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RESEARCH ARTICLE

Inhibition of phospholipase A_2 from *Naja haje* and *Naja nigricollis* venoms by active fraction of *Moringa oleifera* leaves: *in vitro* and *in silico* methods

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ABSTRACT

Phospholipases are one of the principal toxic enzymes in snake venoms inducing a wide variety of pharmacological effects after envenomation. Natural inhibitors from plants are known to inhibit the toxic enzyme activities of snake venoms. In this study, ethanol crude extract of *M. oleifera* leaves was partitioned using n-hexane and ethyl acetate after which fractionation was done using column and thin layer chromatography. Subsequently, the inhibitory activities of the crude extract and sub-fractions of *M. oleifera* were investigated against phospholipases A_2 isolated from *Naja haje* and *Naja nigricollis* venoms using *in vitro* and *in-silico* approaches while EchiTab-PLUS polyvalent antivenom was used as the standard drug. The molecular weight of isolated *N. haje* phospholipase A_2 (NH-PL) and *N. nigricollis* phospholipase A_2 (NN-PL) were 24.11 and 35.22 kDa respectively. NH-PL enzyme had a specific activity of 2.70 μM/min/mg substrate while NN-PL activity was 2.10 μM/min/mg substrate. The K_m of NH-PL was 0.330 μM with V_{max} of 0.085 μM/mL min while NN-PL had V_{max} of 0.198 μM/mL.min and K_m of 0.670 μM. *M. oleifera* n-hexane sub-fraction 5 (MOLH5) exhibited a total inhibition of NN-PL and NH-PL enzyme activities at all concentrations used. Molecular docking of the phytoconstituents of MOLH5 against the catalytic site of phospholipase A_2 revealed 2-Hydrazino-8-hydroxy-4-phenylquinoline as the lead compound and a potential drug candidate with a docking score of −6.789 kcal/mol. Findings indicated that MOLH5 possesses phospholipase A_2 natural inhibitors that could be explored as a therapy for snake envenoming.

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1. Introduction

Phospholipase A2s (PLA2s) are known as an important component of all kinds of snake venoms that were studied with the highest amounts being found in venomous families such as Elapidae, Viperidae and Hydrophidae (Mackessy 2010). Elapids are venomous snakes which include cobras, mammals, marine snakes and coral snakes, found in tropical and subtropical regions of the world (O'Shea 2005). Cobra venoms contain numerous toxins mainly neurotoxins, cardiotoxins, cytotoxins and PLA2 that causes pathophysiological effects after envenoming (Luiselli *et al.* 2002). Studies have documented that PLA2s are the predominant and fatal toxins in most snake venoms (Calvete 2011, Gutierrez and Lomonte 2013).

Naja haje (Egyptian cobra) and *Naja nigricollis* (African spitting cobra) are species known to be among the most dangerous snakes in the world and their envenoming has resulted in a significant number of deaths (Shuting *et al.* 2004, Warrell 2011). Also, these species are among the most medically important snakes associated with envenoming in Nigeria (Habib 2013). Their venoms are complicated mixtures of several toxic enzymes and exhibit significantly higher concentrations of PLA2 (Gutierrez *et al.* 2010). Toxicities associated with *N. haje* and *N. nigricollis* envenoming are largely attributed to the presence of PLA2 enzymes which are the most important and cell-damaging components in cobra venom. PLA2 is a lipolytic enzyme that hydrolyzes fatty acyl ester bonds at the second position of membrane phospholipids producing equimolar amounts of bioactive free fatty acids and lysophospholipids, which are then converted to potent pro-inflammatory mediators like platelet-

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activating factor and eicosanoids (Hasson *et al.* 2010). PLA2 superfamily consists of a wide number of intracellular (calcium-dependent and calcium-independent) or secreted enzymes that have been widely analyzed with various crystal structures (Dennis *et al.* 2011).

Enzymatic PLA2s have been reported to be responsible for various toxic effects, including deformation, kidney failure, mutilation and heart failure as it affects the nervous system by preventing the flow of nerve impulses to muscles and at later stages prevent those sent to the heart and lungs as well, causing death due to total respiratory failure (Kini 2003, Tohamy *et al.* 2014). Furthermore, snake venom PLA2s have been identified as one of the most widely used targets for antidote screening and research has shown that both natural and synthetic PLA2 inhibitors are able to extenuate the morbidity and mortality of snakebite envenomation (Gutierrez and Lomonte 2013).

The conventional treatment of snake bite is the use of antivenom obtained from antisera of cattle and horses (Theakston *et al.* 2003). Antivenom has a limited impact on local hemorrhage or necrosis combined with laborious and costly maintenance and production (Neves-Ferreira *et al.* 2010) Therefore, finding alternative solution to complement the existing antivenom treatment is of necessity for antivenom researchers. Studies have documented that medicinal plants are a rich source of natural inhibitors and pharmacologically active compounds with neutralizing potentials on a broad spectrum of snake venom toxins (Kunjam *et al.* 2012). The importance of medicinal plants for treating snake bites and their toxic effects have been addressed in literatures (Abubakar *et al.* 2000, Yamashita *et al.* 2010) and quite a number of antivenom compounds have been isolated from these plants (Mors *et al.* 2000).

Moringa oleifera (Lam.) is a medicinal plant and its crude extract has been established as potent neutralizing agent against the toxic effects of *N. haje* and *N. nigricollis* venoms using *in vivo* and *in vitro* methods (Adeyi *et al.* 2021, 2020). This present study, further demonstrated the inhibitory action of the crude extract and sub-fractions of *M. oleifera* leaves on phospholipases A_2 isolated from *N. haje* and *N. nigricollis* venoms using *in vitro* and *in silico* approaches.

2. Materials and methods

2.1. Venom and antivenom

N. haje and *N. nigricollis* venoms were procured from herpeterium of the Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria. The adult snake of each species was captured in Zaria town, Northern Nigeria. A polyvalent antivenom (EchiTAb-Plus ICP) produced by Instituto Clodomiro Picado, University of Costa Rica, Costa Rica was used as a standard drug for this study.

2.2. Plant material, extraction and partitioning

M. oleifera leaves were obtained from the University of Ibadan campus and identified in the herbarium of Department of Botany, University of Ibadan, Nigeria with voucher specimen no: UIH-22442. The leaves were air-dried at room temperature (25 °C ± 2 °C) for 14 days. Afterwards, the dried leaves were pulverized from which 2500 g was weighed and extracted in three liters of absolute ethanol using the cold maceration method (World Health 1998). Filtrate obtained was concentrated using a rotary evaporator at 40 ^{0}C. *M. oleifera* crude extract (MOCE) yielded 123.5 g. MOCE was further partitioned using n-hexane and ethyl acetate as described (Wagenen *et al.* 1993). The filtrates (n-hexane, ethyl acetate and ethanol) were later concentrated using a rotary evaporator at 40 °C. The three extract partitions of *M. oleifera* yielded 40.86 g, 37.68 g and 35.18 g for *M. oleifera* hexane partition (MOLH), *M. oleifera* ethyl acetate partition (MOLAC) and *M. oleifera* ethanol partition (MOLET) respectively.

2.3. Fractionation of n-hexane, ethyl acetate and ethanol extract partitions of M. oleifera leaves and gas chromatography mass spectrometry (GC-MS) analysis

Approximately 20 g each of MOLH, MOLAC and MOLET was fractionated into its component sub-fractions using column chromatography over silica gel (60–120 mesh size). Silica gel was used as the stationary phase while hexane, ethyl acetate, ethanol and methanol solvents were combined in increasing proportion as the mobile phase. Thin layer chromatography (TLC) techniques were used to pool the eluted fractions. The TLC plates were observed under UV light at 254 nm and 365 nm wavelength respectively and also sprayed with Ceric Ammonium Sulfate solution followed by heating on a TLC heater at about 150° C for a few minutes (Osamudiamen *et al.* 2017). Three sub-fractions of *M. oleifera,* n-Hexane (MOLH5), Ethylacetate (MOLAC3) and Ethanol (MOLET5) were obtained from the TLC analysis. The elution profiles of the fractions are: For n-hexane sub-fraction: MOLH5-100% ethanol, ethyl acetate sub-fraction MOLAC3-100% ethanol/hexane while ethanol sub-fraction

MOLET 5–80% methanol/ethyl acetate. The sub-fractions obtained were condensed using a rotary evaporator at 40 °C and used as test samples for this study. GC-MS analysis of n-hexane sub-fraction of *M. oleifera* (MOLH5) was carried out using Agilent technologies 7890 A (DB 35 MS Capillary Standard polar column with dimensions of 30 mm × 0.25 mm ID × 0.25 µm film). Helium was used as carrier gas at a low down of 1.0 ml/min. The injector functioned at 250 °C and oven heat was maintained as follows: 60 °C for 15 min, then slowly amplified to 280 °C at 3 min. MS were taken at 70 eV; a scan distance of 0.5 s and fragments starts from 50 to 650 Da. Total GC operation periods were 25 min. The comparative percentage amount of every module was calculated by evaluating its average peak area to the total areas. The software adopted to handle mass spectra and chromatograms was Turbo mass. Interpretation of GC-MS was done by the National Institute Standard and Technology (NIST) database (Perumal *et al.* 2014).

2.4. Isolation of phospholipase A_2 enzyme from N. haje and N. nigricollis venoms

Lyophilized *N. haje* and *N. nigricollis* venoms (200 mg) were reconstituted in 3 ml of 10 mM phosphate buffer (pH 6.5) separately. The reconstituted venoms were applied to a Cellex-CM column (0.8 × 18 cm), equilibrated with the same buffer and eluted at a flow rate of 17 ml h–1. After all unbound protein was cleared from the column, a linear NaCl gradient (0–0.4 M) was started and continued until all protein was cleared from the column. Protein that eluted from the column was determined by recording its absorbance at 280 nm. The 1.4 ml fractions that were collected were tested for phospholipase A_2 activity. Gel filtration was done on Sephadex G-75 (0.9 × 160 cm; flow rate 8.9 ml h–1) and Bio-Gel P-100 (1.6 × 88 cm; flow rate 13 ml h–1) columns, equilibrated with 10 mM phosphate buffer, pH 7.4, containing 100 mM NaCl. Fractions of 1.4 ml were collected.

2.4.1. Determination of enzyme activities

Phospholipase A_2 activity was measured on a Beckman DU 640 spectrophotometric system at 37 °C with egg yolk as a substrate. One unit of activity was defined as the amount of enzyme which produced a decrease in absorbance of 0.01 per minute at 925 nm (Mebs 1970).

2.4.2. Protein determination

Protein concentration was estimated as described (Goa 1953). Bovine serum albumin was used to establish the standard curve.

2.4.3. SDS-polyacrylamide gel electrophoresis

The polypeptide composition of crude venoms and purified enzymes was examined on 12.5% SDS-slab-gels (Laemmli 1970) (Bio-Rad mini electrophoresis system). Electrophoresis was performed at room temperature at a constant voltage of 200 V for about 45 min. The gels were stained with 0.1% Coomassie blue R-250 and destained in a solution of 40% methanol and 10% acetic acid. The molecular weights of the stained protein bands were determined by scanning the gels on a Beckman DU 600 spectrophotometer equipped with a gelscan accessory (standard proteins 14.3–116.4 kDa).

2.4.4. Determination of phospholipase kinetic parameters

The kinetic parameters Michaelis–Mentens constant and the maximum velocity (K_m and V_{max}) of *N. haje* phospholipase (NH-PL) and *N. nigricollis* phospholipase (NN-PL) were determined using the Lineweaver-Burk plot.

2.4.5. Effects of pH and temperature on NH-PL and NN-PL enzyme activity

The phospholipase enzymes were assayed at a temperature between 30 °C and 80 °C, varying the time between 10 min and 60 min, to investigate the effect of heat on the activity of the phospholipase enzymes, to determine the optimum temperature and thermal stability of the enzymes. The optimum pH of the isolated phospholipase was determined by assaying for phospholipase activity while varying pH values between 3.0 and 9.0.

2.5. Enzyme inhibition using crude extract and sub-fractions of M. oleifera leaves

Enzyme assay earlier described was performed in the presence of varying concentrations of crude extract and sub-fractions (MOLH5, MOLAC3 and MOLET5) of *M. oleifera*. Exactly 0.1 g of each test sample were dissolved and used as substrates to inhibit the partially purified phospholipase. The first test tubes labelled T1 were used as a control in all inhibition studies while the last 5 test tubes labelled T2-T6 contained 2 mg/ml, 4 mg/ml, 6 mg/ml, 8 mg/ml and 10 mg/ml of treatment respectively and incubated with 100 µl enzyme

(*N. nigricollis* and *N. haje* phospholipases A_2) at room temperature for the inhibition assay.

2.6. Ligand library generation and preparation

Two-dimensional (2D) structures of the phytoconstituents of n-hexane sub-fraction of *M. oleifera* leaves were obtained from the Pubchem database in SDF format and prepared using ligprep tool (LigPrep, Schrödinger, LLC, New York, NY, 2017), (Using Epik at pH 7.0 with OPLS3 force field) Maestro, Schrodinger Suite 2017.

2.6.1. Target preparation and receptor grid generation

The crystal structure of venom phospholipase A_2 in a complex with a natural fatty acid tridecanoic acid (PDB ID: 1TC8) (Singh *et al.* 2005) was obtained from the Protein Data Bank. The protein, 1TC8 was prepared using Maestro 11.1, Schrodinger Suite. Protein preparation wizard, optimized at pH 7.0, and minimized using OPLS3 force field. The prepared protein grid was generated on the active site through the Receptor Grid Generation tool at the size −0.78, 26.95, and −2.21 A° (X, Y, and Z) to encompass all amino acids at the active site of the protein.

2.6.2. Docking of the phytoconstituents of n-hexane fraction against venom phospholipase A_2

The phytoconstituents of n-hexane sub-fraction of *M. oleifera* (MOLH5) were docked against 1TC8 using Glide tool on maestro 11.1 (Glide, Schrödinger, LLC, New York, NY, 2017). The docking experiment was done by treating the protein as a rigid body, while the ligand's rotatable bonds were set to be free. The prepared ligands were docked to the generated grid of the prepared protein, employing default standard precision (SP) to rank the ligands according to their docking score. Consequently, the result from SP were subjected to extra precision (XP) and ligand sampling to none (refine only). The co-crystallized compound of 1TC8 was also docked using the procedure above and compared with the hit.

2.6.3. Prediction of the drug-likeness and ADME toxicity of the hit compound

Molinspiration server (https://www.molinspiration.com) (Shityakov and Förster 2014) and admetSAR prediction online tool (http://lmmd.ecust.edu.cn/) (Cheng *et al.* 2012) were used to determine the drug-likeness and ADME (absorption, distribution, metabolism, excretion), the toxicity of the hit compound. Violations of Lipinski's rule of five were used to determine the drug-likeness of the compound (Lipinski *et al.* 2001).

2.7. Statistical analysis

All values presented are mean ± SE Statistical analyses were done by one-way ANOVA test followed by Dunnett's multiple comparisons post-hoc tests for differences in mean separation. Values were considered statistically significant at $p < 0.05$.

3. Results

3.1. Phospholipases purification; molecular weight and kinetic parameters determination

The fractionation of *N. haje* and *N. nigricollis* resolved into 30 peaks, only 3 peaks exhibited phospholipase activity. The pooled fractions (13–17 for NH-PL and 21–23 for NN-PL) showed the presence of protein and enzyme (Figure S1 and S2). NH-PL enzyme displayed a specific activity of 2.69 mol/min/mg with 30.21% yield and 1.22 purification fold while NN-PL showed a specific activity of 2.1 mol/min/mg with 2.57% yield and 35.50 purification fold (Table 1). NH-PL and NN-PL enzymes showed one main band with an estimated molecular weight of 24.11 kDa and 35.22 kDa on SDS-PAGE respectively (Figure 1). The molecular weights of proteins as estimated on the graph were presented in Figure S3. NH-PL had a Michaelis–Mentens constant (Km) value of 0.33 μM and a maximum velocity (V_{max})

Table 1. Summary of phospholipase purification from *N. haje* and *N. nigricollis* venoms.

Purification steps	Cobra species	Total activity (U/ml/min)	Total protein (mg/ml)	Specific activity (U/mg)	Purification fold	Percentage yield (%)
Crude sample	*N. haje*	5.184	2.36	2.197	1.00	100
	N.nigrocollis	1021.3	1251.5	0.816	1.00	100
CM sephadex	*N. haje*	1.566	0.5805	2.697	1.228	30.21
Ion exchange chromatography	*N. nigrocollis*	362	173	2.1	35.5	2.57
70% Ammonium Sulfate precipitation	*N. haje*	517.8	502.7	1.03	68.5	1.34
	N. nigrocollis	628.5	455.7	1.38	61.2	1.69
Gel filtration chromatography	*N. haje*	219.4	58.1	3.78	29.1	4.91
	N. nigrocollis	189.1	61.2	3.1	18.5	3.79

Values are represented as the mean of three experiments ($n = 3$).

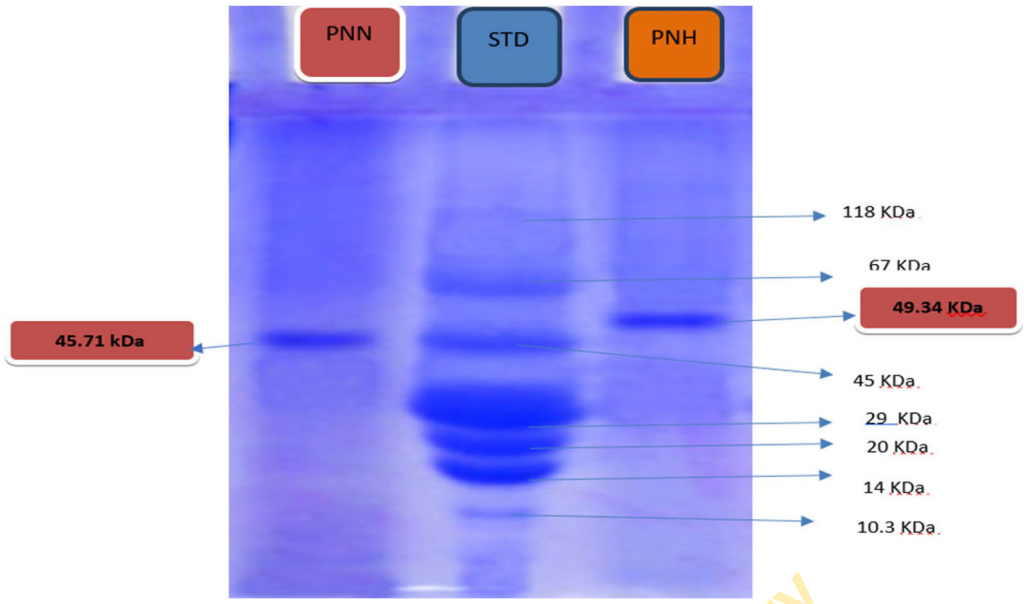

Figure 1. The SDS molecular weight determination for phospholipase from *N. haje* and *N. nigricollis* venoms. Standard proteins were as contained in Sigma Molecular Weight Markers Calibration Kit for SDS polyacrylamide gel electrophoresis ranging from 10.3–117.4 kDa. Lane 1 PNN = Phospholipase of *N. nigricollis* (45.71 KDa), Lane 2 STD = Standard, Lane 3 PNH = Phospholipase of *N. haje* (49.34 KDa), Data are in triplicates ($n = 3$), Significance at $p < 0.05$.

value of 0.08 μM/ml min while NN-PL had K_m value of 0.19 μmol/Ml min and Vmax of 0.67 μM.

3.2. Effects of pH and temperature on activities of NN-PL and NN-PL enzymes

The optimum temperature was observed at 30 °C for both enzymes (Figure S4) while an optimum pH 4.0 and 5.0 ensued for NH-PL and NN-PL respectively (Figure S5).

3.3. Inhibitory effects of crude extract and fractions of M. oleifera on NH-PL enzyme activities

The catalytic activity of NH-PL enzyme was totally inhibited by n-hexane sub-fraction of *M. oleifera* (MOLH5) at all concentrations (Figure 2(B)) while crude extract (MOCE) exhibited complete inhibition of NH-PL enzyme activity at high concentrations (Figure 2(A)). A strong inhibitory effect was displayed against NH-PL enzyme activities by ethanol (MOLET5) and ethyl acetate (MOLAC3) sub-fractions of *M. oleifera* but total inhibition was not achieved at all concentrations (Figure 2(C,D)). Also, EchiTAb polyvalent antivenom showed slightly significant effects on NH-PL enzyme activities (Figure 2(E)).

3.4. Inhibitory effects of crude extract and fractions of M. oleifera on NN-PL enzyme activities

M. oleifera n-hexane sub-fraction (MOLH5) displayed a complete inhibitory effect against NN-PL enzyme activities at all concentrations (Figure 3(B)). Ethyl acetate sub-fractions of *M. oleifera* (MOLAC3) almost completely inhibited NN-PL enzyme activities at all concentrations (Figure 3(C)) whereas ethanol sub-fraction of *M. oleifera* (MOLET5) exhibited a partial inhibition of NN-PL enzyme activities (Figure 3(D)). Also, crude extract of *M. oleifera* and EchiTAb polyvalent antivenom showed total inhibition of NN-PL enzyme activities at high concentrations (Figure 3(A,E)).

3.5. GC-MS profile and inhibition of venom phospholipase A2 by n-hexane sub-fraction of M. oleifera

Phytoconstituents of the n-hexane sub-fraction obtained from the GCMS analysis demonstrate inhibition of the venom phospholipase A2, 1TC8 (Table 2). The lead, 2-Hydrazino-8-hydroxy-4-phenylquinoline possesses a binding energy of −6.789 kcal/mol. The lead forms two hydrogen bonds interactions with ASP 49, two salt-bridge interactions with HIP 48, ASP 49, and one pi-cation interaction with TYR 52 within the catalytic domain of 1TC8 while the co-crystallized ligand forms two salt-bridge interactions with LYS 31 and LYS 56 and one hydrogen bond interaction with LYS 56 within the catalytic domain of 1TC8 (Figure 4(A,B)).

3.6. The lead is a potential drug-like compound

The drug-likeness potential of the lead, 2-Hydrazino-8-hydroxy-4-phenylquinoline was determined through

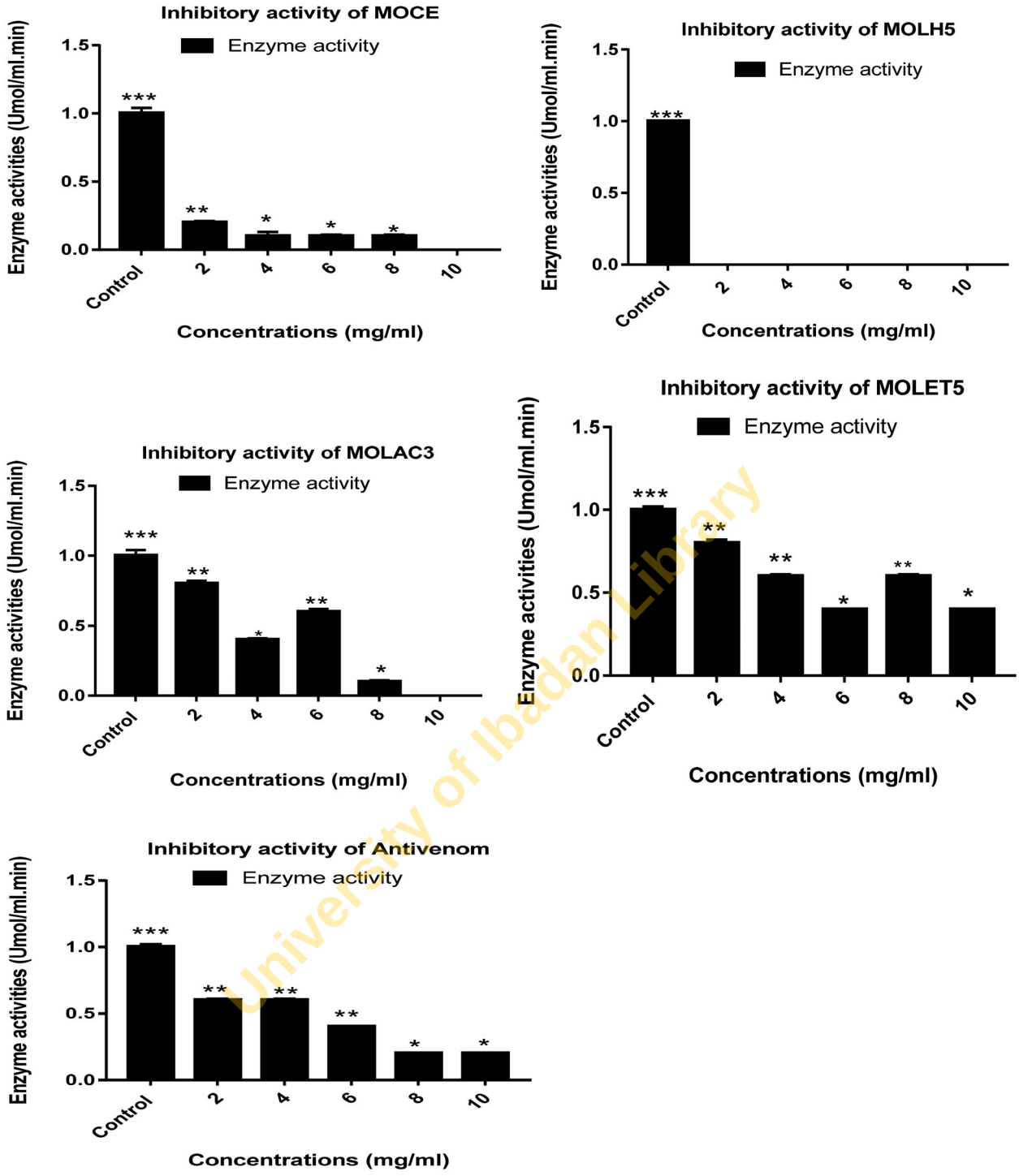

Figure 2. Inhibition of *N. haje* phospholipase enzyme (NH-PL) activities by crude extract and fractions of *M. oleifera*. MOCE: *M. oleifera* crude extract, MOLH5: *M. oleifera* hexane sub-fraction, MOLAC3: *M. oleifera* ethyl acetate sub-fraction, MOLET5: *M. oleifera* ethanol sub-fraction, Antivenom: EchiTAb polyvalent antivenom. Data are in triplicates ($n = 3$), with significance at $P < 0.05$.

in-silico drug-likeness prediction and ADME toxicity screening (Table S1 and S2). The lead violates none of Lipinski's rule of five and it is non-hepatoxic (ADMET toxicity screening) (Cheng *et al.* 2012).

4. Discussion

PLA2 is a toxic enzyme that hydrolyzes free fatty acid from sn-2 location of membrane phospholipids to release polyunsaturated fatty acids and lysophospholipids,

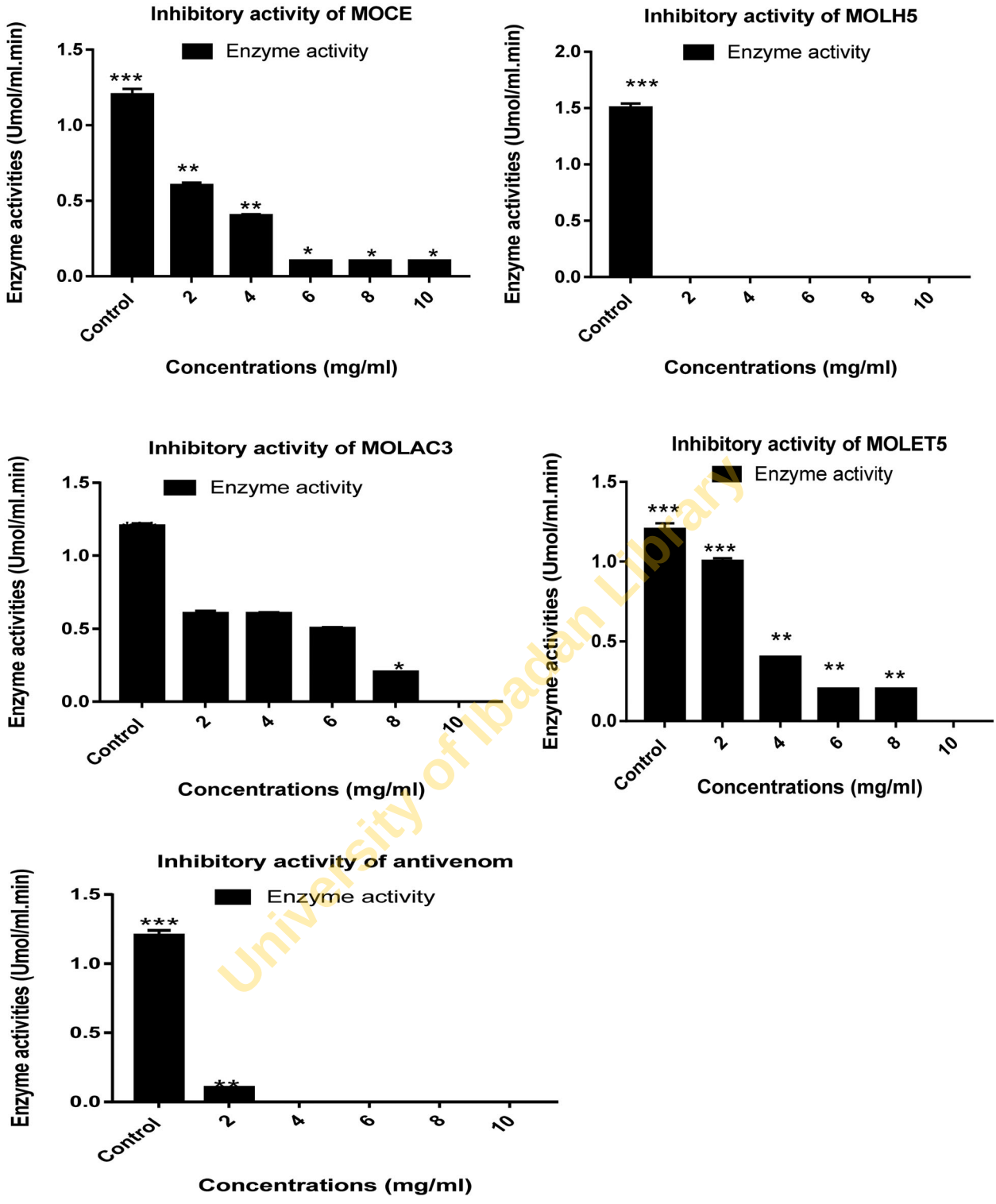

Figure 3. Inhibition of *N. nigricollis* phospholipase enzyme (NN-PL) activities by crude extract and sub-fractions of *M. oleifera*. MOCE: *M. oleifera* crude extract, MOLH5: *M. oleifera* hexane sub-fraction, MOLAC3: *M. oleifera* ethyl acetate sub-fraction, MOLET5: *M. oleifera* ethanol sub-fraction, Antivenom: EchiTAb polyvalent antivenom.

rendering it one of the main toxins that offer snakes their deadly attribute after envenoming (Gutierrez and Lomonte 2013, Ziganshin *et al.* 2015). Combined with their catalytic activity, snake venom PLA2s elicit a wide variety of pharmacological effects that play a pivotal role in envenomation damage. Consequently, inhibition of snake venom PLA2s could lessen the toxic damage exhibited after snake envenoming (Ziganshin *et al.* 2015).

Table 2. Docking scores of the phytoconstituents of n-hexane sub-fraction of *M. oleifera* leaves (MOLH5) against venom phospholipase A2, ITC8.

Phytoconstituents of the n-hexane sub-fraction	Docking score
2-Hydrazino-8-hydroxy-4-phenylquinoline	−6.789
Ethyl-alpha.-d-glucopyranoside	−6.379
n-Hexadecanoic acid	−5.812
Gamma-Tocopherol	−5.739
Alpha-D-Galactopyranose, 2-(acetylamino)-2-deoxy-	−5.719
Acetamide, 2-(5-methoxy-1-methylindol-2-yl)-N-(3-hydroxypropyl)-	−5.665
Methanol, [6,8,9-trimethyl-4-(1-propenyl)-3-oxabicyclo[3.3.1]non-6-en-1-yl]-	−5.535
Co-crystallized ligand	−3.654

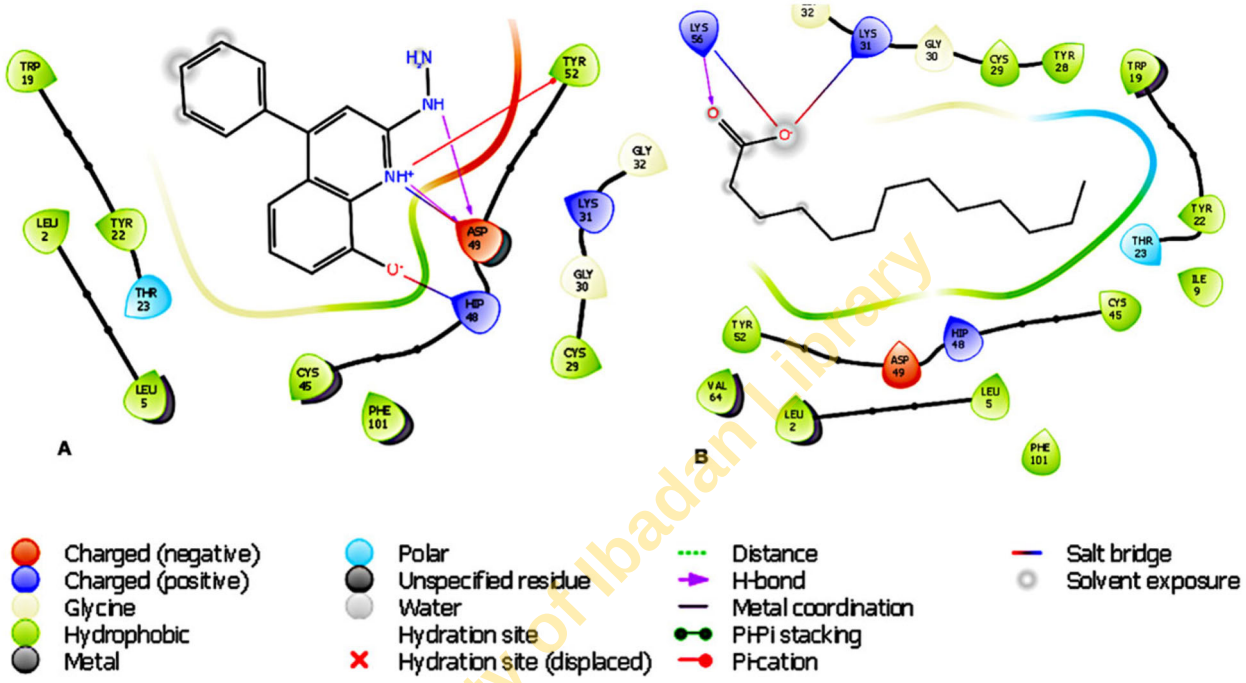

Figure 4. Molecular interactions of the lead, (A) 2-Hydrazino-8-hydroxy-4-phenylquinoline and the (B) Co-crystallized ligand within the 1TC8 catalytic domain.

In this study, a single band was observed on SDS-PAGE for NH-PL and NN-PL indicating the purity of isolated enzymes with an estimated molecular weight of 24.11 kDa and 35.22 kDa respectively similar to the report by Laila *et al.* (2019). The increase in specific activity of both enzymes after purification processes could be due to the removal of other synergistic interacting components of the venom. NH-PL and NN-PL recorded optimal activity at pH 4.0 and 5.0 respectively which is comparable to other reports on the purification of venom PLA_2 enzymes (Sallau *et al.* 2008, Shashidaramurthy and Kemparaju 2006). It has been documented that such pH level could assist in enhancing enzymatic reactions through general catalytic mechanisms to facilitate pathological roles of phospholipase enzymes after envenoming (Scott and Sigler 1994).

The thermal stability result showed the enzymes to be stable up to 30 °C and was denatured after 40 min and 50 min for NH-PL and NN-PL respectively. Interestingly, the low thermostable nature of the venom proteins was evident from the heating experiment. The optimal temperature observed was lower when compared to other studies that demonstrated the thermo-stable nature of phospholipase in other snake venoms (Francis *et al.* 1995, Sallau *et al.* 2008). However, an optimum temperature of below 30 °C has also been reported by Chang *et al.* (1987).

NN-PL and NN-PL enzymes had slightly low K_m values suggesting the enzymes exhibit a fairly high affinity for their substrates which further substantiates the observed toxicities after cobra envenoming due to PLA2 action. In addition, enzymatic PLA2 reaction products are active cytotoxic which are responsible for various toxic effects, as the enzymes are lipolytic and hydrolyze fatty acyl ester bonds on membrane phospholipids (Hasson *et al.* 2010). This action causes lysis of the red blood cells as observed in snake bite

victims due to the heamolytic action of venom PLA2 enzymes combined with inhibition of neurotransmitters (neurotoxicity) resulting in the shutting down of the central nervous system (Caratsch *et al.* 1985).

Findings from the inhibitory studies showed that *M. oleifera* crude extract (MOCE), *M. oleifera* ethyl acetate sub-fraction (MOLAC3) and *M. oleifera* ethanol sub-fraction (MOLET5) exhibited a significant inhibitory effect by effectively reducing the activities of NN-PL and NN-PL enzymes to some extent. However, *M. oleifera* n-hexane sub-fraction (MOLH5) proved to be more effective as it abolished completely the activities of the enzymes at all concentrations indicating that the sub-fraction contains potent active inhibitors of PLA2 enzyme. These results further affirmed and substantiated our previous findings on the neutralizing potentials of *M. oleifera* crude extract and fractions against toxicities induced by *N. nigricollis* and *N. haje* venom *in vivo* (Adeyi *et al.* 2020, 2021). Similarly, other studies have reported the inhibition of phospholipase A2 using natural products from plants (Cavalcante *et al.* 2007, da Silva *et al.* 2008, Diogo *et al.* 2009).

Snake venom PLA2 enzymes require calcium (Ca^{2+}) as co-factor in equimolar concentration at their catalytic site for activeness and also, an increase in Ca^{2+} is needed for both enzyme binding and catalysis (Shashidharamurthy and Kemparaju 2006). Studies have also described calcium-binding loop in the myotoxicity of phospholipase A2 with evidence that calcium binds to His48 of phospholipases situated at the catalytic site of the enzyme leading to the activation of its toxic function (Yang 1994). Mors *et al.* (2000) have reported that active compounds present in plants have metal-chelating abilities on snake venom toxic enzymes thereby inhibiting the activities of such enzymes. The inhibitory actions of *M. oleifera* fractions may be due to the bioactive constituents present in the plant which may have caused the removal of calcium from the catalytic sites of PLA2 thereby rendering the enzyme inactive. On the other hand, EchiTAb Plus-ICP polyvalent antivenom showed a total inhibition NN-PL at high concentrations but failed to completely halt the action of NH-PL enzyme activity at all concentrations. This observation may be due to specificity in the polyvalent antivenom production as it was made majorly for sub-Saharan Africa species of snake envenoming from *Echis ocellatus, Bitis arietans* and *N. nigricollis* excluding *N. haje*. Thus, the potent neutralizing capability exhibited by *M. oleifera* n-hexane sub-fraction (MOLH5) against *N. nigricollis* and *N. haje* phospholipases enzyme activity as compared to antivenom is interesting and worthy of note.

Screening of the phytoconstituents of n-hexane sub-fraction of *M. oleifera* leaves (MOLH5) against venom phospholipases A2, 1TC8 aided in identifying the phytochemical(s) mainly responsible for the activities of n-hexane sub-fraction in this present study. The lead, 2-Hydrazino-8-hydroxy-4-phenylquinoline possesses a binding energy of −6.789 kcal/mol when compared with the co-crystallized ligand, (n-tridecanoic acid) (Singh *et al.* 2005), with a docking score of −3.654 kcal/mol. The lead forms two hydrogen bonds interactions with ASP 49, two salt-bridge interactions with HIP 48, ASP 49 and one pi-cation interaction with TYR 52 within the catalytic domain of 1TC8 while the co-crystallized ligand forms two salt-bridge interactions with LYS 31, LYS 56 and one hydrogen bond interaction with LYS 56 within the catalytic domain of 1TC8. The higher binding energy displayed by the lead when compared with the co-crystallized ligand is probably due to the extensive interactions the lead form within the catalytic domain of 1TC8 (two hydrogen bonds, two salt-bridge, and pi-cation interactions) when compared with the interactions the co-crystallized ligand made within the catalytic domain of 1TC8 (two salt-bridge and one hydrogen bond interactions). This observation corroborated the report of Akinloye *et al.* (2020) that extensive interactions of ligands with key residues within the catalytic domain of enzymes contribute to their inhibitory potentials and binding energies.

The prediction of the drug-likeness of compounds has been shown to be reliable (Schneider 2013, Akinloye *et al.* 2020). A drug candidate should not violate more than one of the five rules as highlighted by Lipinski *et al.* (2001). In the present study, the lead, 2-Hydrazino-8-hydroxy-4-phenylquinoline violated none of Lipinski's rule of five, depicting it as a potential drug candidate. The lead is a Human Intestinal Absorption positive (HIA+) and also human colon carcinoma cell line positive (Caco-2-), this depicts the possibility of its absorption and diffusion into the bloodstream and intestine. The permeability of the lead through the blood-brain barrier (BBB+) is equally visible. It is a non-substrate of the P-glycoprotein (P-gp), suggesting it can pass through the Central Nervous System (CNS) (Davis *et al.* 2014). The lead is a non-substrate and non-inhibitor of CYP3A4 (49), hence the chance for drug-drug interactions is slim. However, it showed a tendency for hepatoxicity, hence, there is a need for the optimization of the lead both to reduce hepatoxicity and increase inhibitory activities against venom phospholipase A2. It is pertinent to note that complete inhibition of NN-PL and

NH-PL enzyme activities at all concentrations by the *M. oleifera* n-hexane sub-fraction 5 (MOLH5) as observed in this study further gives credence to the results obtained from the *in-silico* studies herein.

Conclusion

Findings from this study revealed that *M. oleifera* n-hexane sub-fraction (MOLH5) has the potential to inhibit the enzymatic actions of phospholipases A_2 isolated from *N. haje* and *N. nigricollis* venoms. The inhibitory effect of MOLH5 on the enzymes could be attributed to the presence of 2-Hydrazino-8-hydroxy-4-phenylquinoline- a potential drug candidate as revealed by the *in silico* prediction. Thus, the use of natural products from plants as antivenom agents should be given proper consideration and explored for the management of snakebite envenoming.

Author contributions

Conceptualization: Dr Akindele Adeyi Prof Emuebie Okonji, and Dr Damilohun Metibemu, Methodology: Dr Akindele Adeyi, Prof Emuebie Okonji, Mr Babafemi Ajisebiola and Mr Jimoh Abideen, Formal analysis and investigation: Dr Adeyi Olubisi and Mr Jimoh Abideen; Manuscript Writing - original draft and editing: Mr Babafemi Ajisebiola, Dr Akindele Adeyi and Dr Damilohun Metibemu. All authors have read and approved the final manuscript.

Ethical approval

Not applicable.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data sets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Inhibition of phospholipases A_2 from *Naja haje* and *Naja nigricollis* venoms by active fraction of *Moringa oleifera* leaves: *in vitro* and *in silico* method

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Table S1: Drug-likeness of the lead, 2-Hydrazino-8-hydroxy-4-phenylquinoline

Mw	nrotb	HBA	HBD	XlogP	TPSA	nviolations
221.26	1	2	1	3.57	33..12	0

MW: Molecular weight (<500), nrotb: number of ratable bonds, HBA: number of hydrogen bond

acceptors (<10), HBD: number of hydrogen bond donor (<5), XlogP : the partition coefficient ≤ 5, TPSA: topological polar surface area, nviolations : number of violation of lipinski's

Table S2: ADME Toxicity screening of the lead, 2-Hydrazino-8-hydroxy-4-phenylquinoline

HIA	Caco-2 permeability	BBB	p-gp Substrate/ Inhibitor	CYP3A4 Inhibition/Substrate	Carcinogenicity	Hepatoxicity
HIA+	Caco2+	BBB+	Non-substrate/ non-inhibitor	CYP3A4-non-substrate/ non-inhibitor	Non-carcinogen	Toxic

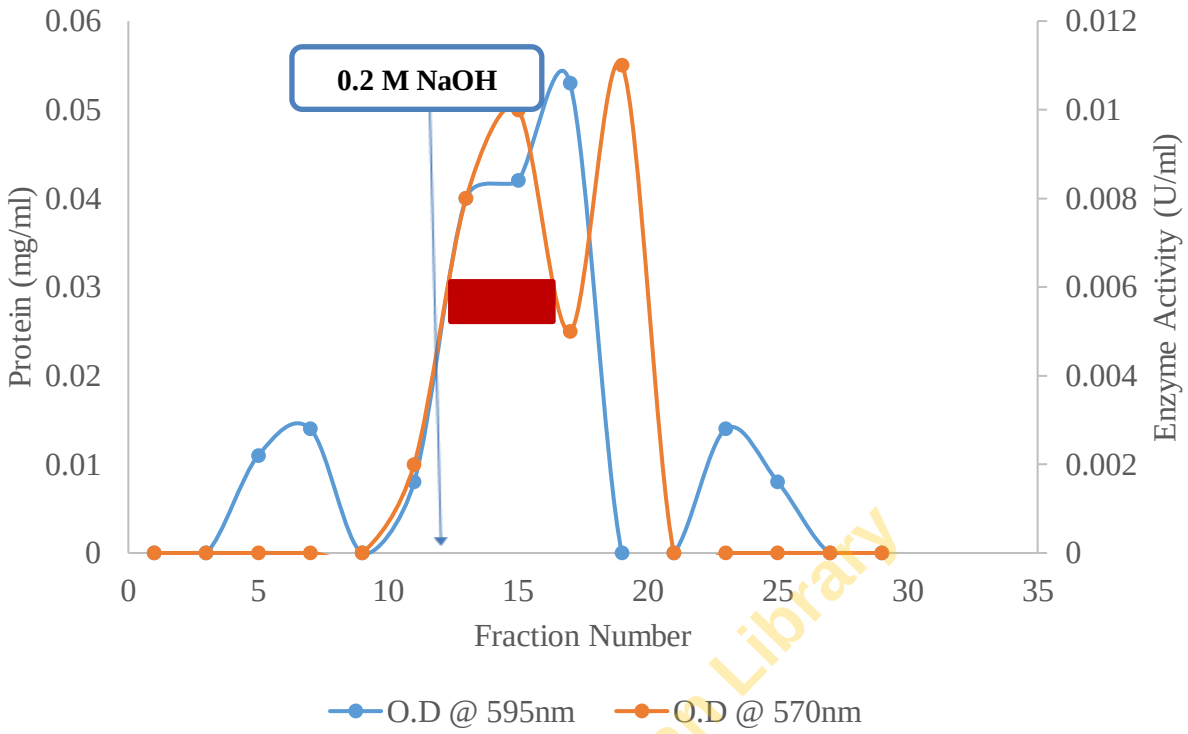


Figure S1: Protein and enzyme concentration of eluents from the venom of *N. haje*.

( ) Protein concentration

( ) Phospholipase concentration

( ) Pooled fractions (13-17)

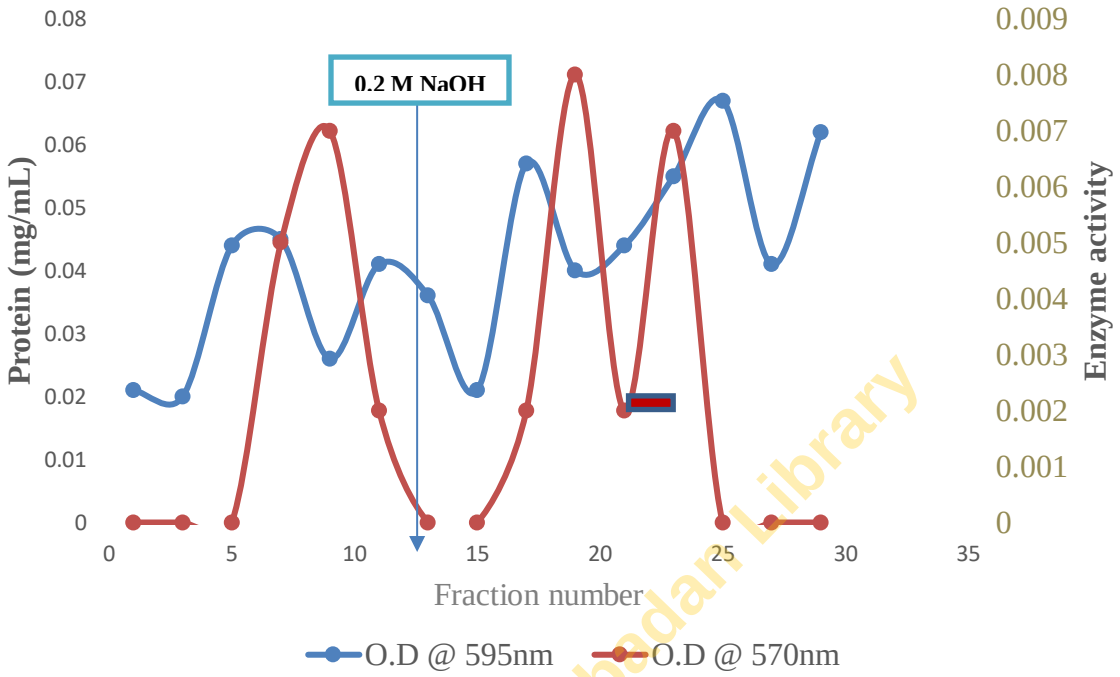


Figure S2: Protein and enzyme concentration of eluents from the venom of *N. nigricollis*

( ) Protein concentration

( ) Phospholipase concentration

( ) Pooled fractions (21-23)

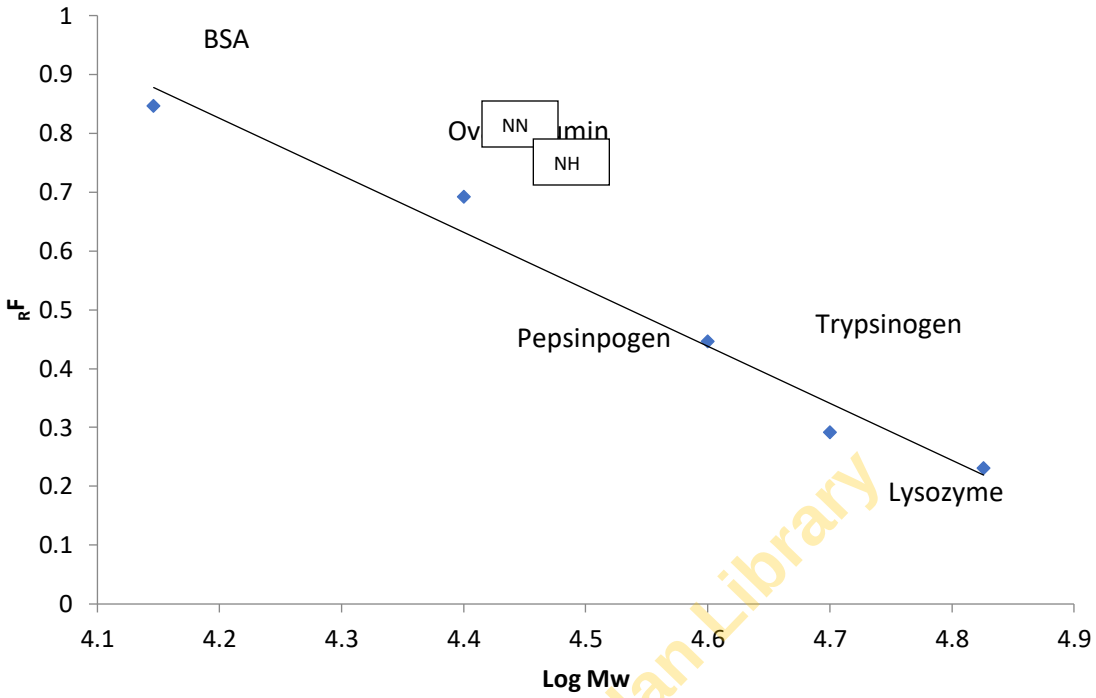
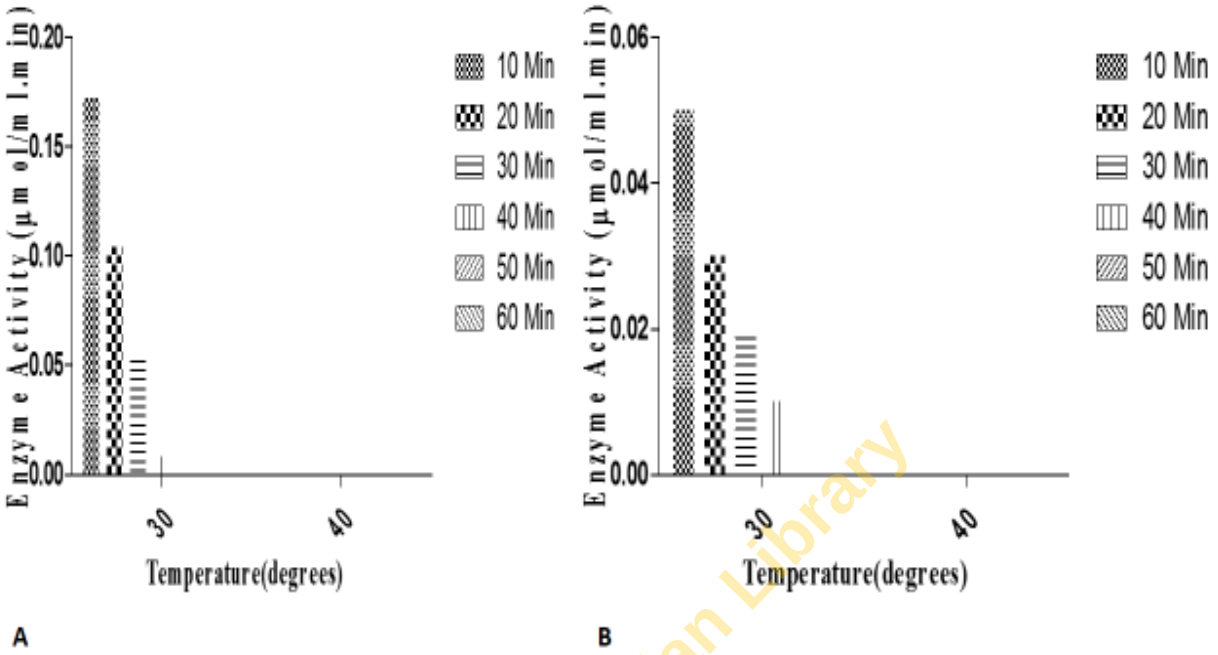


Figure S3: Graph showing Log M_w versus R_f for the estimation of molecular weight of proteins

BSA: Bovine Serum Albumin

NH: *N. haje* phospholipase

NN: *N. nigrocollis* phospholipase

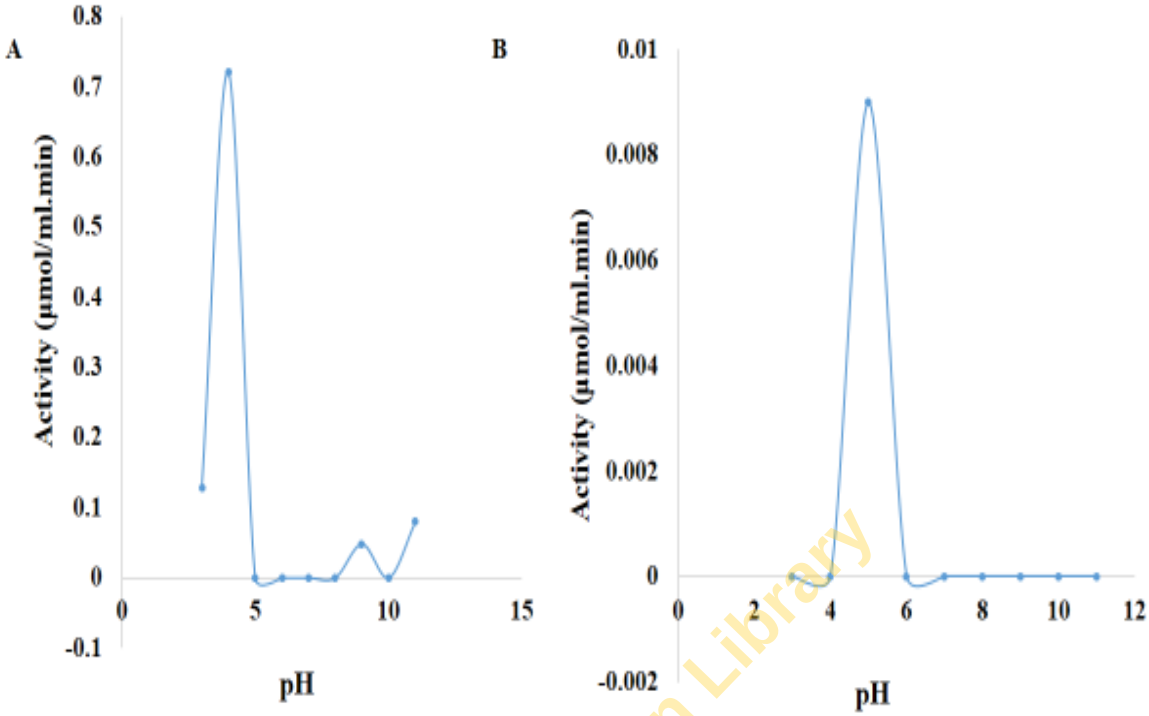


Data are in triplicates (n = 3), Significance at $P < 0.05$.

Figure S4: Effect of temperature on isolated enzymes

Legend:

A. *N. haje* phospholipase (NH-PL) activity, **B.** *N. nigricollis* phospholipase **(**NN-PL) activity



Data are in triplicates (n = 3), Significance at $p < 0.05$.

Figure S5: Effect of pH on isolated enzymes

Legend:

A. *N. haje* phospholipase (NH-PL) **B.** *N. nigricollis* phospholipase (NN-PL)