Multifunctional toxin phospholipase A_2 (PLA_2) in *Naja oxiana* venom a promising target for novel 2,5-disubstituted-1,3,4-oxadiazole derivatives: dry and wet lab approaches

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Abstract

The present work is designed to synthesize 2,5-disubstituted-1,3,4-oxadiazole derivatives **5a-5d** as snake venom phospholipase A_2 (PLA_2) inhibitors. The *p*-nitrophenol was reacted with ethyl chloroacetate to prepare ester **1** which then converted into corresponding hydrazide **2** by treating with hydrazine. The hydrazide **2** was cyclized by reacting with carbon disulfide into 5-(4-nitrophenoxy)methyl-1,3,4-oxadiazole-2-thiol **3**. The precursor **3** was alkylated with different benzyl chlorides **4a-4d** to afford 2,5-disubstituted-1,3,4-oxadiazoles **5a-5d**. The structural characterization of prepared derivatives was accomplished by FTIR, ^{1}H and ^{13}C NMR spectral data. The snake venom was isolated from *Naja oxiana* (*N. oxiana*) by pressing their glands below eyes to perform anti-PLA_2 activity. The inhibitory potential of synthesized derivatives **5a-5d** was evaluated against PLA_2 present in the venom. The synthesized derivatives **5a-5d** showed good PLA_2 inhibitory potential especially **5d** exhibited excellent activity having IC_{50} value 0.002 mM ($0.01>p>0.001$) followed by **5c** having IC_{50} value 0.003 mM ($0.01>p>0.001$). Compound **5a** and **5b** have IC_{50} values 0.027 mM ($p<0.001$) and 0.014 mM ($p<0.001$) respectively. Molecular docking was carried out against PLA_2 (PDBID 1A2A) to evaluate binding interactions of oxadiazole derivatives **5a-5d**. The docking results showed that all compounds have binding interactions with amino acid residues located in active binding site. They have good binding affinities particularly **5d** showed excellent binding interactions with binding energy value of -6.8 kcal/mol in comparison with other analogues. Based on dry and wet lab results it was explored that newly synthesized derivatives, specifically **5d** may act as a potent inhibitor of PLA_2 present in *N.oxiana* venom.

Key words: Snake bite envenomation, Oxadiazoles, Phospholipase A_2 Inhibition

1. Introduction

Snake bite envenomation (SBE) is recognized as category "A" neglected tropical disease on June 2017 by World Health Organization (WHO) attributed to 5.4 million cases and 100,000 mortalities annually worldwide[1]. Especially impoverished populations in rural, tropical and sub-tropical areas are highly vulnerable[2]. South East Asia is the heavily affected area owing to higher density of population, routined agriculture activities and abundance of venomous snake species like cobras, vipers and kraits. Approximately, 300 species of snakes are distributed in Pakistan out of which 40 are deadly poisonous documented to cause 40,000 envenomation cases and 8200 deaths yearly[3]. *N. oxiana* sub-specie of cobra categorized in Elapidae (genus Naja) is one of the most neglected and deadly venomous specie of snakes abundantly reported from Kharan and Chagai (Baluchistan), however, rarely found in northern areas of Pakistan[4,5]. It is responsible for diverse complications from mild to severe like pain, necrosis, hemorrhage, hematuria, edema, renal damage, infected gums, hepatic injury, mucous discharge and proteinuria in the victims. *N. oxiana* venom is a diverse array of proteins and peptides including PLA_2, alkaline phosphatase, serine proteases, metalloproteinases and three finger toxins. PLA_2 is abundantly present in *N. oxiana* venom notorious for number of pharmacological and toxicological effects[6,7]. PLA_2 at the sn-2 position of phospholipid membrane catalyzes fatty acid hydrolysis and liberates free fatty acid particularly arachidonic acid which is the main precursor for the synthesis of inflammatory mediators such as prostaglandin, thromboxane and prostacyclin. These inflammatory mediators were reported to cause inflammation, edema, platelet aggregation and anticoagulant effect[8,9]. PLA_2 stimulates neurons at pre-synaptic and post-synaptic terminals to induce neurotoxicity, however, myotoxicity is associated with destruction of muscle fibrils and sarcoplasmic reticulum of skeletal muscles[10]. Administration of antisera (immunoglobulins) is the standard treatment to neutralize

aforementioned toxicities but it is associated with severe adverse effects (anaphylactic shock, pyrogenic reactions and serum sickness). Furthermore, its high cost, lack of availability, specificity issue and storage problems pose peculiar challenges[11]. Phytomedicine endorsed by local healers provide limited efficacy, superficial effect and liver toxicity[12]. There is an urge to develop novel strategies having anti- PLA_2 activity to overcome its toxicities.

The 1,3,4-oxadiazole nucleus is a versatile heterocyclic moiety gained considerable interest in drug discovery owing to their wide range of pharmaceutical applications[13]. 1,3,4-oxadiazole derivatives have reported to exhibit antifungal, genotoxic[14], antiprotozoal[15] , antibacterial, antitubercular, anti-inflammatory, anticonvulsant, anti-HIV, anthelmintic, antipyretic, antioxidant, antidiabetic, spasmolytic, immunosuppressive, antiallergic, analgesic, antimalarial, sedative, hypnotic[16], antiepileptic, antineoplastic, analgesic and anticancer activities. Reported drugs having 1,3,4-oxadiazole nucleus are nesapidil (antihypertensive), furamizole (antibiotic), zibotenten (anticancer) and raltegravir (antiretroviral)[17]. Our group has already reported a number of oxadiazole and thiadiazole derivatives as these heterocycles established a particular position in medicinal chemistry due to wide range of activities[18,19,20]. The present work is planned to synthesize 2,5-disubstituted-1,3,4-oxadiazole derivatives as potential inhibitors of phospholipase A_2 activity.

2. Experimental

2.1 Snake venom collection

N. oxiana snakes were trapped from Chagai district, Baluchistan Pakistan with the assistance of charmers. Snakes were recognized by the zoologist Dr. Muhammad Latif, Department of Zoology,

University of Education, Lahore (Multan Campus). Snake venom was obtained by pressing their glands below the eyes. Subsequently, venom was lyophilized and kept at 2-8°C.

2.2 Chemistry

All the chemicals and reagents used in this project were obtained from Sigma Aldrich Company and used as received. Melting points were determined on Gallen Kamp apparatus via open capillary tubes. FTIR spectra were recorded on Perkin Elmer spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker NMR spectrometers in chloroform-d at 400 MHz.

2.2.1 Procedure for the synthesis of ethyl 2-(-4-nitrophenoxy)acetate (1)

4-nitrophenol 5g (35.94 mmol) and ethylchloroacetate (2.1 ml) was reflux in the presence of K_2CO_3 in acetonitrile for 5 to 6 hrs. TLC was used to monitor the progress of the reaction. On completion, solvent was evaporated under reduced pressure and residues being taken up in ethyl acetate (60 ml). The organic layer was washed with 1% HCl to remove base then separated and subjected to evaporation on rota evaporator. The solid residue of desired intermediate **1** was purified by recrystallization in ethanol as white crystals.

2.2.2 Procedure for the synthesis of 2-(-4-nitrophenoxy)acetohydrazide (2)

2-(-4-nitrophenoxy)acetate 4.5g (21.32 mmol) and hydrazine monohydrate (1 ml) dissolved in dry ethanol (40mL) and reflux for 5 to 6 hrs. The progress of the reaction was monitored by thin layer chromatography using ethyl acetate and n-hexane 1:1 as mobile phase. After reaction completion solvent was evaporated under reduced pressure and residue was poured into ice cold water, the hydrazide **2** was precipitated out. The crude hydrazide was recrystallized using ethanol to afford pale yellow crystals.

2.2.3 Procedure for the synthesis of 5-(4-nitrophenoxy)methyl)-1,3,4-oxadiazole-2-thiol (3)

2-(-4-nitrophenoxy)acetohydrazide 4g (18.95 mmol), KOH was mixed with 40ml dry ethanol, carbon disulphide (2.3 ml) was added drop wise and reflux for 6 to 8 hrs. The use of KOH facilitates the cyclization by nucleophilic attack of hydrazide on electrophilic carbon of CS_2. The completion of reaction was confirmed by TLC (ethyl acetate and n-hexane 1:1) and after completion of reaction, the solvent was concentrated on rotary evaporator and residue was treated with ice cold water then acidified with HCl to afford yellow crystals of oxadiazole **3** which were filtered and recrystallized in ethanol.

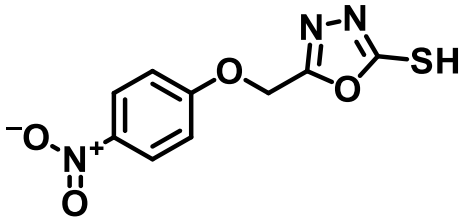
2.2.4 Procedure for the synthesis of 5-(4-nitrophenoxy)methyl)-1,3,4-oxadiazole-2-thiol derivatives (5a-5d)

5-(4-nitrophenoxy)methyl)-1,3,4-oxadiazole-2-thiol) 0.5g (1.97 mmol) was dissolved in ethanol by stirring at 30ºC, then KOH was added into the reaction mixture. The mixture was kept on stirring for 30 min. Subsequently, different benzyl halide derivatives **4a-4d** were introduced into the reaction mixture in equimolar ratios to synthesize title 2,5-disubstituted-1,3,4-oxadiazole derivatives **5a-5d**. The reaction time varied between 2-3 hours for different benzyl halides. TLC was used to monitor the reaction progress. On completion, the solvent was evaporated under reduced pressure. The residue was extracted in ethyl acetate, wash the organic layer with water to afford the final products. The final products **5a-5d** were purified by column chromatography by using ethyl acetate:n-hexane (1:2) as eluents. The silica gel 60 was used as stationary phase to pack the column and the impure compound was mixed with 1-2ml of ethyl acetate and loaded carefully at the top of column. The gradient elution was used to purify the compound, the n-hexane (mobile phase) was employed as single solvent initially and then gradually ethyl acetate was added to n-

hexane for complete elution. The purified compound was obtained by removing the mobile phase under reduced pressure.

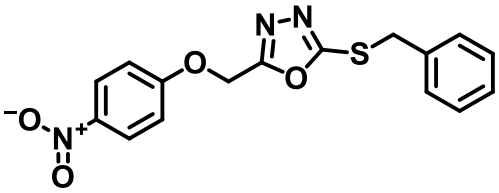
2.3 Spectral Characterizations of Synthesized Compounds

2.3.1 *5-(4-nitrophenoxy)methyl-1,3,4-oxadiazole-2-thiol (3)*



Creamy yellowish crystals; Yield: 77 %; m.p. 205-210 ºC; R_f=0.15(n-hexane:ethylacetate 3:1); Mol. Formula: $C_8H_5N_3O_4S$; FTIR (KBr ν_{max} cm^{-1}): 2359 (S-H), 1654 (C=N oxadiazole ring), 1255 (C-O-C ether linkage), 1519 (N-O); **^{1}HNMR** (400 MHz, $CDCl_3$): δH 5.13 (s, 2H, H-5), 7.06 (d, 2H, H-3, 3'= 8.8Hz), 8.24 (d, 2H, H-2, 2'= 9.2Hz).

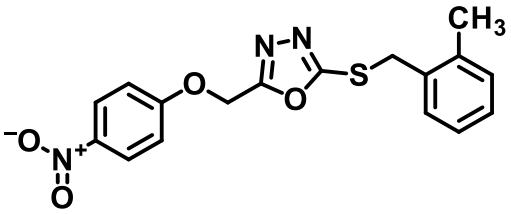
2.3.2 *2-(benzylthio)-5-[(4-nitrophenoxy)methyl]-1,3,4, oxadiazole (5a)*



Pale yellow crystals; Yield: 96.6 %; m.p. 185-195 ºC; R_f=0.65(n-hexane:DCM3:1); Mol.Formula: $C_{15}H_{11}N_3O_4S$; FTIR (KBr ν_{max} cm^{-1}): 2928 (C-H sp^3), 1655 (C=N oxadiazole ring), 1512 (N-O), 1238 (C-O-C ether linkage); **^{1}H- NMR** (400 MHz, $CDCl_3$): δH 8.18 (d, $J_{2/3} = J_{2'/3'}$ = 9.12 Hz, 2H, H-2, 2′), 7.38 (d, $J_{10/11} = J_{10'/11'}$ = 6.67 Hz, 2H, H-10, 10′), 7.28 (ovp, 3H, H-11, 11′, 12), 7.06 (d, $J_{3/2} = J_{3'/2'}$ = 9.12 Hz, 2H, H-3, 3′), 5.28 (s, 2H, H-5), 4.46 (s, 2H, H-8); **^{13}CNMR** ($CDCl_3$, 135 MHz, δppm): 142.7 (C1), 128.4 (C2,2'), 129 (C3,3'), 166.3 (C4), 60. (C5), 162.4 (C6), 162.2 (C7), 36.9 (C8), 126.1 (C9), 130.8 (C10), 129 (C11), 129.2 (C12)

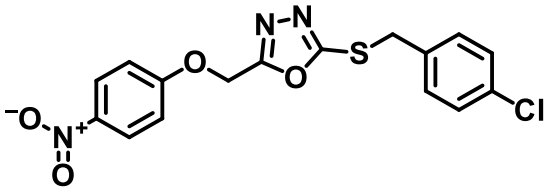
2.3.4 *2-((2-methylbenzyl) thio)-5-[(4-nitrophenoxy)methyl]-1,3,4-oxadiazole (5b)*

Vivid yellowish green powder; Yield: 92.5 %; m.p. 150-151 ºC; R_f=0.75(n-hexane: ethyl acetate 1:1); Mol. Formula: $C_{15}H_{11}N_3O_4SCl$; FTIR (KBr ν_{max} cm^{-1}): 2918 (C-H sp^3), 1653 (C=N oxadiazole ring), 1254 (C-O-C ether linkage), 1517 (N-O); **^{1}H- NMR** (400 MHz, $CDCl_3$): δH 8.21 (d, $J_{2/3} = J_{2'/3'}$ = 9.10 Hz, 2H, H-2, 2′), 7.35 (d, $J_{11/12}$ = 7.46 Hz, 1H, H-11), 7.21-7.13 (m, 3H, H-12, 13, 14), 7.06 (d, $J_{3/2} = J_{3'/2'}$ = 9.12 Hz, 2H, H-3, 3′), 5.30 (s, 2H, H-5), 4.50 (s, 2H, H-8), 2.41 (s, 3H, H-CH_3); **^{13}C-NMR** ($CDCl_3$, 135 MHz, δppm): 142.7 (C1), 137.2 (C2, C2'), 132.7 (C3, C3'), 166.4 (C4), 60 (C5), 162.3 (C6), 162.2 (C7), 35.2 (C8), 126.2 (C9), 115 (C10), 126.5 (C11), 128.8 (C12), 130.4 (C13), 130.9 (C14), 19.3 (C15).



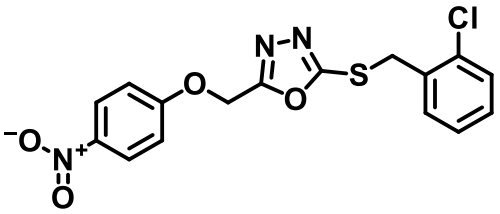
2.3.5 ***2-(4-chlorobenzyl) thio)-5-[(4-nitrophenoxy)methyl]-1,3,4-oxadiazole (5c)***

Yellow crystal; Yield: 90 %; m.p. 168-170 ºC; R_f=0.69(n-hexane:ethylacetate 3:1) Mol. Formula: $C_{15}H_{11}N_3O_4SCl$; FTIR (KBr ν_{max} cm^{-1}): 3082 (C-H sp^2), 2937 (C-H sp^3), 1704 (C=N oxadiazole ring), 1517 (N-O), 1257 (C-O-C ether linkage); **^{1}H-NMR** (400 MHz, $CDCl_3$): δH 8.18 (d, $J_{2/3} = J_{2'/3'}$ = 9.13 Hz, 2H, H-2, 2′), 7.33 (d, $J_{11/12} = J_{14/13}$ = 8.41 Hz, 2H, H-10, 10′), 7.25 (d, $J_{11/12} = J_{14/13}$ = 8.41 Hz, 2H, H-11, 11′), 7.06 (d, $J_{3/2} = J_{3'/2'}$ = 9.12 Hz, 2H, H-3, 3′), 5.28 (s, 2H, H-5), 4.41 (s, 2H, H-8); **^{13}C-NMR** ($CDCl_3$-, 135 MHz, δppm): 142.7 (C1), 134.3 (C2, C2'), 133.9 (C3, C3'), 165.9 (C4), 60 (C5), 162.5 (C6), 162.1 (C7), 36.1 (C8), 115 (C9), 126.1 (C10, C10'), 1329.1 (C11, C11'), 130.6 (C12).



2.3.3 ***2-(2-chlorobenzyl) thio)-5-[(4-nitrophenoxy)methyl]-1,3,4-oxadiazole (5d)***

Creamy yellow crystals; Yield: 75 %; m.p. 100-102 ºC; R_f=0.56 (n-hexane: ethyl acetate 3:1); Mol. Formula: $C_{16}H_{14}N_3O_4S$; FTIR (KBr ν_{max} cm^{-1}): 3079 (C-H sp^2), 1655 (C=N oxadiazole ring), 1515 (N-O), 1251 (C-O-C ether linkage); **^{1}H- NMR** (400 MHz, $CDCl_3$): δH 8.19 (d, $J_{2/3} = J_{2'/3'}$ = 9.25 Hz, 2H, H-2, 2′), 7.55 (dd, $J_{11/12}$ = 7.35 Hz, $J_{11/13}$ = 1.61 Hz, 1H, H-11), 7.36 (dd, $J_{11/12}$ = 7.94 Hz, $J_{11/13}$ = 1.16 Hz, 1H, H-14,), 7.25-7.16 (ovp, 2H, H-12, 13), 7.07 (d, $J_{3/2} = J_{3'/2'}$ = 9.25 Hz, 2H, H-3, 3′), 5.29 (s, 2H, H-5), 4.56 (s, 2H, H-8); **^{13}C-NMR** ($CDCl_3$, 135 MHz, δppm): 142.7 (C1), 134.5 (C2, C2'), 133.4 (C3, C3'), 165.3 (C4), 60 (C5), 162.1 (C6), 162.1 (C7), 34.7 (C8), 115 (C9), 131.6 (C10), 129.9 (C11), 129.9 (C12), 127.2 (C13), 126.1 (C14).



2.4 Molecular docking

2.4.1 Retrieval of phospholipase A_2 structure using Protein database

Snake venom PLA_2 structure (3D) was retrieved by using protein database having PDB ID 1A2A.

2.4.2 Designing of ligands

Structure of synthesized ligands were drawn in ChemDraw Professional 16.0 and database was prepared. Energy was minimized and 3D protonated by using Molecular Operating Environment 2015 (MOE 2015.10) to obtain good structural conformation.

2.4.3 Preparation of Receptor

Enzyme was refined by removing all water molecules and ligands, however, hydrogen atoms were added to calculate partial atomic charges by using MOE 2015.10. Enzyme was 3D protonated and MOE tool energy minimization algorithm was used to minimize energy by following parameters; gradient: 0.1, Force Field: Amber10: EHT, Gas phase Solvation, Chiral Constraint: Current Geometry. Subsequently, prepared enzyme was further employed for docking.

2.4.4 Validation of modeled structure

The stereochemical validation of modeled structure of enzyme is an important part of the comparative molecular docking process. The stereochemical quality of modeled enzyme was checked by Ramachandran plot.

2.4.5 Identification of active site

Site finder was used to identify active sites in the enzyme by using 3D atomic coordinates of the receptor. It is an approach for predicting protein ligand binding sites mainly based upon energy.

2.4.6 Docking

The interaction of the ligand molecule with the protein complex was evaluated by using MOE docking software to determine the correct conformation (structure of molecule is not rigid when the bonds are rotated) and configuration (structure of the molecule remain rigid when the whole molecule is rotated) of the ligand in order to obtain minimum energy structure. The docking parameters were; Iteration limit = 200, Total runs = 30, Cycle/runs = 5, Potential Energy Grid: ON, Annealing Algorithm: Simulated Annealing.

2.5 Phospholipase A_2 inhibition assay

PLA_2 assay was carried out according to acidimetric technique reported by Tan and Tan (1988). Egg yolk suspension was prepared by using equal quantity of sodium deoxycholate (8.1 mmol), calcium chloride (18 mmol) and egg yolk. Mixture was kept on stirring for 10 min to form homogeneous suspension and sodium hydroxide (1M) was added to maintain pH up to 8. Different concentration of venom (1-10 µg/0.01 ml) were added to egg yolk mixture (15 ml) to start hydrolysis and saline was served as control. Decline in pH will be noted after 02 min, drop in 1 pH unit corresponds to the release of 133 µmoles of fatty acid from egg yolk. Phospholipases A_2 activity was estimated by the quantity of free fatty acid released per minute. Snake venom (10 µl, 10mg/10ml) and synthetic compounds in 50 mmol concentrations were pre-incubated to calculate anti-PLA_2 activity in terms of percentage[7]. PLA_2 inhibition activity was calculated by given formula:

$$\text{Activity (units/ml/min)} = \frac{\text{Molar concentration of product released} \times \text{total vol. of assay}}{\text{Vol. of enzyme used} \times \text{time utilized} \times \text{vol. used in measurement}}$$

The IC_{50} values were determined using a dose response curve obtained by plotting the percentage inhibition versus the log concentration using Graph pad prism software (version 5.0).

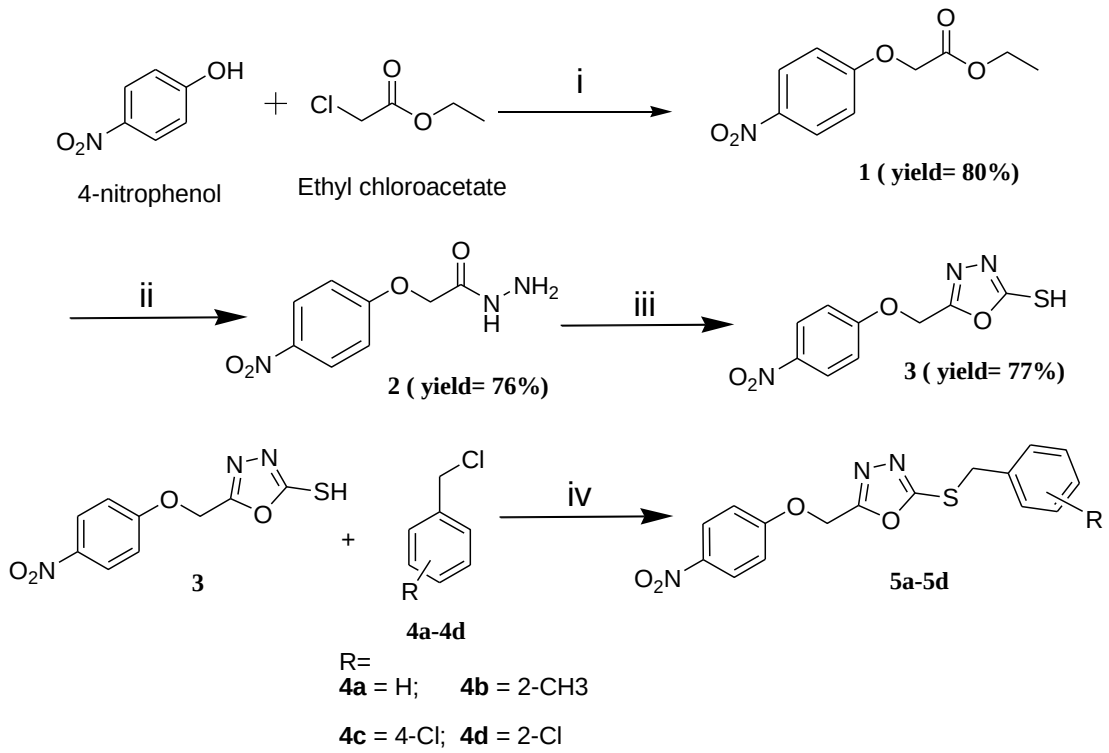
2.6 Statistical analysis

Microsoft excel® (2010) was used to calculate Mean ± S.D. Moreover, to compare the results with the reference standard student's *t*-test was applied while value of probability was set at $p>0.001$.

3.1 Results and discussion

3.1.1 Chemistry

The current work is designed to synthesize novel 1,3,4-oxadiazole derivatives **5a-5d** to evaluate their PLA_2 inhibitory activity. The 4-nitrophenol was reacted with ethyl chloroacetate to prepare the ester **1** which then converted into corresponding hydrazide **2** by treating with hydrazine. The hydrazide **2** was cyclized by reacting with carbon disulfide into 5-(4-nitrophenoxy)methyl-1,3,4-oxadiazole-2-thiol **3**. The precursor **3** was alkylated with different benzyl chlorides derivatives **4a-4d** to afford the final products 2,5-disubstituted-1,3,4-oxadiazole derivatives **5a-5d** (Scheme 1).



Scheme 1 Synthetic route, reagent and conditions for synthesis of 2-5-disubstituted-1,3,4-oxadiazole derivatives **5a-5d**: (i) CH_3CN/K_2CO_3, Reflux 5-6 h; (ii) N_2H_4/C_2H_5OH, Reflux 5-6 h; (iii) CS_2/C_2H_5OH, Reflux 6-8 h; (iv) KOH/C_2H_5OH, Reflux 2-3 h.

In FTIR spectal data stretching frequency of C=N at 1655 cm^{-1} and SH at 2362 cm^{-1} confirmed the cyclization of hydrazide into oxadiazole ring. ^1H NMR spectra were recorded at 400 MHz in deutrated chloroform-$CDCl_3$ gave singlet of two proton at δ 5.13ppm, doublet at δ 8.24ppm with coupling constant 9.2Hz having integration of two proton, another signal displayed at 7.06 *δ*ppm

with coupling constant 8.8 Hz as doublet with integration of two proton. All of the above findings indicated the presence of 1,4-disubstituted benzene ring which was 4-nitrophenoxy group of precursor 5-(4-nitrophenoxy)methyl-1,3,4-oxadiazole-2-thiol **3**. Eventually, the structure of precursor was established as 5-(4-nitrophenoxy)methyl-1,3,4-oxadiazole-2-thiol **3**. Title compounds **5a-5d** were synthesized in reasonable yield by condensing precursor with different benzyl halide derivatives **4a-4d**. The synthesized derivatives were confirmed by TLC and characterized by spectroscopic techniques e.g. FTIR, ^{1}H NMR and ^{13}C NMR.

3.1.2 Molecular docking analysis

3.1.2.1 Comparative binding energy analysis of synthesized derivatives

Computational docking is a meritorious technique for evaluating the conformational bind which really exemplify ligand inside the targeted enzyme's catalytic region. The Ramachandran plot also assured that the amino acid residues located in the favoured region (Fig. 1).

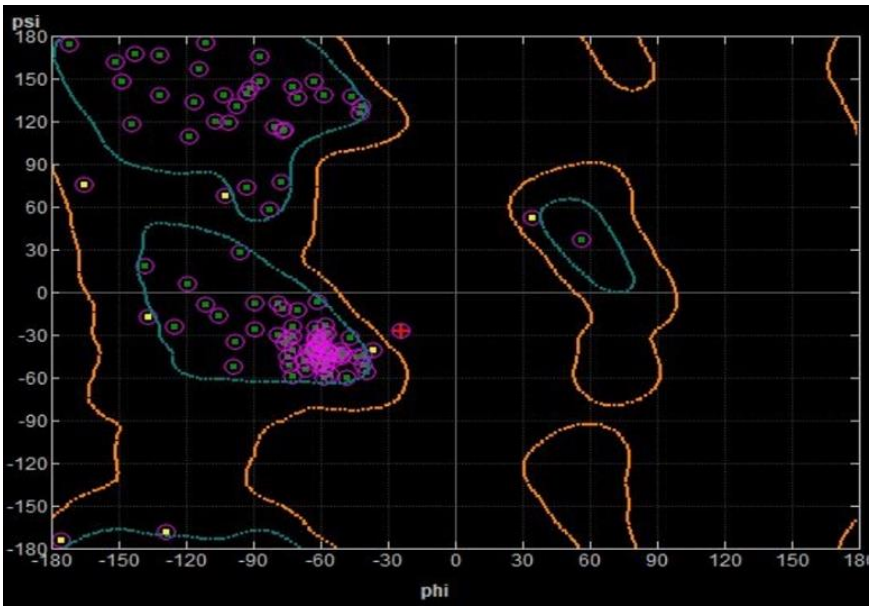


Figure 1 Ramachandran graph of protein predicted by MOE 2015.10.

All the synthesized analogues **5a-5d** were docked against PLA_2 to predict the best positional conformation. Docked complexes were evaluated with respect to their glide docking values (docking energy, kcal/mol) and binding interactions (hydrogen/hydrophobic). Docking analysis showed that glide docking energies were little fluctuated among all ligands as the basic skeleton of all the synthesized derivatives were comparable. The docking results revealed that compound **5d** with excellent binding energy (-6.8 kcal/mol) in comparison with other derivatives suggesting its potential as the best inhibitor of PLA_2. Furthermore, compound **5c** also possessed good binding energy value (-6.6 kcal/mol), however, **5a** and **5b** exhibited binding energy values (-6.4 kcal/mol) and (-6.5 kcal/mol) respectively (Fig. 2).

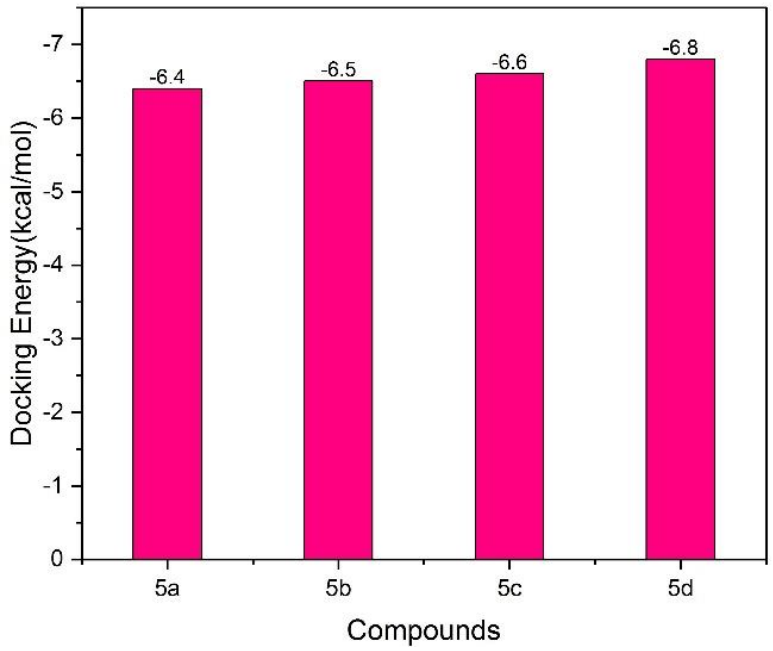


Figure 2 Graphical depiction of docking energy values of compounds **5a-5d**.

3.1.2.2 Critical analysis of binding pocket of PLA_2 against 5d

The compound **5d**-docked pose was selected on the basis of docking outcomes in order to deeply recognize the ligand-protein binding interactions. Molecular docking studies depicted that

analogue **5d** effectively bound within active binding regions of PLA_2. The oxadiazole ring showed intrusion in the inner core of binding pocket while benzyl moiety showed interaction at the entrance of binding region. Binding analysis showed two hydrogen bonding, one hydrophobic interaction and one pi-pi stacking in **5d**-docking complex. One hydrogen bond was formed with the oxygen of nitro group having interaction with Asn 6 with bond length 2.15Å whereas nitrogen of the oxadiazole ring form another hydrogen bond with His 48 with bond length 2.90Å. One hydrophobic interaction was observed with Asp 49 with bond length 2.42Å while pi-pi stacking of oxadiazole ring was shown with Phe 5. Literature study also revealed that residues involved in binding are the active residues of PLA_2 enzyme which strengthened our docking results. The two dimensional (2D) docking pose and binding interaction of most potent compound **5d** is presented in Fig 3.

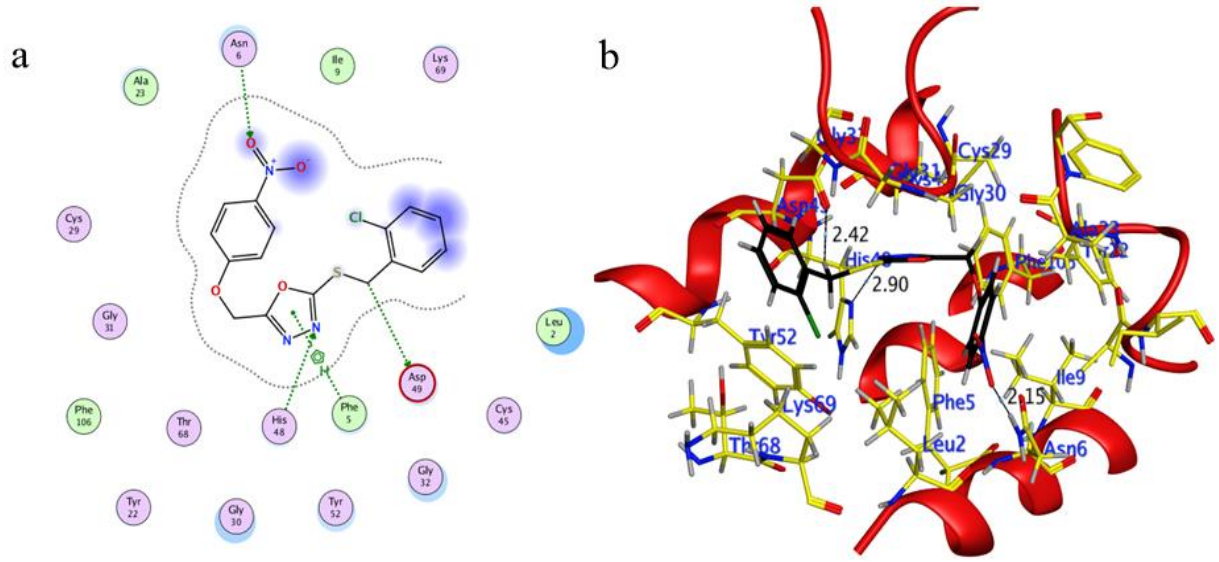


Figure 3 a) Two dimensional and b) Three dimensional poses of compound **5d** with in active binding site of PLA2 enzyme showing interactions with Asp 49, His 48, Phe 5 and Asn 6.

3.1.3 Enzymatic assay for anti-PLA_2 activity

The novel oxadiazole derivatives **5a-5d** were synthesized by introducing a substituent at thiol group in order to evaluate anti-PLA_2 activity. EDTA and standard antidote (antisera) served as a reference standard for comparison purposes. The analogue **5d** possessed most potent PLA_2 inhibitory activity with IC_{50} value of 0.002 mM ($0.01>p>0.001$) (Fig. 6). The derivative **5c** showed good inhibitory activity having IC_{50} 0.003 mM ($0.01>p>0.001$). The compounds **5a** and **5b** showed less activity as compared to other derivatives having IC_{50} 0.027 mM ($p < 0.001$) and 0.014 mM ($p < 0.001$) respectively (Fig. 4).

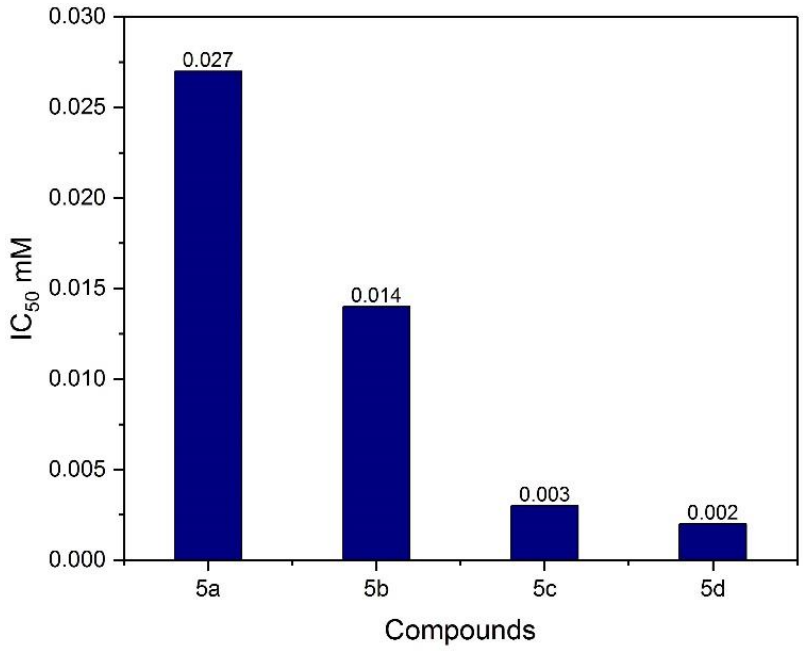


Figure 4 Graphical depiction of IC_{50} values of compounds **5a-5d**.

These compounds possessed different organic, inorganic and heterocyclic moieties. According to preliminary SAR studies, the benzene ring and orientation of Cl moiety plays an imperative role in PLA_2 inhibitory activity. Notably, compound **5d** is more potent as compare to other compounds owing to presence of chlorine molecule at ortho position. Compound **5c** possess chlorine atom at para position, its inhibitory activity was decreased by 10 folds as compare to **5d**, so it is concluded

that halogen at ortho position is imperative to inhibit PLA_2 activity. Moreover, substitution of methyl group at ortho position of benzene ring is not beneficial for PLA_2 inhibition. Based on these outcomes it was concluded that compound **5d** would be designed as an inhibitor of PLA_2. The tables 1 and 2 showed effects of different *N. oxiana* venom concentration in terms of free fatty acids released/min and antidotal effects of synthesized compounds against PLA_2 respectively.

Table 1 Effect of various concentration of *N. oxiana* venom in terms of free fatty acids released/min.

Sr. No	**Conc. of venom (µg/0.01mL)**	**Change in pH (Mean ± S.D)**	**Fatty acid released/min (µM)**	**Enzyme activity (units/ml/min of crude venom)**
1	1	7.82 ± 0.09	13.3	665
2	2	7.8 ± 0.1	26.6	1330
3	4	7.62 ± 0.01	53.2	2660
4	8	7.41 ± 0.2	106.4	5300
5	10	7.0 ± 0.05	133	6650
6	Negative Control (Saline)	8 ± 0	0	0

Table 2 Antidotal effect of various newly synthesized 2-5-disubstituted-1,3,4-oxadiazole derivatives (50 mM) against PLA_2 present in *N. oxiana* venom.

Sr. No.	**Tested compounds**	**Change in pH**	**Fatty acid**	**Maximum protection**

	(50 mM)	(Mean ± S.D)	Released/min	(µM)	(%)	*p* value
1	5a	7.40 ± 0.008	53.2		40	$p<0.001$
2	5b	7.33 ± 0.017	43.89		33	$p<0.001$
3	5c	7.76 ± 0.02	101.08		76	$0.01>p>0.001$
4	5d	7.87 ± 0.01	]107.73		81	$0.01>p>0.001$
5	EDTA	7.90 ± 0.016	119.7		90	$p<0.05$
6	Standard antisera (Immunoglobulins)	7.95 ± 0.014	126.35		95	Select to compare

4. Conclusion

Substituted 5-(4-nitrophenoxy)methyl-1,3,4-oxadiazole-2-thiol derivatives **5a-5d** were synthesized in good to excellent yield. The simple reaction route was adopted to synthesize the title oxadiazole derivatives **5a-5d**. The computational molecular docking studies were performed against PLA_2 (PDBID 1A2A) which revealed that compound **5d** presented outstanding binding affinity with binding energy value (-6.8 kcal/mol). The docking results inferred that the synthesized compunds bound well in the active binding site of the target protein. The snake venom was safely isolated from *N. oxiana* to perform anti-PLA_2 activity and results showed that compounds **5a-5d** exhibited good PLA_2 inhibitory potential especially **5d** displayed excellent activity having IC_{50} value 0.002 mM ($0.01>p>0.001$). The compound **5d** was found to be most

potent phospholipase A_2 inhibitor among all other derivatives. Acidimetric assay for PLA_2 proved that **5d** was the most potent inhibitor as compared to other derivatives. The results of the dry and wet lab were found to be compatible, synthesized derivatives predominantly **5d** may act as a leading molecule to design most effective inhibitor of PLA_2 present in *N.oxiana* venom.

Conflict of interest: The authors declared no conflict of interest.

References

1. Luiselli, L., Sale, L., Akani, G. C. and Amori, G. Venomous snake abundance within snake species' assemblages worldwide. *Divers.*, (2020), **12**(2), 69.
2. Asad, M. H. H. B., Iqbal, M., Rouf, M., Akram, N. R. K., Muneer, S., Shabbir, M. Z., Khan, M. S., Murtaza, G., and Hussain, I. 5´-nucleatidases of Naja Naja Karachiensis snake venom: Their determination, toxicities and remedial approach by natural inhibitors (medicinal plants). *Acta Polo. Pharm.*, (2016), **73**(3), 667-673.
3. Alirol, E., Sharma, S. K., Bawaskar, H. S., Kuch, U., and Chappuis, F. Snake bite in South Asia: a review. *PLoS negl trop dis*, (2010), **4**(1), e603.
4. Masroor, R., Khisroon, M., and Jablonski, D. A case study on illegal reptile poaching from Balochistan. Pakistan. *Herpetozoa*, (2020), **33**, 67.
5. Ebrahim, K., Vatanpour, H., Zare, A., Shirazi, F. H., and Nakhjavani, M. Anticancer activity a of caspian cobra (Naja Oxiana) snake venom in human cancer cell lines via induction of apoptosis. *Iran. J. Pharm. Res*, (2016), **15**(Suppl), 101.
6. Asad, M. H. H. B., McCleary, R. J. R., Salafutdinov, I., Alam, F., Shah, H. S., Bibi, S., Ali, A., Khalid, S., Hasan, S. M. F., Sabatier, J. M., Waard, M. D., Hussain, I., and Rizvanov, A. A. Proteomics study of Southern Punjab Pakistani cobra (Naja naja: formerly Naja naja karachiensis) venom. *Toxicol. Environ. Chem*, (2019), **101**(1-2), 91-116.
7. Asad, M. H. H. B., Durr-e-Sabih., Yaqab, T., Murtaza, G., Hussain, M. S., Hussain, M. S., Nasir, M. T., Azhar, S., Khan, S. A., and Hussain. I. Phospholipases A_2: Enzymatic assay for snake venom (Naja naja karachiensis) with their neutralization by medicinal plants of Pakistan. *Acta Pol. Pharm*, (2014), **71**(4), 625-630.

8. Murakami, M., Taketomi, Y., Miki, Y., Sato, H., Hirabayashi, T., and Yamamoto, K. Recent progress in phospholipase A_2 research: from cells to animals to humans. *Prog. Lipid Res*, (2011), **50**(2), 152–92.
9. Six, D. A., Dennis, E. A. The expanding superfamily of phospholipase A_2 enzymes: classification and characterization. *Biochim Biophys Acta*, (2000), **1488**(1–2), 1–19.
10. Montecucco, C., Gutierrez, J. M., and Lomonte, B. Cellular pathology induced by snake venom phospholipase A_2 myotoxins and neurotoxins: common aspects of their mechanisms of action. *Cell. Mol. life Sci*, (2008), **65**(18), 2897–912.
11. Ahmed, S. M., Ahmed, M., Nadeem, A., Mahajan, J., Choudhary, A., and Pal, J. Emergency treatment of a snake bite: Pearls from literature. *J. Emerg. Trauma Shock*, (2008), **1**(2), 97.
12. Trim, S. A., Trim, C. M., Williams, H. F., and Vaiyapuri, S. The failures of ethnobotany and phytomedicine in delivering novel treatments for snakebite envenomation. *Toxins*, (2020), **12**(12), 774.
13. Kumar, B., Kumar, A., Beheraand, A. K., and Raj, V. Latest update on pharmacological activities of 1, 3, 4-oxadiazole derivatives. *J Cell Sci Ther*, (2016), **7**(233), 2.
14. Maslat, A. O., Abussaud, M., Tashtoush, H., and AL-Talib, M. Synthesis, antibacterial, antifungal and genotoxic activity of bis-1, 3, 4-oxadiazole derivatives. *Pol. J. Pharmacol*, (2002), **54**(1), 55-60.
15. Paruch, K., Popiołek, Ł., and Wujec, M. Antimicrobial and antiprotozoal activity of 3-acetyl-2, 5-disubstituted-1, 3, 4-oxadiazolines: a review. *Med Chem Res*, (2020), **29**(1), 1-16.

16. Kumar, K. A., Jayaroopa, P., and Kumar, G. V. Comprehensive review on the chemistry of 1, 3, 4-oxadiazoles and their applications. *Int. J. Chem. Tech. Res*, (2012), **4**(4), 1782-1791.
17. Iqbal, Z., Iqbal, A., Ashraf, Z., Latif, M., Hassan, M., and Nadeem, H. Synthesis and docking studies of N- (5- (alkylthio) -1, 3, 4-oxadiazol-2-yl) methyl) benzamide analogues as potential alkaline phosphatase inhibitors. *Drug Dev. Res*, (2019), **80**(5), 646-654.
18. Abas, M., Bahadur, A., Ashraf, Z., Iqbal, S., Rajoka, M.S.R. Rashid, S.G., Jabeen, E., Iqbal, Z., Abbas, Q., Bais, A., Hassan, M., Liu, G., Feng, K., Lee, S.H., Nawaz, M., Qayyum, M.A. Designing novel anticancer sulfonamide based 2,5-disubstituted-1,3,4-thiadiazole derivatives as potential carbonic anhydrase inhibitor. *J. Mol. Struct*. (2021), **1246**, 131145.
19. Abbas, S., Afzal, S., Nadeem, H., Hussain, D., Langer, P., S-evigny, J., Ashraf, Z., Iqbal, J. Synthesis, characterization and biological evaluation of thiadiazole amide derivatives as nucleoside triphosphate diphosphohydrolases (NTPDases) inhibitors. *Bioorg. Chem*, (2022), **118**, 105456.
20. Iqbal, Z., Ashraf, Z., Hassan, M., Abbas, Q., Jabeen, E. Substituted phenyl[(5-benzyl-1,3,4-oxadiazol-2-yl)sulfanyl]acetates/acetamides as alkaline phosphatase inhibitors: Synthesis, computational studies, enzyme inhibitory kinetics and DNA binding studies. *Bioorg Chem*, (2019), **90**, 103108.