

Isolation of Phospholipase A₂ Inhibitor from *Cryptolepis oblongifolia* (Meins) Schltr

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Abstract

Chromatographic studies of *Cryptolepis oblongifolia* yielded a triterpene fatty acid (Oleanyl Erucoate) which was identified through spectroscopic means. The inhibitory effect of the isolated compound on PLA₂ of *Naja nigricollis* venom was investigated. The ¹³CNMR of the isolated compound showed carbonyl functional group and oleanane type of structure while EIMS analysis revealed that, the compound was Oleanyl Erucoate with molecular weight of [M+1] 735. The triterpene fatty acid inhibited the PLA₂ in a dose dependent fashion with an inhibition binding constant (*ki*) of 1.74 mg/mL. The activity of this compound might provide a scientific basis for the local use of *Cryptolepis oblongifolia* in traditional medicine for the treatment of inflammation or contribute in the development of novel drugs that could serve as potential antivenin.

Keywords: *Naja nigricollis* venom, Oleanyl erucoate, Inhibition

1. Introduction

Phospholipases A₂ (PLA₂) are enzymes found to catalyze the hydrolysis of fatty ester in the 2- position of 3-phospholipid to release fatty acid and lysophospholipid, the fatty acid so formed may act as either second messenger or a precursor of eicosanoids [1], which are key factors of inflammation. Snake bite envenomations are of public health concern as a result of their high mortality and chronic morbidity. It is also of biomedical importance with social and economic impact on developing regions around the world. Snake venoms are composed of complex mixture of active substances, mainly peptide and proteins which are able to interfere with the course of several biological processes including thrombosis by affecting platelet aggregation and blood coagulation [2]. Some of these proteins include enzymes like phospholipase A₂ and metalloprotease [3]. *Naja nigricollis* belongs to the family Elapidea and is among the snake species dangerous to humans [4]. The snake is one of the two most common snakes found in Northern Nigeria [5] and has been reported that its venom contains metalloprotease and phospholipase [6]. Over years, natural products (medicinal plants) have been used in the treatment of snakebites, particularly in rural areas of Nigeria, however the mode of action and the active components of these plants are almost unknown [7]. *Cryptolepis oblongifolia* is a shrub indigenous to the West Africa it grows wild along the West Coast of Africa and some parts of Central Africa. To the best of our knowledge, to date, there has been no scientific report on either the biological or chemical effect of the plant on any phospholipase activity taking into account the relevant role the enzyme play in the dynamics of inflammation. The aim of this study was to provide scientific basis for the use of the plant in traditional treatment of inflammation and also to examine whether it harbours some snake venom PLA₂ inhibition potential.

Methods

Plant material: The aerial parts of *C. oblongifolia* were collected from Dagachi district of Zaria, Nigeria. It was identified at herbarium unit of Department of Biological Science, Ahmadu Bello University Zaria, by comparison with a voucher specimen number of 302. The plant part was dried at room temperature, ground to powder and stored in sealed container until needed.

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

Preparation of extract from *C. oblongifolia*

The powdered plant (500 g) was extracted in hexane (2.5 L) and concentrated under reduced pressure with a yield of 9%.

Silica- gel Column Chromatography of *C. oblongifolia* Hexane Extract

The hexane extract (4 g) was chromatographed over silica-gel (100g, 60-230 μm). Hexane (100%) was used as the initial eluent followed by ethyl acetate gradient (0 – 10%). Fractions of 40 mL each were collected, concentrated and dried under reduced pressure. Column fractions were monitored on TLC (Merck F 254) visualizing under UV (254 nm and 365 nm), *P*-anisaldehyde reagent or vanillin/sulphuric acid were used as spraying reagent.

Identification of Compound Isolated

The isolated compound was identified using NMR and Electron Impact Mass Spectroscopy. The Nuclear magnetic resonance (NMR) Spectra conducted include 1D (1H, 13C, and DEPT-135) and 2D (H-H COSY,

HSQC, and HMBC). The spectra were obtained at 300 K on ARX-400 MHz (Bruker/ TOPSPIN). The Electron Impact Mass Spectroscopy (EIMS) was carried out at 70 ev.

Preparation of Snake Venom extract

Freeze dried venom of *Naja nigricollis* (20 mg) was dissolved in 1 mL of saline.

Phospholipase assay: This was carried out as described by [3]. Here, 0.5 mL of egg yolk suspension (2 mg mL^{-1}) was introduced into a clean test tube containing $50 \mu\text{L}$ of 1 mM CaCl_2 . To this, $100 \mu\text{L}$ of 20 mg mL^{-1} venom solution was added and incubated at 37°C for 1 hour. Thereafter, the enzyme was inactivated by heating at 100°C for 2 min, a drop of phenolphthalein then added and titrated against 2 mM NaOH solution to an end point. The same procedure was carried out in the presence of inhibitor in order to obtain titre value for the standard for adequate comparison to deduce effect of the enzyme on the yolk (deduction of any FFA released). The activity of the phospholipase (PLA_2) was defined as the amount of enzyme required to hydrolyse 1 mg of free fatty acid (FFA) from the lecithin present in the egg yolk under the standard assay conditions.

Results

Chemical shifts ($^1\text{H NMR}$ and $^{13}\text{C NMR}$) of the isolated triterpene fatty acid in CDCl_3 at 400 MHz. The compound was obtained as white amorphous substance with m.p 80°C and gave positive Liebermann-Burchard test for terpenoids. The $^{13}\text{C NMR}$ signal at 173.7 ppm indicating the presence of carbonyl group and olefinic carbon bond at $\delta 129, 142.6 \text{ ppm}$ indicating terminal methylene and also signal at $\delta 80.5$ indicate the presence of methine carbon bearing hydroxy groups.

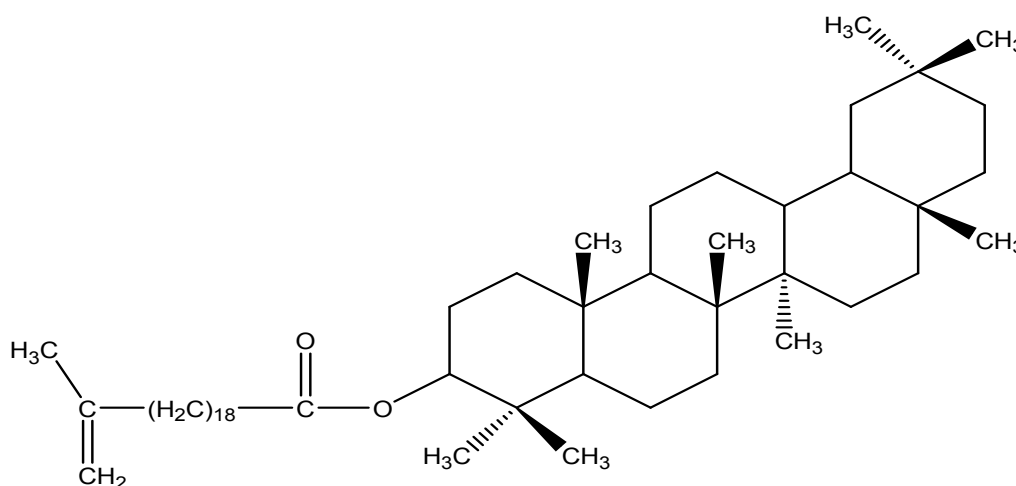


Figure 1: Structure of Triterpene fatty acid

Oleanyl erucoate: White amorphous powder (CHCl_3); mp $78-80^\circ\text{C}$; EIMS m/z 735 [M^+] (20), 409 (10), 231 (25), 219 (35), 121 (45), 177 (65), 189 (70), 204 (100); $^1\text{H NMR}$ (400MHz, CDCl_3 δ) 1.05 (2H, m, H-1), 1.26 (2H, m, H-2), 4.48 (1H, t, H-3), 1.30 (2H, m, H-6), 1.52 (2H, m, H-7), 1.57 (1H, m, H-9), 1.34 (2H, m, H-15), 1.39 (2H, m, H-21), 0.80 (3H, s, H-23), 0.94 (3H, s, H-24), 0.92 (3H, s, H-25), 0.91 (3H, s, H-26), 1.07 (3H, s, H-27), 0.85 (3H, s, H-28), 0.84 (3H, s, H-29), 0.88 (3H, s, H-30), 2.28 (2H, t, H-2'), 1.74 (2H, m, H-2'), 1.29 (2H, br, s, H-4'-H-16'), 2.28 (3H, s, H-21'), 4.84 (2H, s, H-22'); $^{13}\text{C NMR}$ (400MHz, CDCl_3 δ) 38.5 (C-1), 80.5 (C-3), 51.1 (C-9), 38.3 (C-4), 40.7 (C-8), 23.0 (C-11), 26.9 (C-12), 29.3 (C-20), 16.0 (C-25), 28 (C-28), 18.0 (C-28), 16.0 (C-26), 15.0 (C-29), 173.7 (C-1'), 34.4 (C-2'), 29.1-29.7 (C-4'-C-16'), 32.4 (C-18'), 142.6 (C-20'), 55.5 (C-19'), 77.6 (C-21'), 129.7 (C-22').

Figure 2. Showed line-Weaver Burks plot described the effect of compound P at varying concentration of 0 mg/ml , 0.625 mg/ml , 1.25 mg/ml and 2.5 mg/ml using egg yolk as substrate. From the figure it is evident that, the compound P inhibits the enzyme in non competitive manner as all the lines converged at the X-axis. The Dixon's plot (figure 3) determine the inhibition binding constant k_i to be 1.74 mg/ml from the intercept using straight line equation, while figure 1 showed the structure of isolated compound P, which is a triterpene attached to fatty acid using an ester linkage.

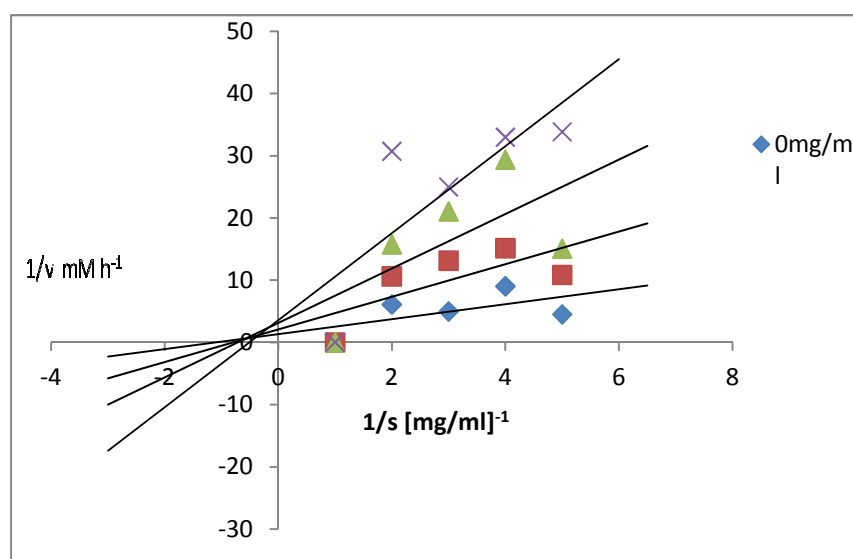


Figure 2: Line-Weaver-Burks plot showing Effect of Compound P on *Naja nigricollis* venom phospholipase A₂ Activity.

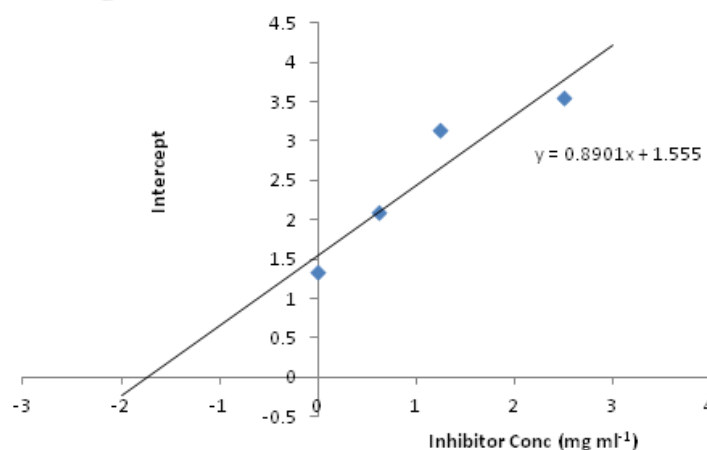


Figure 3: Dixon's plot to determine inhibition binding constant (k_i) for Compound P toward crude of *Naja nigricollis* venom phospholipase A₂

Discussion

In this research, the active component of *C. oblongifolia* responsible for anti-phospholipase A₂ activity was studied against the crude venom PLA₂ of *Naja nigricollis*. Our findings revealed that, the isolate P have haemolytic factor or it has the ability of blocking the venom-induced haemolysis. ¹³CNMR at δ 173.7 ppm indicated the presence of carbonyl ester. The ¹HNMR spectrum showed the presence eight methyl singlets at δ 0.80, 0.85, 0.91, 0.84, 0.94, 0.92, 0.88 and 1.07 ppm and a multiplet at δ 4.49 ppm suggesting the basic skeleton of 3 β substituted derivative of triterpenoids, also a multiplet at δ 2.28 indicating a side chain attached to carbonyl carbon atom. The above ¹³CNMR data was almost the same as that of Oleanane. In addition to an ester carbonyl that was found at δ 173.7 ppm and an olefinic proton at 4.86 ppm was observed, further HMBC spectrum showed correlation between Methylene protons adjacent to carbonyl of an ester suggesting the presence of a side chain at C-3. The HSQC spectrum showed long range correlation between proton at δ 2.28 ppm and δ 173.7 ppm which is a carbonyl, this confirm that it is part of the ester side chain. A broad singlet at 1.68 ppm for β -methylene proton of carbonyl correlate with carbon at δ 25.2 ppm, also a broad singlet at 1.6 ppm integrated for 38 protons indicating that the side chain at C-3 contains 22 carbon atoms as in erucic acid. These arguments was supported by the intense mass fragment ion peak observed at m/z 409 formed by loss of side chain from molecular ion peak. Based on the above spectra data, the compound P was proposed to be Oleanyl erucate.

The lineweaver plot (Fig. 2) showed the effect of varying concentration of the inhibitor on snake venom PLA₂, the result indicated that, the oleanyl erucoate inhibit the phospholipase A₂ activity in a non-competitive manner and the Dixon's plot (Fig. 3) showed the minimum concentration of inhibitor that inhibit the enzyme (ki) to be of 1.74 mg/ml. Isolated edunol, a pterocarpan from *Harpalyce brasiliiana*, with antimyotoxic and antiproteolytic activities against *B. jararacussu* venom beside expressive phospholipase A₂ inhibitory properties, [9] also the synthetic pterocarpan was found to inhibit the myotoxic and phospholipase A₂ of *B. jararacussu* [10]. Angulo and Lomonte, (2003) reported the antivenom activity of fucoidan a polyssacharide sulphate from the brown marine alga *Fucus vesiculosus*. Interesting result obtained by [11] indicated that *Casearia syvestris*, significantly inhibit the activity of PLA₂ isolated from *Bothrops crotalus* and *Micrurus* snake venoms. Fifteen compounds isolated from plants were also reported as snake venom antidote. They were shown to protect mice to a significant degree against the lethal action of the venom of *Bothrops jararaca* snakes [12].

Several other plants extract were known to have antiphospholipase A₂ and antivenoms neutralizing properties. Few example include the extract of the leaves of *Guira senegalensis* was found to detoxify (*in vitro*) venom from two common northern Nigerian snake species *Echis carinatus* and *Naja nigricollis*. [13], similarly the crude venom from *Bothrops jararaca* has pro-coagulant platelet aggregating and phospholipase A₂ activities [14].

Since the ¹³CNMR analysis revealed that, the isolated (anti venom) compound in *C. oblongifolia* is a triterpene with a fatty acid attached using an ester linkage and EIMS result showed that, the triterpene fatty acid compound is Oleanyl erucoate as given in Fig.1. Studies on the interaction of oleanyl erucoate with snake venom PLA₂ provide an additional dimension to understanding of the mechanism of compounds on PLA₂ toxicity. These interactions occur at specific sites and hence are capable of recognizing subtle structure that is responsible for its biological activities. These finding support the hypothesis that PLA₂ (toxin) have a specific site responsible for its enzymatic and pharmacological action. This isolated compound (inhibitor) interacts with the active site of PLA₂ to inhibit its action on its natural substrate.

The possible mechanism of inhibitory effect of Oleanyl erucoate is that, the carbonyl group as a result of catalysis of PLA₂ experience electron pull by Ca²⁺ at pH of 7 providing nucleophilic centre in the presence of imino group of histidine 48 residue at the active side of the PLA₂, similar effect is repel back by the inhibitor through carbonyl group there by forming complex and rendering the enzyme inactive, similarly this effect can also be experience through the olefinic bond present in the inhibitor. Or the Calcium ion (Ca²⁺) that is required for PLA₂ activity can chelate electron from carbonyl group there by inhibiting PLA₂ to occur, these goes in similar way with the olefinic bond present in the inhibitor. This observation is supported by [15] in a related development, reported that an organic acid isolated and purified from the root extract of an Indian medicinal plant Sarsaparilla *Cryptolepis buchanni* R.Br possessed viper venom inhibitory activity, similar work was reported by [16] showed the antivenom activity of fucoidan a polyssacharide sulphate, isolated from brown marine alga *Fucus vesiculosus*. Isolated 4-neolidyl catechol from the extracts of *Piper umbelantum* and *Piper peltatum* effectively neutralized toxic effect of *Bathrops* myotoxins, inhibiting their PLA₂, myotoxin and edema inducing activities [17]. However one or more mechanism can explained this inhibitory activity perfectly, although no structural or inhibitory spectrum information of the isolated compound is available to date. However it is important to note that this active compound opens the possibility of its application for therapeutic purposes.

Conclusion

Cryptolepis oblongifolia, (Meisn) Schltr contained bioactive compounds such as stigmasterol and Oleanyl erucoate. Isolation and identification of these compounds have provided bases for the use of the plant in traditional medicine. Continuous efforts toward the study of new natural anti-venoms are relevant for the understanding of snake venom biology as well as the physiopathology involving snake bite effects in different organisms. Regarding PLA₂s, these studies could serve as a valuable input on comprehension of its mode of action, classification and their structure-function relationship, so extending the protein science field.

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Declaration of Interest

There are no conflicts of interest

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