as follows: distilled water only, 250, 500 and 1000 mg/kg, immediately after the last dose on day 5, 3 X LD₅₀ of the *Bitis arietans* venom was administered to individual animal across all groups intra peritoneal. The duration of survival and survived animal were recorded for 24 hours.

Data Analysis

Data are presented as the mean ± SD. The data were analyzed by one-way analysis of variance via a statistical software package (SPSS, Version 20.0, IBM Corporation, NY, USA) one-way ANOVA using Duncan multiple range *posthoc* test (DMRT). Values were considered to be significantly different at $p < 0.05$.

III. Results

Phytochemical screening

The phytochemical screening revealed that the extract contains alkaloids, saponins, flavonoids, tannins, cardiac glycosides, carbohydrates and terpenoids as displayed on Table 1.

Acute toxicity (LD₅₀)

The LD₅₀ of the extract was found to be more than 5000 mg/kg body weight. No animal died across the groups (Table 2).

Acute toxicity LD₅₀ of *Bitis arietans* Venom

The acute toxicity of *Bitis arietans* venom was calculated using Probit analysis. The Probit table was used to populate Table 3 and the information from this table was used in constructing Figure 1. The LD₅₀ correspond to Probit 5 and the log of the dose is 3.25 the dose is 1.78 mg/kg.

***In vivo* Neutralization of *Bitis arietans* Venom by *E. cotinifolia* Leaf Extract**

In the first protocol, only one animal that received 2500 mg/kg extract and the venom survived. The percentage survival is 20 % even though the mean survival time and the protection factor increased dose dependently this was not statistically significant. In the second protocol, none of the animal survived. Similarly, the protection factor and the mean survival time increase dose dependently. However, the protection factor was statistically significant at the 1000 mg/kg extract dose (Table 4 and 5).

IV. Discussion

Plants are sources of potent biochemical's. These are obtained from various parts of the plant Herbal remedies in traditional folk medicine provide a still largely unexplored field for the development of potentially new drugs (Ojo *et al.*, 2018). Preliminary phytochemical investigation has been done in ethanolic extracts of *E. cotinifolia* leaves and showed the presence of phytochemical constituents namely alkaloids, flavonoids, glycosides, saponins, tannins and terpenoid in Table 1. The phytochemical screening in the present study has revealed the presence of alkaloids, flavonoids, glycosides, saponins, tannins and terpenoids which may be responsible for the therapeutic properties of *E. cotinifolia*. Tannins and Flavonoids are phenolic compounds that are acting as principal antioxidants or free radical scavengers (Ojo *et al.*, 2013). Since these phenolic compounds were originated to be present in the extracts, it might be accountable for the potent antioxidant capacity of *E. cotinifolia*. These phytochemicals of medicinal plants have primarily been reported for their medicinal value, which can be valuable for therapeutic index. For instance, saponins and glycosides proved as hypotensive and cardio depressant properties (Olaleye, 2007), which are helpful for the treatment of congestive heart failure and cardiac myopathy (Brian, *et al.*, 1985). The leaves of *E. cotinifolia* contains, terpenoid the active constituent of which is diterpene and is responsible for ASV property by modifying the actions of proteins, and enzymes also inhibit snake venom phospholipase A₂ activities (Meier *et al.*, 1991).

The plant is considered to be safe as the dose as higher as 5000 mg/kg produced no mortality as described by International Research Animal Committee (IRAC 2004).

In this study, lyophilized (freeze dried) snake venom were used because venom is easily perishable and in the solid form, it is easy to handle. LD₅₀ of *Bitis arietans* was calculated because the lethality of the same snake venom varies from place to place. The LD₅₀ was found to be 1.78 mg/kg body weight. This LD₅₀ correspond to the LD₅₀ value range published by Mallow *et al.*, 2003 administered through intra peritoneal route (0.9 – 3.7 mg/kg). This shows that the *Bitis arietans* venom is very potent and has not been mis- handled during the course of the study.

Furthermore, it was observed that the ethanolic extract of plant *E. cotinifolia* when given to the rats after they received snake venom of *Bitis arietans* significantly increased mean survival time and protection fold but could not protect all the rats from death with only 20 % survival at dose 2500 mg/kg and the results were found better when it was used at higher dose. This could be possible due to inactivation or precipitation of active

venom components by the plant extract. This result is similar to previous studies by Nazimuddin *et al.* (1978) reported that ethanolic extract could inhibit lethal activity of *Bitis arietans* venom.

However, the plant extract was further given to the rats orally for 5 days, and it also showed the protection from the snake venom. This suggests that it has some prophylactic value. But the exact cause could not be ascertained. These results were also similar to the study done by Fatepur and Gawade (2007) on the preliminary screening of herbal plant extract for ASV activity against common sea snake. It was observed that extract of the plant *E. cotinifolia* provides some protection against the lethal dose of venom. Certain naturally occurring substances such as flavonoids are known compounds possessing protein-binding and enzyme-inhibiting properties.

The pharmacological properties of snake venom are mainly associated with proteins, particularly enzymes. Venom of *Bitis arietans* is also the mixture of different proteins. It contains powerful postsynaptic neurotoxins which are low molecular weight and diffuses rapidly through blood stream. It also contains toxic phospholipase A₂ with presynaptic neuromuscular blocking activity. PLA₂ is almost invariably the most toxic component of the venom and responsible for wide range of pharmacological effects, including neurotoxicity, cardiotoxicity, hemotoxic, and damage to biological membranes (Otten, 1998). The toxins of viper are composed of hematoxin, enzymes, and proteins.

Furthermore, anti-venom sometimes does not provide enough protection against snake envenomation, especially local poisoning. The use of plants against the effect of snakebite has long been recognized, even in modern times. Only for last 20 years, it has merited to closer scientific attention. Although, quite a number of reports from different geographic areas mention plants reputed to neutralize the action of snake venom, only a few attribute such activity to certain chemical compounds identified in them, and even less are concerned with a possible mechanism of action. However, in Northern Nigeria, many plants are recognized against snake envenomation.

V. Conclusion

The venom neutralizing potential of the extract was statistically not significant at ($p < 0.05$) when the animals were challenged with the venom before the extract. When the animals were pretreated with the extract before being challenged with the venom, the neutralizing potency was statistically significant at ($p < 0.05$). However, the extract produced an increase in mean survival time and protection fold but could not protect the animals from death in both protocols. These results suggest that the ethanolic extract is orally safe in laboratory animals and possesses significant anti-venom potential against *Bitis arietans* venom.

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Conflict of Interest

Authors declare no conflict of Interest

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Neutralization of the Lethal Effect of *Bitis arietans* Venom by *Euphorbia cotinifolia* Leaf Extract

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Table 1: Phytochemical Analysis of *Euphorbia cotinifolia* Leaf Extract

| Phytochemical | Results |
|--------------------|---------|
| Alkaloids | ++ |
| Saponins | + |
| Tannins | +++ |
| Flavonoids | +++ |
| Carbohydrates | ++ |
| Terpenoids | +++ |
| Anthraquinones | - |
| Cardiac glycosides | + |

- = Absent, + = present, ++ = more present, +++ = highly present.

Present % yield = 38/100=38%

Table 2: Acute Toxicity of *Euphorbia cotinifolia* Leaf Extract

| Dose (mg/kg) | No of animals | No of survivors | No of death |
|----------------|---------------|-----------------|-------------|
| Phase 1 | | | |
| 10 | 3 | 3 | 0 |
| 100 | 3 | 3 | 0 |
| 1000 | 3 | 3 | 0 |
| Phase 2 | | | |
| 1600 | 1 | 1 | 0 |
| 2900 | 1 | 1 | 0 |
| 5000 | 1 | 1 | 0 |

Table 3: Acute Toxicity of *Bitis arietans* Venom (LD₅₀)

| Dose (mg) | Dose (ng) | Log dose | N | Dead | % dead | Probits |
|-----------|-----------|----------|---|------|--------|---------|
| 0.2 | 200 | 2.30 | 5 | 0 | 5 | 3.36 |
| 0.6 | 600 | 2.77 | 5 | 0 | 5 | 3.6 |
| 1.8 | 1800 | 3.25 | 5 | 0 | 60 | 5.25 |
| 5.4 | 5400 | 3.73 | 5 | 0 | 100 | 6.64 |
| 16.2 | 16200 | 4.20 | 5 | 0 | 100 | 6.64 |

Table 4: *In vivo* Neutralization of Anti-Venom Effect in First Protocol

| Treatment | Mean survival time (minutes) | Protection fold | Total animal survival/no of animals in group | Percentage survival |
|------------------------------------|------------------------------|-----------------|--|---------------------|
| 3xLD ₅₀ SV only | 132±12.00 | - | 0/5 | 0 |
| 3xLD ₅₀ SV + 250 mg/kg | 168±29.39 | 1.27 | 0/5 | 0 |
| 3xLD ₅₀ SV + 1000 mg/kg | 192±22.45 | 1.45 | 0/5 | 0 |
| 3xLD ₅₀ SV + 2500 mg/kg | 492± 244.75 | 3.73 | 1/5 | 20 |

Legends: n=5; LD₅₀ = the lethal dose that kill 50% of the animal; SV= snake venom; NS = not significantly difference from the control at p<0.05

Table 5: *In vivo* Neutralization of Anti-Venom Effect in Second Protocol

| Treatment | Mean survival time (minutes) | Protection fold | Total animal survival/no of animals in group | Percentage survival |
|------------------------------------|------------------------------|-----------------|--|---------------------|
| 3xLD ₅₀ SV only | 120 ± 18.97 | - | 0/5 | 0 |
| 250 mg/kg + 3xLD ₅₀ SV | 228 ± 22.44 | 1.90 | 0/5 | 0 |
| 500 mg/kg+3xLD ₅₀ SV | 240 ± 32.86 | 2.00 | 0/5 | 0 |
| 1000 mg/kg + 3xLD ₅₀ SV | 552 ± 162.55 | 4.60* | 0/5 | 0 |

Legends: = Significantly difference from control at p<0.05; SV= snake venom; LD₅₀= the lethal dose that kill 50% of the animal; n= 5

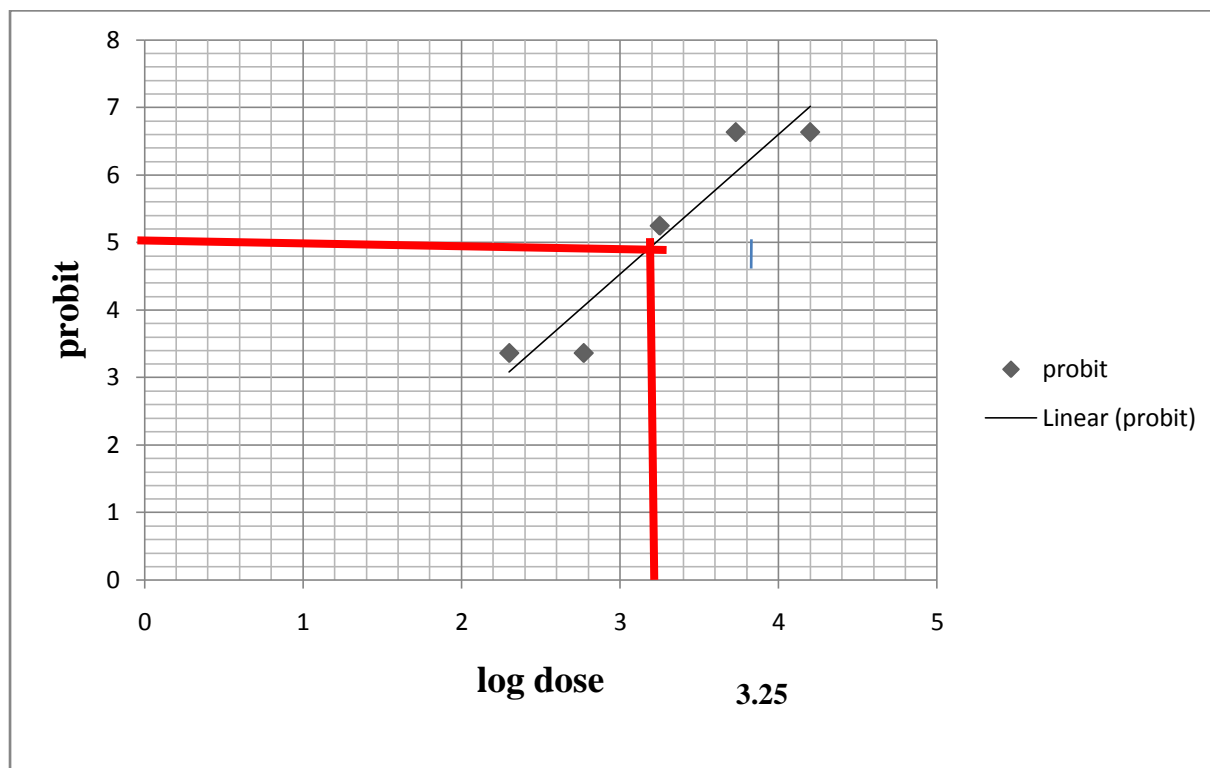


Figure 1: Neutralization of *Bitis arietans* Venom by *Euphorbia cotinifolia* Leaf Extract: graph of Probit versus log doses

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