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INHIBITORY ACTIVITY OF LUPEOL FROM *Cryptolepis oblongifolia* (MEINS) SCHLTR ON PARTIALLY PURIFIED PHOSPHOLIAPASE A2 VENOM OF *Naja nigricollis* (ELAPIDAE)

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ABSTRACT

The research is design to provide scientific evidence for the use of *Cryptolepis oblongifolia* in traditional treatment of inflammation and also to examine whether it have some snake venom phospholipase A_2 inhibition potential. A triterpene was isolated from *C. oblongifolia* using column chromatography and identified via spectroscopic means. The inhibitory activity of the isolate on phospholipase A_2 (PLA_2) of *Naja nigricollis* venom was evaluated. The proton nuclear magnetic resonance (^1H NMR) of the isolated compound showed the presence of olefinic protons while the carbon nuclear magnetic resonance (^{13}C NMR) showed the presence of exomethylene carbon atoms. By comparison with the reported literature the compound was found to be lupeol, the inhibitory activity of the isolate on PLA_2 of *Naja nigricollis* venom showed that, the isolate inhibits the PLA_2 at dose dependent manner with inhibition binding constant (*ki*) of 0.015 mg/ml. The activity of the compound isolated supports the local usage of *C. oblongifolia* in traditional treatment of inflammation.

Keywords: Inhibition, Lupeol, *Naja nigricollis*, Phospholipase A_2

INTRODUCTION

Cryptolepis oblongifolia belongs to the family periplocaceae, the plant was found to be in the same genus with *Cryptolepis sanguinolenta* and *Cryptolepis buchananii* which were reported to be used in treatment of snake bite traditionally (Verma *et al*., 2007). *Naja nigricollis* belongs to the family Elapidea, one of the common snake found in Northern part of Nigeria that is dangerous to human being (Reid, 1982), its venom contains metalloprotease and phospholipase. Phospholipase A_2 are group of enzymes that hydrolyzes the 2-position of 3-phospholip there by releasing eicosanoids or its derivatives which were reported to be agents of inflammation (Fuly *et al*., 2002). PLA_2 as the major component of snake venom, being responsible for several pathophysiological effects caused by snake envenomation, such as neurotoxic, cardiotoxic, cytotoxic, hypotensive and anti-coagulant activities. In many parts of rural areas of Africa and Asia, medicinal plants have been used as alternatives to conventional antisnake venom (ASV) due to scarcity of ASV, high cost of ASV and poor access to healthcare services (Gomes *et al*., 2010). Herbal treatments using medicinal plants are the most popular form of traditional medicine and 80% of people worldwide rely on herbal medicines for some aspect of their primary healthcare needs (WHO, 2013). Many plants constituents have beneficial activity against snake venom, probably because of the inhibitory effect of the constituents on phospholipase A_2 (PLA_2) (Umar *et al*., 2014). The aim of this research was to carry out inhibitory studies of plant isolate on purify PLA_2 venom of *Naja nigricollis* that caused death in some part of Nigeria.

MATERIALS AND METHODS

Collection of Plant Material

Samples of *C. oblongifolia* were collected in the month of September, 2019 at Dagachi district of Zaria and identified at Herbarium unit of Biological Science, Ahmadu Bello University Zaria by comparison with a voucher specimen number 302. The roots parts were ground to powder and 1000 g was obtained as the net weight.

Preparation of Extracts from Roots Part *C. oblongifolia*

The powdered plant (1000 g) was extracted via maceration with hexane (5.0 L).The extract was concentrated under reduced pressure with rotary evaporator.

Silica –gel Column Chromatography of hexane extract

The extracts (5 g) of each was loaded on to packed adsorbent and allowed to stabilize for 3-hours before elution started. Hexane (100 %) was used as the initial eluent followed by ethyl acetate gradiently from 0-50 %. Fractions of 40 ml each were collected, allowed to concentrate/dry under room temperature. Columns fractions were monitored on TLC plate (Merck F_{254}) visualized under UV 254 nm and 365 nm / p-anisaldehyde reagent or vanillin/sulphuric acid used as spraying reagent.

Melting point determination for isolated compound

The melting points of isolated compound was determined using Gallenkamp melting point apparatus and the melting point was ascertained from the thermometer when the sample melted.

Test for Terpenoids (Liebermann - Burchard test)

The isolated compound was dissolved in hexane and spotted on TLC plate developed in Hexane: Ethyl acetate (7:3) solvent system. The plates were sprayed with Lieberman - Buchard solution.

Spectroscopic analysis

***Nuclear Magnetic Resonance*:** Nuclear magnetic resonance (NMR) Spectra conducted include 1D (^1H, ^{13}C, and DEPT-135) and 2D (H-H COSY, HSQC, and HMBC). NMR Spectra was obtained on ARX-400 MHZ (Brucker/ TOPSPIN) at University of Pretoria, South Africa.

***Snake venom*:** Freeze dried Naja nigricollis venom was a gift from Dr. Y.P Ofemili of the Department of Physiology and Toxicology, Faculty of Veterinary Medicine Ahmadu Bello University Zaria.

Partial purification of *Naja nigricollis* crude venom

Gel filtration

Crude venom of *Naja nigricollis* (200 mg) was dissolved in 1 ml distilled water and loaded on Sephadex G-75 column (1 cm x12 cm) that was pre-equilibrated with 50 mM phosphate buffer (PH 7.1). The column was eluted with 50 mM Phosphate buffer at flow rate of 1 ml/min. Fifty fractions of 3 ml each were collected and assayed for protein and phospholipase A_2 activity. The PLA_2 active fractions were pooled together as one fraction.

Rechromatography on Phenyl Sepharose

The pooled PLA_2 active fractions from the process of gel filtration were subjected to hydrophobic interaction on phenyl sepharose column (2 cm x 60 cm) that was pre-equilibrated with 50 mM phosphate buffer, (PH 7.1). The column was then eluted with stepwise gradient of NaCl (0.0, 0.2 and 0.3 M) in 50 mM phosphate buffer. Fractions were assayed for protein and phospholipase A_2 activity. Fractions with the same PLA_2 activity were pooled as one.

Rechromatography on Sephadex G-25 (Gel Filtration)

The pooled fractions from phenyl sepharose column were chromatographed on Sephadex G-75 column (1 cm x12 cm) pre – equilibrated with 50 mM phosphate buffer (PH 7.1). The column was eluted with the same buffer. Fractions were assayed for protein by measuring absorbance at 280 nm and PLA_2 activity. The fractions (single peak) from this step were used for all subsequent analysis.

RESULTS AND DISCUSSIONS

Silica-gel chromatography of hexane extract yielded a compound A1 which appeared as white amorphous substance which shows green colour with Lieberman Burchard and melt at 175 ^{0} C -176^0 C. The ^{1}HNMR spectrum of compound A1 showed signals due to six methyl singlets at δ_H 0.78 (3H), 0.81(3H), 0.86 (3H), 0.99 (3H), 1.05 (3H) and 1.28 (3H) integrated for 3H each, an olefinic methyl group at δ_H 1.67 (3H), and a doublet due to a terminal methylene protons at δ_H 4.66, H-29b and 4.54 ppm H-29a, which are typical of triterpenoids. The ^{1}HNMR spectrum also showed double doublet at δ_H 3.20, H-3 due to a methine proton attached to hydroxyl group. The structure assignment of A1 was further substantiated by the ^{13}C NMR experiment which shows seven methyl group [$\delta_{C:}$ 28.2 (C-23), 18.2 (C-28), 16.2 (C-25), 16.2 (C-26), 15.5 (C-25), 14.9 (C-27) and 19.5 (C-30)]; the presence of exomethylene was observed at [δ_C 151.17 (C-20) and δ_C 109.5(C-29)] and methine hydroxy bearing carbon atom at δ_C 79.2 ppm, C-3. Ten methylene, five quartenary carbons were assigned with the aid of DEPT 135^0. The structure was further established with the aid of 2D NMR experiment (^{1}H-^{1}H COSY and HMBC). The COSY spectrum of A1 exhibited some cross peaks such as between (δ_H 2.14, H-19) and one SP^3 methylene proton signal (δ_H 1.37, H-21) and between oxygenated methine proton (δ_H 3.2, H-3) and sp^3 methylene signal (δ_H 1.61, H-21). The HMBC spectrum showed cross peaks between methine proton signal at δ_H 3.2 (H-3) and methyl carbon signal (δ_C 28, C-23), the pair of broad singlets of olefinic proton at δ_H 4.55 and 4.66 showed cross peak with methylene carbon signal [δ_C 48.2 (C-19) and 19.5 (C-30)]. By comparison with reported data literature as showed in table 1 the compound A1 was propose to be lupeol, a pentacyclic tri-terpenoid (Fig.1). The venom of *Naja nigricollis* was purified to apparent homogeneity using three purification steps as showed in Fig. 2, 3 and 4. Lupeol (isolate from *C. oblongifolia*) was studied against purified phospholipase A_2 of *Naja nigricollis* venom. Our finding revealed that, the isolate inhibit the enzymes in a non-competitive pattern with inhibition binding constant of 0.015µg/mol. However Umar *et al*., 2014 reported the inhibition of PLA_2 of *Naja nigricollis* crude venom by Oleanyl erucoate (Triterpene fatty acid). Similarly Abubakar *et al*., 2000 reported the detoxification (in-vitro) of two common Northern Nigeria snakes venom by the leaf extract of *Guiera senegalensis*. Compound(s) like lupeol, oleanyl erucoate can be used as template for the development of novel drugs that can be used in treatment of snakebite.

Table 1: ^{1}HNMR and ^{13}CNMR Spectral Data of Compound A1 and Literature reported in ppm (in CDCl3)

C/H S/NO	^{13}CNMR	^{1}HNMR	^{13*}CNMR	^{1*}HNMR
1.	38.8	-	38.7	
2.	27.4	-	27.4	
3.	79.2	3.21	79.0	3.21
4.	38.9	-	38.9	
5.	55.5	0.71	55.5	0.71
6.	18.5	1.39	18.5	1.39
7.	34.4		34.2	
8.	41.0		40.9	
9	50.6	1.28	50.5	1.28
10.	37.3		37.2	
11.	21.1	1.53	21.0	1.53
12.	25.3	1.28	25.2	1.29
13.	38.3	1.67	38.1	1.67
14.	42.5	1.42	42.9	1.42
15.	27.2	1.05	27.1	1.05
16.	35.8		35.5	
17.	43.0		43.0	
18.	48.5	0.91	48.3	0.91
19.	48.2	2.41	48.0	2.14
20.	151.1		151.0	
21.	30.0	1.33	29.9	1.33

22.	40.0		40.2	
23.	28.0	1.63	28.2	1.64
24.	15.5	1.61	15.5	1.61
25.	16.2	0.83	16.1	0.84
26.	16.2		16.0	
27.	14.9	0.97	14.8	0.97
28.	18.2	0.78	18.0	0.79
29a.	109.5	4.55	109.0	4.61
29b	109.5	4.66	109.0	4.71
30.	19.5	1.67	19.5	1.69

Note: 13*CNMR and 1 *HNMR = Abdullahi *et al*., 2013

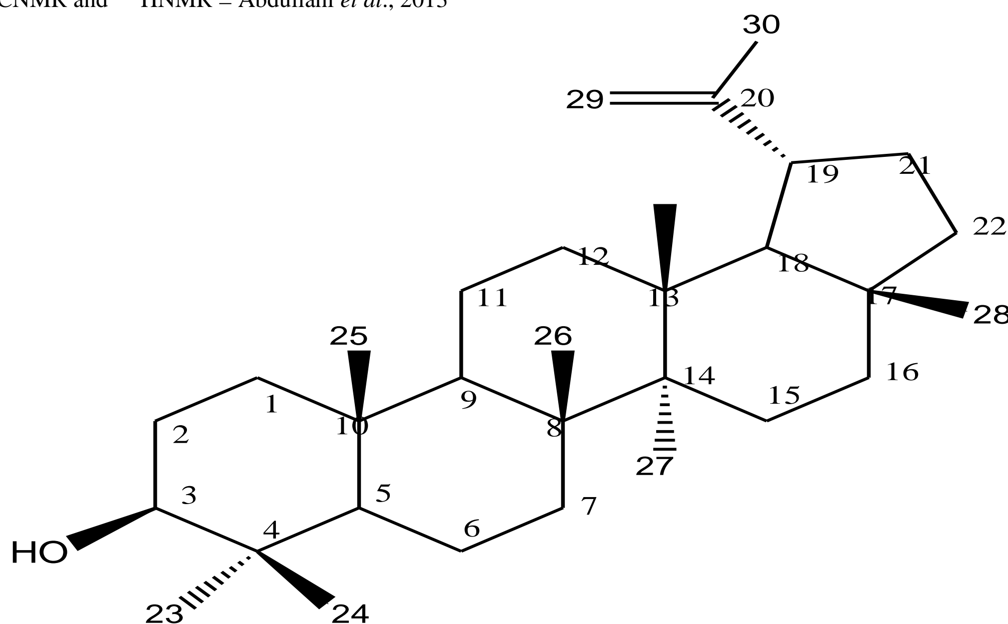

Figure 1: Proposed Structure of Compound A1 (Lupeol)

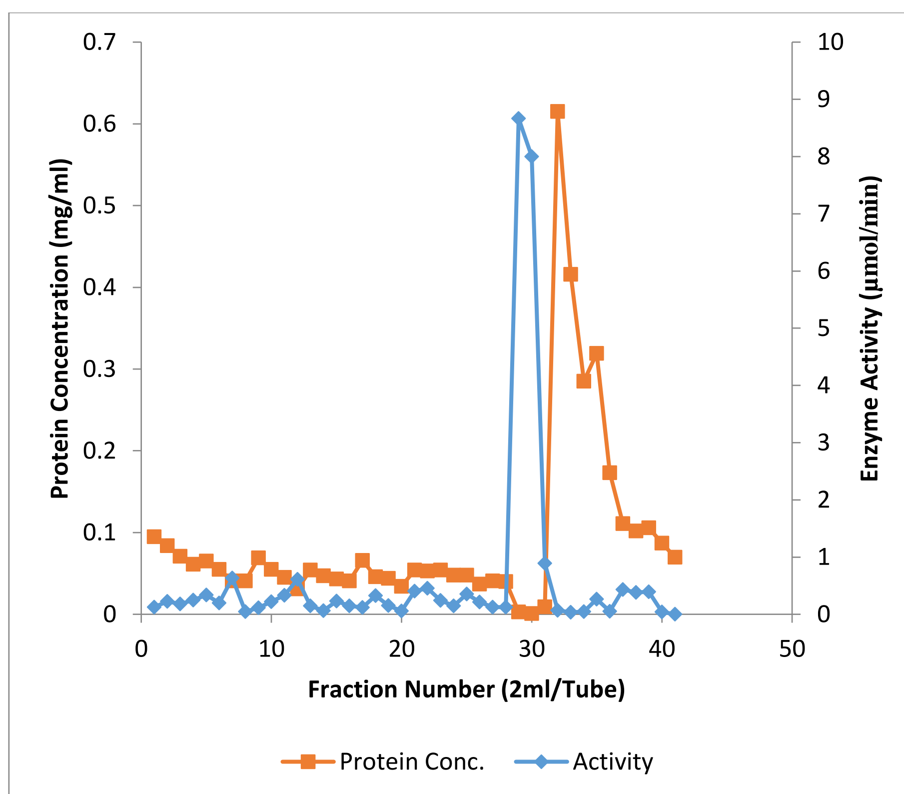

Figure 2: Elution profile of crude *Naja nigricollis* Venom from a Sephadex G-75 column

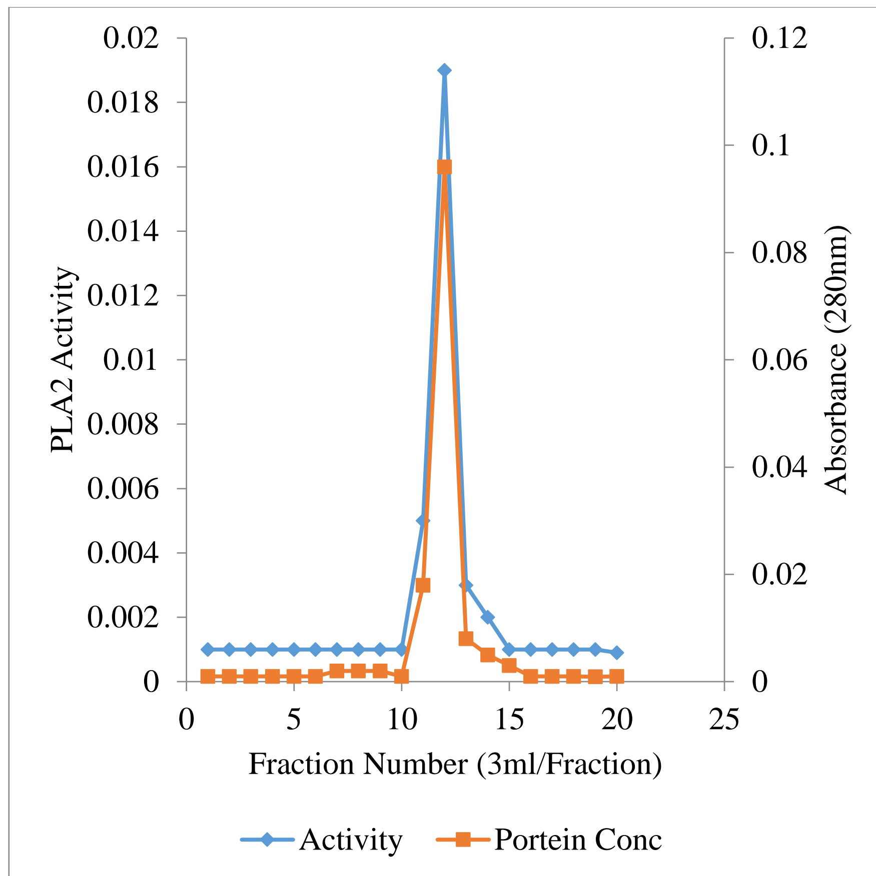

Figure 3: Elution profile of PLA_2 Active Fractions from First step on Phenyl sepharose column

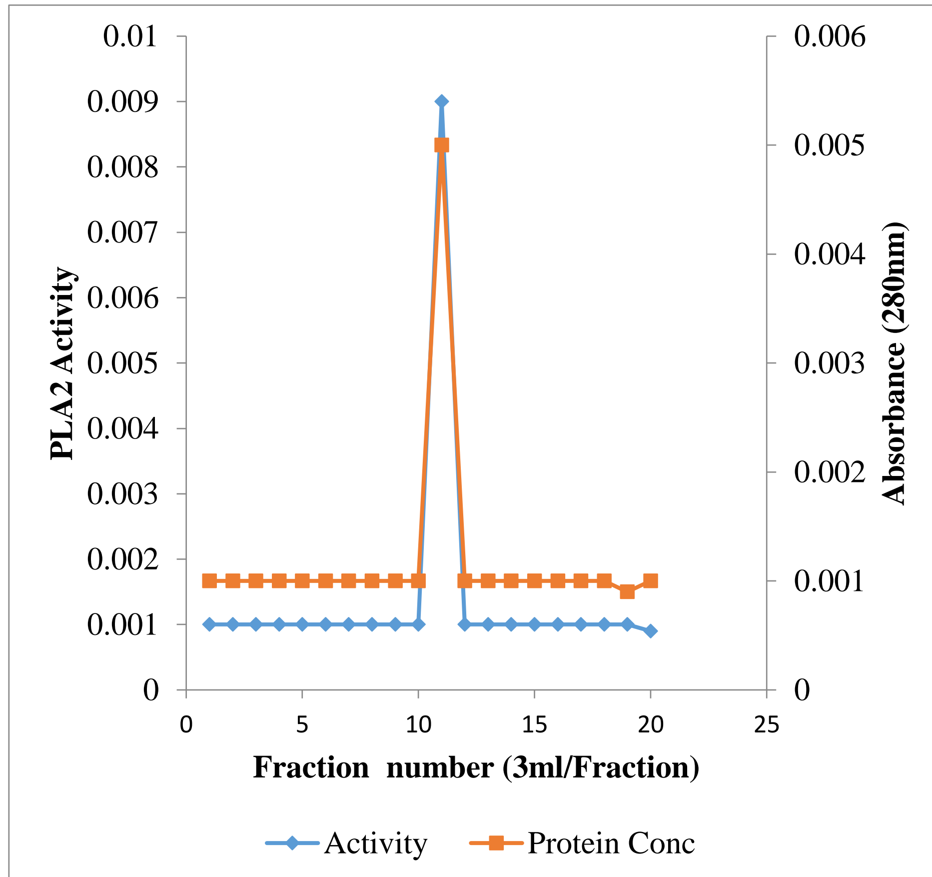

Figure 4: Elution profile of PLA_2 Active fraction from second step on Sephadex G-25 column

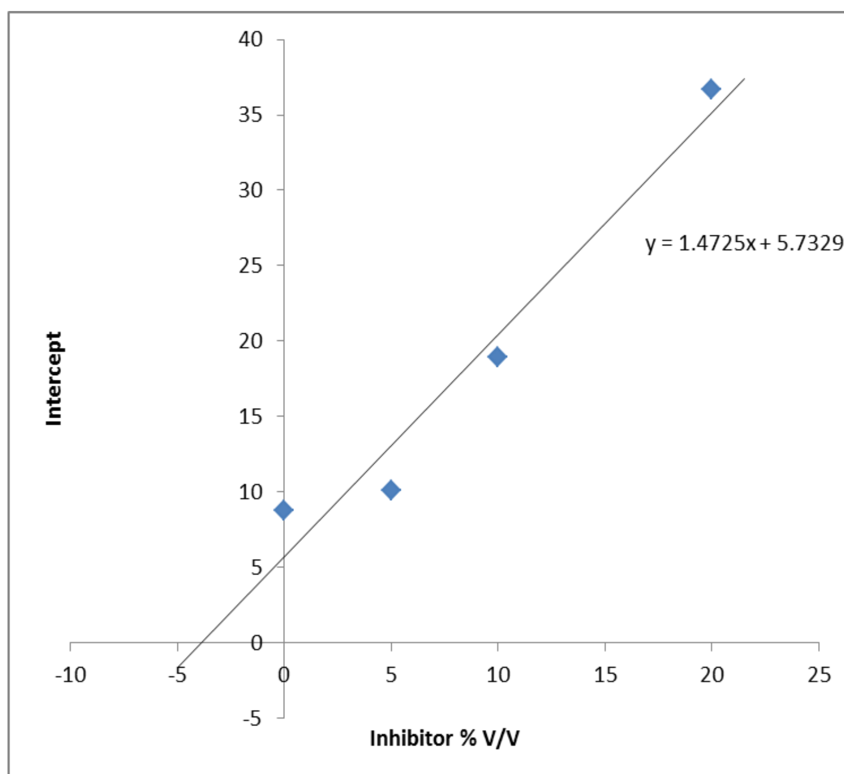

Figure 5: Dixon's plot of Phospholipase A2 in the presence of Lupeol (Compound A1)

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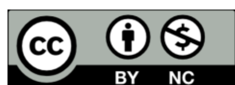

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