Phospholipase A2 from Naja nigricollis and Inhibitory Activity of Extracts from Commiphora africana (A. Rich.) Engl. (Burseraceae)

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Introduction

Snakebite envenoming is a neglected global health problem responsible for substantial mortality, disability and psychological morbidity especially in rural tropical and subtropical regions of the world. Phospholipase A2 (PLA2) enzymes constitute a major toxic venom component especially the venoms from elapids. Naja nigricollis is of the family Elapidae. Commiphora africana is a species of the family Burseraceae which is traditionally used in many parts of Africa for the treatment of number of ailments including snake envenomation, cancer and some inflammatory diseases. The present study was designed to determine the hydrolytic actions of PLA2 present in the N. nigricollis venom in an egg yolk suspension model and to access the PLA2 inhibitory effects of extracts of C. africana by acidimetric assay method. The snake venom PLA2 was found to release free fatty acids from the egg yolk suspension in a dose-dependent manner. The venom concentration of 0.1 mg/mL was found to liberate 13.3 µmol of free fatty acids with a decline of pH of 0.1 which may be attributable to the phospholipase A2 in the venom. Higher liberations of 133 µmol levels of free fatty acids was achieved with a decrease of pH of 1.0 at concentrations of 4.0 mg/mL of the venom. The PLA2 inhibitory activity observed from the extracts and fractions also showed concentration-dependent inhibition as depicted by the IC50 values. These results may provide some scientific basis for the usefulness of the plant in the traditional management of snakebite envenomation across Africa.

Keywords: Phospholipases A2, Commiphora africana, Naja nigricollis, inhibition.

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snake venom PLA₂ have been identified as one of the most widely used target for antidote screening and researches have shown that both natural and synthetic svPLA₂ inhibitors are able to attenuate the morbidity and mortality of snakebite envenomation. Thus, the search for complementary therapies to treat snakebites is relevant and medicinal plants could be a rich source of natural inhibitors and pharmacologically active compounds with ability to neutralize a broad spectrum of venomous toxins. Commiphora africana has been a useful medicinal plant in the management of snake envenomation across Africa and thus the desire to carry out this study.

Materials and Methods

Plant Identification
The herbarium specimen of the plant sample was identified and authenticated by cross matching with an existing specimen voucher number no. 2848 as Commiphora africana by Taxonomist Sanusi Namadi of the herbarium unit of the Department of Botany, Faculty of Life Sciences, Ahmadu Bello University, Zaria - Nigeria.

Collection and preparation of plant material
The leaf and stem bark of Commiphora africana were collected around the vicinity of Ahmadu Bello University Dam, Samaru campus, Zaria in the month of September, 2017. The collected samples were washed with distilled water, air dried under shade for 10 days, after which they were pulverized with the aid of a milling machine, sieved and packaged into tightly closed plastic containers and kept at room temperature until required for use. They were subsequently referred to as the leaf and stem bark powdered plant materials, respectively.

Extraction and Fractionation
The extraction was carried out according to the modified method of Su et al. using cold maceration technique. The dried powdered leaf and stem bark of Commiphora africana (50 g each) were extracted with 80% aqueous ethanol (100 mL) using a mechanical shaker for 12 hours and the mixture filtered using Whatman No.1 filter paper. The filtrates were concentrated using rotatory evaporator and completely dried on a water bath to obtain residues which were transferred into a glass container, labeled as Commiphora africana leaf extract (CALE) and Commiphora africana stem bark extract (CASE), respectively.

The glass containers were kept in desiccators at room temperature prior to use. The stem bark extract (CASE) (10 g) was properly dissolved in distilled water (50 mL) to obtain a suspension which was filtered using the Whatman No.1 filter paper, the filtrate was subsequently fractionated according to the modified method of Woo et al. by partitioning with ethyl acetate and n-butanol solvents which were subsequently concentrated using rotatory evaporator and completely dried on a water bath to obtain residues which were transferred into a glass container, labeled as ethyl acetate fraction (EAF), saponin-rich fraction (SRF), flavonoid-rich fraction (FRF) and residual aqueous fraction (RAF), respectively (Figure 1).

Source of snake venom
The proﬁled freeze-dried venom of Naja nigricollis was obtained from the laboratory of Prof. M. S. Abubakar of the Venom, Anti-Venom and Natural Toxins Research Centre, Ahmadu Bello University, Zaria, Nigeria.

Determination of the hydrolytic activity of the phospholipase A₂ enzyme in N. nigricollis venom
Phospholipase A₂ enzymes are enzymes found to catalyze the hydrolysis of fatty ester in the 2-acyl ester bond in phosphoglycerides leading to the release of free fatty acids. The acidimetric assay for PLA₂ enzymes was conducted as described by Yap et al. An egg yolk suspension mixture (15 mL) was prepared by mixing three equal parts consisting of one-part chicken egg yolk, one part 18 mM calcium chloride and one part 8.1 mM sodium deoxycholate. The pH of the egg yolk suspension was adjusted to 8.0 with 1 M sodium hydroxide and stirred for 10 minutes to ensure homogenous suspension. Thereafter, 0.1 mL of N. nigricollis venom (1 mg/mL) was added to the egg yolk mixture to initiate the process of hydrolysis. A decrease in pH of the suspension was noted after two minutes with the help of a pH meter. The above procedure was repeated for (0.5, 1.0, 2.0 and 4 mg/mL) venom concentrations. The normal saline was used as control. A decline of 1 pH unit from (8.0 to 7.0) corresponds to 133 μmoles of fatty acid release. Enzyme activity was expressed as μmoles of fatty acid released/minute. The procedure was done in triplicates.

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**Figure 1:** Schematic chart for the fractionation of C. africana stem bark extract using a modified method of Woo et al.²⁴
Determination of the phospholipase A\(_2\) inhibitory activity of the extracts and fractions of \(C.\) africana

The inhibitory assay was carried out according to the method of Asad et al.\(^{27}\) with little modification. One milliliter of \(N.\) nigricollis venom (0.1 mg/mL) was incubated with (1 mL) each of various concentrations (0.1, 0.25, 0.50 and 1mg/mL) of the extracts and fractions of \(C.\) africana for thirty minutes at 37°C. Thereafter, 0.1 mL of the preincubated mixture was added to each egg yolk mixture (15 mL) to initiate hydrolysis and normal saline was used as control. A decrease in pH of the suspension was noted after two minutes using a pH meter. From the hydrolytic activity studies of the phospholipases A\(_2\), it was observed that 0.1 mL of \(N.\) nigricollis venom (1 mg/mL) was able to lower the pH of the egg yolk mixture from 8.0 to 7.5 and thus was adopted as reference. Results are indicated as inhibition percentages, where 100% is the activity induced by the crude venom alone and the IC\(_{50}\) was determined from the linear portion of the response dose curves. All tests and readings were carried out in triplicates.

Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of the three analytical triplicates by using Microsoft Excel version 2007 and comparisons between groups was carried out using the Student’s t-test. Level of significance was set at \(P \leq 0.05\).

Results and Discussion

Extractive profiles of the extracts and fractions of Commiphora africana

The extractive profile of 50 g each of \(C.\) africana powdered leaf and stem bark yielded 14 g of Commiphora africana leaf extract (CALE) and 15 g of Commiphora africana stem bark extract (CASE), while the fractionated portions of the stem bark extract yielded 1.0 g of ethyl acetate fraction, 0.2 g of saponin-rich fraction and 0.7 g of flavonoid-rich fractions.

Hydrolytic activity of the phospholipases A\(_2\) in \(N.\) nigricollis venom

Snake venom (0.1 mg) was found to liberate 13.3 umol of free fatty acids. However, on increasing the quantity of venom (0.5, 1.0 and 2.0 μg) larger amounts of fatty acids (39.9, 66.5 and 93.1 μmol/min) were liberated, respectively. One unit drop in pH from 8.0 to 7.0 was observed at concentration of 4.0 mg of \(N.\) nigricollis venom (Table 1). The hydrolytic activity of snake venom PLA\(_2\) at the 2-acetyl ester bond in phospholipids or lecithin in the egg yolk mixture lead to the release of free fatty acids which can be measured in terms of pH changes of between pH 8.0 to 7.0. The assay is adjudged to be highly reproducible and as sensitive as most established assays for phospholipase A\(_2\).\(^{28}\) Snake venom PLA\(_2\)'s, in particular, play a critical role in early morbidity and mortality from snakebite, causing death by paralysis as well as destruction of tissues and derangement of homeostatic mechanisms critical for regulation of coagulation and oxygen transport. They also play a very important role in neurotoxicity and myotoxicity which are of major clinical relevance in snake envenomation.\(^{28}\) The phospholipases from venoms of elapid family for example \(N.\) nigricollis exert pronounced neurotoxic effect resulting to flaccid paralysis as a major clinical manifestation.\(^{29}\) The enzymatic activity of PLA\(_2\) was observed to positively correlate with increased quantity of venom (Table 1). PLA\(_2\) can also interact with other components in the cells particularly the lipids.\(^{28}\) Both specific and nonspecific PLA\(_2\) develop protein - phospholipid interaction with covalent, non-covalent or disulfide interactions leading to hydrolysis of phospholipids and release of free fatty acids.\(^{30}\)

Inhibitory properties of \(C.\) africana leaf and stem bark extracts against \(N.\) nigricollis phospholipases A\(_2\)

The PLA\(_2\)'s inhibitory activity was observed from the leaf and stem bark extracts of Commiphora africana which showed concentration-dependent inhibition with the leaf extract having IC\(_{50}\) value of 576.49 μg/mL while the stem bark had IC\(_{50}\) value of 213.56 μg/mL (Table 2). The stem bark extract was observed to exhibit a more significant PLA\(_2\) inhibition when compared to the leaf extract as depicted by its lower IC\(_{50}\) value. The half maximal inhibitory concentration (IC\(_{50}\)) represents the concentration of a substance that is required for 50% inhibition in vitro. The IC\(_{50}\) value is inversely proportional to inhibitory activity, thus the lower the value, the more active is the extract. There have been documented evidence of use of herbs as remedy against snakebite especially in the tropical and subtropical snake prone regions of Africa, Asia and South America.\(^{31}\) These medicinal plants and other biological products having antitodal efficacy against snake envenomation have been evaluated using various models.\(^{32}\) Most plant antivenom agents have been proposed to function to neutralizing snake venom PLA\(_2\)'s toxicity.\(^{33}\) The inhibitory property exhibited by the leaf and stem bark extracts of \(C.\) africana may be due to the presence of the active chemical constituent(s) in the extract and the relative abundance of these constituents determines the inhibitory activities of the extracts as reflected by the IC\(_{50}\) values.

Due to the superior phospholipase A\(_2\) inhibitory activity exhibited by the stem bark extract as compared to the leaf extract and coupled with the reported traditional uses of the stem bark for the management of snake bite envenomation,\(^{4,15}\) the stem bark extract was further fractionated (Figure 1).

Inhibitory activity of fractions from stem bark extract of \(C.\) africana against \(N.\) nigricollis phospholipases A\(_2\)

The fractionated portions (ethyl acetate, saponin-rich and flavonoid-rich fractions) from the stem bark extract exhibited a much higher inhibition as depicted by the IC\(_{50}\) values of 21.36, 26.47 and 17.52 μg/mL, respectively (Table 3). The flavonoid-rich fraction was found to be the most potent fraction, then the ethyl acetate fraction and the saponin-rich fractions as indicated by their lower IC\(_{50}\) values (Table 3). Polyphenols such as terpenoids, xanthenes, flavonoids, rosmarinic acid, aristolochic acid and α-tocopherol have been reported to inhibit PLA\(_2\)'s from snake venoms.\(^{36}\) Other compounds such as ferulic acid, caffeic acid and gallic acid have shown an inhibitory ability against the activities induced by whole snake venoms.\(^{38}\) Recent researches have also shown that human secretory PLA\(_2\) inhibitors such as quercetin, bioflavonoids and other flavonoids isolated from plant extracts can also inhibit snake venom PLA\(_2\).\(^{33,35}\) Similar results were observed with other report (flavonoid compounds (flavonoids, flavones, isoflavones) for inhibiting telomerase and aromatase.\(^{39}\) Commiphora africana have been reported to contain some of these phytoconstituents such as flavonoids, tannins, triterpenes and saponins.\(^{4,13}\) Medicinal plant extracts as traditional antidotes have long been used in countries around the world and especially in communities where the access to effective antivenins is a challenge, therefore traditional herbal treatments are often used as adjuvant to therapies before or along with the antivenin treatment.\(^{39}\) The inhibition of the venom phospholipase A\(_2\) activity in the presence of the extract implies that the active constituent(s) or component in the extract could have an effect on the action of this enzyme and thus higher amounts or concentrations of the extract may lead to lowered enzyme activity and consequently alleviate or ameliorate the clinical symptoms related to the physiological activity of the enzyme. Although the component(s) of the extracts responsible for the observed PLA\(_2\) inhibitory activity in this study has not yet been identified, it is likely that the extract is acting through a direct physical interaction with the venom or through some enzymatic processes. Direct physical interaction is a characteristic mode of action of many polyphenolic compounds found in plants examples of which includes tannins ((p)galloylcafechin, (epi-)catechin), flavonoids, saponins and long-chain esters of some organic acids.\(^{32}\) Medicinal plants are endowed with abundance of these constituents and therefore may have therapeutic properties in application as natural inhibitors. The inhibitory effect of the extract in this assay may be attributed to this pathway, however further work is necessary to isolate and examine such component(s) and also conduct other assay models to validate these speculations.
Table 1: Effect of various concentration of *N. nigricollis* venom on the amount of free fatty acids released in terms of change in pH of egg yolk suspension.

| Concentration of Venom (mg/mL) | Change in pH (Mean ± SD) | Fatty acid released/min (µmole) |
|-------------------------------|--------------------------|-------------------------------|
| Control (saline)              | 8.0 ± 0.02               | 0                             |
| 0.1                           | 7.9 ± 0.02               | 13.3                          |
| 0.5                           | 7.7 ± 0.03               | 39.9                          |
| 1.0                           | 7.5 ± 0.01               | 66.5                          |
| 2.0                           | 7.3 ± 0.04               | 93.1                          |
| 4.0                           | 7.0 ± 0.03               | 133.0                         |

Table 2: Antidotal properties of the leaf and stem bark extracts of *C. africana* against *N. nigricollis* phospholipases A$_2$ in terms of an increase in pH of an egg yolk suspension.

| Sample          | Concentration µg/mL | pH     | Percentage inhibition (%) | IC$_{50}$ (µg/mL) | r$^2$  |
|-----------------|---------------------|--------|---------------------------|-------------------|-------|
| CALE            | 100                 | 7.5 ± 0.03 | 0                          |                   |       |
|                 | 250                 | 7.6 ± 0.03 | 20                         |                   |       |
|                 | 500                 | 7.8 ± 0.01 | 60                         | 576.49            | 0.9001|
|                 | 1000                | 7.9 ± 0.03 | 80                         |                   |       |
| CASE            | 100                 | 7.6 ± 0.04 | 20                         |                   |       |
|                 | 250                 | 7.7 ± 0.03 | 40                         | 213.56            | 0.6846|
|                 | 500                 | 8.0 ± 0.04 | 100                        |                   |       |
|                 | 1000                | 8.0 ± 0.01 | 100                        |                   |       |
| Crude venom     | 100                 | 7.5 ± 0.01 | reference                 |                   |       |
| Normal saline   | 8.0 ± 0.02          | Control |                           |                   |       |

CALE = *C. africana* leaf extract, CASE = *C. africana* stem bark extract. Values are expressed as mean ± SD. r$^2$ = regression co-efficient.

Table 3: Antidotal properties of some fractions from *Commiphora africana* stem bark extract (CASE) against *N. nigricollis* phospholipases A$_2$ in terms of an increase in pH of an egg yolk suspension.

| Sample          | Concentration µg/mL | pH     | Percentage inhibition (%) | IC$_{50}$ (µg/mL) | r$^2$  |
|-----------------|---------------------|--------|---------------------------|-------------------|-------|
| EAF             | 10                  | 7.6 ± 0.04 | 20                         | 21.36             | 0.6846|
|                 | 25                  | 7.7 ± 0.03 | 60                         |                   |       |
|                 | 50                  | 8.0 ± 0.04 | 100                        |                   |       |
|                 | 100                 | 8.0 ± 0.01 | 100                        |                   |       |
| SRF             | 10                  | 7.6 ± 0.04 | 20                         |                   |       |
|                 | 25                  | 7.7 ± 0.03 | 60                         | 26.47             | 0.6846|
|                 | 50                  | 8.0 ± 0.04 | 100                        |                   |       |
|                 | 100                 | 8.0 ± 0.04 | 100                        |                   |       |
| FRF             | 10                  | 7.6 ± 0.04 | 20                         |                   |       |
|                 | 25                  | 7.9 ± 0.03 | 80                         | 17.52             | 0.6284|
|                 | 50                  | 7.9 ± 0.04 | 80                         |                   |       |
|                 | 100                 | 8.0 ± 0.04 | 100                        |                   |       |
| Crude venom     | 100                 | 7.5 ± 0.01 | Reference                 |                   |       |
| Normal saline   | 8.0 ± 0.02          | Control |                           |                   |       |

EAF = Ethyl acetate fraction, SRF = Saponin-rich fraction and FRF = Flavonoid-rich fraction. Values are expressed as mean ± SD. r$^2$ = regression co-efficient.

Conclusion

The significant phospholipase A$_2$ inhibitory activity exhibited by the extracts of *Commiphora africana* may suggest that the plant contain some constituents with antivenin properties with the relative variation in concentration between the leaf and stem bark. These results could provide some scientific basis for the effectiveness of the plant for the traditional management of snake envenomation in Northern Nigeria and around Africa.

Conflict of interest

The authors declare no conflicting interest.
Authors’ Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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