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ISOLATION, PARTIAL PURIFICATION, CHARACTERIZATION OF PHOSPHOLIPASE A₂ FROM *TRYPANOSOMA EVANSI* AND THE EFFECT OF SOME INDIGENEOUS PLANT EXTRACTS ON ITS ACTIVITY

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ABSTRACT

The enzyme calcium-dependent phospholipase A₂ (E C 3.1.1.4) was isolated from *Trypanosoma evansi* and purified to electrophoretic homogeneity. It was solubilized from *T. evansi* cells recovered from white albino rat which were previously inoculated by intraperitoneal injection of infected camel blood. This was chromatographically purified resulting to a single active peak which produced a final purification fold of 1.28 for the phospholipase activity and 93.3 % recovery. The enzyme was optimally active at pH 7.2 and 37°C. It had a K_m and V_{max} of 4.87 x10⁻² mg/ml and 8.34x10² μmol/min.mg protein respectively, and pK_a values of 6.7 and 7.2 which absolutely implicated ionisable group such as imidazole group of histidine and hydroxyl of tyrosine in the active site respectively. The Arrhenius plot was linear with activation energy of 10.33kcal/mol. PLA₂ was inhibited in the presence of heparin and EDTA noncompetitively; though very low distinct activity was observed in the presence of EDTA. The enzyme was inhibited by Zn²⁺, Mn²⁺ and Cu²⁺. Double reciprocal plots of the initial velocity data of the inhibition by the indigenous plant extracts revealed a noncompetitive pattern of inhibition for the *Ipomoea asarifolia* and a competitive for *Mimosa pigra* in a dose dependent fashion. The inhibition binding constant (K_i) of these extracts were found to be 2.0x10² μg/ml and 1.12x10² μg/ml respectively. The low K_i values obtained for these extracts towards this enzyme is an indication of high affinity of the active principles present in the plants extracts. These could be explored to serve as a cost effective source of *T. evansi* PLA₂ antidote and as well help in designing a novel drug with high efficacy for management and treatment of trypanosomiasis.

Keywords: Partial Purification, *trypanosoma evansi*, inhibition, Phospholipase A₂.

INTRODUCTION

Trypanosomiasis is a debilitating, severe and often fatal disease (Micheal, *et al.*, 1999) affecting both humans and animals and caused by a protozoan parasite called trypanosome. The disease is transmitted by biting flies, which have acquired their infection from animals harboring the pathogenic parasites (Holland *et al.*, 2001, 2003; Omer *et al.*, 2007; OIE, 2008; Otto *et al.*, 2010 and Nathan *et al.*, 2011). In humans, the disease is called sleeping sickness while in domesticated animals it is referred to as nagana in Africa, sura or chagas disease in United States. It is estimated that about 30,000 to 50,000 people are infected by the disease every year (Furaga, 2002). Anemia is the main clinical sign in man and animal trypanosomiasis

(Fiennes 1970, Kobayashi *et al.*, 1976). Many theories are proposed as regards the cause of anemia in trypanosomiasis (Trizzard *et al.*, 1978). One of these postulations implicated phospholipase A₂ (E.C. 3.1.1.4). These are group of hydrolytic enzymes that catalyse the specific cleavage of fatty acid ester bond producing free fatty acids and lysolecithin. The hemolytic properties of these products are due to their detergent-like actions and powerful surface acting agent leading to cell lyses (Nok *et al.*, 2003). The primary causative agent in camel and horses sleeping sickness (sura) has been blamed on *Trypanosoma evansi*; one of the most common and widespread trypanosomal disease. This

strain of parasite infects most mammals although horses and camels are the principal host and represent the most significant source of loss in those animals, with consequential gross economic implication. This is because some communities in the northern part of Nigeria depend on the keeping and selling of camels and horses for their major source of income. Loss of these animals to trypanosomiasis invariably results in economic problems to the affected communities and even to the government which may benefit from revenue derived from the animals. Sura is endemic in many parts of Africa, Asia and South America where thousand of animals die during disease outbreak each year (Sarah *et al.*, 2006). Recently a case of human infection with *T. evansi* has been documented in India (W.H.O. Epidemiol 2005).

This work attempts to Isolate, partially purify, and Characterize phospholipase A₂ from *Trypanosoma evansi*; and in addition, establish the inhibitory (Trypanocidal) effect of *Ipomoea asarifolia* and *Mimosa pigra* used among the nupe ethnic group of Nigeria to treat animal and human trypanosomiasis

The use of chemoprophylactic drugs is expensive, time consuming and thus unsatisfactory to the long time problem of African animals' trypanosomiasis. Hence the need for investigation into cost effective and more efficacious targets such as plant extracts that have been believed to have trypanocidal effect. Over the years, these people have learnt from experience, the clinical signs of the disease and have developed method of treatment through the use of herbal medicine, but the efficacy has not been scientifically proven.

Pathogenesis and Clinical Manifestation

Changes in essential amino acid profiles are observed in animals infected with trypanosomiasis. The amino acids depleted include leucine, isoleucine, serine, valine, arginine asparagine, threonine, tyrosine, and tryptophan (Anosa, 1988). Implication of the amino acid deficiencies include immunosuppression the major clinical feature in trypanosomiasis, thus, lowering the host resistance to other opportunistic infection, resulting in secondary diseases which complicate greatly both the clinical and pathological features of trypanosomiasis.

Tryptophan, being a substrate for the synthesis of nicotinamide while tyrosine, a precursor for the synthesis of catecholamine and thyroid hormone, any alteration in their level of production will affect the synthesis of these hormones with consequential pathological implication (Igbokwe, 1989).

Trypanosomiasis also results in physiological changes in the affected host. Decrease in liver glucose-6-

phosphatase and cytochrome oxidase activity have been demonstrated (Shertzer *et al.*, 1982). The toxic effect of trypanosome catabolites like p-hydroxyphenyl pyruvate and phenylpyruvate lead to the alteration of mitochondrial function and decrease in gluconeogenesis. As part of deleterious effect of trypanosomiasis are a decrease in glycogen content and an increase in lipid deposits in the liver of the infected animal (Seed and Hall, 1985). Also serum lipid concentration is shown to be elevated in infected rabbits (Diehl and Ribsy, 1974). These reports suggest that changes in the lipid and carbohydrate metabolism which lead to an energy deficit in the infested host, may be the reasons for the protein catabolism and muscle wasting characteristic in trypanosomiasis (Seed and Hall, 1985). Liver dysfunction in infected host has been suggested to decrease in serum albumin and cholinesterase, increase in the activities of alanine amino transferase (ALT), aspartate amino transferase (AST) and lactate dehydrogenase (Anosa, 1988). Generally, there are two recognized stages in the clinical presentation of human Africa trypanosomiasis, namely the early haemolymphatic stage and late encephalic stage when the CNS is involved (Peter, 2004).

In the haemolymphatic stage, the onset is variable, which usually occurs 1-3 weeks after bite, the episode of fever lasting 1-7 days occur together with generalized lymphadenopathy, the symptoms could be malaise, headache general weakness and weight loss (Apted 1970). In this stage, multiple organs may be infected including spleen, liver, skin, cardiovascular system, endocrine system, and the eyes. This underscores invariably, the wide spectrum of systemic dysfunction that may be implicated (Atougua *et al* 2000). The clinical features involved in the late encephalic stage are psychiatric, motor, sensory abnormalities and sleeping disturbances. Mental disturbances may include irritability, lassitude, and headache, apparent personality change overt psychiatric presentations such as violence, hallucination, suicidal tendencies and mania (Duggan *et al.*, 1966). The characteristic sleep disturbance include lassitude distractibility and spontaneous uncontrollable urges to sleep, along with a reversal of the normal sleep-wake cycle in which day time somnolence alternates with nocturnal Insomnia (Duggan *et al.*, 1966). If untreated this may progress to final stage which is characterized by severe somnolence, double incontinence cerebral edema, coma and inevitable death.

Clinical signs of disease may vary in individual or between species. Animals with *T. evansi* infection

usually have a short clinical course of disease and die within week to months of infection. In contrast, some chronic infections of *T. evansi* may persist for years. In horses, donkeys, and mules, the incubation period of disease ranges from 60 days. Signs may include weight loss, lethargy, anemia, and enlarged lymph nodes, dependent edema, exudation, or necrosis of the coronary vessels. Abortions and still births may occur freely sometimes infection with *T. evansi* may not always cause overt signs or disease.

Phospholipases A₂

PLA₂ (EC 3.1.1.4) are upstream regulators of many inflammatory processes. This particular phospholipase specifically recognizes the SN-2 acyl bond of phospholipids and catalytically hydrolyzes the bond releasing arachidonic acid and lysophospholipids. Upon downstream modification by cyclooxygenases, arachidonic acid is modified into active compounds called eicosanoids. Eicosanoids include prostaglandins and leukotrienes which are categorized as inflammatory mediators (Dennis, *et al.*, 2004). PLA₂ are commonly found in mammalian tissues as well as insect and snake venom. Venom from both snakes and insects is largely composed of melittin which is a stimulant of PLA₂. Due to the increased presence and activity of PLA₂ resulting from a snake or insect bite, arachidonic acid is released from the phospholipid membrane disproportionately. As a result, inflammation and pain occur at the site. There are also prokaryotic A₂ phospholipases.

Phospholipase A₂ (PLA₂) is a calcium-dependent enzyme which hydrolyses the 2-acyl ester bond of phospholipids to release fatty acids and lysophospholipids. The fatty acids released, most notably arachidonic acid, have a role in inflammation as the precursors of prostaglandins and leukotrienes (Burgoyne, and Morgan, 1990) while the other products, lysophospholipids, are the precursors of platelet aggregating factor and other physiologically important compounds. PLA₂ is therefore a potential target for anti-inflammatory drugs. Several PLA₂ have been identified as based on their gene sequences.

Functions of phospholipase

Clearly, the functions of phospholipases are diverse. Basically, two types of functions are considered. First, there is the action of phospholipases in digestion; Digestion of extra cellular phospholipids can serve as mechanism by which bacteria can derive a source of phosphate. While digestion of dietary neutral lipid and phospholipids is dependent on the action of phospholipases in the intestine this action provides the essential fatty acid required for normal metabolism.

And secondly, the regulatory Phospholipases have low catalytic activity and are present in small quantities in cells relative to the digestive enzymes. In addition, they are under stringent regulation that limits their activity, thereby protecting the cell from membrane degradation and cytolysis. These Phospholipases have come under intense investigation of late with the recognition that the formation of a number of bioactive lipids such as the eicosanoids (e.g., prostaglandins and leukotrienes) is dependent on the action of Phospholipases. Likewise, the very fundamental acyl composition of the phosphoglycerides that constitute the bilayer of membranes is dependent on Phospholipase actions in a deacylation-reacylation cycle (Moseley, 1987)

Classes of phospholipase A₂

They are classified mainly into three groups:

(i) cytosolic PLA₂ (cPLA₂); (ii) Ca²⁺-independent intracellular PLA₂ (iPLA₂) and (iii) Ca²⁺-dependent secreted PLA₂ (sPLA₂) (Ben Bacha, *et al.*, 2007). They differ from each other in terms of substrate specificity, Ca²⁺ requirement, and lipid modification. There is also a PLA₂ class which hydrolyses the platelet-activating factor (PAF) and the oxidized lipids, called PAF acetylhydrolases (PAF-AH) [Dennis and Six, 2000; Balsinde *et al.*, (1999)]. The sPLA₂ group forms a growing family of dependent Ca²⁺ lipolytic enzyme with a conserved Ca²⁺ binding loop and His-Asp dyad at the catalytic site [Valentin, and. Lambeau (2000); Murakami, (2001)]. They are low molecular mass enzymes (14–18 kDa) with a rigid tertiary structure, having five to eight disulfide bonds, which confers both stability against proteolysis and denaturing conditions (Dennis and Six, 2000); Balsinde *et al.*, 1999). Eleven sPLA₂ have been identified in mice (groups IB, IIA, IIC, IID, IIE, IIF, V, X, III, XIIA and XIIB) that display distinct yet partial overlapping tissue distribution (Valentin, and. Lambeau, 2000; Murakami, (2001). Humans contain all of these except IIC, which occurs as a pseudogene (Dennis and Six, 2000). There has been intense interest in the role of sPLA₂ in the liberation of arachidonic acid from cellular phospholipids for the formation of the eicosanoid (Murakami, *et al.* 1997). Also, given the molecular diversity of sPLA₂ and their distinct distribution patterns, it is likely that these enzymes are involved in other physiological responses besides inflammation. Some of these additional known functions include digestion of dietary phospholipids in the gastrointestinal tract (Verheij *et al.*, 1981; Richmond *et al.*, 2001) and other sPLA₂ have bactericidal action (Buckland, *et al.*, 2000; Beers *et al.*, 2002) and release free fatty acids for formation of the permeability barrier of skin. (Mao-Qiang, *et al.*, 1996). The group IB PLA₂

is known as the pancreatic type sPLA₂. In the pancreas, however, this enzyme is present mainly as an inactive precursor, proPLA₂, a single-chain zymogen which is converted by trypsin into the active enzyme (De Haas, *et al.*, (1968). The major physiological function of sPLA₂-IB has been thought to be digestion of glycerophospholipids in nutrients, given its abundance in digestive organs (De Haas, *e .al.*, 1968). However, the group IB PLA₂ is also found in non-digestive tissues, including lung, spleen, gonad and kidney (Seilhamer *et al.*, 1986). Receptors of this enzyme have been identified in various tissues, and group IB PLA₂ is reported to play a role in cell proliferation and hormone release via these receptors (Seilhamer *et al.*, 1986). sPLA₂-IB have been isolated from pancreases of various mammal species. In the contrast, no study has been performed on bird PLA₂, in the exception of some assays to purify the turkey PLA₂ (TPLA₂) (Ben Salah 2003; Abir et al 2007). However, the purified TPLA₂ was found totally inactive and all biochemical and enzymatic analysis were done with partially purified enzyme. The extracellular forms of phospholipases A₂ which have been isolated from different venoms (snake, bee, and wasp), from virtually every studied mammalian tissue (including pancreas and kidney) as well as from bacteria require Ca²⁺ for activity (Ben Salah, 2003).

Pancreatic PLA₂ serve for the initial digestion of phospholipid compounds in dietary fat. Venom phospholipases help to immobilize prey by promoting cell lysis. Cytosolic phospholipases A₂ (cPLA₂). The intracellular PLA₂ are also Ca-dependent, but they have completely different 3-dimensional structure and significantly larger than secreted PLA₂ (more than 700 residues). They include C2 domain and large catalytic domain. These phospholipases are involved in cell signaling processes, such as inflammatory response. The produced Arachidonic acid is both a signaling molecule and the precursor for other signalling molecules termed eicosanoids. These include leukotrienes and prostaglandins. Some eicosanoids are synthesized from diacylglycerol, released from the lipid bilayer by phospholipase C.

Method of Assay

The use of titration is applicable to all types of phospholipase since a proton is liberated by the action of each. A minimum enzyme activity that can be detected is about 100nmols of (H⁺) liberated per minute. This clearly eliminates the use of titration for the assay of intracellular phospholipases. The lowest pH that can be used for assaying is about 6.0. Below that pH the pKa of the fatty acid is approached and the proportion of the undissociated fatty acid becomes too

high, however, since most phospholipases with sufficient activity for titration have optimal activity about pH 6.0 it is not a major problem. Prior to using titration as an assay method for phospholipase activity, it is essential to establish the type of activity (A₁ A₂ etc). For example, an enzyme preparation may contain more than one phospholipase that results in multiple sites of attack on the substrate. One of the most straight forward titration assays was described by (Dettaas, *et al.*, 1968) where egg Yolk is homogenized in water deoxycholate and CaCl₂ were added and the uptake of alkali at a specific pH was measured. It was interesting that whole egg yolk was a better substrate than purified egg lecithin primarily due to the acidic phospholipids present in the egg-yolk emulsion.

Indigenous plant

Plant a

Botanic Name: *Ipomoea asanfolia*

Common Name Ginger-leaf morning glory leyd

Remor Kada or Romon Rafi (Hausa).

Family: Convolvulaceae

Description: A lawless Succulent perennial, trainings on ground usually several meter long, it reproduces from seeds and stem shoots. The stem is solid near the base but below nearer the top of the plant.

Habitat. A common weed of hydromorphic soils, low lying and inland valleys, streams river banks. (Irvine, 1961).

Distribution – They occur in the tropics of the world although some species are also reach temperate zone. The greatest species diversity occurs in America and Africa. (Rosado-Valado, *et al.*, 2000).

Uses: The leaves and stem are use by herbal traditional practitioner as herbal medicine to treat various ailments

Plant b

Botanical Name: *Mimosa pigra*:

Family: Leguminosae: Sub-family mimosaceae.

Synonyms: M. Pellita: Common names (English) bashful plant giant sensitive plant, (Hausa) Kaidagi.

Based on literature *Mimosa pigra*, is used for the treatment of snake bite in Africa (Irvine, (1961). In Somalia, roasted and ground mimosa leaves are made into an infusion which is drunk to treat a weak heart or weak pulse (Grosvenor *et a.l.*, 1995). In Mexico, an infusion of *Mimosa pigra* was traditionally used in Mayan Medicine for treatment of diarrhea. Extracts of *Minosa pigra* has antimicrobial activity (Rosado-Valado, *et al.*, 2000). It is traditionally used along others leaves to treat trypanosomiasis in Africa.

MATERIALS AND METHODS

Materials

Chemical/Reagents

DEAE-Cellulose (DE 52), heparin, EDTA, Bovine serum albumin (BSA) and Ammonium sulphate were products of Hopkins and Williams, England. Hydrochloric acid, disodium hydrogen phosphate, sodium dihydrogenphosphate, sodium chloride, magnesium chloride coppersulphate, cobalt chloride, and calcium chloride were supplied by BDH chemicals, Triton-x-100, was a product of Boehringer, Mannheim Germany. Acrylamide Bis-acrylamide, N'N'N'N. – Tetra ethylene diamine (TEMED). Ammonium persulphate and Coomassie brilliant blue were supplied by Shandon, Southern England and also Bromophenol and Mercaptoethanol.

Equipment - Microscope -phase contrast, electrophoretic machine, Bench Centrifuge, and spectrophotometer

.Animal – Fifteen mature albino rats free from infection were purchased from the department of Pharmacology, Ahmadu Bello University, Nigeria.

Infection of Rat with *T. evansi*

The parasite *Trypanosoma evansi* STIB 731-AA (IL-1392) was obtained from the Department of Veterinary Parasitology ABU Zaria. Parasites were passaged into a white albino rat (donor rat) by intraperitoneal inoculation. The levels of parasitaemia of the infected rats were monitored daily. The parasitaemia was monitored daily on a wet film preparation from tail vein blood.

Preparation of Wet Blood Film

This was carried out by taking blood (tail vein blood) from the infected animal into an EDTA treated eppendorf tube. A drop of these mixture (EDTA–blood), was placed in a clean slide covered with a cover slip to ensure even spread of the blood in the slide. The slide was examined under a microscope with X400 magnification using phase contrast lens for the presence of *Trypanosome evansi*.

Preparation of Thin Blood Smear

Here, a small drop of the infected blood was placed on a slide with a capillary tube. The blood was then spread evenly with a spreader along the slide in a rapid and steady movement, the slide waved in air to dry. The dried slide smear was fixed in methanol for about 5 minute and air dried. This was immersed in Giemsa stain prepared in 1:10 dilutions with the phosphate buffer pH 7.6 into a coupling jar for 30 minutes. The slide was rinsed in running water until the smear appeared bluish-pink which was used for the detailed morphological studies.

Infection of Experimental Animals

New set of previously uninfected white albino rat five (5) were infected by intra peritoneal injection with about 10^3 parasites/ml sourced from tail vein blood of

the *T evansi* infected donor rat reservoir at a massive parasitemia (of 1×10^6 parasite/ml).

Monitoring Parasite/parasite counting

Parasitemia was monitored each day by bleeding the tail of each animal, about one drop of blood was collected on slide and covered with a cover slide which was observed under X400 magnification of a phase contrast microscope.

Sometimes the blood could be diluted with equal amount of normal saline solution (0.9% NaCl). Fields were examined and the mobile parasites are counted. The infected animals were killed when the parasitaemia was massive and the blood pooled in heparin.

Estimation of Parasitemia.

Estimation of parasitemia was done by counting an average of 2000 parasites in a field infected blood cells through thin blood smears made from the tail-blood of infected rats stained with Giemsa as described by Garcia, (2001).

Equilibration of DEAE – Cellulose Column

The column was prepared according to Lanham and Godfrey (1970). About five grams of DE-Cellulose (DE-52) was weighed into and dissolved in 25ml of phosphate buffered saline glucose (PSG) pH 8.0 to form a slurry and stirred to dissolve the clump and ensure evenly mixing of slurry. During this mixing the pH was observed to have risen to about pH 8.7 which was equilibrated to pH 8.0 by addition of phosphate acid. The cellulose (slurry) was then loaded into a column (2x20cm) which has been stocked with cotton wool plug at its out let. The cellulose or column was further packed by washing several times with the PSGB.

Isolation of Trypanosome

The isolation of the trypanosome from the infected blood was carried out by method described by Lanham and Godfrey (1970). The infected albino rats were scarified. About 5.0ml of their blood was collected in a heparinized syringe diluted 1:1 with PSG and then loaded into a buffer pre-equilibrated DEAE -Cellulose column and chromatographically eluted with 0.10M PSG into test tubes. Each of the eluent collected was observed under microscope, using wet film mount method to detect the presence of the trypanosome. The eluents which contained the parasites were pooled and centrifuged at 105,100xg for 10 minutes. The pellets was reconstituted in 10ml of the PSG at pH 7.2 and kept at -4°C , until needed.

Solubilization of the Trypanosome membrane

The frozen trypanosomes pellets was removed from storage and allowed to thaw at room temperature. This is followed by three (3) cycles of freezing and thawing for complete solubilization of the trypanosomes

membrane. This lysate is now the source of crude enzyme.

Ion exchange Chromatography

The ion exchange was carried out by method described by Nok *et al.*, (1993). All operations were maintained at about 4 degree centigrade. The crude enzyme was loaded on a buffer (pH 6.8) pre-equilibrated DEAE-cellulose column (2.0X30cm) and eluted by linear gradient between 0.02 and 0.3M phosphate saline buffer (pH 6.8) Forty fractions (40) of 5.0ml each was collected and assayed for phospholipase activity and protein content. The dialysate was labeled as partially purified PLA₂.

Characterization of partially purified PLA

Determination of phospholipase (PLA₂) activity of *Trypanosoma evansi*.

The phospholipase activity of *Trypanosoma evansi* was determined using the egg yolk coagulation method of Habermann and Neumann which was modified by Gomes and Pallabi, (1999) Phospholipase activity was measured quantitatively (titration) by the initial rate of free fatty acid produced in micro moles per minute. The assay mixture contains 500 μ l of egg yolk lecithin 50 μ l of Calcium Chloride, 100 μ l of enzyme solution (in Phosphate buffer 7.2) to give a total volume of 650 μ l. The reaction was always initiated by the addition of the enzyme last. There after, the enzyme inactivated by heating at 100°C for 1 minute. The liberated fatty acid was titrated to an end point against 20mm NaOH using phenolphthalein as indicator. The phospholipase A₂ activity was given as the number of moles of free fatty acid (average of 3 determination $\pm$ SD) liberated by 1mg of the enzyme.

Effect of substrate concentration

The substrate concentration was varied over the range of 2-10 μ mg/ml of lecithin. The effect of substrate concentration on phospholipase A₂ activity was carried out as outlined in the standard enzyme assay. The kinetic constant k_M and V_{max} for the enzyme were determined from line weaver – burk (double-reciprocal) plot. Similarly, the effect of varying concentration of Ca²⁺ on the activity of the purified enzyme was also carried out as described in this section.

Effect of pH on the purified enzyme.

The activity profile of the purified enzyme (phospholipase A₂) was determined as a function of pH using lecithin as substrate. The buffer (sodium phosphate buffer) was prepared at different pH in the range (pH 5.0 - 8.0). The enzyme activity was then assayed at each pH level. A plot of the pH range versus the phospholipase activity was prepared to determine optimum pH.

Effect of pH on K_m and V_{max} of partially purified PLA₂

The enzyme activity was assayed quantitatively by filtration and at varying concentrations of lecithin. The assay procedure was essentially the same except for the change in buffer pH for each initial rate study. The initial rate studies was carried out using pH, 6, 6.4, 6.8, 7.2, 7.6 and 8.0. Line weaver burk plots of the values at different pH were used to obtain the K_m and V_{max} at each corresponding pH. The values of V_{max}/k_m and $\log V_{max}/k_m$ were also determined and the results used in the Dixon-webb plot.

Effect of temperature on enzyme activity

The effect of temperature on the rate of hydrolysis of phospholipids (lecithin) to release free fatty acid was studied at 25°C, 30°C, 35°C, 37°C, 40°C, 45°C, 50°C, and 60°C as described by Stauffer (1969). The purified enzyme (0.1ml) was added to the substrate solution and incubated at each of the temperatures above for 30 minutes as described and modified by Dickson and Messon (1979).

Thermo stability of the purified enzyme

In this experiment, equal volume (0.1ml) of the enzyme was incubated at intervals of 10°C for 30 minutes at 20, 30, 40, 50, and 60°C in water bath as described by (Stauffer (1969). The various tubes were cooled to room temperature and treated as previously described for determination of phospholipase activity. Also a plot of logarithm of enzyme activity ($\log V$) versus reciprocal of temperature in Kelvin (1/k) was carried out to determine Arrhenius plots of energy of activation of the purified enzyme.

Effect of incubation time

The effect of incubation time on the hydrolysis of substrates (lecithin) was carried out. The substrate 0.5ml was incubated with the purified enzyme (0.1ml) at 20min 30 min, 40 min, 50 min, 60 min, 80 min after the incubation the mixture was boiled in 100°C boiling water to stop the reaction and then assay for phospholipase A₂ activity.

Effect of Temperature on the activity of PLA₂

A solution (0.1ml) of the partially purified enzyme was dispensed into 6 different test tubes and sealed with aluminum foil. Each tube was incubated in a water bath for 30 minutes at the following temperatures 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C.

The incubated enzyme solutions were then assayed for activity after denaturizing by dipping the reaction mixture into 100% boiling water for one minute and then allowing cooling at room temperature. The activity of the enzyme at different temperature were plotted to determine the optimum temperature. The activation energy (E_a) was also obtained from the slope

of the plot of log of initial velocity versus the reciprocal of the temperature in Kelvin (Arrhenius plot).

Determination of protein content

The protein content of the enzyme solution was determined by employing Biuret method using bovine serum albumin as standard. Exactly 4.0ml of Biuret reagent was added to 1ml of the enzyme solution. The solution was thoroughly mixed and the color was allowed to develop at room temperature for 30 minutes, after which the absorbance of the purple colored solution was measured at 540nm in spectrophotometer, zeroing the instrument with a blank at every instance.

Kinetic studies

The partially purified phospholipase A₂ was assayed at various concentrations of (0.02M, 0.04 M, 0.06 M, 0.08 M, and 0.1 M) at a fixed substrate concentration of 500µl. This was followed by initial velocity studies which was carried out by varying the concentrations of egg yolk lecithin as follows 2mg/ml, 4mg/ml, 6mg/ml, 8mg/ml and 10mg/ml. This pattern of varying the concentration of phospholipase was maintained throughout the studies.

Effect of Divalent Cations on the Activity of PLA₂

The enzyme was assayed for the effect of divalent cations at pH 7.2 and all other conditions remaining constant, except that, Ca²⁺, the cofactor required for PLA₂ activity was replaced with, copper, magnesium, and zinc. In any case, the activity was measured quantitatively by titration.

Effect of anticoagulant

Substrate was prepared at concentrations between 2mg/ml-10mg/ml in triplicate. Each preparation was inoculated with 100µl of PLA₂ and the activity assay as described previously. Inhibition analysis was made on similar preparations in the presence of 5x10⁻³ M of EDTA and Heparin.

Effect of indigenous plant extract on PLA₂ activity

Mimosa pigra and *Ipomea asarifolia* are two indigenous plants used traditionally for the treatment of trypanosomiasis. Ethanolic extract of these plants were prepared in duplicate. Vg: 10 mg/ml (10%), 20mg/ml (20%). Initial velocity studies were carried out. Results obtained from the initial velocity studies were used to determine K_M and K_i for the various extracts.

In all cases, the reaction was initiated by adding the enzyme last and measured quantitatively by titration.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

(SDS-PAGE) SDS-PAGE of the partially purified enzyme PLA₂ was carried out as described by Webster *et al.*, (1972); 0.5ml of the partially purified enzyme sample, the crude enzyme and enzyme marker were

treated with 0.5ml of the treatment buffer (1% SDS, 1% Mercapto-ethanol and 0.02M sodium phosphate at pH 6.8) and incubated in water bath at 70°C for one minute. 15µl of bromophenol blue was added as the tracking dye to visualize the fastest migrating component in the mixture.

The prepared samples were then dispensed onto the gel (12% running gel and 6% stacking gel prepared in precision bore gel tube) and the electrophoresis was performed concurrently at a current of 5mA/gel tube for about eight hours. Tris-glycine buffer pH (8.9) was used as the running buffer.

During the electrophoresis a high specific gravity sample, glycerol was added to concentrate the sample. After the electrophoresis, the gels were removed from the gel tubes and stained with 0.25% Coomassie brilliant blue overnight in glass test tubes. The gels were then rinsed and also destained many times in 7% acetic acid. This was to allow for the visualization of the position of the partially purified and the crude enzyme.

RESULTS

In the purification of phospholipase from the trypanosoma *evansi* the steps followed were, isolation of the trypanosoma (*T. evansi*) from blood of infected albino rat by the principle of ion exchange chromatography as described by Lanham and Godfrey (1970), quantification of trypanosome, solubilization of trypanosome membrane, and, ion-exchange chromatography using DEAE-52 cellulose. The degree of purification of PLA₂ activity for each step of the purification was tested by routine determination of total protein and enzyme activity as shown in purification data table one.

Isolation of *Trypanosoma evansi*

From 7ml of the blood drawn from infected white albino rat, only three out of five collected contained the trypanosome, each fraction contained 5ml of the eluate of the trypanosome.

The number of the trypanosome in each fraction was quantified by using wet mount method. Fractions number 3, 4 and 5 contained 22.3, 12.7 and 7.8 x10⁷ trypanosome respectively. The 15ml pooled eluate was further concentrated to 8ml using high speed centrifuge and reconstituted in 0.1M of sodium phosphate buffer pH 7.2.

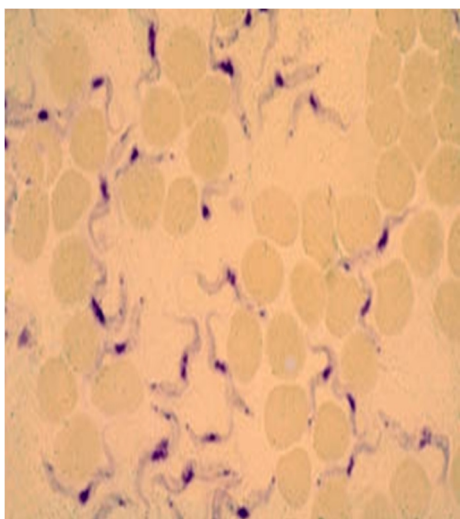


Plate 4.1 Photograph of *T. evansi* in the infected blood
Solubilization of *T. evansi*

After three cycles of freezing and thawing and addition of triton X100 the crude PLA₂ was released into solution. The total activity of the crude PLA₂ was determined to be 101.6μmoles/min and total protein activities to be 5.23mg as shown the purification table.

Table I :Purification scheme of PLA₂

Purification step	Total protein [mg]	Total PLA ₂ Activity μmoles fatty acid released	Specific PLA ₂ Activity [μmoles/min mg protein]	Purification fold	Recovery [%]
Crude Extract	5.23	101.60	19.42	1.00	100
Ion-Exchange on DEAE-52 CELLULOSE	3.85	94.78	24.62	1.27	93.33

4.3Ion exchange chromatography

The chromatography separation of the enzyme preparation phospholipase A₂ from *T evansi* by elution on DEAE-52 cellulose column resulted in single active peak. The elution was performed with liner gradient (0.01M-0.3M) NaCl in sodium phosphate buffer PH7.2 the active peaks produced a final purification fold of 1.27 for phospholipase A₂ activities and 93.33% recovery.

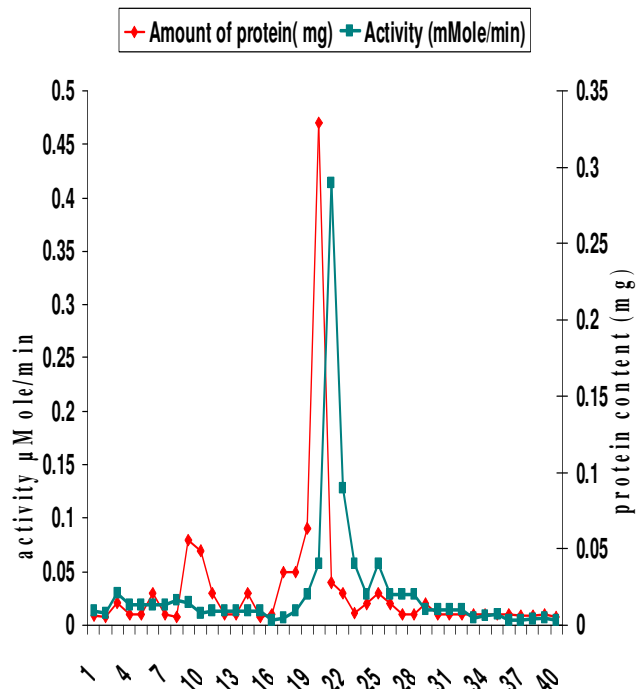
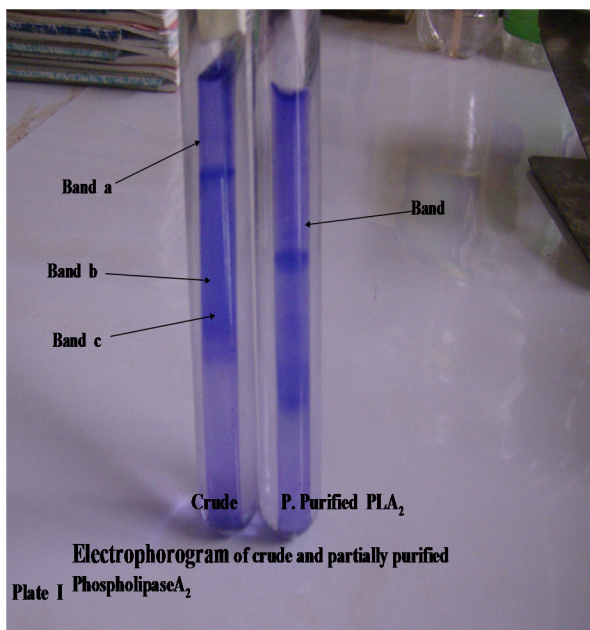


Fig III: Elution profile of *T. evansi* PLA₂ on DEAE-52 ion exchange column



Effect of incubation time on phospholipase A₂

The time course of the reaction for one hour 30 minute is shown in figure. The amount of free fatty acid released by the catalytic activity of the active enzymes is apparently proportional to the incubation time in the given experiment. The enzymes activity after 20 minutes of incubation continuously and significantly increased up to one and half hours incubation period.

Thus means that the amounts of the fatty acid liberated or hydrolyzed from the substrate, lecithin was a function of incubation time for up to one & half hours as shown in the present study

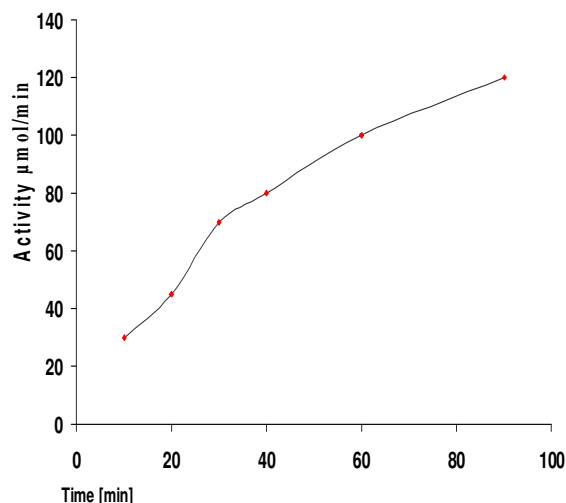


Figure IV: Effect of incubation time on PLA₂

Effect of incubation temperature on phospholipaseA₂ activity

The value obtained in this plot for the relationship between temperature and phospholipase activity describe a typical dumb-bell shape curve the partially purified enzymes produce a very broad temperature range at activities with optimum at 37°C. Also at incubation temperature of 50°C the enzymes still retained more than 60% of activity. However, the activity dropped by more than 60% when the enzymes was incubated at 60°C.

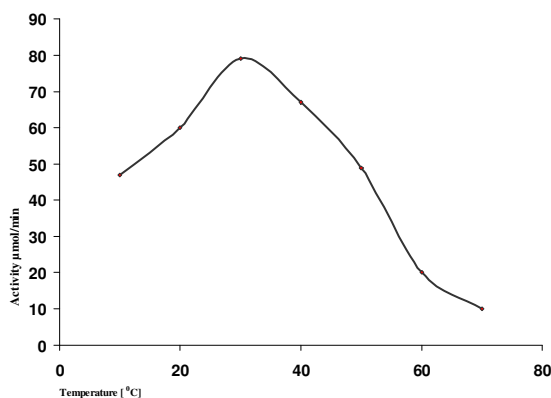


Figure 4.4: Effect of incubation temp on PLA₂

Thermostability of the enzyme

For thermostability of the enzymes, it was found to be stable at 40°C. A plot of logarithm of velocity ($\log_{10} V$) against the reciprocal of the standard temperature in Kelvin ($1/k$), was performed to determine the energy of activation (E_a) of phospholipase for the catalysis.

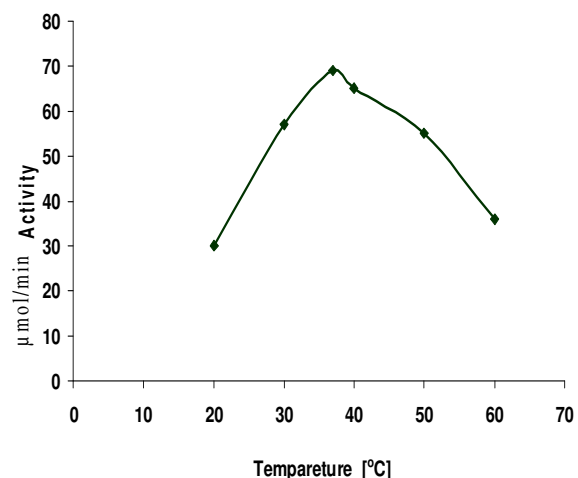


Figure 4.5: Thermostability of *T. evansi* PLA₂

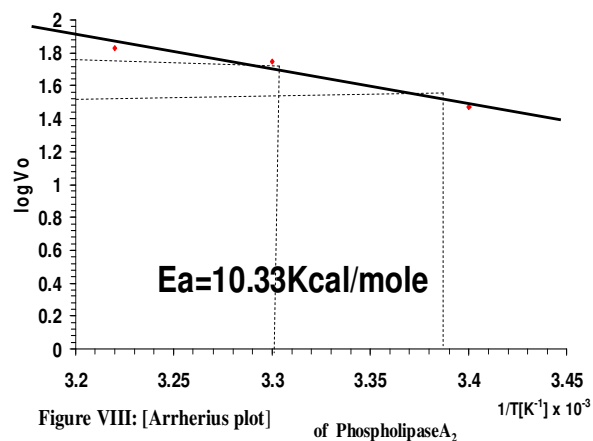


Figure VIII: [Arrhenius plot] of PhospholipaseA₂

Effect of pH on the rate of reaction of PLA₂

When the purified PLA₂ was examine from pH. 5.0 to 8.0 in 0.4 intervals and using lecithin as the substrate it produced an optimum pH of 7.2 using 0.1M sodium phosphate buffer as the reacting buffer. The enzymes was highly active over a narrow pH range from 6.4-7.6. The pH activity curve shows vary low enzyme activity in alkaline pH above 8 as well as acidic pH below 6.

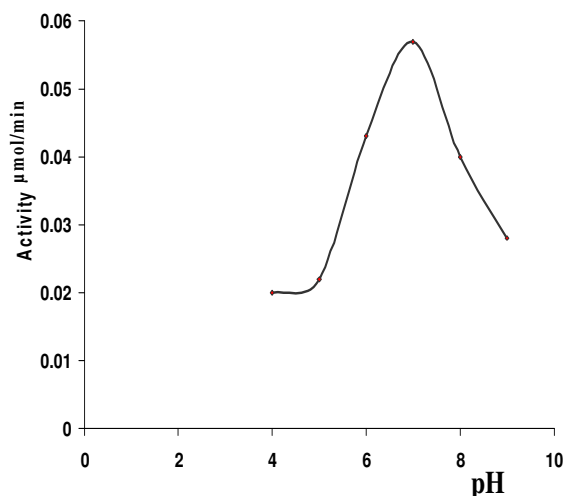


Figure IX: Effect of pH on PhospholipaseA₂ activity.

Identification of amino acid residue in enzyme active site

Again, in order to estimate the amino acid residue in the active site of the enzyme, the pK_a of the residue were estimated as pHs. This was done by determining the kinetic constant (k_m and V_{max}) of the enzymes at every pH level used. Thus, the pH ranging from (5-8) was used at interval of 0.4 when Dixon plot of $\log V_{max}/k_m$ ($1/\text{slope}$) versus pH was carried out. PK_a values of 6.7 and 7.2 were obtained

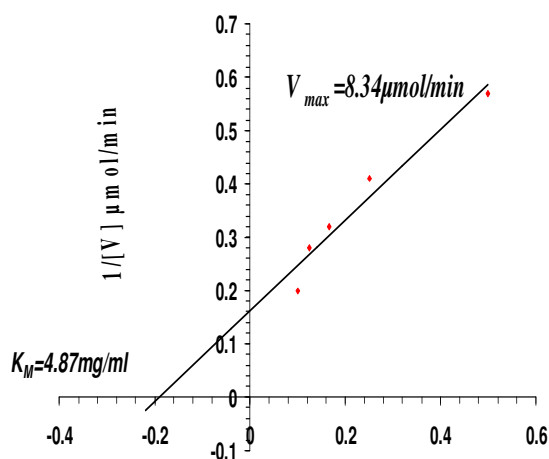


Figure X: Double reciprocal plot of $1/S$ against $1/V$ at pH 7.2

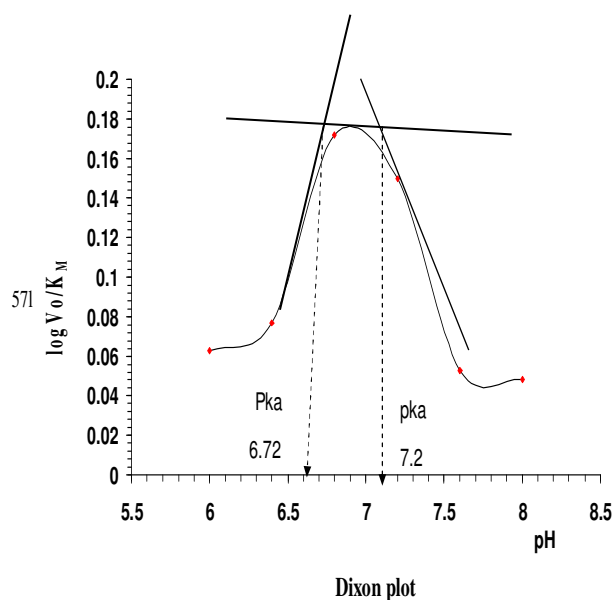


Figure XI: Plot of $\log V_o/K_M$ against pH

Effect of divalent cations

The enzyme did not show any absolute requirement for divalent cations since moderate activity was recorded even in the absence of the cofactor i. e. Ca^{2+} and while Mg^{2+} was an activator Cu^{2+} , Zn^{2+} , Mn^{2+} , were inhibitors.

Effect of anticoagulants

Inhibition studies with EDTA and heparin showed that the inhibition was noncompetitive in all cases. That the inhibition by E.D.T.A, a metal chelator, was most prominent is to be expected since PLA₂ is known to require the divalent cation Ca^{2+} for activation. Zn^{2+} , Cu^{2+} , Mn^{2+} are inhibitors.

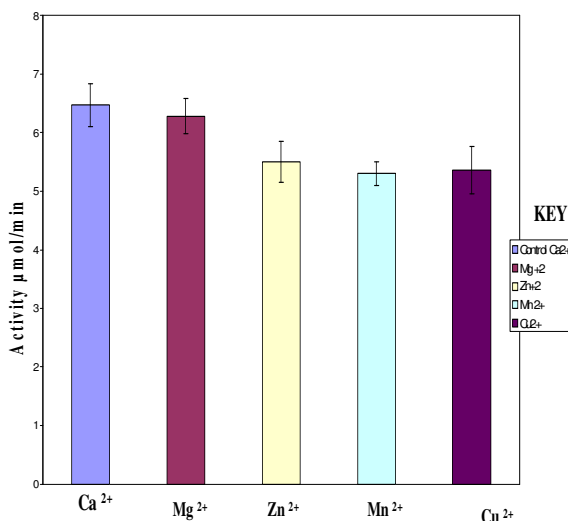


Fig XIII: The effect of divalent cations on PLA₂

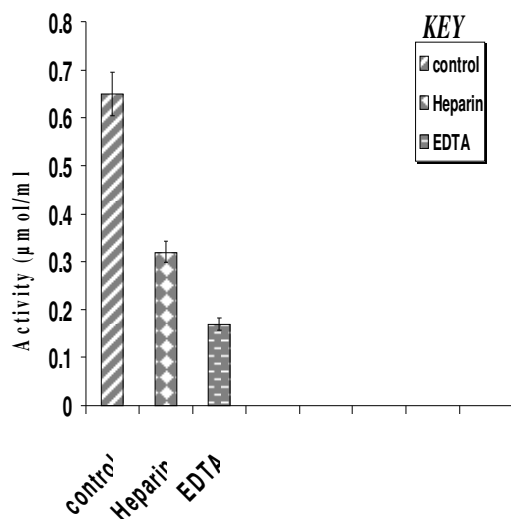


Figure XIV: Effects of anticoagulant on PhospholipaseA₂

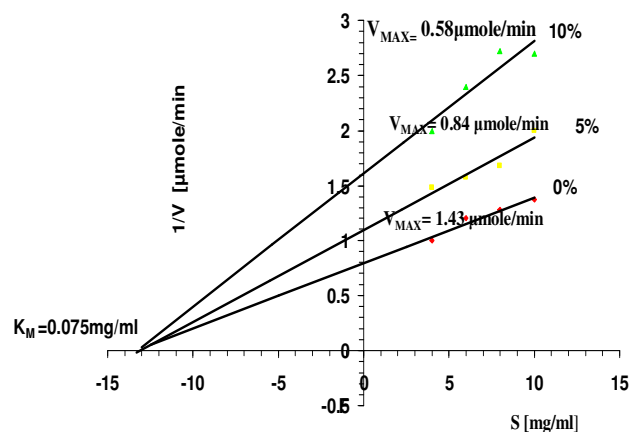


Figure XVIII: Effect of extract of *Ipomoea asarifolia* on PhospholipaseA₂

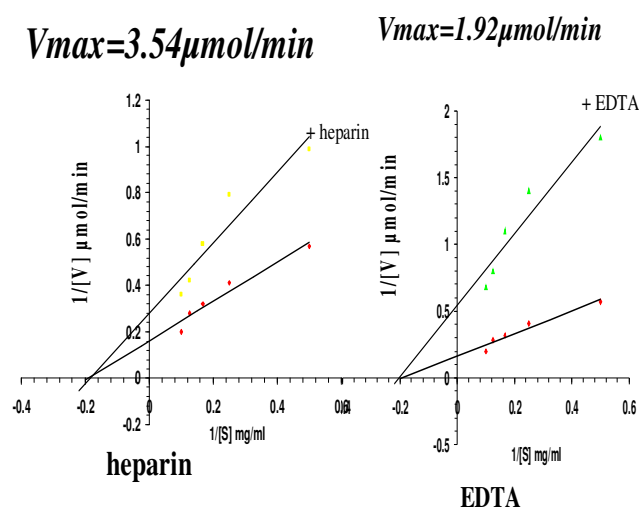


Figure 4:12 Effect of EDTA and heparin on PLA₂

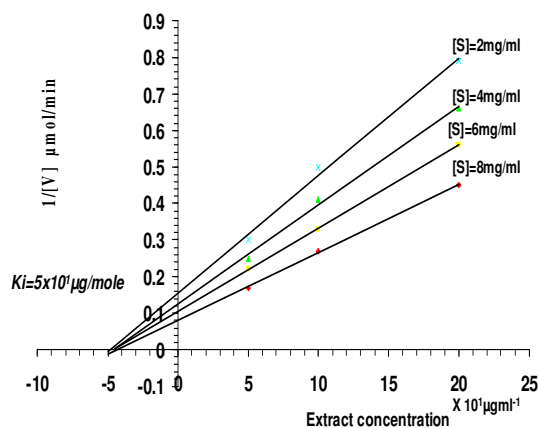


Fig 4.14: Determination of inhibition constant [Ki] of *Ipomoea asarifolia* extract on PhospholipaseA₂

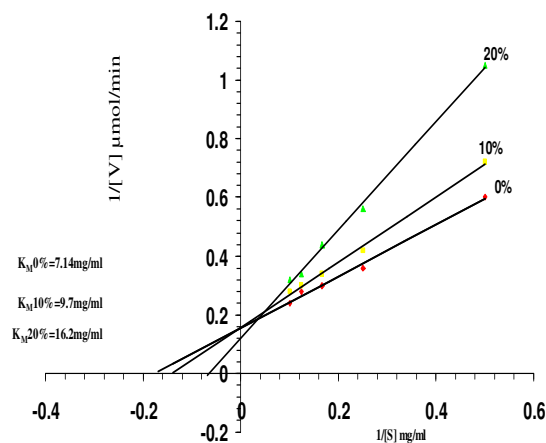


Figure415 : Effect of extract of *Mimosa pigra* on PhospholipaseA₂

Effect of indigenous plant extract

Double reciprocal plots of the initial velocity data of the inhibition by the extracts reveal a non competitive pattern of inhibition for the *Ipomoea asarifolia* and a competitive inhibition for *Mimosa pigra* in a dose dependent fashion PLA₂ Extrapolated inhibition binding constant k_i were found to be $50.0 \mu\text{g mL}^{-1}$ and $87.11 \mu\text{g mL}^{-1}$ respectively.

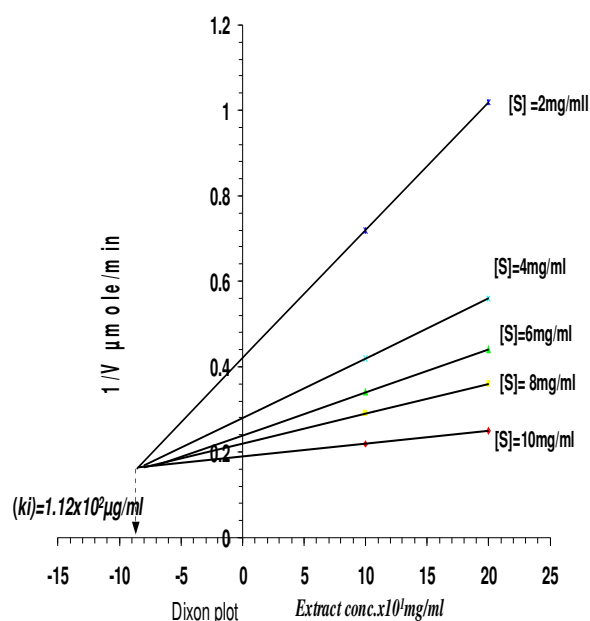


Figure 4.17: Determination of inhibition binding constant $[K_i]$ of *Mimosa pigra* for Phospholipase A_2 .

DISCUSSION

PLA₂ has been isolated from various animals and plant source such as pancreas, snake and bee venoms (Chan *et al.*, 1982; Forst *et al.*, 1986; Couland *et al.*, 1987) and (Horigone *et al.*, 1987) reported on platelet and phospholipase A₂. The primary structure of human pancreatic PLA₂ has been reported by (Verheij *et al.*, 1983).

Phospholipases are involved in lipid metabolism and are important probe of structure function relationship in biological membranes (Dawson, 1973). The enzyme was purified at one cycle purification step as indicated by the single active peak obtained. This could be attributed to the fact that large percentages of the protein are membrane bound. During the experiment on temperature stability of *T. evansi* PLA₂, the enzyme tolerated wide range of temperature due to the extensive conservation of PLA₂ throughout all contemporary organisms and was found to be highly stable at temperature range between 20-50 °C. The decline in activity could be due to denaturation of the enzyme at that temperature. The enzyme is stable at 90°C and pH 3.0 for at least five minutes (Saito 1962, Uthe 1971). Also by relating the range 20-50°C within the enzymes expresses optimum activity, the result is not unexpected, because for parasite to survive, it must be in equilibrium with physiological body temperature of the host which is between 35-39°C for animal.

Data from the temperature studies subjected to Arrhenius plot of log initial velocity as a function of reciprocal absolute temperature gave an activation

energy of 10.33 kcal/mole. For Such low activation energy to be thermodynamically favorable implies that less frequency of collision is required to surmount the activated complex to form products. In a chemical reaction, reactants achieve their E_a by collision, but in the presence of catalyst the E_a is reduced and less energy is required for the reaction to progress. It would be deduced therefore that for PLA₂ from *T. evansi* to effectively function, activation energy of 10.33 kcal/mole is required otherwise the reaction will not proceed. In fact, Nok *et al.*, (1993) obtained much lower activation energy of 6.35 kcal/mole in their work on PLA₂ from *T. Congolense*.

The pH optimum of 7.2 from *T. evansi* agrees closely with the findings of Horigome *et al.*, 1987; who determined the pH of PLA₂ from rat platelets to shows a broad pH optimum (>pH 7.0) But this value deviate from the value obtained by (Nok *et al.*, 1993) where he reported the optimum pH of 6.8 for PLA₂ from *T. congolense*. Traditionally PLA₂ calcium dependent is stable throughout a broad pH range, could be acid, neutral or basic. The plot of $\log V_{max}/K_M$ vs pH predict that ionable group with pKa values of 6.7 and 7.2 are responsible for the catalytic activities or bounding of the substrate to the enzymes. These values are in agreement to the values obtained by (Nok *et al.*, 1993) who found that groups with pKa values of 6.5 and 7.2 are responsible for the catalysis or binding of substrate to the enzyme *T. congolense* PLA₂. Therefore it could be informed that the group with pKa value of 6.7 i.e imidazole group of histidine and the second group with pKa value 7.2 that is hydroxyl group of tyrosine are likely to be found in the catalytic site of the PLA₂ of *T. evansi* these findings could be supported by the work of (Nok *et al.*, 1993) PLA₂ was inhibited in the presence of heparin and EDTA non competitively, though very low distinct activity was observed even in the presence of EDTA. The enzyme is also inhibited by Zn^{2+} Mn^{2+} and Cu^{2+} while Mg^{+2} is an activator.

A double reciprocal plot for the evaluation of inhibition type by *Ipomoea pigra* revealed non-competitive pattern with K_i values of 9.4 and 16.2mg/ml, respectively. At these concentrations the physiological index of efficiency of the enzyme (V_{max}/K_m) decreased from 22.4 h⁻¹ to 16.5 and 9.8 h⁻¹ respectively. The non-competitive inhibition pattern suggest that sites other than the active could be involved in the inhibition. Since pre-incubation of the enzyme with *Ipomoea asarifolia* followed by dialysis failed to reverse the inhibition, these suggest that the *Ipomoea asarifolia*-PLA₂ interaction involved strong covalent forces affecting the structure.

The pattern of PLA₂ inhibition by the extract of *Mimosa pigra* shows that the active site of the enzyme is involved in the inhibitory action and that the extract is likely to contain some compound or small molecules that serve as analogues of the PLA₂ substrate, by competing for the enzyme active site. Since the substrate (lecithin) and extract are competing for the same site on the enzyme, the K_M for the substrate an apparent increase in the presence of the extract. This can be seen in a double reciprocal plots as a shift in the X intercept (-1/K_m) and in the slope K_m/V_{max}).

PLA₂ is a pathological enzyme found in several species of protozoa and snake venom. It is known to be toxic to cell membrane, causing local cell and tissue damage as well as inflammatory effect. The hydrolysis of the glycerophospholipids by sPLA₂ has great importance in the release of lipid mediators (Dennis, 1978) however; high phospholipase activity can lead to pathological implications. More recently the phospholipase activity has been associated with angiogenesis, apoptosis and cancer (Sved, *et.al.* 2004). Arachidonate is metabolized into prostaglandins and to leukotrienes, potent mediators of immune suppression, cellular proliferation, tumor motility as well as invasion (Sved *et al.*, 2004) regulating tumor vascularization and metastasis in animal models. Beside enzymatic activity, sPLA₂ from snake venoms can be neurotoxic (Priatelj *et al.*, 2006), myotoxic (Fuly *et al.*, 2000), cardiotoxic Chiou, (1993) and can inhibit platelet aggregation and blood coagulation (Andriao-Escarso *et al.*, 2002). Thus, PLA₂ induce various pharmacological effects including myotoxic and anticoagulant effect. The inhibition of PLA₂ can therefore be significant in ameliorating the cell damaging effect by the PLA₂ on red blood cells which leads to anaemia.

The low K_i values obtained for these extracts towards this enzyme is an indication of high affinity the extract or the active components (present in the plants) have for this enzyme and therefore, an indication that it could be explored to serve as a good source of *T.evansi* PLA₂ antidote and as well help in designing a novel drug, with high efficacy

CONCLUSION AND RECOMMENDATION

The finding from this research has shown, that phospholipase A₂ from *Trypanosoma evansi* has activity over broad range of pH and temperature. The enzyme was inhibited by indigenous plants (*Ipomea asarifolia* and *Mimosa pigra*) extracts. However, the purification and characterization of an enzyme are not enough information or data as regards the role it plays in the overall survival of the parasite. This makes further work imperative. Therefore, we recommend the technique of recombinant DNA and / or gene silencing

technique i.e. (RNA interference) be employed to validate whether or not PLA₂ could be used as a potent therapeutic target. It is equally important to carry out a comparative studies of the host and parasite PLA₂ so as to map out the differences and similarities existing which could be highlighted and applied when using PLA₂ in *T. evansi* as a drug target.

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