Multifunctional toxin phospholipase A_2 (PLA_2) in *Naja oxiana* venom, a promising target for 2,5-disubstituted-1,3,4-oxadiazole derivatives

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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 snake venom was isolated from *Naja oxiana* by pressing their glands below eyes to perform anti-PLA_2 activity. The compounds 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 ($0.01 > p > 0.001$). Compounds 5a and 5b have IC_{50} values 0.027 mM ($p < 0.001$) and 0.014 mM ($p < 0.001$) respectively. The docking results showed that all compounds have binding interactions with amino acid residues in active binding site. They have good binding affinities, particularly 5d has binding energy –6.8 kcal/mol compared to other analogues. On the basis of dry and wet lab results, it may proposed that 5d may act as a potent inhibitor of PLA_2 in *N. oxiana* venom.

Keywords: *Naja oxiana*, phospholipase A_2 inhibitors, oxadiazoles, snake bite envenomation.

SNAKE bite envenomation (SBE) was recognized as a category 'A' neglected tropical disease in June 2017 by the World Health Organization (WHO), Geneva, attributed to 5.4 million cases and 100,000 mortalities annually worldwide[1]. Especially impoverished populations in rural, tropical and subtropical areas are highly vulnerable[2]. Southeast Asia is significantly affected owing to higher population density, routine agriculture activities and an abundance of venomous snake species like cobras, vipers and kraits. Approximately 300 species of snakes are distributed in Pakistan, of which 40 are highly poisonous, documented to cause 40,000 envenomation cases and 8200 deaths annually[3]. *Naja oxiana* sub-species of cobra categorized in Elapidae (genus *Naja*) is one of the most neglected and deadly venomous snake species abundantly reported from Kharan and Chagai in Balochistan; however, it is rarely found in the northern areas of Pakistan[4,5]. It causes complications like mild to severe pain, necrosis, haemorrhage, hematuria, oedema, renal damage, infected gums, hepatic injury, mucous discharge and proteinuria in the victims. *N. oxiana* venom has a diverse array of proteins and peptides, including phospholipase A_2 (PLA_2), alkaline phosphatase, serine proteases, metalloproteinases and three-finger toxins. PLA_2 is abundantly present in *N. oxiana* venom and responsible for several pharmacological and toxicological effects[6,7]. PLA_2 at the sn-2 position of the phospholipid membrane catalyses 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 are reported to cause inflammation, oedema, 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 the destruction of muscle fibrils and the sarcoplasmic reticulum of skeletal muscles[10]. Administration of antisera (immunoglobulins) is the standard treatment to neutralize the 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 provides limited efficacy, superficial effect and liver toxicity[12]. There is a need to develop novel strategies having an anti-PLA_2 activity to overcome its toxicity.

The 1,3,4-oxadiazole nucleus is a versatile heterocyclic moiety that has gained considerable interest in drug discovery owing to its wide range of pharmaceutical applications[13]. 1,3,4-Oxadiazole derivatives have exhibited antifungal, genotoxic[14], antiprotozoal[15], antibacterial, antitubercular, anti-inflammatory, anticonvulsant, anti-HIV, anthelmintic, antipyretic, antioxidant, antidiabetic, spasmolytic, immunosuppressive, antiallergic, analgesic, antimalarial, sedative,

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hypnotic[16], antiepileptic, antineoplastic, analgesic and anticancer activities. The reported drugs having 1,3,4-oxadiazole nucleus are nesapidil (antihypertensive), furamizole (antibiotic), zibotenten (anticancer) and raltegravir (antiretroviral)[17]. We have reported several oxadiazole and thiadiazole derivatives, as these heterocycles have established a key position in medicinal chemistry due to their wide range of activities[18–20]. The present study was designed to synthesize 2,5-disubstituted-1,3,4-oxadiazole derivatives as potential inhibitors of phospholipase A_2 activity.

Experimental procedure

Snake venom collection

N. oxiana snakes were trapped in Chagai district, Balochistan, with the assistance of snake charmers. They were recognized by Muhammad Latif (Department of Zoology, University of Education, Lahore (Multan Campus, Pakistan)). Snake venom was obtained by pressing the glands below their eyes. Subsequently, venom was lyophilized and kept at 2–8°C.

Chemistry

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

*Synthesis of ethyl 2-(-4-nitrophenoxy)acetate (**1**):* 4-Nitrophenol 5 g (35.94 mmol) and ethylchloroacetate (2.1 ml) were refluxed in the presence of K_2CO_3 in acetonitrile for 5–6 h. Thin-layer chromatography (TLC) was used to monitor the progress of the reaction. On completion, the solvent was evaporated under reduced pressure and residues were taken up in ethyl acetate (60 ml) to extract the product. The organic layer was washed with 1% HCl to remove the base, then separated and subjected to evaporation on a rota evaporator. The solid residue of the desired intermediate (**1**) was purified by recrystallization in ethanol as white crystals.

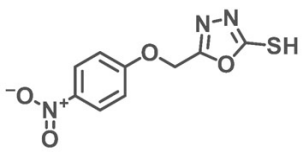
*Synthesis of 2-(-4-nitrophenoxy)acetohydrazide (**2**):* 2-(4-Nitrophenoxy)acetate 4.5 g (21.32 mmol) and hydrazine monohydrate (1 ml) were dissolved in dry ethanol (40 ml) and refluxed for 5–6 h. The progress of the reaction was monitored by TLC using ethyl acetate and *n*-hexane 1 : 1 as the mobile phase. After completion of the reaction, the solvent was evaporated under reduced pressure and the residue was poured into ice-cold water; and hydrazide (**2**) was precipitated out. The crude hydrazide was recrystallized using ethanol to produce pale yellow crystals.

*Synthesis of 5-(4-nitrophenoxy)methyl-1,3,4-oxadiazole-2-thiol (**3**):* 2-(4-Nitrophenoxy)acetohydrazide 4 g (18.95 mmol) and KOH were mixed with 40 ml dry ethanol, then carbon disulphide (2.3 ml) was added dropwise and refluxed for 6 to 8 h. The use of KOH facilitates cyclization by nucleophilic attack of hydrazide on the electrophilic carbon of CS_2. The completion of the reaction was confirmed by TLC (ethyl acetate and *n*-hexane 1 : 1). Thereafter, the solvent was concentrated on a rotary evaporator and the residue was treated with ice-cold water and then acidified with HCl to produce yellow crystals of oxadiazole (**3**), which were filtered and recrystallized in ethanol.

*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 0.5 g (1.97 mmol) was dissolved in ethanol by stirring at 30°C. Then KOH was added into the reaction mixture and stirred for 30 min. Subsequently, different benzyl halide derivatives (**4a–4d**) were introduced into the reaction mixture in equimolar ratios to synthesize the 2,5-disubstituted-1,3,4-oxadiazole derivatives (**5a–5d**). The reaction time varied between 2 and 3 h for different benzyl halides. TLC was used to monitor the progress of the reaction. On completion, the solvent was evaporated under reduced pressure. The residue was extracted in ethyl acetate and the organic layer was washed with water to give the final products. The final products (**5a–5d**) were purified by column chromatography using ethyl acetate : *n*-hexane (1 : 2) as eluents. Silica gel 60 was used as the stationary phase to pack the column and the impure compound was mixed with 1–2 ml of ethyl acetate and loaded carefully at the top of the column. The gradient elution method was used to purify the compound. *n*-Hexane (mobile phase) was used as a single solvent initially and then gradually ethyl acetate was added to *n*-hexane to complete the elution. The purified compound was obtained by removing the mobile phase under reduced pressure.

Spectral characterizations of synthesized compounds

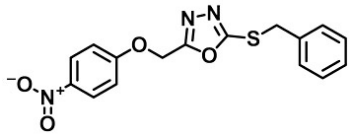
*5-(4-Nitrophenoxy)methyl-1,3,4-oxadiazole-2-thiol (**3**):* Creamy-yellowish crystals; yield: 77%; m.p. 205°–210°C; R_f = 0.15 (*n*-hexane : ethylacetate 3 : 1); molecular formula: $C_8H_5N_3O_4S$; FTIR (KBr ν_{max} cm^{-1}): 2359 (S–H), 1654 (C=N



oxadiazole ring), 1255 (C-O-C ether linkage), 1519 (N-O); ^{1}H NMR (400 MHz, $CDCl_3$): δH 5.13 (s, 2H, H-5),

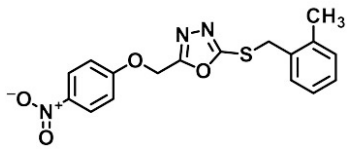
7.06 (d, 2H, H-3, 3′ = 8.8Hz), 8.24 (d, 2H, H-2, 2′ = 9.2Hz).

*2-(Benzylthio)-5-(4-nitrophenoxy)methyl-1,3,4, oxadiazole (**5a**):* Pale yellow crystals; yield: 96.6%; m.p. 185°–195°C; $R_f = 0.65$ (*n*-hexane : DCM 3 : 1); molecular 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



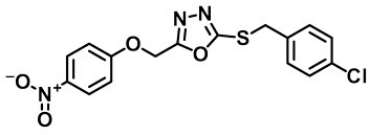
(d, $J_{2/3} = J_{2'/3'} = 9.12$ Hz, 2H, H-2, 2′), 7.38 (d, $J_{10/11} = J_{10'/11'} = 6.67$ Hz, 2H, H-10, 10′), 7.28 (ovp, 3H, H-11, 11′, 12), 7.06 (d, $J_{3/2} = J_{3'/2'} = 9.12$ Hz, 2H, H-3, 3′), 5.28 (s, 2H, H-5), 4.46 (s, 2H, H-8); ^{13}C NMR ($CDCl_3$, 135 MHz, δ ppm): 142.7 (C1), 128.4 (C2, 2′), 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-(2-Methylbenzyl)thio-5-(4-nitrophenoxy)methyl-1,3,4-oxadiazole (**5b**):* Vivid yellowish-green powder; yield: 92.5%; m.p. 150°–151°C; $R_f = 0.75$ (*n*-hexane : ethyl acetate 1 : 1); molecular 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



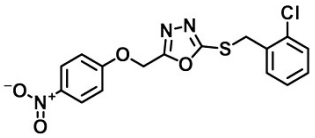
(C–O–C ether linkage), 1517 (N–O); ^{1}H NMR (400 MHz, $CDCl_3$): δH 8.21 (*d*, $J_{2/3} = J_{2'/3'} = 9.10$ Hz, 2H, H-2,2′), 7.35 (*d*, $J_{11/12} = 7.46$ Hz, 1H, H-11), 7.21–7.13 (m, 3H, H-12, 13, 14), 7.06 (*d*, $J_{3/2} = J_{3'/2'} = 9.12$ Hz, 2H, H-3, 3′), 5.30 (s, 2H, H-5), 4.50 (s, 2H, H-8), 2.41 (s, 3H, H-CH_3); ^{13}C NMR ($CDCl_3$, 135 MHz, δ ppm): 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-(4-Chlorobenzyl)thio-5-(4-nitrophenoxy)methyl-1,3,4-oxadiazole (**5c**):* Yellow crystals; yield: 90%; m.p. 168–170°C; $R_f = 0.69$ (*n*-hexane : ethylacetate 3 : 1), molecular 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, $CDCl_3$): δ H 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, δ ppm): 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-(2-Chlorobenzyl)thio-5-(4-nitrophenoxy)methyl-1,3,4-oxadiazole (**5d**):* Creamy yellow crystals; yield: 75%; m.p. 100°–102°C; $R_f = 0.56$ (*n*-hexane : ethyl acetate 3 : 1); molecular 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, δ ppm): 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).

Molecular docking

Retrieval of phospholipase A_2 structure using Protein Data Bank: Snake venom PLA_2 structure (3D) was retrieved from the Protein Data Bank (PDB ID 1A2A).

Designing of ligands: The structure of the synthesized ligands was drawn in ChemDraw Professional 16.0 and the database was developed. The energy was minimized and 3D protonated using Molecular Operating Environment 2015 (MOE 2015.10) to obtain good structural conformation.

Preparation of receptor: The enzyme was refined by removing all water molecules and ligands; however, hydrogen atoms were added to calculate partial atomic charges using MOE 2015.10. The enzyme was 3D protonated and MOE tool energy minimization algorithm was employed to minimize the energy using the following parameters; gradient: 0.1, force field: Amber10, chiral constraint: current geometry. Subsequently, the prepared enzyme was employed for docking.

Validation of the modelled structure: Stereochemical validation of the modelled structure of the enzyme is an

important component of the comparative molecular docking process. The stereochemical quality of the modelled enzyme was checked using the Ramachandran plot.

Identification of active site: A 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 on energy.

Docking: The interaction of the ligand molecule with the protein complex was evaluated using MOE docking software to determine the correct conformation (structure of the molecule is not rigid when the bonds are rotated) and configuration (structure remains 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 and annealing algorithm: simulated annealing.

Phospholipase A_2 inhibition assay

The PLA_2 assay was carried out according to the acidimetric technique reported by Tan *et al.*[21]. Egg-yolk suspension was prepared using equal quantities of sodium deoxycholate (8.1 mmol), calcium chloride (18 mmol) and egg yolk. The mixture was continuously stirred for 10 min to form a homogeneous suspension and sodium hydroxide (1 M) was added to maintain pH up to 8. Different concentrations of venom (1–10 μg/0.01 ml) were added to the egg yolk mixture (15 ml) to start hydrolysis, and saline served as control. A decline in pH was noted after 2 min; a drop in 1 pH unit corresponds to the release of 133 μmol of fatty acid from the egg yolk. Phospholipases A_2 activity was estimated by the amount of free fatty acid released per minute. Snake venom (10 μl, 10 mg/10 ml) and synthetic compounds in 50 mmol concentrations were pre-incubated to estimate anti-PLA_2 activity in terms of percentage[7]. PLA_2 inhibition activity was calculated using the following formula

$$\text{Activity (units/ml/min)} = \frac{\left(\begin{array}{c}\text{Molar concentration of product released} \times \\ \text{total volume of assay}\end{array}\right)}{\left(\begin{array}{c}\text{Volume of enzyme used} \times \text{time utilized} \times \\ \text{volume used in the measurement}\end{array}\right)}.$$

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

Statistical analysis

Microsoft Excel® (2010) was used to calculate the mean ± SD. To compare the results with the reference standard, Student's *t*-test was employed, while probability was set at $P > 0.001$.

Results and discussion

Chemistry

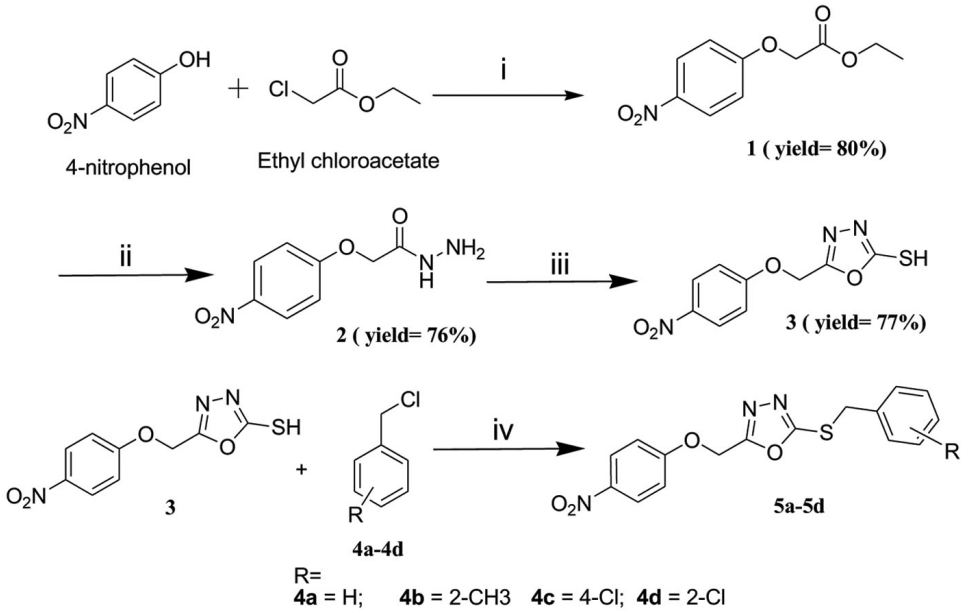
The present study was designed to synthesize novel 1,3,4-oxadiazole derivatives (**5a–5d**) to evaluate their PLA_2 inhibitory activity. 4-Nitrophenol was treated with ethyl chloroacetate to prepare the ester (**1**), which was then converted into the corresponding hydrazide (**2**) by treating with hydrazine. The compound (**2**) was cyclized by treating with carbon disulphide 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 give the final products 2,5-disubstituted-1,3,4-oxadiazole derivatives (**5a–5d**; Scheme 1).

In FTIR spectral data, the stretching frequency of C=N at 1655 cm^{-1} and SH at 2362 cm^{-1} confirmed the cyclization of hydrazide into the oxadiazole ring. ^1H NMR spectra were recorded at 400 MHz in deuterated chloroform-$CDCl_3$, giving a singlet of two protons at $\delta = 5.13$ ppm, doublet at $\delta = 8.24$ ppm with coupling constant of 9.2 Hz having integration of two protons and another signal at $\delta = 7.06$ ppm with coupling constant 8.8 Hz as a doublet with the integration of two protons. The above findings indicate the presence of 1,4-disubstituted benzene ring which is the 4-nitrophenoxy group of the precursor 5-(4-nitrophenoxy)-methyl-1,3,4-oxadiazole-2-thiol (**3**). The structure of the 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 the precursor with different benzyl halide derivatives (**4a–4d**). The synthesized derivatives were confirmed by TLC and characterized using spectroscopic techniques, e.g. FTIR, ^1H-NMR and ^{13}C-NMR.

Molecular docking analysis

Comparative binding energy analysis of synthesized derivatives: Computational docking is a good technique to evaluate the binding affinity of synthesized compounds in activity binding site of the target enzyme. The Ramachandran plot also confirmed that the amino acid residues were located in the favoured region (Figure 1).

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 revealed that glide docking energies fluctuated less among all ligands as the basic skeleton of all the synthesized derivatives was comparable. The docking results revealed that compound (**5d**) had excellent binding energy (–6.8 kcal/mol)



Scheme 1. Synthetic route, reagent and conditions for the 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 and (iv) KOH/C_2H_5OH, reflux 2–3 h.

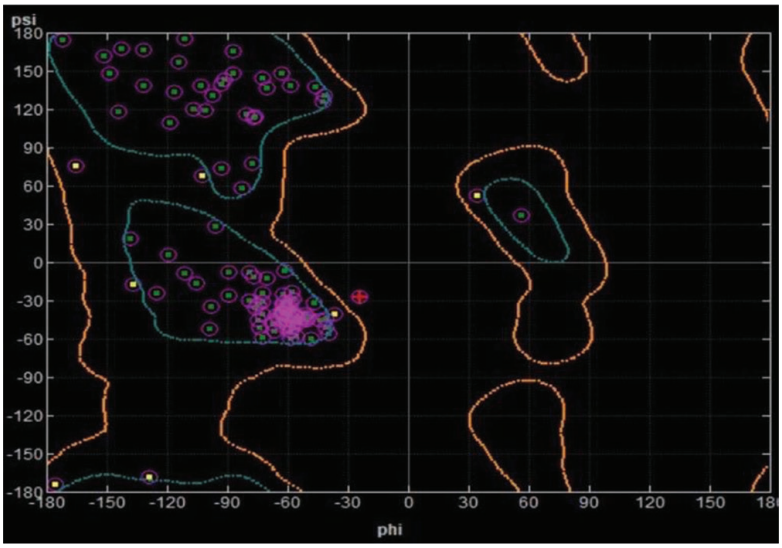


Figure 1. Ramachandran plot of protein predicted by MOE 2015.10.

in comparison with the other derivatives, suggesting its potential as the best inhibitor of PLA_2. Furthermore, compound (**5c**) also possessed a good binding energy value (–6.6 kcal/mol), while (**5a**) and (**5b**) exhibited binding energy values of –6.4 and –6.5 kcal/mol respectively (Figure 2).

Critical analysis of the binding pocket of PLA_2 against (5d): The (**5d**) docked pose of compound was selected on the basis of docking outcomes to examine the ligand–protein binding interactions. Molecular docking studies revealed that analogue (**5d**) showed effective binding within the active binding regions of PLA_2. The oxadiazole ring showed intrusion in the inner core of the binding pocket, while benzyl moiety showed interaction at the entrance of the binding region. Binding analysis showed two hydrogen bonds, one hydrophobic interaction and one pi–pi stacking in the (**5d**) docking complex. One hydrogen bond was formed with the oxygen of the nitro group having interaction with Asn 6 with a bond length of 2.15 Å, whereas nitrogen of the oxadiazole ring formed another hydrogen bond with His 48 with a bond length of 2.90 Å. One hydrophobic interaction was observed with Asp 49 with bond length 2.42 Å, while pi–pi stacking of oxadiazole ring was observed with Phe 5.

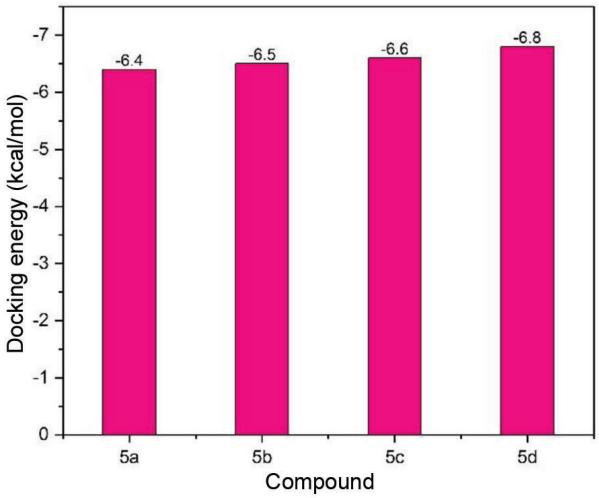


Figure 2. Graphical depiction of docking energy values of compounds (**5a–5d**).

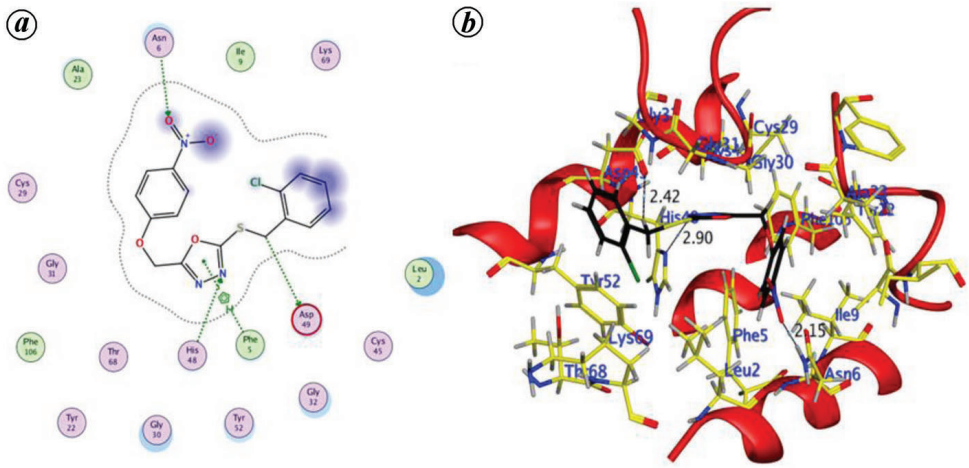


Figure 3. (***a***) Two dimensional and (***b***) three-dimensional structures of compound (**5d**) within the active binding site of PLA2 enzyme showing interactions with Asp 49, His 48, Phe 5 and Asn 6.

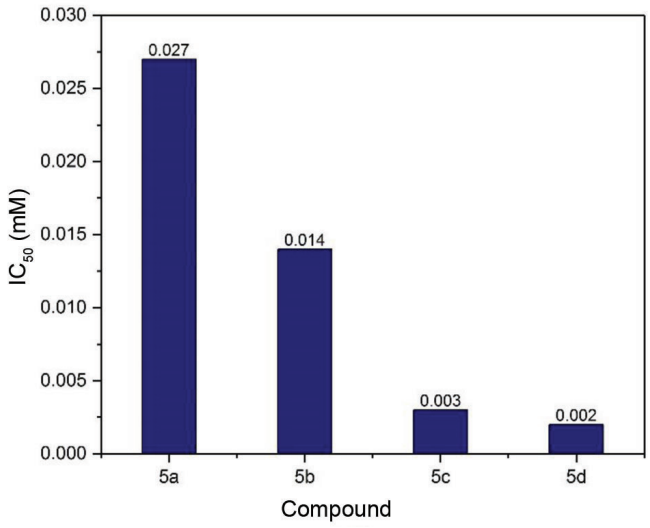


Figure 4. Graphical depiction of IC_{50} values of compounds (**5a–5d**).

The literature reveals that the active residues of the PLA_2 enzyme are involved in the binding which strengthens our docking results. Figure 3 presents 2D docking pose and binding interaction of the most potent compound (**5d**).

Enzymatic assay for anti-PLA$_2$ activity

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

Table 1. Effect of various concentrations of the *Naja oxiana* venom with respect to free fatty acids released/min

Concentration of venom (μg/0.01 ml)	Change in pH (mean ± SD)	Fatty acid released/ min (μM)	Enzyme activity (units/ml/min of crude venom)
1	7.82 ± 0.09	13.3	665
2	7.8 ± 0.1	26.6	1330
4	7.62 ± 0.01	53.2	2660
8	7.41 ± 0.2	106.4	5300
10	7.0 ± 0.05	133	6650
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

Tested compounds (50 mM)	Change in pH (mean ± SD)	Fatty acid released/min (μM)	Maximum protection (%)	*P* value
5a	7.40 ± 0.008	53.2	40	$P < 0.001$
5b	7.33 ± 0.017	43.89	33	$P < 0.001$
5c	7.76 ± 0.02	101.08	76	$0.01 > P > 0.001$
5d	7.87 ± 0.01	107.73	81	$0.01 > P > 0.001$
EDTA	7.90 ± 0.016	119.7	90	$P < 0.05$
Standard antisera (immunoglobulins)	7.95 ± 0.014	126.35	95	$P < 0.05$

These compounds possessed various organic, inorganic and heterocyclic moieties. According to preliminary structure–activity relationship studies, the benzene ring and orientation of the Cl moiety play an important role in PLA_2 inhibitory activity. Notably, compound (**5d**) is more potent than the other compounds owing to the presence of chlorine molecules at the ortho position. Compound (**5c**) possesses chlorine atom at para position, and its inhibitory activity had decreased ten-fold compared to (**5d**). So it can be concluded that halogen at the ortho position is imperative to inhibit PLA_2 activity. Moreover, substituting methyl group at the 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. Tables 1 and 2 show the effects of different *N. oxiana* venom concentrations in terms of free fatty acids released per minute and antidotal effects of the synthesized compounds against PLA_2 respectively.

Conclusion

In this study, 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 them. Computational molecular docking studies were performed against PLA_2 (PDB ID 1A2A), which revealed that compound (**5d**) exhibited outstanding binding affinity (binding energy of –6.8 kcal/mol). The docking results revealed that the synthesized compounds showed effective binding in the active binding site of the target protein. Snake venom was safely isolated from *N. oxiana* to examine the anti-PLA_2 activity. The results revealed that compounds (**5a–5d**) exhibited good PLA_2 inhibitory potential, especially (**5d**) displayed excellent activity with IC_{50} value of 0.002 mM ($0.01 > P > 0.001$). The compound (**5d**) was found to be the most potent phospholipase A_2 inhibitor among all the derivatives. Acidimetric assay for PLA_2 proved that (**5d**) was the most potent inhibitor compared to other derivatives. The results of the dry and wet laboratory approaches were found to be compatible. Thus, the synthesized derivatives, particularly (**5d**) may act as a leading molecule to design the most effective inhibitor of PLA_2 present in *N. oxiana* venom.

Conflict of interest: The authors declare that they have no conflict of interest.

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