**ABSTRACT**

*Solanum dasyphyllum* belongs to the family of plants called Solanaceae, it is commonly called "Africa eggplant" and one of the medicinal plants used in the treatment of snake envenomation in the southwestern part of Nigeria, but investigation concerning its anti-venom activity has not been established. The present study evaluates the in-vitro enzyme inhibition potential of *S. dasyphyllum* leaf and fruit extracts against *Naja nigricollis* (Black-necked spitting cobra) venom. The inhibitory potential of *S. dasyphyllum* leaf and fruit on proteases, acetylcholinesterase, phospholipase A<sub>2</sub> and hyaluronidase enzymes present in the snake venom was evaluated. The methanolic leaf and fruit extracts of *S. dasyphyllum* inhibited the activity of all enzymes evaluated, however, the leaf extract exhibited better enzyme inhibitory effect on *N. nigricollis* venom when compared with the fruit. This could be due to the presence of various phytochemicals in leaf and fruit extract. This result substantiates the ethnomedicinal usage of *S. dasyphyllum* and would help to develop potent antidote therapy against *N. nigricollis* envenomation.
Keywords: Envenomation; protease; phospholipase A₂; acetylcholinesterase; hyaluronidase.

1. INTRODUCTION

Snakebite is a serious medical problem worldwide, especially in the tropics. According to the World Health Organization, snake envenomation was listed as the highest priority (Category A) of neglected tropical disease in July 2017 [1]. It was estimated that 5.4 million snakebites occurred annually with subsequent 1.8–2.7 million cases of envenomation Hegde et al. [2]. Mortality rates due to snakebite poisoning are from 81,410 to 137,880 yearly and the incidence of permanent disabilities and limb amputations are three times as much as morbidity. Habib [3]. It is not every snake that is poisonous. Reid [4]. however, poisonous snakes belong to the following 5 families: Viperidae, Crotalidae, Elapidae, Colubridae and Hydrophidiae Adyemi [5]. N. nigricollis belongs to elapidae family, and its venom retains the typical elapid neurotoxic properties while combining these with cytotoxins. The lethal effect of cobra bites is mainly neurotoxic Meenatchi Sundaram & Michael [6], which act on synaptic nerve terminals. N. nigricollis venom also have affinity for cardiac tissue and act to depolarize cardiac cell membrane, which leads to systolic arrest. N. nigricollis envenomation is characterized by painful and progressive swelling with blood-stained tissue fluid leaking from the bite wound, hypovolemic shock, blistering and bruising Sharma et al. [7]. Antivenom (ASV) is the best antidote specific for snake envenomation Meenatchi Sundaram et al. [8]. Antivenoms are usually hyperimmune sera collected from animals which bind and inactivate venom enzymes. However, the production and supply of antivenom is associated with logistical, storage and economic difficulties. The side effects of antivenom are anaphylactic shock, pyrogen reaction and serum sickness, these side effects may be due to the action of high concentrations of non-immunoglobulin proteins present in commercially available hyper immune antivenom immunotherapy. Verma et al. [9]. In recent years, the subject of plants used to treat snakebite has attracted the attention of several researchers. Plants and their extracts have been used for the treatment of snake bite in most areas where venomous species are endemic. Solanum dasyphyllum belong to the family of Solanaceae. According to Obade et al. [10], scoparone, aesculin, scopoletin, lifetime, and p-coumaric were active compounds in the ethanolic extract of the fruit of S. dasyphyllum, and they are used ethnomedically to counter envenomations and also to treat tooth ailments. The plant is reported to possess anticonvulsant and neuromuscular properties. In the south western part of Nigeria, the fruit of S. dasyphyllum mixed with local black soap is usually apply to incisions at sites of snakebites, presumably to remove venom from bite site and reduce its absorption into the systemic circulation. This practice lacks scientific verification. The objective of this study is to evaluate the in-vitro antivenom activity and venom neutralizing potential of methanolic leaf and fruit extracts of S. dasyphyllum.

2. MATERIALS AND METHODS

2.1 Chemical and Reagents

Trichloroacetic acid (TCA), 5,5’-dithiobis-2-nitrobenzoic acid (Ellman’s reagent), Tris-HCl, Tris base, Cetyltrimethylammonium bromide, Hyaluronic acid, Lecithin, Acetylthiocholine iodide, Calcium chloride were obtained from Sigma-Aldrich U.S.A. All other chemicals and reagents were of analytical grades.

2.2 Plant Material

Fresh leaves and fruit of Solanum dasyphyllum were collected from Odeomu town in Ayedaade Local Government, Osun State. The plant was authenticated at the herbarium in the Department of Botany, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria where a voucher specimen number IFE-17489 was deposited. Both the leaves and fruits were cleaned, air-dried, pulverized into powder and stored in an airtight container.

2.3 Venom Sample

Lyophilized N. nigricollis (Cobra) venom was procured from the Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria, Nigeria, and was preserved at 4°C. Before use, the venom was reconstituted in phosphate buffer, pH 7.2, centrifuged at 2000 rpm for 10 mins and the supernatant was used for anti-venom studies.

2.4 Extracts Preparation

The powdered leaves and fruits (150 g) were soaked with 95% methanol for 72hrs with
constant stirring and was filtered using Whatman No1 filter paper. The filtrate was concentrated using a rotary evaporator, and lyophilizing to dryness.

2.5 Preliminary Phytochemical Screening

The extracts were evaluated qualitatively for the presence of phytochemicals according to the method described by Sofowora [11] to ascertain the presence of secondary metabolites such as alkaloids, tannins, saponin, carbohydrate, flavonoids, terpenes, and cardiac glycosides.

2.6 Evaluation of the Anti-Venom Effect of *Solanum dasyphyllum* Extracts

2.6.1 Protease activity

Protease activity of crude venom was performed according to the Greenberg [12] method with slight modification. A solution of 1% casein, 20 mM phosphate buffer (containing 150 mM NaCl, pH 8) was incubated with cobra venom for 1 hr at 37°C. At the end of the 1st hour, the reaction was stopped by adding 0.5 ml of 10% trichloroacetic acid (TCA) and filtered. The filtrate (1.0 mL) was used for protein estimation by the method of Lowry [13], using L-tyrosine as a standard. In the above investigation, one unit of enzyme activity was defined as the amount that yields 0.02 μmole of tyrosine/hour under experimental conditions described. For the inhibition studies, venom was re-incubated with different concentrations (200-1000 μg/ml) of the plant extracts for 45 minutes at 37°C.

2.6.2 Phospholipase A₂

Phospholipase A₂ assay was determined according to the acidimetric method of Tan and Tan [14] with slight modification. Lecithin suspension was prepared by mixing proportionately 1% lecithin, 18 mM calcium chloride and 8.1 mM sodium deoxycholate. The pH of the suspension was adjusted to 8.0 with 0.02 M sodium hydroxide and stirred for 10 minutes to ensure homogenous mixing. Next, 0.1 ml venom solution was added to 15 ml of lecithin suspension to initiate the hydrolysis. The initial decrease in pH was measured by a pH meter. Inhibition study was carried out by pre-incubating the venom with different concentrations (200-1000 μg/ml) of the plant extracts for 45 min at 37°C.

2.6.3 Hyaluronidase

Hyaluronidase assay of crude venom was determined turbidometrically by the method of Pukrittayakamee et al. [15]. The assay mixture contained buffer of Tris – HCl (pH 8.0), 50 mg hyaluronic acid (0.5 mg/ml in buffer) and venom (1 mg/ml) in the same buffer in a final volume of 1.0 ml. The mixture was incubated for 15 min at 37°C and the reaction was stopped by the addition of 2 mL 2.5% (w/v) cetyltrimethyl ammonium bromide in 2% (w/v) NaOH. The absorbance was read at 400 nm (within 10 min) against a blank containing 1 mL of the same buffer and 2 ml 2.5% (w/v) cetyltrimethyl ammonium bromide in 2% (w/v) NaOH. Inhibition study was carried out by pre-incubating venom with different concentrations (200-1000 μg/ml) of the plant extracts for 45 min at 37°C.

2.6.4 Acetylcholinesterase

Acetylcholinesterase enzyme activity was assayed according to the method described by Ellman et al. [16]. 0.1 ml of 0.01 M DTNB was added to 2.6 ml of 0.1 M phosphate buffer (pH 8.0), 0.04 ml of the venom was added to the above mixture followed by incubation for 5 min, after incubation, 0.04 ml of the substrate (0.075 M acetylcholine iodide) was added to the reaction mixture. Absorbance readings were taken at 420 nm continuously for 3 min at 30 s intervals. For the inhibition studies, venom was pre-incubated with different concentrations (200-1000 μg/ml) of the plant extracts for 45 minutes at 37°C.

3. Results

The phytochemical analysis revealed that soluble starch and anthraquinone are absent in both the leaf and fruit, whereas the other secondary metabolites were present in both extracts as shown in Table 1.

The effect of methanolic leaf and fruit extracts of *Solanum dasyphyllum* on *Naja nigricollis* venom protease is given in Fig 1. All concentrations of *S. dasyphyllum* significantly inhibit the activity of the enzyme in the venom. The effect of the extracts with maximal inhibition was recorded at 400 μg/ml for both the leaf and fruit extract.

Snake venom contains enzymes called Phospholipase A₂ (PLA₂) which causes breakdown lecithin to lysolecithin and fatty acids. This study demonstrated the capability of both the leaf and fruit extract of *S. dasyphyllum* to inhibit the activity of phospholipase A₂ enzyme as shown in Fig 2. However, the methanolic leaf extract shows more inhibitory capacity with its best activity concentration at 400 μg/ml when compared with the fruit extract.
Fig. 3 and Fig. 4 shows the effect of different concentrations of *S. dasyphyllum* on *N. nigricollis* venom hyaluronidase and acetylcholinesterase activity. All the extracts were able to inhibit the enzymes activity in a concentration-dependent pattern. In Fig 3, the fruit extract has more inhibitory activity with its best activity at 800 µg/ml than the leaf extract. However, in Fig 4, the leaf extract has more inhibitory activity on the acetylcholinesterase enzyme when compared to the fruit extract with its best activity at 600 µg/ml concentration.

4. DISCUSSION

The application of herbal remedy in the treatment of various diseases is growing at a faster rate globally, the low toxicity and easy accessibility are one of the major contributors to this global acceptance Adewunmi et al. [17]. The most effective and acceptable therapy for snakebite victims is the immediate administration of anti-venom following envenomation. Although the use of plants against the effects of snakebite has been recognized, more scientific attention has been given to since last 20 years Alam and Gomez, [18]. Like plants, snake venom is a complex mixture of biologically active compounds capable of exhibiting various pharmacological actions. The qualitative phytochemical screening of *S. dasyphyllum* leaf and fruit extract revealed the presence of alkaloids, tannin, flavonoids, Terpenoids, Cardiac glycosides, and carbohydrate. However, soluble starch, anthraquinone, and monosaccharides were not detected in both the leaf and fruit extract. Mors et al. [19] reported that phenolic compounds, saponins, flavonoids and tannins can bind to proteins and can directly act on venom constituents. Similarly, in 2001, Lans et al. [20] reported that plant alkaloids are effective against snake bites. Aristolochic acid, 8-methyloxy-6-nitophenanthro,12-methoxy-4-methyl voachalotine and atropine are all isolated alkaloids compounds that inhibit the lethal effect of snake venom Singh et al. [21]. Furthermore, cardiac glycosides are known to act Na+/K+ pump inhibitor. This causes an increase in the level of sodium ions in the myocytes, which lead to a rise in the level of calcium ions which may be important in counteracting the hemorrhagic effects of snake venoms Denwick, [22].

*N. nigricollis* venom contains several proteases such as serine protease, metalloprotease that can proteolyzed several important proteins and lead to erythrocyte membrane degradation. Previous research has revealed that protease is a mediator for edema, local tissue damage, inflammation, and haemorrhage Markland [23]. *S. dasyphyllum* leaf and fruit extract shows significant inhibition of the proteases in *N. nigricollis* venom. However, the leaf exhibited more inhibitory effect than the fruit extract. The percentage inhibition of the varying concentrations of the leaf extract were all above 50% (50.8%-59.6%), while that of fruit ranges from (33.5%-46.4%). The best inhibitory effect was observed in the leaf at 400 µg/ml.

Table 1. Qualitative phytochemical analysis of the extracts

<table>
<thead>
<tr>
<th>Test</th>
<th>Constituent</th>
<th>Leaf</th>
<th>Fruit</th>
</tr>
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<tbody>
<tr>
<td>Dragendorff</td>
<td>Alkaloid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Wagner’s test</td>
<td>Alkaloid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Shinda’s test</td>
<td>Flavonoid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ferric chloride</td>
<td>Flavonoid</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lead acetate</td>
<td>Flavonoid</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ferric chloride</td>
<td>Tannin</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lead acetate</td>
<td>Tannin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>Terpenoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Keller Killinai</td>
<td>Cardenolites</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Soluble starch</td>
<td>Soluble starch</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Free anthraquinone</td>
<td>Free anthraquinone</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Combined anthraquinone</td>
<td>Combined anthraquinone</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Salkowki’s test</td>
<td>Cardiac glycoside</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lieberman</td>
<td>Cardiac glycoside</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Monosaccharide</td>
<td>Carbohydrate</td>
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<td>-</td>
</tr>
<tr>
<td>Free reducing sugar</td>
<td>Carbohydrate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Molish</td>
<td>Carbohydrate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Frothing test</td>
<td>Saponin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phlobatatin</td>
<td>Saponin</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ indicates present, - indicates absent
Fig 1. Graphical representation of % inhibition of protease enzyme

Fig 2. Graphical representation of % Inhibition of phospholipase A$_2$ enzyme

Fig. 3. Graphical representation of % Inhibition of hyaluronidase enzyme
PLA₂ are a group of esterolytic enzymes present in snake venoms that typically catalyze the breakdown of glycerophospholipids, the main component of biological membranes, into lysophospholipids and a fatty acid [24]. Phospholipase A₂ (PLA₂) can cause hemolysis of Red Blood Cells by acting on Human Red Blood Cell (HRBC) membrane-associated phospholipids liberating lysolecithin. Injury to Red Blood Cell membrane, in turn, will render the cell more susceptible to secondary damage through free radicals. In the present study, the extract of *S. dasyphyllum* at varying concentrations shows a significant inhibitory effect on phospholipase A₂ enzyme. The results showed that leaf extract at 400 µg/ml (61% inhibition) was more potent than other concentration. Although the quantitative estimation of each of the phytochemicals in the extracts was not evaluated, phospholipase A₂ inhibition activity suggests that the leaf extract might contain more quantitative phytochemicals than the fruit, which probably bind to the Phospholipase A₂ enzyme, thus preventing it from binding to its substrate, thereby leading to its inhibition.

The diffusion of toxins from the site of a bite into the circulation is essential for successful envenomation. Hyaluronidase, otherwise called venom spreading factor is a class of enzyme found in snake venom that causes local tissue damage like the destruction of extracellular matrix and connective tissues [25]. The different concentrations of *S. dasyphyllum* leaf and fruit extracts ameliorated the toxic effect of hyaluronidase enzyme. The percentage inhibition of the leaf ranges from 19.6%-39.4% in a dose-dependent pattern, while that of the fruit ranges from 11.9%-49.4% in a non-dose dependent pattern. The best inhibitory effect was observed in the fruit extract of *S. dasyphyllum* at 800 µg/ml.

Acetylcholinesterase (AChE) plays a key role in cholinergic transmission. In this study, all the different concentrations of both the leaf and fruit extract of *S. dasyphyllum* significantly inhibit the neurotoxic effect of acetylcholinesterase. The percentage inhibition of leaf extract of *S. dasyphyllum* ranges from 58%-82.5%, while that of the fruit is from 35%-66.3% in dose-dependent manner. However, the best anti-acetylcholinesterase activity was observed in the leaf extract at 600 µg/ml (82.5%). The significant inhibition of acetylcholinesterase enzyme by *S. dasyphyllum* extract was also reported by Obade et al. [10]. *S. dasyphyllum* can be classified among anti-cholinesterase plants and this activity might be as a result of various phytochemicals present in the plant, such as alkaloids, flavonoids, coumarin and steroids, which have been reported to possess anticholinesterase activity [26].

5. CONCLUSION

The *in-vitro* enzymatic study of methanol leaf and fruit extracts of *S. dasyphyllum* reveals that the extracts could inhibit most of the toxic enzymes in the *N. nigrigollis* venom. The inhibitory activities of *S. dasyphyllum* against *N. nigrigollis* venom should be further confirmed by *in-vivo* studies using animal models. Isolation of
bioactive compound from *S. dasypyllum* could lead to the development of new natural alternative antidote for snake envenomation.

**CONSENT**

It is not applicable.

**ETHICAL APPROVAL**

It is not applicable.

**COMPETING INTERESTS**

Authors have declared that no competing interests exist.

**REFERENCES**


