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Efficacy of Di-n-octyl Phthalate Anti Venom Isolated from *Ceiba pentandra* Leaves Extract in Neutralization of *Echis ocellatus* Venom

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Abstract: The aim of the study is to investigate the efficacy of Di-n-octyl phthalate isolated from *Ceiba pentandra* leaves extract for its anti *Echis ocellatus* venom properties. The leaves of *Ceiba pentandra* was extracted with aqueous methanol, partitioned on column chromatography and subjected to TLC and preparative TLC. The isolate was tested for its anti venom activity and submitted to IR and GCMS for structural elucidation. Our findings revealed that the isolate was biologically active in inhibiting PLA_2 activity in a dose dependent manner. The IR analysis showed that the isolate has a carbonyl functional group and aromatic ring and the GCMS analysis revealed that the compound isolated was Di-n-octyl phthalate with molecular weight 391. The inhibitor (Di-n-octyl phthalate) isolated during this research might become a highly effective therapeutic agent for the reducing of snake bite envenomation. The relevance of these findings would be of importance in the area of development of anti snake bite drugs that are going to be effective, cheap, accessible and less allergic plant compound that can be scientifically explored.

Keywords: *Ceiba pentandra*, di-n-octyl phthalate, *Echis ocellatus*, PLA_2 activity

INTRODUCTION

The indigenous knowledge on medicinal plants plays a vital role in the discovery of new herbal drugs and new sources of pharmaceuticals. The use of medicinal plants has been in practice throughout human history, whose knowledge gathered through experience of many generations. With this in context, it is important to point out that plants have contributed to the abstention of many drugs; substances with interesting activities are often present in plants. Over years, medicinal plants have been used in the treatment of snakebites, particularly in rural areas. However, the mode of action and the active components of these plants are almost unknown (Nok *et al*., 2001). Kambaska *et al*. (2007) reported that the exploration of ethno medicinal data may serve as a useful source of information to the chemist and pharmacologists so that it can provide ways of identifying the bioactive components of the plant extract for the purpose of drug development. We have discovered that the traditional medicine practitioners use a particular herb from a tree called *Ceiba pentandra* linn for the treatment of cattle snakebite victims. As search for new drugs continue, there is also a need to assess locally available herbs as source for anti venom drugs. In this study, the active principle of *Ceiba pentandra* responsible for its anti venom activity was studied against the venom of *Echis ocellatus* especially against the purified PLA_2 components of the venom. We have also attempted to identify the functional group of the phytochemical and elucidated the mechanism of action of inhibitory effects of phospholipase A_2 with the view of identifying the active constituent of the extract.

Reagents, solvents and equipment: All reagents and solvents used were of analytical grade and supplied by Sigma chemicals Co. St. Louis or BDH chemical Company, England. IR spectroscopy (8400S, Japan) GC/MC spectroscopy (GCMS-QP2010 PLUS Shimadzu, Japan)

Experimental animals: Adult albino mice (20-30 g) of either sex were used for the research. They were procured from Animal house of faculty of Pharmaceutical Sciences Ahmadu Bello University, Zaria. The animals were housed, fed with normal animal feeds and maintained in Animal house of faculty of Pharmaceutical Sciences Ahmadu Bello University, Zaria.

METHODOLOGY

Collection and preparation of plant material: Fresh leaves of *Ceiba pentandra* were obtained from Samaru, Sabon Gari local government, Zaria, Kaduna state Nigeria during the month of June. The plant collected was brought to the Department of Biological Science of Ahmadu Bello

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University, Zaria and identified by officer in charge of the herbarium with voucher number 5079. The leaves removed from the plant were air dried in a shade at room temperature for a period of three weeks. The dried parts were powdered by pounding in mortar with pestle and sieved to fine powder. The fine powder was packed in air tight labelled container and stored in refrigerator until required for use.

Capturing of *Echis ocellatus* and milking of venom: The snake was captured by snake charmer identified by officer in the Department of Biological Sciences Ahmadu Bello University, Zaria. The venom was collected in a standard way as described by Bucherl *et al*. (1968). Briefly, the snake was held by snake charmer. The mouth of the snake was opened and its poisonous fangs were placed on the inner edge of the fixed glass and the milking of the venom was facilitated by pressing on and off of the tail of the snake and venom drops into the glass at time intervals. The venom was collected, dried at room temperature and kept until needed.

Preparation of extracts of *Ceiba pentandra*:
Extraction of the leaves with aqueous methanol (60/40): Two hundred grams portions of the dried powdered *Ceiba pentendra* leaves was defated with 60/80 petroleum ether using soxhlet extractor. The petroleum ether mack was dried and then extracted with aqueous methanol (60/40 v/v) exhaustly. The extract was concentrated in rotary evaporator.

Partitioning of the aqueous methanol (60:40) extract of *Ceiba pentandra* plant extract with different solvents: Column chromatography of the n-butanol fraction was conducted as described by Nok *et al*. (1993). A slurry was prepared by dissolving 30 g silica gel in 100 mL methanol: water (1:1) and packed in a column (1.5×30 cm). The bioactive fraction was subjected to column packed with silica gel pre-equilibrated with N-butanol/water 4:5 and the samples were eluted, using running solvent system: n-butanol and water (4:5). and ten fractions were collected, dried and the fractions were tested for antivenom activity.

The bioactive fractions were collected, dried and submitted to thin layer chromatographs analysis on silica gel (0.25 mm) using different running solvents, chloroform methanol (2:98), chloroform: ethyl acetate (4:1). The fractions were tested for antivenom.

Analytical thin layer chromatographic analysis of fraction of the *Ceiba pentandra*: Precoated analytical plate was used for the thin layer chromatography. Capillary tube was filled by dipping one end into the fraction to be examined and lightly applied to the thin layer plate. Ascending chromatography was conducted in a wide chromatographic tank containing chloroform: ethyl acetate (4:1) as mobile phase. The chamber was saturated with the same solvent system before the loaded plates. After that, the plates were dried spots were detected. The spots were scraped and the phytochemical was then eluted with suitable solvent. The eluates were tested for activity and active one used for structural analysis.

Preparative thin layer chromatographic analysis of fraction of the *Ceiba pentandra*: Preparative thin layer chromatographic analysis of the bioactive fraction of the *Ceiba pentandra* was also carried out and the spot region was detected. Spots were scrapped and eluted. The eluates were tested for activity and active one used for structural analysis.

Phospholipase A_2 assay: Phospholipase A_2 neutralization has been determined using the method described by Simuzu *et al*. (1980). Thus 50 mL of the crude venom was incubated with 50 mL of egg yolk at 37ºC for 30 min. At the end of the incubation, the reaction was stopped by immersing in boiling water for five minutes. The reaction mixture was titrated using 20 mM sodium hydroxide and phenolphthalein indicator. The same procedure was repeated using extract preincubated venom. The volume of sodium hydroxide used to neutralize free fatty acids was recorded and the activity of Phospholipase A_2 was calculated as detailed below:

Volume of 20 mMNaOH that neutralized free fatty acid = ymL
$\therefore$ 0.02 x y x 10^{-3} = k moles/30 min
Enzyme activity = k moles/30 min = Z moles/min

In vitro studies of effect of *Ceiba pentandra* leaves partitioned extracts on the venom of *Echis ocellatus*:
Red blood cell fragility test: This test reveals subtle abnormality not detectable with standard osmotic fragility test (Dougterty, 1976). The tail tips of clean healthy mice were clipped and 200 µL of blood were collected in heparinised capillary tubes and transferred to 5 mL of phosphate buffered saline PH 7.2 and centrifuged. After centrifugation the cells were re suspended in 5 mL of the same buffer and one milliliter of the cell suspension was added a duplicate to test tube containing 4 mL of distilled water. This was repeated for the test samples. All samples were incubated at 37ºC for 6 h then mixed and centrifuged. Percentage heamolysis was determined by comparing A_{540} of the supernatant of the control or test samples with A_{540} of completely heamolysed sample (distilled water treated sample).

Evaluation of the isolated *Ceiba pentandra* principle incubated with *Echis ocellatus* venom's phospholipase A_2: Phospholipase A_2 neutralization has been determined using the method described by Simuzu *et al*. (1980). Thus 50 mL of the crude venom (0.1 mg/mL) was incubated with 50 mL of egg yolk at 37ºC for 30 min. At the end of the incubation, the reaction was stopped by immersing in

boiling water for 5 min. The reaction mixture was titrated using 20 mM sodium hydroxide and phenolphthalein as indicator. The same procedure was repeated using extract preincubated venom. The volume of sodium hydroxide used to neutralize free fatty acids was recorded from graduated micro-pipette and the activity of Phospholipase A_2 was calculated.

Effects of isolated *Ceiba pentandra* compound on purified phospholipase A_2 activity: The procedure describe by Simuzu *et al*. (1980) was used. However various concentrations of the substrate (0.3, 0.4, 0.5 and 0.6 mg/mL, respectively) were added to the reaction mixture used. The same procedure was used but the isolated inhibitor was added in the reaction mixture. A reciprocal graph of phospholipase A_2 actvity againt substrate concentration (egg yolk) was plotted.

Evaluation of effects of isolated *Ceiba pentandra* principle on *Echis ocellatus* venom's phospholipase A_2: Phospholipase A_2 neutralization has been determined using the method described by Simuzu *et al*. (1980). The same procedure was repeated except here we used purified (0.1 mg/mL) Phospholipase A_2 and isolate from *Ceiba pentandra.* Different concentrations (0.2, 0.3, 0.4, 0.5 and 0.8 mg/mL, respectively) were added to the reaction mixture. The volume of sodium hydroxide used to neutralize free fatty acids was recorded and compared. The activity of Phospholipase A_2 calculated.

Partial identification of active constituents of leaves exract of *Ceiba pentandra*:

Infrared spectroscopy for identification of functional groups in isolated active: The IR spectroscopy analysis was carried out by using IR equipment (model 8400S, Japan) The purified *Ceiba pentandra* was dissolved in methanol placed on slice to allow the solvent to evaporate. The sample was placed in the sample vial, the machine was set on and the sample was scanned ten times. The print out of the machine was obtained used to identify the functional groups present in the sample. The result was produced based on transmittance/frequency peak type.

Gas Chromatography/Mass Spectrometry (GC/MS): The procedure was carried used using GCMS machine (QP 2010 plus shimad zu, Japan) The purified The purified *Ceiba pentandra* was dissolved in methanol and 2 mL of the sample was transferred into sample vial placed in a chamber of the GCMS machine, the injection of the sample into the machine was programmed with syringe injection position at 250ºC temperature and 100.2 kpa pressure and at 60% column oven. As the separated substances emerge from the column opening, they flow into the Mass spectrometry which identifies compounds by the mass of the analyte molecule. The machine is coupled to computer that has a "library" of known mass spectra, covering several thousand compounds which helps to identify the likely compound that generates the data presented to. After that the print out of the result was obtained and used.

Table 1: Inhibition of hemolysis due to phospholipase A_2 by purified *Ceiba pentandra*

Parameters	Heamolysis (%)
Water and blood only	100
Venom + Purified *Ceiba p.* + Blood	27.4±0.02[a]
Venom and blood only	66.1±0.04[b]

Mean±SD for five determinations; Values with superscripts are significantly different at $p<0.05$ one way ANOVA

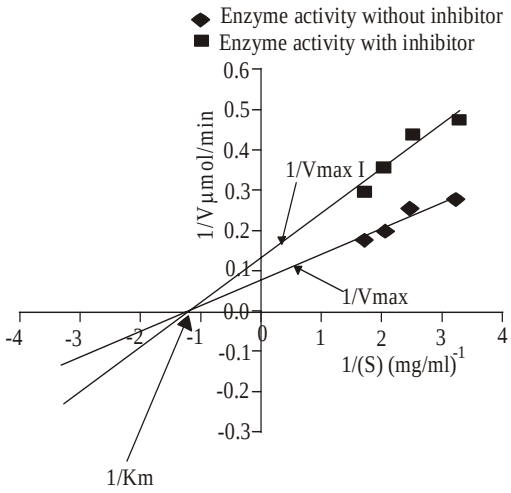

Fig. 1: Lineweaver-burk plot showing the effects of purified *Ceiba pentandra* on phospholipase A_2 activity

Thin layer chromatography of the purified fraction of *Ceiba pentandra*: The bioactive pale yellow fraction of *Ceiba pentandra* was found to contain one major spot and a minor spot. The major spot and the minor have R_f values of 0.112 and 0.866.

Large Scale Preparation of Purified Fraction of *Ceiba pentandra* by Preparative Thin Layer Chromatography revealed that there was only one spot detected using solvent system benzene, n-butanol and ethyl acetate as running solvent (8:5:2).

Inhibition of purified *Echis ocellatus* venom hemolysis of mice blood by purified *Ceiba pentandra* extract: The inhibitory effects of the plant isolate on the purified phospholipase A_2 from *Echis ocellatus* venom showed that the purified *Ceiba pentandra* leaves fraction has potentials of reducing the hermolysis due to venom from 66.1 to 27.4%, suggesting that the plant leaves fraction has inhibitory effect on phospholipase A_2 activity as shown in Table 1.

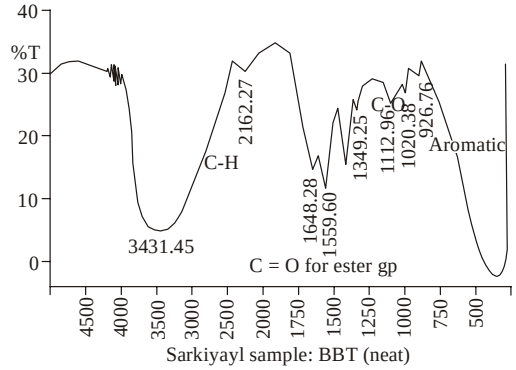

Fig. 2: IR spectrum of the purified anti *Echis ocellatus* principle of *Ceiba pentandra* leaves GC/MS spectrum of purified anti *Echis ocellatus* venom of *Ceiba pentandra*

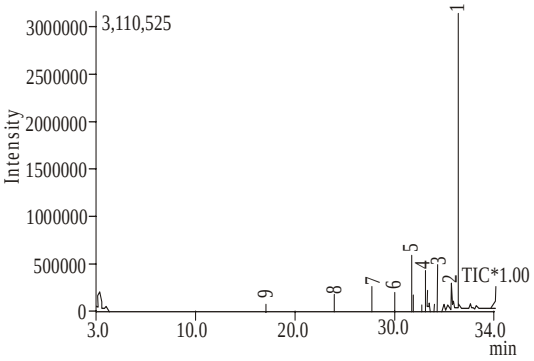

Fig. 3: The GC/MS spectrum of purified anti *Echis ocellatus* venom of *Ceiba pentandra* showing fragments peaks

Lineweaver-burk plot of the inhibition of purified phospholipase A_2 activity by purified fraction of *Ceiba pentandra*: The Inhibition of purified phospholipase A_2 activity by the purified fraction of *Ceiba pentandra* extract shown in Fig. 1 revealed that increase in the concentration of substrate incubated with 0.1 mg/mL of purified extract on phospholipase activity has non competitively. Some inhibitory effect with Km value of 0.8.

Inhibition of purified *Echis ocellatus* phospholipase A_2 activity by the purified fraction of *Ceiba pentandra*: Phospholipase A_2 purified from *Echis ocellatus* venom was inhibited by the principal active agent of the *Ceiba pentandra* leaves in dose dependent manner. Suggesting that isolated compound can neutralise the PLA_2 activity in the snake venom, in Fig. 2.

Infrared spectrum of purified anti venom principle of *Ceiba pentandra*: The IR spectrum of *Ceiba pentandra* is shown in Fig. 2 .The diagnostic band for ester carbonyl group 1648.23 cm can be observed indicating the presence of this functional group. The other prominent

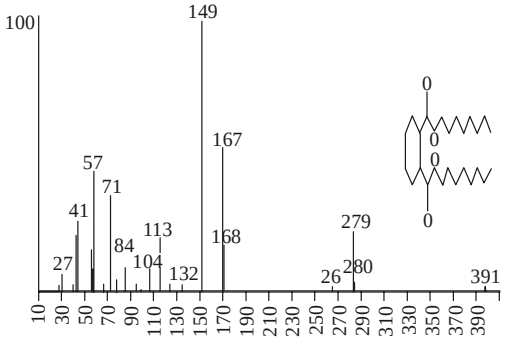

Fig. 4: GC/MS showing spectrum showing possible identity of purified anti *Echis ocellatus* venom principle of *Ceiba pentandra* extract with likely compound is Di –n- octyl phthalate

peaks are seen at C-O stretching of O-CH_2 (1020.16 cm) and less than 928 cm out of plane bending (aromatic), C-H stretching at 3431.48 cm. The characteristic frequencies for various types of bonds in organic compounds is found to absorb in a particular region for instance, the purified compound found to absorb in the region 1400-1750 cm^1 can be deduced to contain the carbonyl functional group.

The chromatogram of *Ceiba pentandra* given in Fig. 3 indicated only one major peak at 3110525 intensity and retention time of 32 min, suggesting that the sample is a pure compound.

The mass spectrum of sample in Fig. 4 indicated that the $(M+H^+)$ ion at m/z 391 and the base peak at m/z 149. The other prominent peaks are those at m/z 57, 71, 167 and 279. The mass fragmentation pattern of the compound isolated revealed that the major ions m/z 167, 279 and 149 are base peaks. The results revealed that the predominant compound present in *Ceiba pentandra* that has inhibitory effects on PLA_2 is Di-n-octyl phthalate with molecular mass 391 as shown in Fig. 4.

DISCUSSION AND CONCLUSION

In this work, the active principle of *Ceiba pentandra* responsible for its anti venom activity was studied against the venom of *Echis ocellatus* especially against the purified PLA_2 components of the venom. Our findings showed that haemolysis of blood due to venom was 66%, where as for that of venom incubated with extract, the haemolysis was reduced to 27.4% as given in Table 1 for the purified sample. The findings suggest that the plant extract may have anti hemolytic factor or it is capable of blocking the venom-induced haemolysis.

The line weaver plot showed on varying concentration of the substrate, the results indicated that, the extract inhibit the phospholipase A_2 activityin a non competitive manner with a K_m value of 0.84 M as shown in Fig. 1. In a related study, (Sallau *et al*., 2008) reported the fairly low Km value for the natural substrate of the

enzyme is an indication of moderately high affinity of the enzyme for phospholipids which further substaintiates the observed toxicities in viperid snakes as a result of PLA_2 activities Consequently, the decrease in Vmax due to inclusion of the plant extract in the substrate mixture suggests that the plant leaves extract has some inhibitory effects on PLA_2. Abubakar *et al*. (2006) reported that extracts of *Indigofera pulchra* and *Aristolochia albida* gave 33.3 and 44.4% protection to mice treated with minimum lethal dose of venom; some gross pathologic symptoms of envenomation were alleviated. However, minimal activities were shown by *Guiera senegalense* and *Sterculia setigera.* Again both *Indigofera pulchra* and *Aristolochia albida* were found to neutralize the anticoagulant, hemolytic and phospholipase activity of crude venom (Abubakar *et al*., 2006). Fifteen compounds isolated from plants were 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 (Pereira *et al*., 1994). Zingali *et al*. (1990) reported that crude venom from *Bothrops jararaca* has pro-coagulant platelet aggregating and phospholipase A_2 activities. Meenatchisundaram *et al*. (2009) showed that *Echis carinatus* venoms have the enzymes (PLA_2) that has the ability to lyses sheep RBC's. *Andrographis paniculata* and *Aristolochia indica* Plant extracts were capable of inhibiting PLA_2 dependent hemolysis of sheep RBC's induced by *Echis carinatus* venom in a dose dependent manner. The result reveals that the plant extract was effective in the inhibition of phospholipase A_2. This inhibitory effect may be responsible for the effect of *Annona cassifiora* as a remedy against snake venom (Weinberg *et al*., 1993). The extract of the leaves of *Guiera senegalensis* was found to detoxify (*in vitro*) venom from two common northern Nigerian snake species, *Echis carinatus* and *Naja nigricollis*, in separate experiments. There was a remarkable reduction in the mortality of albino mice after intra-peritoneal (i.p.) administration of reconstituted venom incubated with the extract, when compared to those challenged with the venom only. The survival of the animals exposed to the venom incubated with the different concentrations of the extract was used as the *in vitro* detoxification parameter (Abubakar *et al*., 2000)

These research findings identified that the leaves of *Ceiba pentandra* linn contains many constituents of organic nature. The IR analysis reveals that the bioactive (anti venom) compound in *Ceiba pentandra* leaves is an aromatic organic ester with carbonyl functional group. The GC/MS result showed that the aromatic organic ester compound is Di octyl phthalate as given in Fig. 4. The result has shown that *Ceiba pentandra* leaves extract possess potent snake venom neutralizing compound. Studies on the interactions of this plant extract with snake venom PLA_2 provide an additional dimension to understanding of the mechanism of action of the compound on PLA_2 toxicity. These interactions occur at specific sites and hence are capable of recognizing subtle structures that are responsible for its biological activities. These investigations support the hypothesis that PLA_2 (toxin) have a specific site responsible for its enzymatic and pharmacological action observed. This isolated inhibitor is likely to interact with the active site of PLA_2 to non competitively inhibits its action on its natural substrate. From the structural analysis conducted the isolated inhibitor is Di-n-octyl phthalate wtith molecular mass 391 as shown in Fig. 4.

Possible mechanism of action of di-n-octyl phthalate: The possible mechanism of inhibitory effect of Di-n-octyl phthalate is that: the carbonyl group of the compound can react with imino group of histidine 48 residue at the active site of the PLA_2 forming complex rendering the enzyme inactive or the carboxylic acid functional group can chelate calcium that is required for PLA_2 activity. This Di-n-octyl phthalate is capable of inhibiting PLA_2 and hence used as an anti venom. This observation is supported by earlier work by Rosalind *et al*. (1988) using mono (2-methyl) phthalate (MEHP) to inhibits PLA_2. In a related study, Labow (1988) revealed that the platelet was used to show that MEHP inhibits phospholipase A_2 (PLA_2). In a related development (Alam *et al*., 1994) reported that an organic acid, isolated and purified from the root extract of an Indian medicinal plant sarsaparilla *Hemidesmus indicus* R. Br, possessed viper venom inhibitory activity. Similar studies was conducted by Ushanandini *et al*. (2006) which indicated that *Tamarind* seed extract inhibited the PLA_2 (Phospholipase A_2), protease, hyaluronidase, l-amino acid oxidase and 5'-nucleotidase enzyme activities of venom in a dose-dependent manner. Another compound with neutralizing ability against the myotoxic action of PLA_2s from the venoms of Viperidae snakes is fucoidan, a sulphated polysaccharide extracted from a variety of seaweeds, such as *Fucus vesiculosus* (Angulo and Lomonte, 2003). No structural or inhibitory spectrum information on these active principles is available to date. However, it is important to note that the presence of bioactive compounds opens the possibility of their application for therapeutic purposes.

CONCLUSION

The aqeous methanol extract of *Ceiba pentandra* was biologically active in inhibiting the phospholipase A_2 which is one of the toxic agents in *Echis ocellatus* venom. Antivenom properties in the plant has been identified, therefore this study gave first hand information on the antivenom properties of *Ceiba pentandra.* The compound responsible for the pharmacological action has been shown to be Di-n-octyl phthalate which inhibits the venom's phospholipase A_2. This leads support to the traditional use of *Ceiba pentandra* in treating snake bites. The identification of the active constituents responsible for anti PL A_2 activity may help in developing drug in future. Finally, the relevance of these findings would be

of importance in the area of drugs of medical interest with a view to actualize conventional snake bite therapy options with effective, cheap, accessible and less allergic plant compound and that can be scientifically explored.

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