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Effect of Methanol Extract of *Mangifera indica* Linn. Kernel on Partially Purified Phospholipase A_2 (PLA_2) from Venom of *Naja nigricollis:* An *in vitro* Study

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ABSTRACT

This study was aimed at evaluating the effect of methanol extract of *Mangifera indica* on phospholipase A_2 from *Naja nigricollis*. Phospholpase A_2 was first isolated from the venom of *Naja nigricollis* and then partially purified using ion-exchange and gel filtration chromatography. The inhibition effect of methanol extract of *Mangifera indica* kernel against PLA_2 was investigated. The extracted PLA_2 showed purification folds of 2.85 and 13.15 for ion-exchange and gel filtration chromatography respectively while the percentage yields are 44.12 and 70% for ion-exchange and gel filtration chromatography, respectively. Extract of *Mangifera indica* kernel showed peak inhibition of 66.67 and 90.00% on the partially purified PLA_2 using arithmetic and geometric variations in the concentrations of the extract, respectively. The concentrations of the extract (inhibitor) at which 50% of the enzyme activity was inhibited (IC_{50}) are 6.67 and 2.5 mg mL^{-1} for arithmetic and geometric progression, respectively. The double reciprocal plot of the enzyme in the presence of the extract at 8 and 32 mg mL^{-1} revealed K_M values of 0.29 and 0.28 mg mL^{-1} and V_{max} values of 6.70 μmoles min^{-1} and 4.5 μmoles min^{-1}, respectively while K_M and V_{max} of 0.29 mg mL^{-1} and 7.6 μmoles min^{-1}, respectively were obtained in the absence of the extract which suggests non-competitive inhibition. Phytochemical analysis of *Mangifera indica* kernel extract revealed the presence of alkaloids, carbohydrates, tannins, flavonoids, saponins and glycosides. The results of this study suggest that *Mangifera indica* extract is a potential anti-venom agent against *Naja nigricollis*.

Key words: Snake venom, phospholipase A_2, *Mangifera indica*, inhibition

INTRODUCTION

Snakes are elongate legless carnivorous reptiles of the suborder serpentes that can be distinguished from legless lizards. About 15 families have been recognized comprising of 456 genera and over 2900 species (Integrated Taxonomic Information System, http://www.itis.gov). Some snakes have developed specialized glands that produce venoms (Coppola and Hogan, 1992). These venoms are composed of complex mixture of active substances mainly peptides and proteins and some of the proteins present in these venoms include enzymes like PLA_2 and metallo-proteases (Sallau *et al*., 2005).

Phospholipase A_2 (PLA_2) EC 3.1.1.4 are enzymes that release fatty acids from the second carbon group of glycerol. It usually recognizes the sn-2 acyl bond of phospholipids and catalytically hydrolyzes the bond releasing arachidonic acid and lysophospholipids (Fuly *et al*., 2002). They are amongst the most toxic venom constituents that perform highly regulated roles in lipid metabolism. The two major modes of toxicity expressed by venom PLA_2 are neurotoxicity and myotoxicity (Sallau *et al*., 2005).

Treatments with anti-venoms are usually effective and recommended but it is not always accessible to rural dwellers necessitating the need to search for alternatives such as ethnomedicine (Otero *et al*., 2000). Plants have been used in the treatment of poisonous snake bites worldwide (Pereira *et al*., 1994). Some of the plant extracts like *Mucuna pruriens, Parkia biglobosa, Aristolochia albida* and many others have already shown positive effect against snake venoms (Alam and Gomes, 2003; Asuzu and Harvey, 2003; Abba *et al*., 2013).

Mangifera indica, a large evergreen tree native to tropical Asia and belonging to the family Anacardiaceae, has been used widely as source of food, medicine and timber. When used for medicinal purposes, different parts are considered. Its leaves have been used locally in the treatment of diarrhea and haemorrhages. This is usually based on the bioactive principle present in it.

The methanolic extract of the bark of *Mangifera indica* has been used for anti-bacterial studies and has proved effective in the treatment of dysentery, gastrointestinal tract infection and respiratory and urinary tract infection (Bala, 2006).

Though medicinal plants remain largely neglected and unrecognized in most parts of the world, study has shown that they are effective in the treatment and management of diseases associated with bacterial infection, snake bite and other diseases (Toth *et al*., 2003). Therefore, there is need to study the efficacy of the kernel of *Mangifera indica* against activities of partially purified PLA_2 from venom on *Naja nigricollis*.

MATERIALS AND METHODS

Chemicals and reagent: Important reagents and chemicals such as DEAE-cellulose, Sephadex G-50, 3, 5- Dinitrosalicylic acid (DNS) reagent and methanol were obtained from Sigma Chemical Company, St. Louis, USA. All other reagents and chemicals were obtained from reputable chemical companies worldwide.

Venom collection: Freeze dried *Naja nigricollis* venom was a gift from Prof. M.S Abubakar of the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria.

Plant collection and identification: Fresh kernels of *Mangifera indica* were obtained from the Samaru market, Sabo Gari Local Government Area, Zaria and identified at the Herbarium unit of the Department of the Biological Sciences, ABU, Zaria.

Preparation of extract/phytochemical screening: *Mangifera indica* extract was prepared by crushing the air dried kernels into fine powder and then macerating for 72 h using a combination of methanol and water as solvent in a 1:1 ratio. A cream coloured powder was obtained after desiccation and the extract was expressed as dry weight and phytochemical screening was carried out by standard method of Evans (2009).

Phospholipase A_2 (PLA_2) assay: The PLA_2 activity was assayed as described by Bhat and Gowda (1989). About 25 μL of 1 mg mL^{-1} L-α-lecithin substrate was incubated with 10 μL of the enzyme for 10 min at 37°C. The reaction was then terminated by immersing the tube in a boiling water bath for 2 min and the amount of released free fatty acid was measured titrimetrically at pH 8.0. The activity of phospholipase A_2 was defined as the amount of enzyme that hydrolyzes 1 μmol of fatty acids from L-α-lecithin per minute under standard conditions.

Determination of total protein: Here, biuret reagent (4 mL) was added into various test tubes and different volumes of albumin dissolved in distilled water was added into biuret reagent and made upto 5 mL with distilled water in 5 different test tubes. Blank was devoid of albumin and incubated at room temperature for 30 min at 37°C. The absorbance was measured at 540 nm after incubation. The same procedure was carried out for crude venom but the venom was diluted further with distilled water to reduce the sensitivity of biuret reagent. For both standard and crude venom duplicates of the reactions were done and the mean obtained. Standard curve was used to extrapolate the value of protein present in crude venom.

Enzyme purification: Two milliliter of 10 mg mL^{-1} of crude *N. nigricollis* venom was loaded onto DEAE cellulose (1.3×36 cm) pre-equilibrated with 50 mM phosphate buffer pH 6.8. The column was eluted stepwise with NaCl gradient (0.01-0.3 M) at a flow rate of 4 mL min^{-1}. Twenty five fractions of 4 mL were collected and assayed for phospholipase A_2 activity and total protein. The PLA_2 active fractions were pooled together and loaded on Sephadex G-50 column equilibrated with phosphate buffer (pH 6.5). The column was eluted with the same buffer, maintaining a flow rate of 2.5 mL min^{-1}. Fractions were collected and assayed for PLA_2 and total protein concentration.

Inhibition and kinetic studies: The total assay mixture composed of 100 μL of 0.02 M phosphate buffer (pH 6.8 containing 6 mM sodium chloride), 25 μL of phospholipase A_2 and extracts at concentration from 2-10 mg mL^{-1} arithmetically and 2-32 mg mL^{-1} geometrically were incubated at 37°C for 10 min. After pre-incubation, the reaction was terminated with 1.0 mL 3, 5-dinitrosalicylic acid (DNS) reagent, placed in boiling water bath for 2 min, cooled to room temperature, diluted and the amount of released free fatty acid was measured titrimetrically at pH 8.0. The control reaction representing 100% enzyme activity did not contain any plant extract. The percentage inhibition was determined as follows:

$$\text{Relative enzyme activity (\%)} = \frac{\text{Enzyme activity of test}}{\text{Enzyme activity of control}} \times 100$$

$$\text{Inhibition in the } PLA_2 \text{ activity (\%)} = (100\text{-relative enzyme activity\%})$$

The determination of the inhibitor concentration at which 50% inhibition occurred (IC_{50}) was extrapolated from plots of percentage inhibition versus inhibitor concentration. The IC_{50} values were defined as the concentration of the extract, containing the phospolipase A_2 inhibitor that inhibited 50% of the PLA_2 activity.

To determine the pattern of inhibition, the enzyme was assayed with varying concentrations of the substrate (0.2-1 mg mL^{-1}), L-α-lecithin in the presence and absence of the inhibitor. Initial velocity values obtained were then used to plot Lineweaver-Burk's plot to determine the K_M and V_{max}.

RESULTS

The purification scheme of phospholipase A_2 from *Naja nigricollis* venom is summarized in Table 1. The crude enzymes had a specific activity of 2.19 μmol min^{-1} mg^{-1} which increased to 6.29 and 28.80 μmol min^{-1} mg^{-1} after passing through DEAE- cellulose and DEAE Sephadex 50 column, respectively.

Preliminary qualitative phytochemical analysis was performed to determine the active ingredients present in the extract. The results showed the presence of different types of active constituents such as tannins, saponins, flavonoids, alkaloid, glycosides, anthraquinones and carbohydrates (Table 2).

Appropriately diluted plant extract was used for enzyme inhibition assay and the activity obtained with each extract was normalized to percent relative activity from which the percentage inhibition was calculated (Fig. 1). Less than 50% PLA_2 inhibition was observed in extract concentrations of 2, 4 and 6 mg mL^{-1} for extracts for which arithmetic progression was employed while extract concentrations of 4, 8, 16 and 32 mg mL^{-1} exhibited inhibitions greater than 50% on PLA_2 activity for extracts for which geometric progression was employed.

Table 1: Purification profile of PLA_2 isolated from *Naja nigricollis*

Purification step	Protein (mg)	Total activity (μmol min^{-1})	Specific activity (μmol min^{-1} mg^{-1})	Purification fold	Yield (%)
Crude	6.40	14.00	2.19	1.00	1.00
Ion exchange on DEAE-cellulose	1.20	7.50	6.25	2.85	44.12
Gel filtration on sephadex G-50	0.34	9.80	28.80	13.15	70.00

Table 2: Phytochemical constituents of methanolic extract of *Manifera indica* kernel

Phytochemical compound	*Manifera indica* kernel
Tannins	+
Saponins	+
Cardiac glycosides	+
Flavonoids	+
Alkaloids	+
Anthraquinones	+
Carbohydrate	+
Glycosides	+

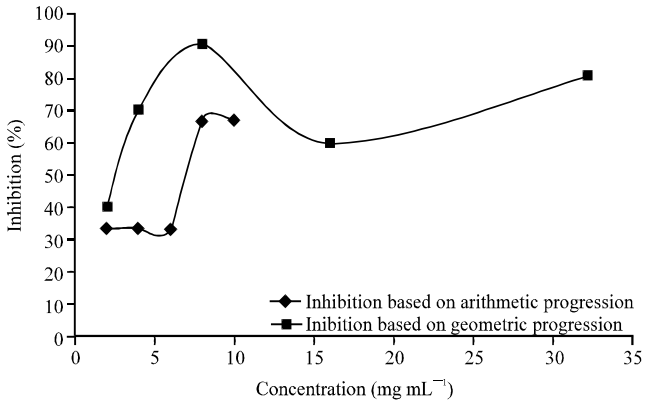

Fig. 1: Percentage inhibition of phospholipase A_2 using varying concentrations (arithmetic and geometric progression) of *Mangifera indica* extract

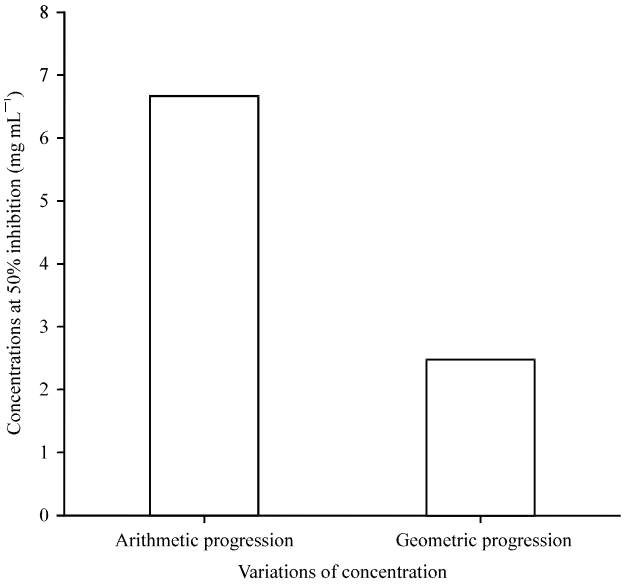

Fig. 2: Inhibitor concentration at which 50% inhibition occurred (IC_{50}) of the *Mangifera indica* kernel extract for the two separate variations of concentrations

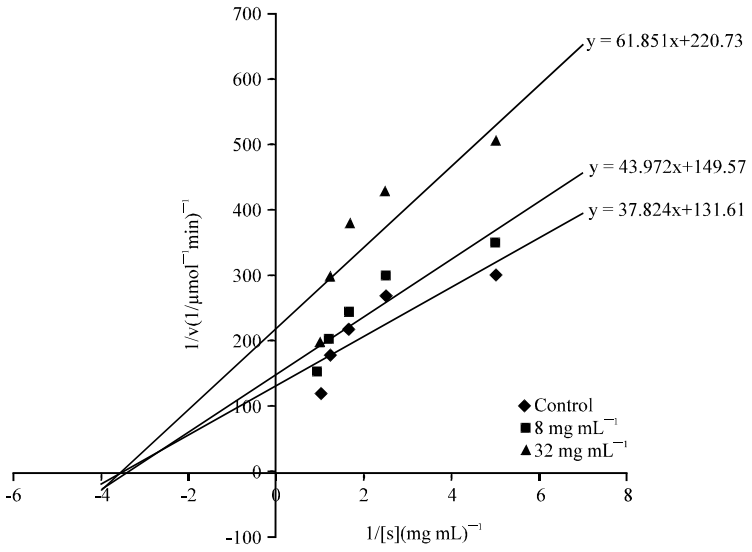

Fig. 3: Lineweaver-Burk plot of phospholipase A_2 enzyme activity in the presence and absence of extract/inhibitor exhibiting non-competitive inhibition

The highest inhibition activities were obtained with the extract concentrations of 8 and 32 mg mL^{-1}. The IC_{50} values of the arithmetic and geometric concentration were 6.67 and 2.5 mg mL^{-1}, respectively (Fig. 2).

Figure 3 shows the Lineweaver-Burk (double reciprocal) plot of phospholipase A_2 in the presence and absence of the extract/inhibitor using the concentrations with the highest inhibitions (8 and 32 mg mL^{-1}). The double reciprocal plot of the enzyme in the presence of the extract at 8 and

32 mg mL^{-1} revealed K_M values of 0.29 and 0.28 mg mL^{-1} and V_{max} values of 6.70 and 4.5 μmoles min^{-1}, respectively while K_M and V_{max} of 0.29 mg mL^{-1} and 7.6 μmoles min^{-1}, respectively were obtained in the absence of the extract which suggests non-competitive inhibition.

DISCUSSION

Understanding the characteristics of PLA_2 from venom is a serious concern for venom researchers, as it would help in designing effective therapeutic anti-venoms. This study reveals that the isolation and partial purification of PLA_2 from venom of *Naja nigricollis* increased in specific activity of the crude PLA_2 after the two-step purification which could be due to the removal of synergistically interacting components of the venom. From this study, the high percentage yield of 70% obtained was higher when compared to ostrich pancreatic PLA_2 and dromedary PLA_2 which could be an indication that the purification steps used here are more appropriate for this enzyme (Bacha *et al.*, 2007).

Even though, several plants have been shown to antagonize the snake venom action, only few are reported to be effective in inhibitory hemostatic disturbance induced by the venom which is a characteristic of Viperine and Elapidae envenomation (Borges *et al.*, 2000; Mors *et al.*, 2000; De Almeida *et al.*, 2004; Hung *et al.*, 2004). This study shows that the extract of *Mangifera indica* kernel has inhibitory effect on the phospholipase A_2 which is in agreement with the study of Abba *et al.* (2013). The therapeutic potential of medicinal plants is traditionally attributed to classes of active constituents including flavonoids, alkaloids, sesquiterpenes, lignins and others (Soares *et al.*, 2005). From our study, it was evident that extract of *Mangifera indica* contain flavonoid, alkaloid, tannin and saponin which could be responsible for the inhibitory effect, observed on the extract. The most likely mechanism of action of antagonist activity of the extract against PLA_2 could be due to the binding of the PLA_2 with flavonoid and alkaloid which have been reported by Soares *et al.* (2005) that flavonoid and alkaloid have shown to antagonize snake venom. Since the extract contains the presences of different active constituents, the mechanism of action could also be as a result of interaction of these different active constituents with PLA_2 in bringing about inhibition.

The concentrations at which 50% of the enzyme has been inhibited (IC_{50}), suggest that the extract could inhibit phospholipase A_2 at much lower concentrations. A double reciprocal plot in the presence and absence of extract of *Mangifera indica* revealed non-competitive pattern which suggests that sites other than the active site could be involved in the inhibition (Abba *et al.*, 2013).

CONCLUSION

The venom of *Naja nigricollis* has PLA_2 activities which could be inhibited by the methanolic extract of *Mangifera indica* kernel extract *in vitro*.

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