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Original Article

Effects of Jatropha Curcas and Acacia Nilotica Plants Extract Phenolics on the Activity of Partially Purified Phospholipase A_2 Enzyme of Naja Katiensis Snake Venom

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Abstract - Snake envenomation issues have been for long a neglected public health problem in Nigeria and other West African countries, causing a high number of human fatalities yearly. It is, however, still a serious social, pharmaceutical, economic and medical issue. This experimental research is aimed at the biocharacterization of the partially purified Phospholipase A_2 enzyme of Naja katiensis venom. The venom enzyme is extracted using a two-way purification step (Gel filtration using G-75 and ion exchange chromatography on CM-Sephadex). The molecular weight of the enzyme is determined using Sodium Dodecyl-Sulphate Electrophoresis. The extracted enzyme kinetic parameters were also determined, after which its relative optimum affinity/activity was also determined related to different temperature ranges, pH, metal ions and salts. The partially purified PLA_2 gave a total enzyme activity of 3.11 μmol/min, with an estimated molecular weight of 17.5 KDa. Initial velocity data of the enzyme was used to compute the kinetic velocity of the enzyme, where the Km and Vmax of the enzyme were estimated to be 12.6 mg/ml and 3.32 μmoles/min, respectively. The enzyme's optimum temperature and pH were found to be 35°C and 7.0. Ca^{2+} ions were revealed to increase enzyme activity and affinity. Enzyme inhibition analyses done for the two plants' phenolic fractions reveal that the two plant fractions have some level of inhibitory capability against the snake venom PLA_2 enzyme. The research revealed that data could provide an alternative natural way of producing a more friendly pharmaceutical formulation for managing and treating snake envenomation.

Keywords - Antiserum, Chromatography, Envenomation, Kinetic, Naja Katiensis, Venom.

1. Introduction

Snakebite is one of the most serious detrimental public medical issues as it normally results in chronic morbidity plus a high death rate, especially among people from rural areas. It is also of medical and economic importance, with a high social impact on semi-developing areas around the world [1]. Snakebite is one of the most serious human health concerns up to date, as venom-treatment specificity is always a problem when it comes to the treatment and therapeutical management of snake envenomation [2]. Another serious fact is the present treatment storage requirements, which are unavailable in most remote areas in developed and developing countries. Understanding the velocity, nature and contents of snakes' venom is one of the best-starting point-way towards seeing an end to this medical and social paradox [3].

Naja katiensis is one of the most poisonous predominant kinds of snakes found in northern Nigeria. The venoms matter output by snakes post-bite is usually composed of a complex cocktail of active substances, which includes; mainly small peptides and proteins (mainly enzymes), which have the natural ability to interfere with the natural course of many biochemical processes, including thrombosis by changing normal platelet aggregation and blood biochemical coagulation [5]. Many of these proteins include enzymes like proteases, phospholipase A_2, metalloprotease, hyaluronidase, L-amino oxidase, lyases and oxido-phosphodiesterase [6].

Snake envenomation-negative hemostatic effects were reported to have been directly involved in proteolytic processes caused by the protease and phospholipase A_2 enzymes present in snake venom, which ultimately results in blood bio-coagulation and fibrinolysis, among others. Snake venom proteolytic enzymes basically interpose with many other biological reactions, nervous system and organs biochemical inflammation, among others [7].

Phospholipase A_2, also known as lecithinase, is one of the most ubiquitous of all snake venomous enzymes, basically found in all snake venoms [8]. It usually acts by damaging the body's cellular mitochondria, erythrocytes, white blood cells, platelets, peripheral nerve endings, skeletal body muscles, vascular endothelium and other numerous cellular membranes [9]. Its activity normally produces a kind of presynaptic neurotoxic conditioning and opiate-like sedative effects, leading to the autopharmacological release of histamine and anti-coagulation [10]. Snakebite is also seen as one of the most undervalued public health issues in Nigeria, instigating around 100,000 human fatalities seasonally [4]. It, however, remains a grave, budgetary, convivial and medical problem in Nigeria and the entire African continent.

Jatropha curcas is a plant that belongs to the Euphorbiaceae family, which is a perennial, monoecious tree shrub up to six meters high. Jatropha curcas is traditionally used to treat wounds, malaria and snake envenomation [11]. The plant is also used to control soil degradation, erosion, desertification and increase soil fertility [12]. At the same time, Acacia nilotica is another evergreen plant with an average size of about 2.5 - 25 m. The tree usually has a short, thick and cylindrical trunk and grey bark [13]. Acacia nilotica (bagaruwa in Hausa) has been appropriated and recycled as medicinal plants in different sections of Northern Nigeria, West and North Africa, and other regions of the world. It is used to delight infections such as diarrhea, dysentery, malaria, cancers and diabetes [14].

2. Materials and Method

2.1. Plant Sampling and Authentication

Leaves of Jatropha curcas and Acacia nilotica were collected from different parts of Katsina and Kaduna states, Nigeria. All selected plants were authenticated by a qualified plant scientist of the herbarium section of the Biological Sciences Department, NDA Kaduna. Each plant sample was properly cleaned using tap water and cleansed with distilled water before air parching was obfuscated. Each desiccated plant sample was then crumbled separately into powder and hoarded in elegant polythene bags at $4^{o}C$ till required for use [15].

2.2. Plant Material Extraction

A portion (500 g) of each of the respective processed, stored powder of the plant materials was separately percolated in a maceration bottle using 300 ml of chloroform. The powdered plant sample was then allowed to macerate for one week in the solvent inside the percolator. Each extract was then filtered using a pressure suction pump and then dissipated to dryness at 40 ^{0}C using rotovap. Individual remnants produced were then allowed to cool, weighed and stored in a refrigerator until use [4].

2.3. Phytochemical Screening

Qualitative and quantitative phytochemical analysis was done using standard protocols described by [16, 17].

2.4. Extraction and Purification of Phenolics Compounds

2.4.1. Column Chromatographic Fractionation

Plants extracts were subjected to tin layer and column chromatography using respective kinds of silica gels, hexane, sea sand and numerous solvent in order of polarity as described by [18].

2.4.2. Determination of Total Phenolic Compounds

The total phenolic content analyses were done using standard protocols described by [19].

2.5. Partial-Purification of PLA$_2$ from Naja Katiensis Venom

2.5.1. Crude Venom Prep-Analysis

About 30 mg of the *Naja katiensis* lyophilized crude venom was dissipated in phosphate buffer (20 Mm, pH 7.60), blended and centrifugated at 3000 xg for ten mins, then stored at 4ºC until use [20].

2.5.2. Phospholipase A$_2$ Purification

Naja katiensis Phospholipase A$_2$ enzyme was extracted using a two-step purification protocol; Gel filtration using sephadex G-75, ammonium acetate buffer and a pH of 7.4 [4]. While the ion exchange chromatography was done using C-50 sephadex, a Tris buffer and a pH 8.2 described by [9].

2.5.3. Determination of PLA$_2$ Activity

Phospholipase A$_2$ activity was determined using the egg yolk lecithin method described in [4].

2.5.4. Total Protein Content Tenacity

Bradford protein determination protocol was used in calculating the protein content of all fractions, as reported by [21].

2.6. Characterization of Partially-Purified PLA$_2$ from Naja Katiensis Venom

2.6.1. Effect of Temperature and pH

The isolated enzyme pH and temperature optimum tolerance range were analyzed using standard protocols described by [22, 23]. While the enzyme kinetics velocity was ascertained using the Lineweaver-Burk method to pinpoint the enzyme K_m and V_{max} values [4].

2.6.2. Effect of Some Metal Ions on Partially-Purified PLA$_2$ Activity

Various metals were dissolved in deionized water, and about 1ml of the sample was added to 1ml of each metal. These were brooded for 20 minutes at 73.4 ^{o}F before determining the aerobics of the PLA$_2$ [24].

2.6.3. Molecular Weight Extrapolation

The molecular weight of the enzymes was analyzed using the SDS page electrophoresis protocol [25].

2.6.4. Enzyme Inhibition Studies

The inhibitory effects of the two plants' phenolics were analyzed using standard procedures [26]. The activities of Phospholipase A_2 (V_0) were resolved in the company, and the absence of various fixations of the phenolic fractions (0.4, 0.6 and 0.8mg/ml) [4].

3. Result and Discussion

3.1. Result

Phospholipase A_2 enzyme was partial purification from the venom of naja katiensis using a pair-way purification step; Gel filtration using G-75 and CM-Sephadex for the anion exchange chromatography (Figure 1 and Figure 2). The raw venom had a specific activity of 12.20 mol/min/mg at a hundred percent yield. Prior to gel filtration, it was 21.94 µmol/min/mg at 93% yield. Further purification, the specific activity was reduced to 34.56 µmol/min/mg at 86% yield with a purification overlap of 2.05 (Table 1).

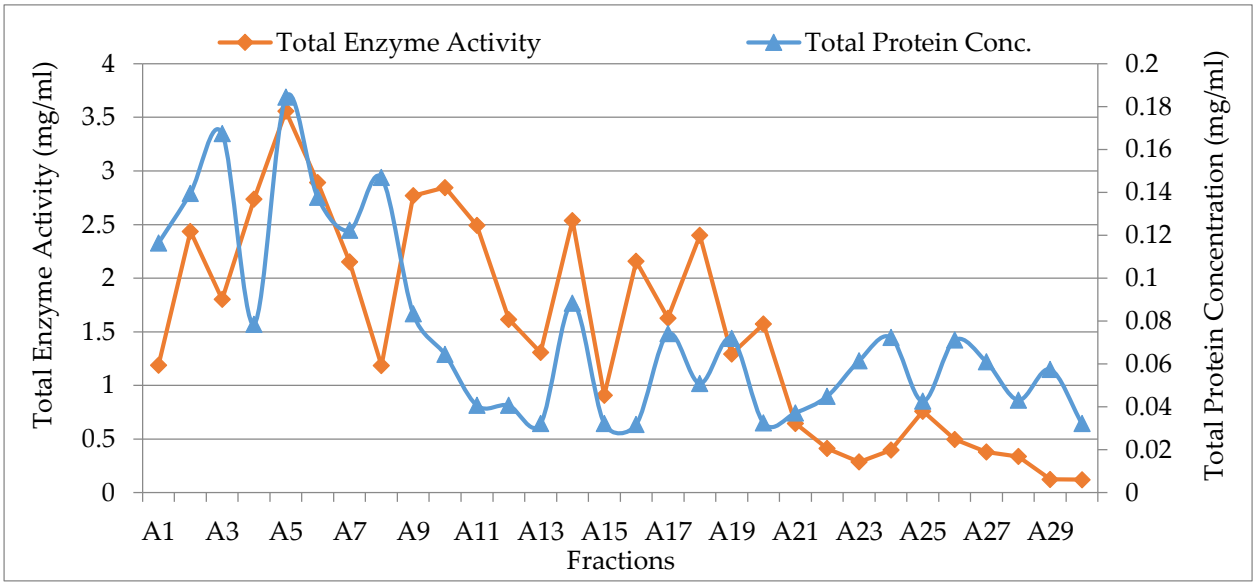


Fig. 1 Elution profile of phospholipase A_2 on sephadex G-75

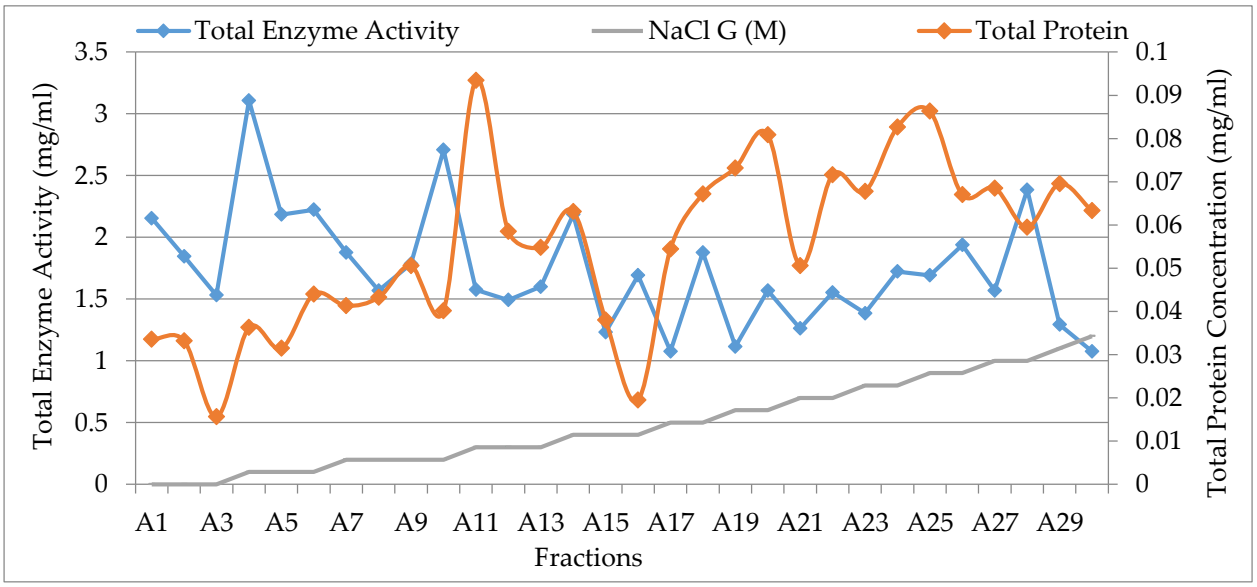


Fig. 2 Elution profile of phospholipase A_2 on CM-sephadex

Thirty fractions were accumulated at a cardiac output of 1ml / 108sec at 25°C from the gel-filtration chromatography. Fractions 5 & 6 were found to have the highest enzyme activity, which was then pooled and subjected to an ion-exchange chromatography on CM-Sephadex wash out with 90 ml of NaCl gradient 0.1-1.2M at a flow output of 1ml / 120sec at 25°C (Figure 2). Characterization of the extracted enzyme reveals the aerobics of the semi-purified phospholipase A_2 enzyme, which rises as temperature increases, up to an optimum temperature of 35°C. Above this temperature range, activity decreased as the temperature went up (Figure 3). Moreover, the aerobics of the enzyme increased with increasing pH up until an optimum pH of 7, a point where the enzyme activity was at its peak; further increases in pH resolve in a reduction in the enzyme activity (Figure 4). Initial velocity data of the enzyme was used to resolve the kinetic velocity of the enzyme, where the Km of the enzyme was estimated to be 12.6 mg/ml, while its Vmax is 3.32 µmoles/min. SDS-PAGE Electrophoresis done on the partially purified Phospholipase A_2 from naja katiensis venom estimate its molecular weight is shown on lane 3, with an extrapolated weight of 17.5 kDa (Figure 5).

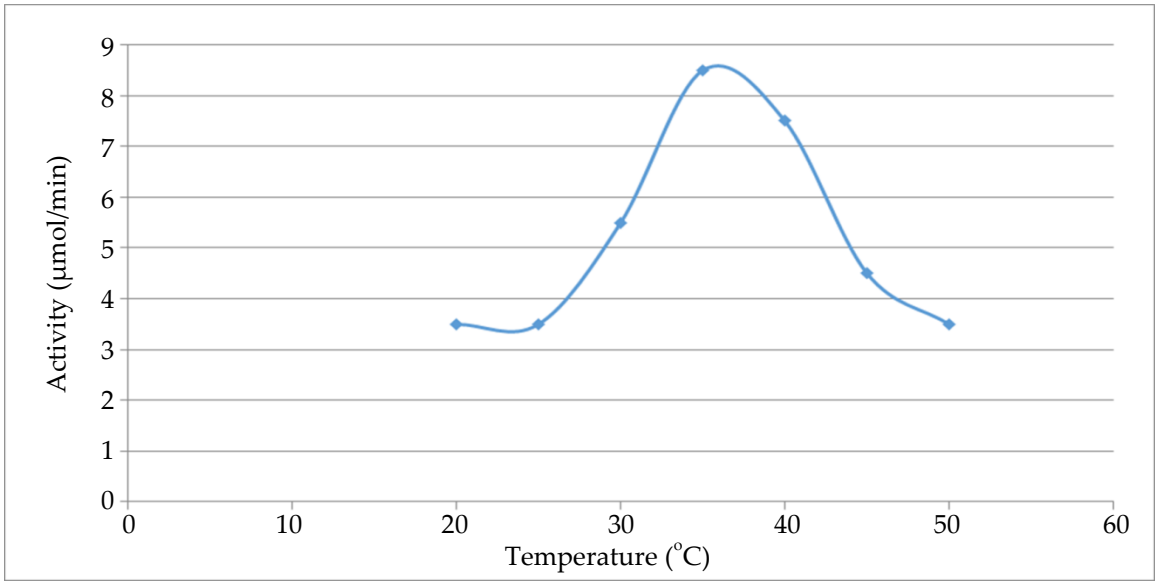


Fig. 3 Temperature effect on the PLA_2

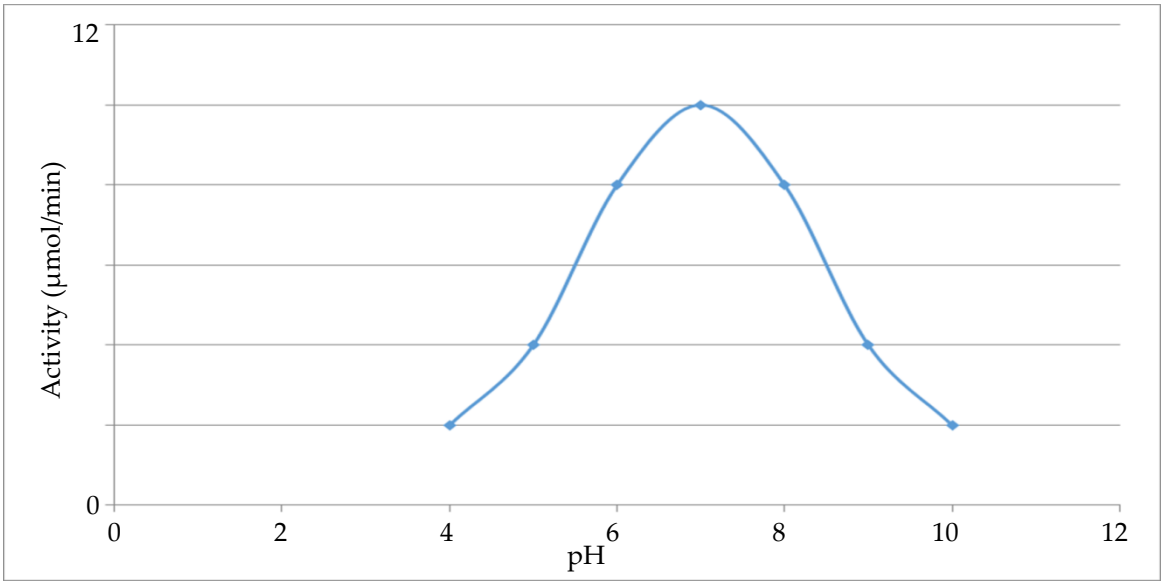
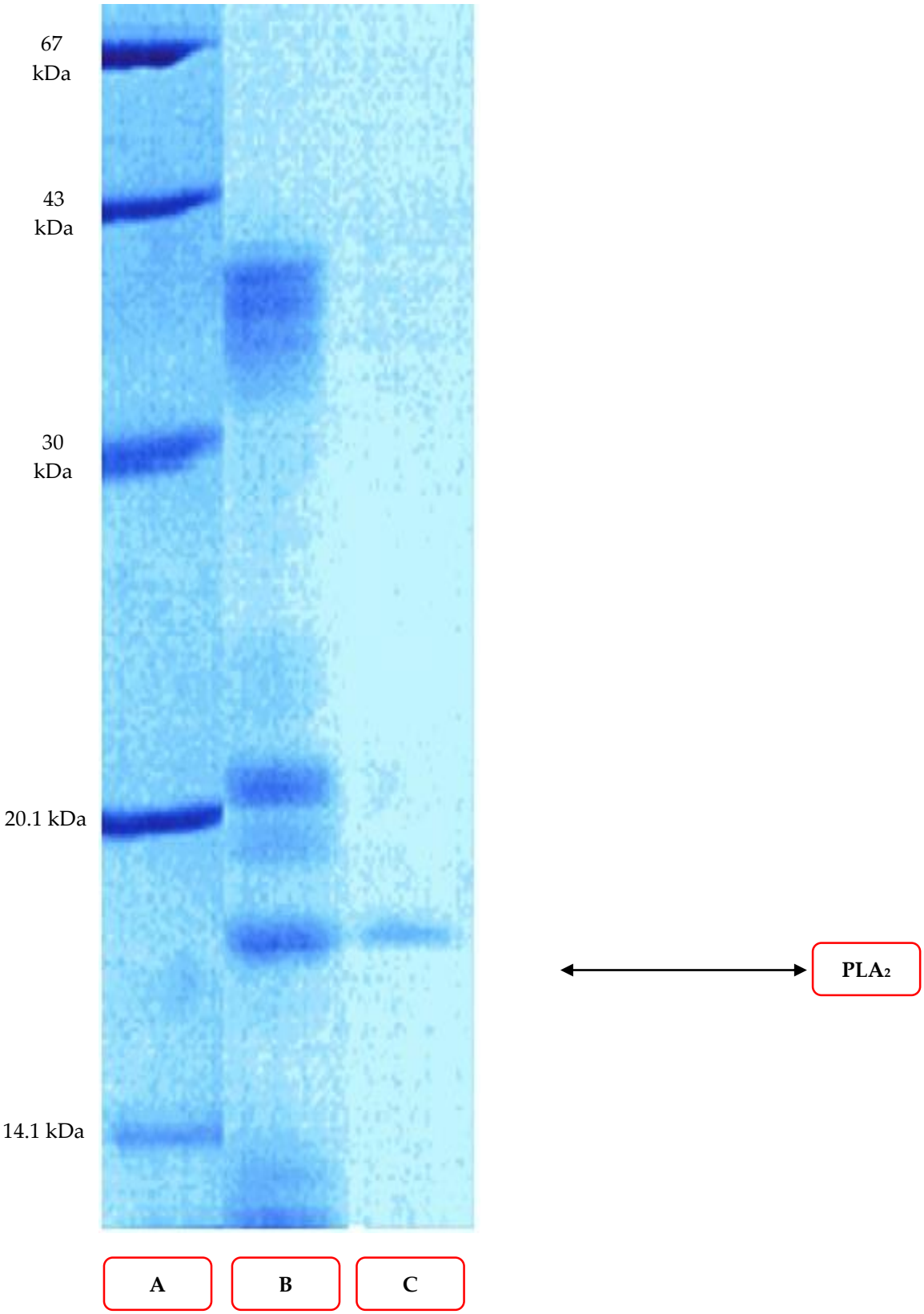


Fig. 4 pH effect on the PLA_2



KEY: Route A = Molecular weight marker, Route B = Raw Venom Route C = Extracted Phospholipase A_2 (extrapolated to be 17.5 kDa)

Fig. 5 SDS-page of partially purified phospholipase A_2 of naja katiensis venom

Meanwhile, the effects of different kinds of metal ions and salt were ascertained, as shown in Table 2. Phyto-metabolic screening of the plant materials reveals that the plant materials have numerous kinds of phytochemicals in different proportions (Tables 3 & 4). Effects of the two selected plant phenolics were tested in-vitro, where J. curcas fractions showed a more inhibitory effect on the activity of isolated enzymes than the other plant phenolic (from the activity of 3.03 µmol/min to 0.73 ± 1.37 µmol/min) (Table 5).

Table 1. Purification table of naja katiensis venom phospholipase A_2

Enzyme	Step	Total Protein (mg/ml)	Total Enzyme Activity (µmol/min)	Specific Activity (µmol/min/mg)	Purification Fold	Yield (%)
PLA_2	Crude	0.35	4.27	12.20	1.00	100
	Gel filtration on sephadex G-75	0.18	3.95	21.94	1.70	93
	Ion exchange on CM-Sephadex	0.09	3.11	34.56	2.05	86

Table 2. Effect of some metal ions/salt on partially-purified PLA_2 aerobics

Ions (10mM)	PLA_2 Activity	
	Control	Activity
Ca^{2+}	3.03 µmol/min	5.28 µmol/min
Mg^{2+}		1.63 µmol/min
Cd^{2+}		0.37 µmol/min
Zn^{2+}		3.12 µmol/min
Mn^{2+}		2.12 µmol/min
Ba^{2+}		1.50 µmol/min

Table 3. Qualitative and quantitative phytochemical content of chloroform extract of jatropha curcas plant parts

S/N	Phytochemical	Leave Extract (mg/g dry wt)	
		Qualitative	Quantitative
1	Flavonoid	+	4.05 ± 1.28
2	Alkaloid	+	9.27 ± 0.70
3	Saponins	+	2.90 ± 2.92
4	Phytosterols	+	0.63 ± 0.73
5	Phenols	+	9.55 ± 2.35
6	Terpenoids	+	1.68 ± 0.94
8	Triterpenoids	+	
9	Tannins	+	2.53 ± 1.36
10	Cardiac glycoside	+	0.18 ± 0.18
11	Anthraquinones	+	0.59 ± 1.77
12	Anthocyanins	-	
13	Phlobatannins	+	
14	Flavonols/flavones	+	

15	Coumarins	-	
16	Quinones	+	
17	Resins	+	
18	Amino acids	-	
19	Chalcones	-	
20	Vitamin A	-	
21	Vitamin D	-	
22	Acidic compound	+	

Key: + = Presence - = Absence

Results are presented as mean ± standard deviation.

Table 4. Qualitative and quantitative phytochemical content of chloroform extract of acacia nilotica plant parts

S/N	Phytochemical	Leave Extract (mg/g dry wt)	
		Qualitative	Quantitative
1	Flavonoid	+	1.97 ± 1.64
2	Alkaloid	+	1.53 ± 2.85
3	Saponins	+	1.32 ± 2.75
4	Phytosterols	+	0.29 ± 1.64
5	Phenols	+	9.92 ± 1.15
6	Terpenoids	+	1.32 ± 1.74
8	Triterpenoids	-	
9	Tannins	+	1.36 ± 1.82
10	Cardiac glycoside	+	0.83 ± 1.28
11	Anthraquinones	+	0.06 ± 0.94
12	Anthocyanins	-	
13	Phlobatannins	+	
14	Flavonols/flavones	+	
15	Coumarins	-	
16	Quinones	+	
17	Resins	-	
18	Amino acids	-	
19	Chalcones	-	
20	Vitamin A	-	
21	Vitamin D	-	
22	Acidic compound	-	

Key: + = Presence - = Absence

Results are presented as mean ± standard deviation.

Table 5. Effects of jatropha curcas and acacia nilotica phenolic fractions on the activity of naja katiensis venom PLA_2 enzyme

PLA_2 Initial Activity (Vo)	Plants Fraction	Phenolic Fraction + PLA_2 (μmol/min)
3.03 μmol/min	Jatropha curcas	0.73 ± 1.37
	Acacia nilotica	1.95 ± 0.52

Key : ± = Standard deviation

3.2. Discussion

Envenomation by snakes has become a serious global detrimental issue, especially with the numerous counts of antiserum treatment specificity, storage and availability issues [27]. The only remedy accessible so far is just the dynamic anti-serum which adheres to the content of the venom and dissociates them but does not have any consequence on the impairment already prompted by the venom [28].

Another issue with snake antivenom is the fact that it is expensive, has high specie-venom specificity, and ideal storage conditions and facilities, which are really not available, especially in rural and even some developed areas of Nigeria and other African countries [4]. Having more information about snake venom enzymes, their features, and their bio-gram profile is the best starting focal point for developing other more suitable pharmaceutical formulations that will serve as a better alternative to the current snake antisera.

Naja katiensis is one of Nigeria's most detrimental venomous snakes, responsible for the maximum number of deaths. In this study, the characterization of partially purified phospholipase A_2 enzyme of naja katiensis venom was done. The ubiquity of the PLA_2 enzyme has been portrayed in numerous human cells/tissues, animal bio-venoms, pathogenic organisms and cancer cells [29]. It, however, has been shown to be present in N. katiensis venom used in this study. PLA_2 being one of the most predominant enzymes in snake venom, aids in damaging cellular mitochondria, erythrocytes, white blood cells, platelets, skeletal cellular contents and vascular outer endothelium. Hence produces presynaptic neuro-toxic aerobics and sedative outcomes [30].

In this study, the enzyme Phospholipase A_2 was partial purification using a two-step purification, where the most active fractions from the first step are pooled together and applied to the second step under the NaCl gradient. The partially purified PLA_2 was active on lecithin, with an activity of 3.11 μmol/min and a purification yield of 86%, which shows that a relatively higher relative extraction was achieved. This corresponds with the study of [26, 4] on different isolated snake venom PLA_2 enzymes.

The Kinetics of enzyme inhibition helps to show the different kinetics velocities for enzymes in residence and the truancy of an inhibitor. The relative enzyme kinetics study shows that the Km and V_{max} value of the enzyme was 12.6 mg/ml and 3.32 μmoles/min, respectively. Characterization studies show that the enzyme has an optimum temperature and pH of 35^{o}C (Figure 3) and 7.0 (Figure 4), respectively. However, adjustment of these parameters proof lethal to the venom enzyme. This corresponds with a similar snake venom research report by [31].

This enzyme characterization study shows that increasing the biosystem temperature also increased the enzyme affinity up to an optimum temperature of 35^{o}C as stated above; however, a higher temperature increase above this also decreased the enzyme activity. The same scenario was seen as per pH increased and decreased. [32] also reported similar findings regarding snake venom enzyme kinetics and affecting factors. SDS-PAGE Electrophoresis done on the semi-purified Phospholipase A_2 from Naja katiensis snake venom extrapolated its molecular weight to about 17.5 kDa (Figure 5). In related research done by [33] revealed that the PLA_2 extracted from snake venom has a molecular weight of 15.6 KDa, similar to the one isolated from B. Jararacussu from Brazil 16.7 KDa and [34] revealed that PLA_2 isolated has 15 KDa molecular mass.

Meanwhile, the effects of different kinds of metal ions and salt were ascertained as shown in Table 2, where the study reveals that most metal ions have detrimental effects on the isolated enzyme, except Ca^{2+} ions which tend to increase the enzyme activity and affinity. However, Ba^{2+} and Cd^{2+} metal ions have the highest recorded inhibitory effects on the isolated enzyme.

In-vitro analyses done to find out the effects of the two selected plants' phenolic segments show that both fractions have some level of anti-enzyme activity against the Snake venom Phospholipase A_2; however, J. curcas fraction shows the highest inhibitory effects on the enzyme as it was able to rapidly reduce the activity of the isolated enzyme from 3.03 μmol/min to 0.73 ± 1.37 μmol/min (Table 5). This study was compared to the study by Chinyere et al. 2016 on the consequence of hydro-root extract of Annona senegalensis on Bitisarietans venom protease and phospholipase A_2 aerobics. This research also reports similar findings to ours, even though it involves different kinds of snake venoms.

4. Conclusion

J. Curcas Phenolics can be used in formulating alternative pharmaceutical agents for managing and treating Naja Katiensis snake envenomation. However, higher or lower temperatures (above 60°C or below 20°C) and pH above or below 7.0, plus the presence of some heavy metal ions and salt, can be used against the activity and affinity of Naja Katiensis PLA_2 venom. The data from this research can also aid in formulating a friendlier therapeutical agent for treating and managing snake envenomation.

Authors' Contributions

Conceptualization, Hassan Abba Umar and Nkechi Eucharia Egbe; Methodology, Hassan Abba Umar; Validation, Ugochukwu Okechukwu Ozojiofor and Onuh Kingsley; Formal Analysis, Madu Adulkarim Him; Data Curation, Kereakede Ebipade and Umar Sani Inuwa; Writing - Original Draft Preparation, Hassan Abba Umar and Ugochukwu Okechukwu Ozojiofor; Writing - Review & Editing, Fatima Abdullahi Harun and Supervision, Nkechi Eucharia Egbe.

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