

Molecular docking, Computational and antithrombotic studies of novel 1,3,4-oxadiazole Derivatives

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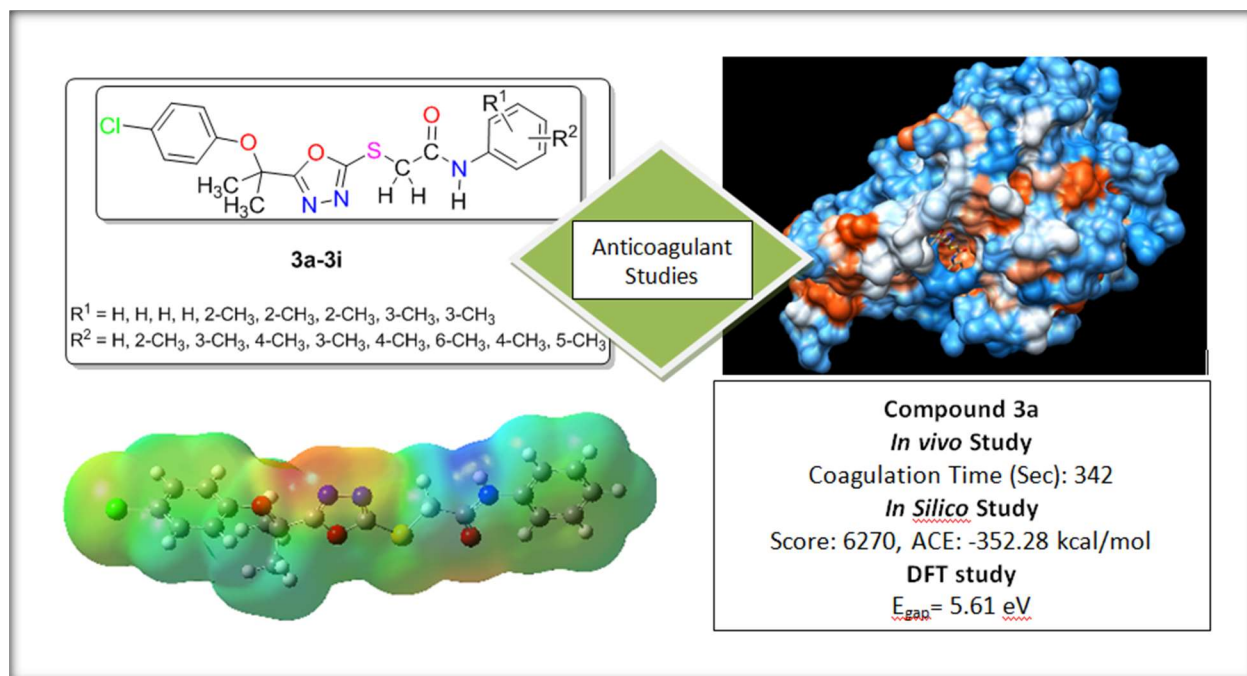
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ABSTRACT

A new series of 1,3,4-oxadiazoles derivatives was synthesized, characterized and evaluated for their *in vitro* and *in vivo* anti-thrombotic activity. Compounds (**3a-3i**) exhibited significant clot lysis with respect to negative control and reference drug streptokinase (30,000 IU) while enhanced clotting time (CT) values were observed (130-342 sec) for these tested compounds than the standard drug heparin (110 sec.). High affinity towards 1NFY with greater docking score was observed for the compounds (**3a, 3i, 3e, 3d and 3h**) than the control ligand RPR200095. In addition, very good inhibitory potential against factor Xa (F-Xa) was observed with higher docking scores (5612-6270) with ACE values (-189.68 to -352.28 kcal/mol) than the control ligand RPR200095 (Docking score 5192; ACE -197.81 kcal/mol). *In vitro*, *in vivo* and *in silico* results proposed that these newly synthesized compounds can be used as anti-coagulant agents.

Keywords: Factor Xa (F-Xa), Cardiovascular diseases (CD), Coronary heart disease (CHD), Tissue plasminogen activator (t-PA), Urokinase (UK), Streptokinase (SK), N,N-Dimethyl formamide (DMF)

Graphical Abstract



1. INTRODUCTION

Now-a-days cardiovascular diseases (CD) like coronary heart disease CHD, atherosclerosis, hypertension and acute myocardial infarction are main causes of death in humans. Among all kind of these CD, thrombosis is one of the frequently found symptom [1]. Almost 20 million people are being affected by thrombotic event worldwide every year. Agents that enhance the fibrinolytic activity and inhibit thrombus formation are important for the treatment and prevention of cerebrovascular and cardiovascular diseases [2]. After an injury or trauma, thrombin (EC.3.4.21.5) is activated and subsequently fibrinogen starts forming fibrin which clots the blood. Accumulation of fibrin in blood vessels increases thrombosis resulting in various CVDs and myocardial infarction [3].

Thrombolysis, also known as clot busting is the breakdown of blood clots by pharmacological action of drugs [4]. Thrombus disturbs blood flow by hindering the vein consequently denying the tissues of ordinary blood stream and oxygen supply. Thrombolytic agents such as Urokinase

(UK), Streptokinase (SK), and t-PA are frequently used to dissolve the clot in the management of thrombosis [6]. Tissue plasminogen activator (t-PA) enacts the plasmin that digests the fibrin strands supporting the blood clot and restores the normal blood flow to the affected tissues [5]. Their usage is associated with many side effects such as hyper risk of hemorrhage, lack of specificity and anaphylactic reaction [7]. These drugs have short half-lives (3-20 minutes) in the body with greater toxicity levels which may cause systemic bleeding [2]. Because of all these drawback and side effect, thrombolytic agents are required with many clinical advantages. In addition heparin and warfarin also used frequently as anti-coagulatory agents to prevent the formation of thrombus by inhibiting the factor Xa which is present at the junction of intrinsic and extrinsic pathway and thrombin activation via an anti-thrombin (AT) dependent mechanism.

1,3,4-Oxadiazole moiety, is a versatile pharmacore for designing potentially biological active agents because of its significant role in medicinal chemistry and wide range of applications as a pharmacological and pharmaceutical agent [9]. These have been found to exhibit various biological activities such as hypoglycemic [10], anti-HIV [11], anti-convulsant [12], anti-malarial [13], anti-tubercular [14], analgesic [15], lipid peroxidation inhibition [16] and genotoxic affects [17]. 2,5-Disubstituted 1,3,4-oxadiazol-2-thiol and its derivatives possess anti-microbial, anti-inflammatory, anti-viral [12] and anti-coagulation activities [18]. Oxadiazole involve in the inhibition of Factor-Xa (F-Xa) which is one of the pharmaceutical approaches that is directly involved in thrombin formation. . In coagulation process one molecule of F-Xa activates many molecules of prothrombin to thrombin by signal amplification [34, 35]. Therefore the inhibition of F-Xa is considered to be an effective treatment for many clot lysis events with low risk of bleeding as compared to direct thrombin inhibition. Amplified generation of thrombin

can be suppressed by F-Xa inhibitor without disturbing the levels necessary to primary homeostasis [8].

Keeping in view the biological activities of 1,3,4-oxadiazoles, we have report the *in vitro* and *in vivo* clot lysis efficacy of its derivatives along with their evaluation by molecular docking and computational studies. Novel compounds and their inhibitory effect on F-Xa were investigated by molecular docking for visualizing the efficiency of clot lysis agents.

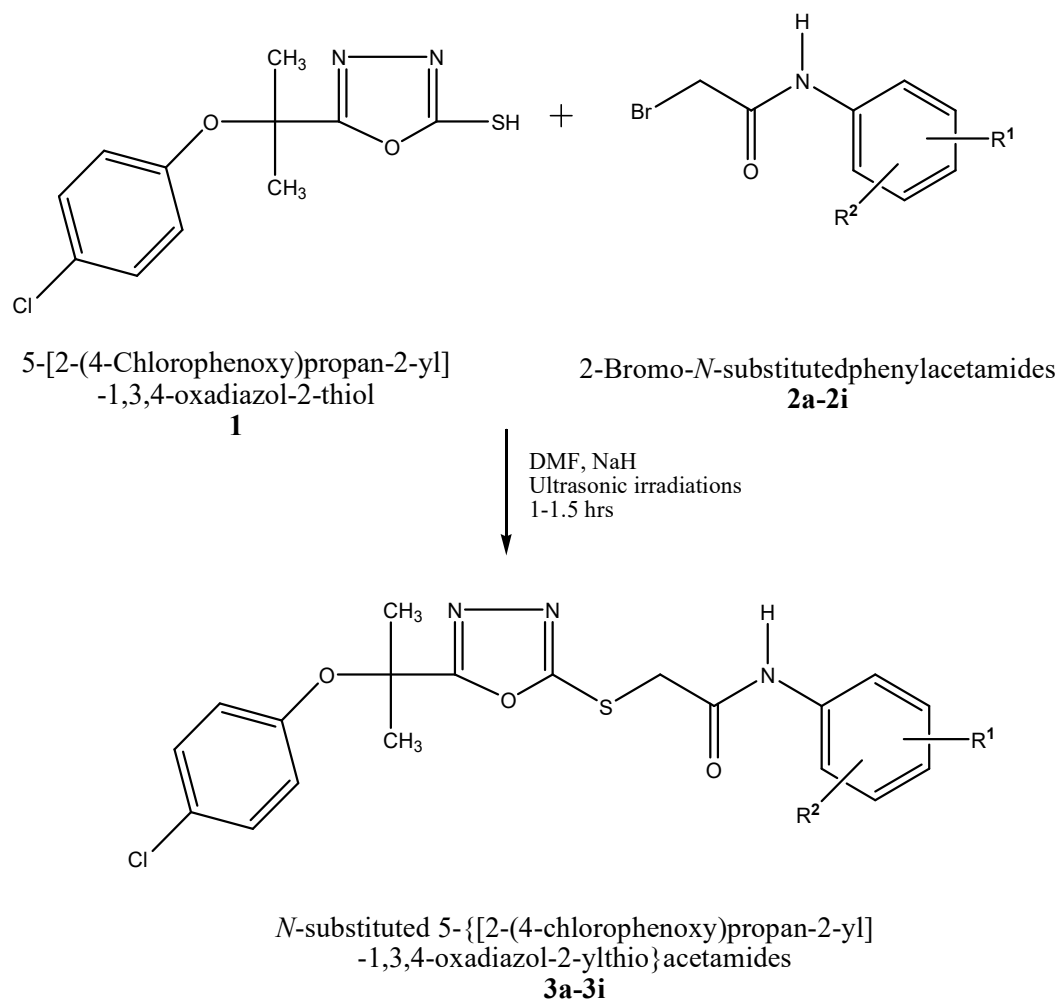
2. Results and Discussion

2.1 Chemistry

The targeted compounds **3a-3i** (table 1) was synthesized as depicted in (Scheme). The key intermediate compounds 5-[2-(4-chlorophenoxy)propan-2-yl]-1,3,4-oxadiazol-2-thiol (**1**) and *N*-substituted-2-bromoacetamides **2a-2i** were prepared according to reported procedure [12]. The nucleophilic substitution reaction of **1** with *N*-substituted-2-bromoacetamides **2a-2i** was carried out using lithium hydride as a base under ultrasonic irradiations at room temperature to obtain the *N*-substituted 5-{{2-(4-chlorophenoxy)propan-2-yl}-1,3,4-oxadiazol-2-ylthio}acetamides **3a-3i**. This modified method gave the better yield in less time than conventional reported method where only stirring is used to precede the reaction [33]. The supposed structures of targeted compounds were confirmed by spectral data.

The compound **3a** was obtained as white shining white fluffy amorphous flakes. The EI/MS spectra showed molecular ion peak at m/z 403 M^+ [$C_{19}H_{19}ClN_3O_3S$]⁺. The IR spectrum demonstrated the absorption band at 3138 (N-H stretching), 3000 (C-H aromatic stretching), 1671 (C=O amide stretching), 1552 (C=C aromatic ring stretching.). In ¹H-NMR spectrum the appearance of singlet at δ 10.42 represents 1 proton of NH. In aromatic region the appearance of two doublets one at δ 7.57, J = 8 Hz having integration for two protons (H-3' & H-5') and other at

δ 6.75, $J=9$ Hz for two protons (H-2' & H-6') revealed that aromatic ring is disubstituted at para (1,4) positions. Similarly the appearance of triplet at δ 7.33, $J=7.8$ Hz for two protons (H-2'' & H-6''), a doublet at δ 7.28, $J=8.5$ Hz for two protons (H-3'' & H-5'') and a triplet at δ 7.08, $J=7.3$ Hz integrate one proton (H-4'') can be assigned to the unsubstituted benzene ring of amide. A singlet at δ 4.33 was due to methylene group (CH₂) linking the amide and sulfanyl group. In aliphatic region of the spectrum a singlet at δ 1.71 was assigned to 6H of two methyl groups. Therefore on the basis of above cumulative evidences the structure of **3a** was confirmed as 2-{5-[2-(4-chlorophenoxy)propan-2-yl]-1,3,4-oxadiazol-2-ylthio}-*N*-phenylacetamide. The structures of other *N*-substituted acetamides were also characterized in a similar manner. All the signals in ¹H-NMR spectrum of **3a-3i** thoroughly affirmed the successful substitutions on the parent 1,3,4-oxadiazole core.



Scheme: Outline for the synthesis of acetamides (3a-3i). Reagents & conditions: *N*-substituted-2-bromoacetamide (2a-2i, one in each case), DMF, LiH, sonication for 1-1.5 hours at RT.

Table 1: Different substitutions in 3a-3i

Codes	3a	3b	3c	3d	3e	3f	3g	3h	3i
R¹	H	H	H	H	2-CH ₃	2-CH ₃	2-CH ₃	3-CH ₃	3-CH ₃
R²	H	2-CH ₃	3-CH ₃	4-CH ₃	3-CH ₃	4-CH ₃	6-CH ₃	4-CH ₃	5-CH ₃

2.2. Anti- coagulation activity

2.2.1. In vitro clot lysis effect

Results showed that compound **3i** showed good results (41%) as compared to SK (38 %). The order of *in vitro* clot lysis effect of all tested compound is **3i>SK>3e>3a>3d=3f>3h>3g>3b>1>3c**. The results of all synthesized compounds are presented in Table 2.

Table 2: Clot lysis effect of synthetic compounds on human blood

Sr. No.	Compounds	% Clotlysis
1	1	20.2
2	3a	27
3	3b	20.5
4	3c	11
5	3d	25
6	3e	32
7	3f	25
8	3g	20.6
9	3h	21
10	3i	41
11	Distilled water	4
12	Streptokinase (SK)	38

2.2.2. *In vivo* Anti-coagulation activity

All the synthesized compounds were evaluated for anti-coagulation activity in rats. Whole blood after removal from vascular system and on exposure to external environment was converted into solid clot. Within limits, time required for conversion of blood into solid clot is a measure of coagulation system. All oxadiazole derivatives showed prominent prolongation in clotting time except **1**. Compounds **3a** (342 sec), **3i** (214 sec.), **3e** (167 sec.), **3d** (132 sec.) and **3h** (130 sec.) showed clotting time (CT) values greater than standard drug heparin (110 sec.). The order of *in vivo* CT of all the tested oxadiazole derivatives is $3a > 3i > 3e > 3g > 3d > 3h > 3b > 3f > 3c > 1$. The CT result of all tested compounds is presented in Figure 1.

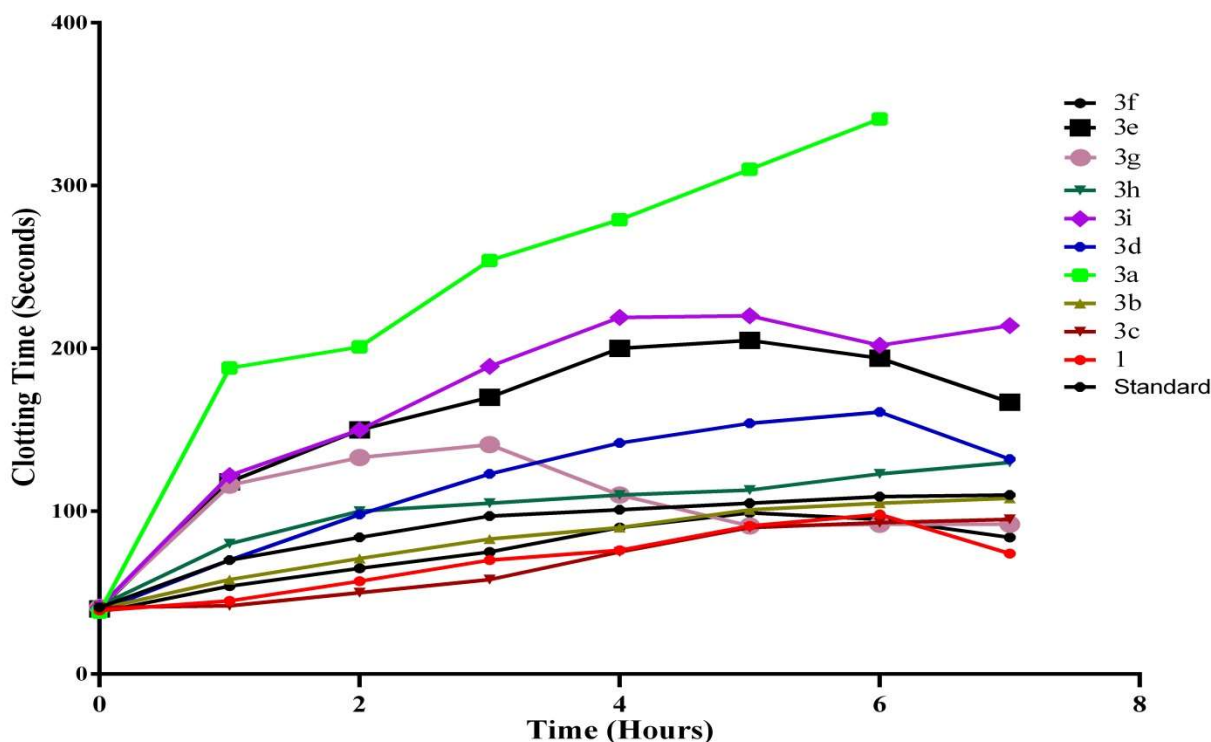


Figure1. Graph showing antithrombotic activity (CT) of the tested compounds and their comparison to standard.

2.3. Molecular Docking

The serine protease F-Xa plays a crucial role in blood coagulation process by converting prothrombin to thrombin. This F-Xa is located at the conjunction point of extrinsic and intrinsic pathway. In coagulation process one molecule of F-Xa activates many molecules of prothrombin to thrombin by signal amplification [34, 35]. Therefore the inhibition of F-Xa is considered to be an effective treatment for many thrombotic events with low risk of bleeding as compared to direct thrombin inhibition [36].

There are four binding pockets labeled as S1 to S4 within the active site. The most important are S1 and S4 binding pockets that are exploited by Factor Xa inhibitor. The S1 pocket is in the form of a narrow cleft and it usually favors positively charged moieties such as benzamidine, amine and guanidine [37]. The second main binding pocket is S4 and is shaped by different amino acid residues [38, 39]. The energy score (S) was the main criterion to evaluate the binding affinity of ligand (table 3). The compound which showed highest binding affinity forms the most stable ligand-enzyme complex. The result of docking studies; energy score, involved factor Xa active site amino acid residues, interacting ligands moieties for each compound and reference inhibitor are given in Table 3 and Figure 2-5. Analysis of docking results showed that:

Standard **RPR200095** was also docked with F-Xa for comparison and has depicted score 5192 with an ACE value -197.81 kcal/mol (Figure 2). **RPR200095** has exhibited hydrophobic contact potential with pocket amino acids Lys²⁴³, Arg²⁵, Met²⁴².

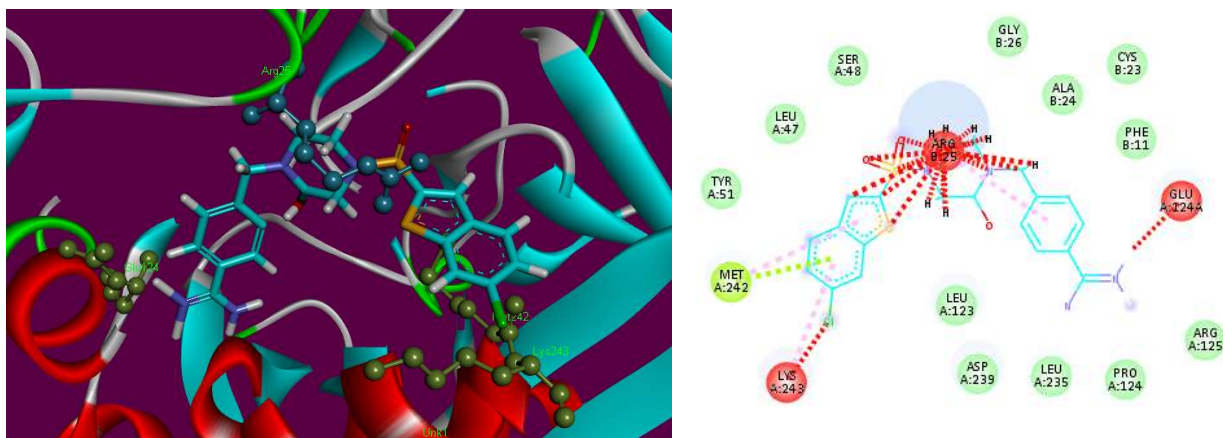


Figure 2. Binding site interaction of standard **RPR200095**

In the initial assessment of the docked complexes of F-Xa (**1**, **RPR200095**, **3a-3i**) revealed that five ligands **3a**, **3d**, **3e**, **3h** and **3i** showed significant interaction patterns even better than standard **RPR200095**. These were found to bind near the entrance of active site gorge (Figure 2). However, as compared to binding of standard **RPR200095**, ligands **3a**, **3d**, **3e**, **3h** and **3i** do not penetrate deep into the binding pocket like **RPR200095** instead due to the bulkiness of these ligands they fit on the top of the binding pocket, possibly blocking the substrate entry or the product release from the active site, in that way showing anticoagulant activity.

Ligand **3a** showed most potent interaction with F-Xa active site with a score of 6270 and an ACE of -352.28 kcal/mol (table 3). Visual inspections of these complexes predicts a binding conformation of ligand **3a** showed significant interaction with F-Xa binding site compared to the other 10 ligands. The interacting residue of this complex is Arg²⁵ (Figure 5). Ligand **3a** has shown a potential hydrogen bond between *NH* adjacent to carbonyl and phenyl-groups and Arg²⁵. The length of the hydrogen bond was 2.94 Å indicating significant interaction. Similarly, ligand **3a** exhibited hydrophobic contact potential with pocket amino acids Ala²⁴, Pro⁴³ and also depicted pi-sulphur contact potential with Cys⁴⁴.

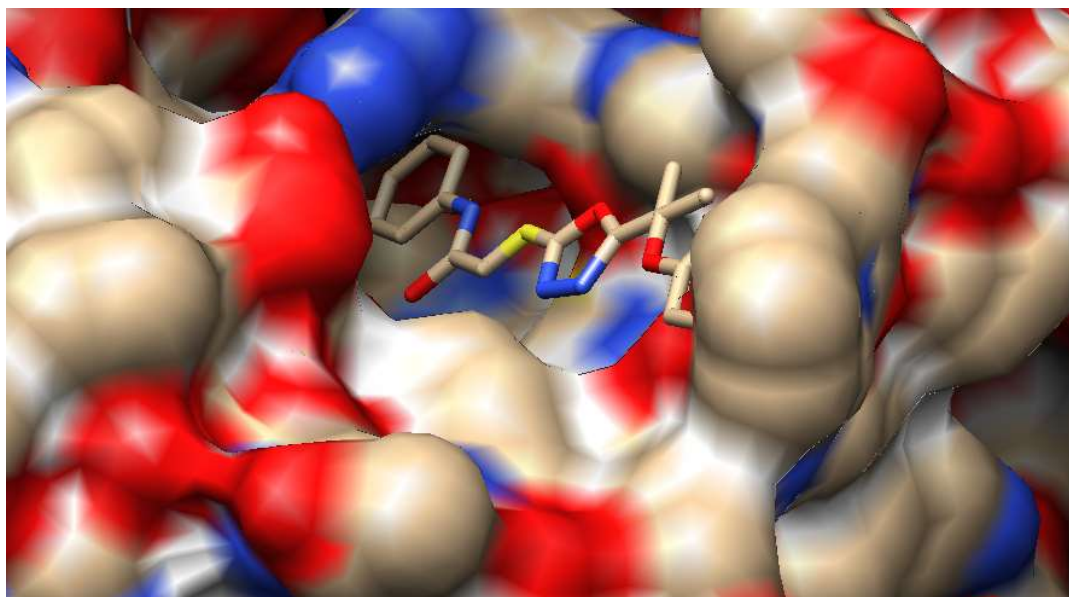


Figure 3. Hydrophobic affinity analysis of most active inhibitor **3a**.

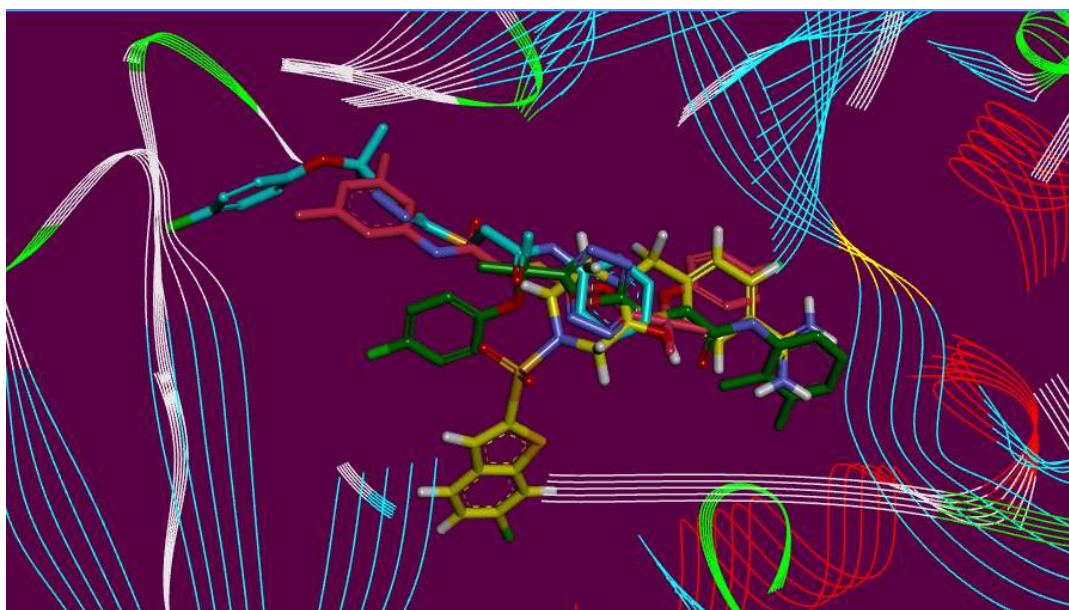


Figure 4. Overlap of bound conformations of RPR200095 (yellow) with compounds **3a** (blue), **3i** (pink) and **3e** (green).

Interestingly, ligand **3i** showed no hydrogen bond interaction with F-Xa receptor but instead found significant geometric fit of this ligand in the receptor and hence scoring in *Patch Dock* being based on shape complementarity principles it gave a score of 5612 and an ACE of -312.12 kcal/mol with Factor Xa (Figure 5). Ligand **3i** exhibited pi-cation contact potential with Arg²⁵ and 1,3,4-oxadiazole, and hydrophobic interaction found between Arg²⁵ and 4-chlorophenyl- group. Ligand **3e** showed interaction with F-Xa active site with a score of 5518 and an ACE of -189.68 kcal/mol. Ligand **3e** showed three hydrogen bonds between Arg²⁵ and oxygen of 1,3,4-oxadiazole (2.98 Å), Leu¹²³ and nitrogen of 1,3,4-oxadiazole (3.03 Å), Pro¹²⁴ and NH adjacent to carbonyl and 2,3-dimethylphenyl groups (3.17 Å). Similarly, ligand **3e** has exhibited hydrophobic contact potential with pocket amino acids Lys²³⁶, Ala²⁴, Arg²⁵, Leu¹²³ and Cys⁴⁴ respectively. Ligand **3e** has shown potential for pi-stack interaction with Leu²³⁵ and 2,3-dimethylphenyl moiety (Figure 5).

In case of ligands **3d** and **3h** depicted scores 5646 and 5612 with an ACE values -287.52 and -311.48 kcal/mol, respectively. This ligand **3d** showed a potential to accept a hydrogen bond from the backbone amino group Arg²⁵ and sulphur attached with 1,3,4-oxadiazole (3.19 Å), Leu¹²³ and nitrogen of 1,3,4-oxadiazole (3.25 Å) and also exhibited hydrophobic contact potential with pocket amino acids Cys⁴⁴, Pro¹²⁰, Arg²⁵, Leu²³⁵, Lys²³⁶ and can play an important part to give this ligand good binding affinity than other ligands of this series. Ligand **3h** has shown potential for van der Waals contact and such interactions involve the hydrophobic contact with Pro⁴³, Leu¹²³ and Phe¹¹⁴. This ligand depicted arene-cation contact with Arg²⁵, also exhibited pi-sulphur contact with Met²⁴² and 1,3,4-oxadiazole and was unable to show polar interactions with the pocket amino acids.

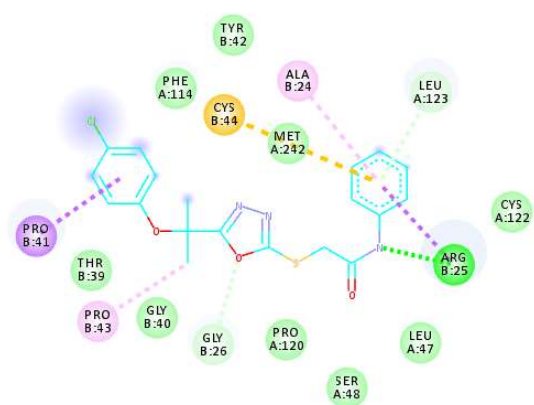
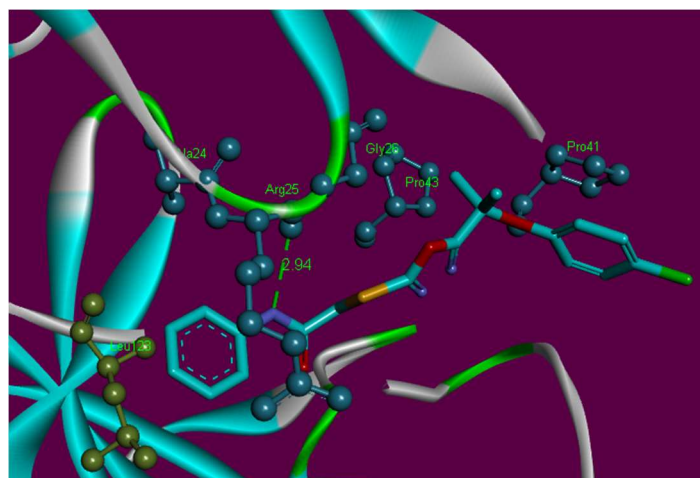
Table 3: Docking results for the highest ranked biologically active ligand F-Xa.

Compd. No.	Score	<i>In-vivo</i> anticoagulant activity at 7 th h (Sec)	ACE Kcal/mole	Amino acids showing hydrogen bond contacts	Distance (Å)	Amino acids showing van der Waals contacts within 4 Å	Amino acids showing hydrophobic contacts lie within 4 Å	Amino acids showing arene-cation contacts
1	3562	74	-197.87	Glu ⁴⁹	3.22	Ser ⁴⁸ , Leu ⁴⁷ , Met ²⁴² , Ala ²⁴ , Cys ¹²² , Pro ⁴³ , Tyr ⁴²	Pro ¹²⁰ , Phe ¹¹⁴ , Cys ⁴⁴ , Arg ²⁵ , Leu ¹²³	Arg ²⁵
3a	6270	342	-352.28	Arg ²⁵	2.94	Tyr ⁴² , Met ²⁴² , Phe ¹¹⁴ , Cys ¹²² , Leu ⁴⁷ , Ser ⁴⁸ , Pro ¹²⁰ , Gly ²⁶ , Gly ⁴⁰ , Thr ³⁹	Ala ²⁴ , Pro ⁴³	-
3b	5312	108	-370.05	-	-	Leu ⁴⁷ , SER ⁴⁸ , Pro ¹²⁰ , Tyr ⁴² , Phe ¹¹⁴ , Pro ⁴¹ , Gly ⁴⁰ , Pro ¹²⁴ , Glu ¹²⁴	Cys ⁴⁴ , Pro ⁴³ , Gly ²⁶ , Pro ¹²⁴ , Met ²⁴² , Phe ¹¹ , Ala ²⁴ , Leu ¹²³	Arg ²⁵
3c	5324	95	-253.57	Arg ²⁵	2.83	Arg ¹²⁵ , Glu ¹²⁴ , Pro ¹²⁴ , Ala ²⁴ , Cys ¹²² , Phe ¹¹⁴ , Glu ⁴⁹ , Ser ⁴⁸ , Leu ⁴⁷ , Met ²⁴²	Leu ¹²³ , Arg ²⁵ , Pro ¹²⁰ , Leu ²³⁵ , Lys ²³⁶	-

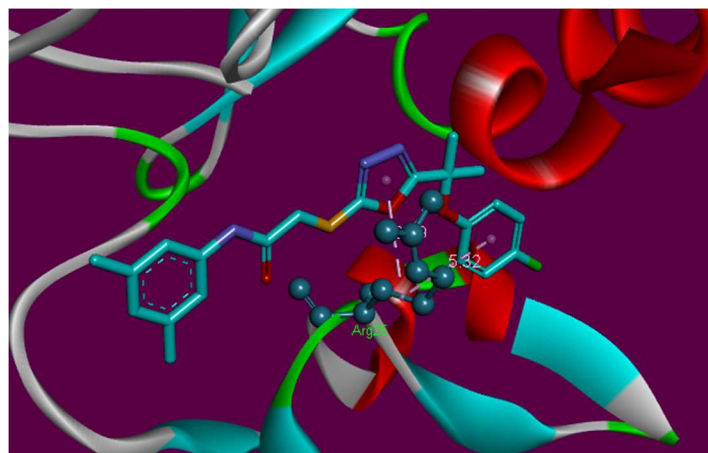
						Asp ²³⁹			
3d	5646	132	-287.52	Arg ²⁵ , Leu ¹²³	3.19, 3.25	-	Cys ⁴⁴ , Pro ¹²⁰ , Arg ²⁵ , Leu ²³⁵ , Lys ²³⁶	-	
3e	5658	167	-189.68	Arg ²⁵ , Leu ¹²³ , Pro ¹²⁴	2.98, 3.03, 3.17	Arg ¹²⁵ , Glu ¹²⁴ , Phe ¹¹⁴ , Glu ⁴⁴ , Ser ⁴⁸	Lys ²³⁶ , Ala ²⁴ , Arg ²⁵ , Leu ¹²³ , Cys ⁴⁴	-	
3f	5626	84	-340.54	Arg ²⁵ , Arg ²⁵	2.99, 274	Ala ²⁴ , Gly ²⁶ , Phe ¹¹⁴ , Gly ⁴⁰ , Pro ⁴³ , Tyr ⁴² , Leu ⁴⁷ , Met ²⁴² , Glu ¹²⁴	Phe ¹¹ , Leu ¹²³ , Leu ²³⁵ , Cys ⁴⁴ , Pro ⁴¹ , Pro ¹²⁰	-	
3g	5442	92	-262.48	-	-	Asp ²³⁹ , Pro ¹²⁴ , Glu ¹²⁴ , Trp ¹²⁷ , Cys ¹²²	Ala ²⁴ , Phe ¹¹ , Cys ⁴⁴ , Arg ²⁵ , Lys ²³⁶ , Arg ¹²⁵ , Leu ²³⁵	-	
3h	5612	130	-311.48	-	-	Thr ³⁹ , Gly ⁴⁰ , Pro ⁴¹ , Gly ²⁶ , Pro ¹²⁰ , Cys ⁴⁴ , Leu ⁴⁷ , Asp ²³⁹ , Ala ²⁴ , Phe ¹¹ , Glu ¹²⁴	Pro ⁴³ , Leu ¹²³ , Phe ¹¹⁴	Arg ²⁵	
3i	5662	214	-312.12	-	-	Pro ⁴¹ , Tyr ⁴² , Pro ¹²⁰ , Leu ⁴⁷ , Cys ⁴⁴ , Met ²⁴² , Ala ²⁴ , Asp ²³⁹ , Gly ²⁶ , Pro ⁴³ , Thr ³⁹ , Gly ⁴⁰	Arg ²⁵	Arg ²⁵	
RPR200095	5192	110	-197.81	-	-	Leu ⁴⁷ , Ser ⁴⁸ , Cys ²³ , Tyr ⁵¹	Lys ²⁴³ , Arg ²⁵ , Met ²⁴²	-	

						Cys ¹²² , Gly ²⁶ ,		
						Ala ²⁴ , Phe ¹¹ ,		
						Pro ¹²⁴ , Leu ²³⁵ ,		
						Asp ²³⁹ , Leu ²³⁵		

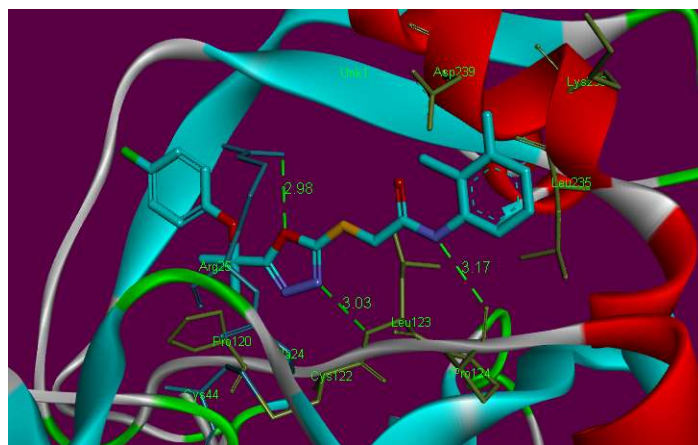
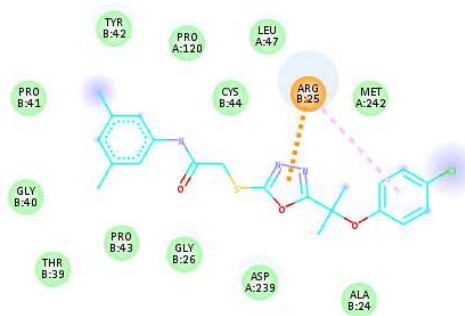
No. = Specific code assigned to ligand; ACE= Atomic contact energy calculated by *Patchdock* (kcal/mol); Distance = hydrogen bond length calculated from docked pose by using *Ligand interaction* tool of *Patch Dock*.



(3a)



(3i)



(3e)

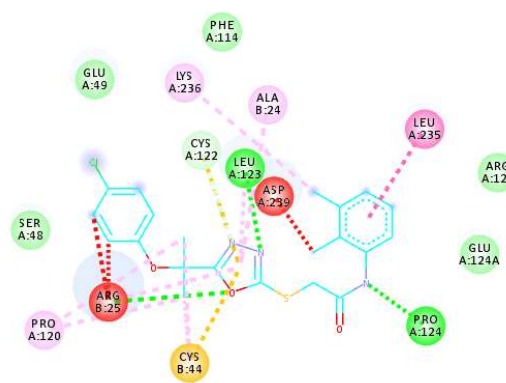


Figure 5. 2D and 3D binding site interactions of the most probable docked ligands **3a**, **3i** and **3e** within F-Xa active site showing their binding interactions with *Patch Dock*, depicting unfavorable bump (red), carbon hydrogen bond (light green), pi-alkyl (light pink), pi-cation (brown) and amide pi-stack (pink) interactions.

In order to elaborate the structural elements liable for the observed inhibitory effect against the binding site F-Xa, the most active ligand **3a** bound to protein was focused to binding

affinity assessment using Hyde utility of Lead IT software. Hyde allows visual approximation of favorable and unfavorable contributions due to the structure/bound conformation of inhibitor with the neighboring amino acids. The favorably contributing structural elements (atoms and torsions) to the overall binding energy are visually color in green, similarly the structural elements that are not contributing favorably are colored in red, and neutral elements are in white (Figure 3). The aromatic phenyl moieties substituted *1,3,4-oxadiazole* are contributing favorably to the binding energy. The only unfavorable structural element was the unsubstituted nitrogen atoms of *1,3,4-oxadiazole* ring. This lead to the assumption that if these nitrogen atoms are substituted by some other atoms *i.e.*, carbon or other heteroatoms it may reason of even better binding affinity and thereby showing anticoagulant activity.

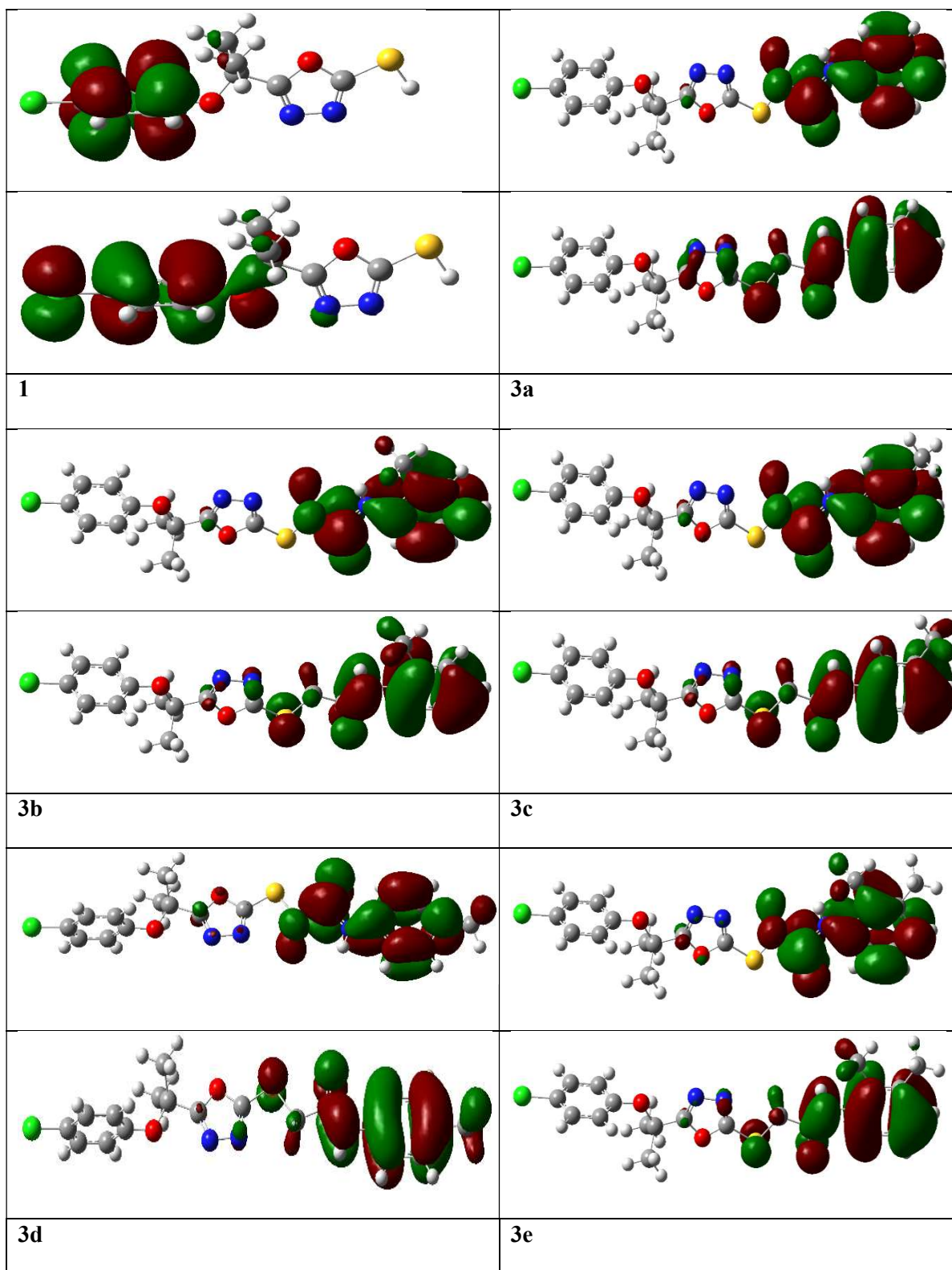
2.4. Computational investigations

3.4.1. Frontier molecular orbital (FMO) analysis

The energy of frontier orbital's, namely, the Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO) are very popular parameters from quantum chemistry calculations provide valuable information about molecular systems. The energy gap between HOMO and LUMO (E_{gap}) measures the kinetic stability of the molecule [40]. A large value of the energy gap implies high kinetic stability and low chemical reactivity. Furthermore, the energy gap between HOMO and LUMO explains the intermolecular charge transfer (ICT) within the molecule, which is responsible for the bioactivity of the molecule. The distribution pattern of the FMOs has been illustrated in Figure 6. In all the studied derivatives **3a-3i**, except **1** in which HOMOs delocalized on 4-chlorophenoxy group, the HOMOs had leading contribution from *N*-arylamide and the adjacent Sulfur atom. Similarly, LUMOs had also dominating contribution from *N*-arylamide moiety except **3g**, **3h** and **1** in which major

contribution from 4-chlorophenoxy group. However in case of **3i** HOMOs delocalized on both benzene rings along with acetamide groups. The ICT has been observed from *N*-arylacetamide to the 4-chlorophenoxy group units in **3g** and **3h**.

The E_{HOMO} , E_{LUMO} and HOMO–LUMO energy gaps (E_{gap}) at the B3LYP/6-31G** level of theory has been tabulated in Table 4. The highest E_{gap} in **1** and **3g** decrease their biological activity. While the low energy gap in **3a**, **3e**, **3d**, **3i** and **3h** makes them potent inhibitor of F-Xa due to which they show high anti-coagulant activity. Other electronic parameters such as Ionization potential (I), Electron affinity (A), hardness (η), softness (s), Chemical potential (μ), absolute electronegativity (χ), electrophilicity index (ω) and dipole moment (D) of the synthesized compounds were also calculated which were represented in Table 4. The electrophilicity index will be useful to explain the binding capacity with biomolecules [41].



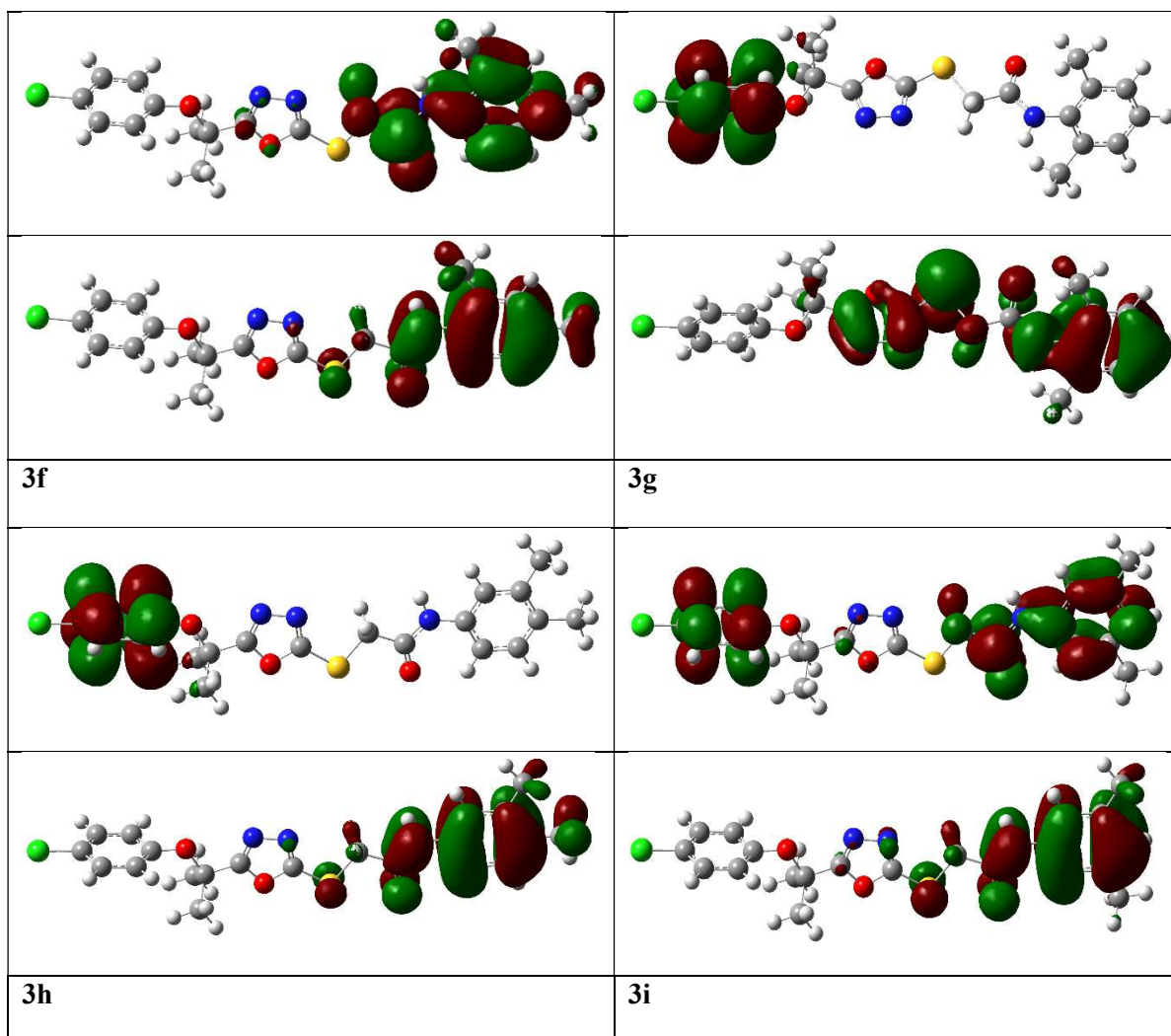


Figure 6. The charge density distribution of the HOMOs (bottom) and LUMOs (top) of the oxadiazoles derivatives (**3a-3i** and **1**).

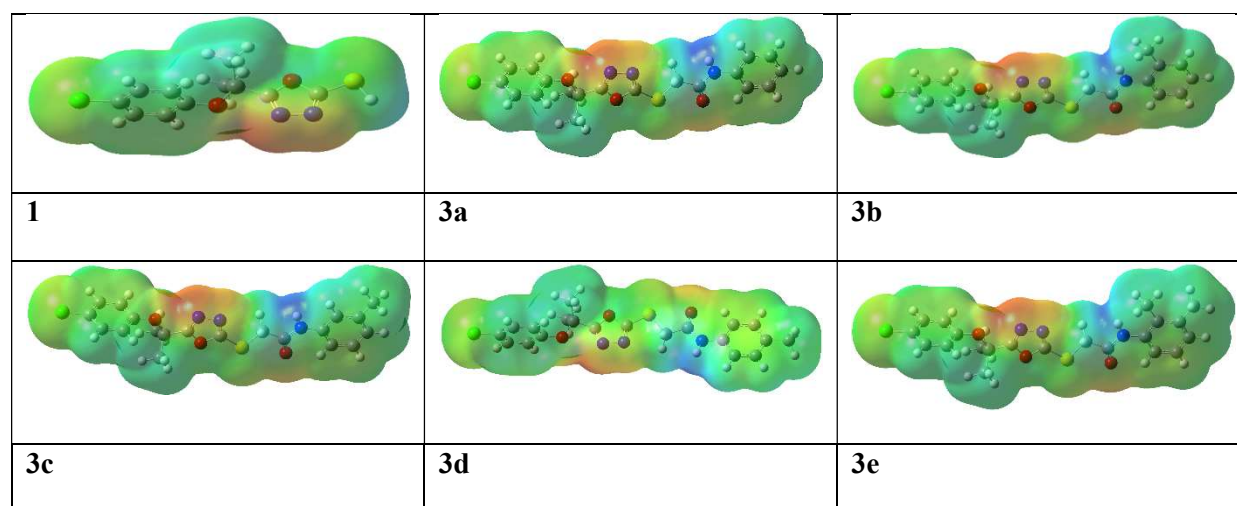
Table 4. DFT computed molecular properties (all in eV, except dipole moment which is in the units of Debye) for oxadiazoles derivatives obtained at B3LYP/6-31G** level of theory

Sr. No.	Compound name	E_{HOM}	E_{LUM}	E_{gap}	Ionization potential (I)	Electron affinity (A)	Chemical hardness (η)	Chemical softness (S)	Chemical potential (μ)	Electronegativity (χ)	Electrophilicity (ω)	Dipole moment (D)
1	1	-6.60	-0.58	6.02	6.60	0.58	3.01	0.17	-3.59	3.59	2.14	3.66
2	3a	-6.25	-0.64	5.61	6.25	0.64	2.80	0.18	-3.44	3.44	2.12	1.84
3	3b	-6.14	-0.64	5.51	6.14	0.64	2.75	0.18	-3.39	3.39	2.09	1.83
4	3c	-6.15	-0.60	5.54	6.15	0.60	2.77	0.18	-3.37	3.37	2.06	2.20
5	3d	-6.05	-0.57	5.48	6.05	0.57	2.74	0.18	-3.31	3.31	1.99	2.39
6	3e	-6.07	-0.58	5.49	6.07	0.58	2.75	0.18	-3.32	3.32	2.01	2.19
7	3f	-5.95	-0.57	5.39	5.95	0.57	2.69	0.19	-3.26	3.26	1.97	2.36
8	3g	-6.41	-0.54	5.87	6.41	0.54	2.94	0.17	-3.47	3.47	2.05	2.14
9	3h	-5.97	-0.53	5.43	5.97	0.53	2.72	0.18	-3.25	3.25	1.94	2.67
10	3i	-6.08	-0.54	5.54	6.08	0.54	2.77	0.18	-3.31	3.31	1.98	2.37

2.4.2. Molecular Electrostatic Potential (MEP)

Undoubtedly MEP is a very useful tool to understand the molecular interactions. Especially its 3-D mapping is widely used to explain the relative reactive sites for the electrophilic (negative region) and nucleophilic (positive region) attack in a molecule. It also provides visual understanding of the relative polarity of the molecule. To predict reactive sites for electrophilic and nucleophilic attack of all synthesized compounds, the MEP surface maps have been calculated and illustrated in Figure 7. The different colors represent the different values of the electrostatic potential at the surface. Negative and positive Electrostatic Potential (EP) regions indicated by red and blue color respectively, while the neutral potential regions represented by green color.

Careful analyses of the MEP revealed that oxadiazole moiety would be favorable site for electrophile attack in all the studied compounds. Further in all synthesized derivatives, the carboxamide group showed both positive and negative potential that makes them good F-Xa inhibitors and anti-coagulants.



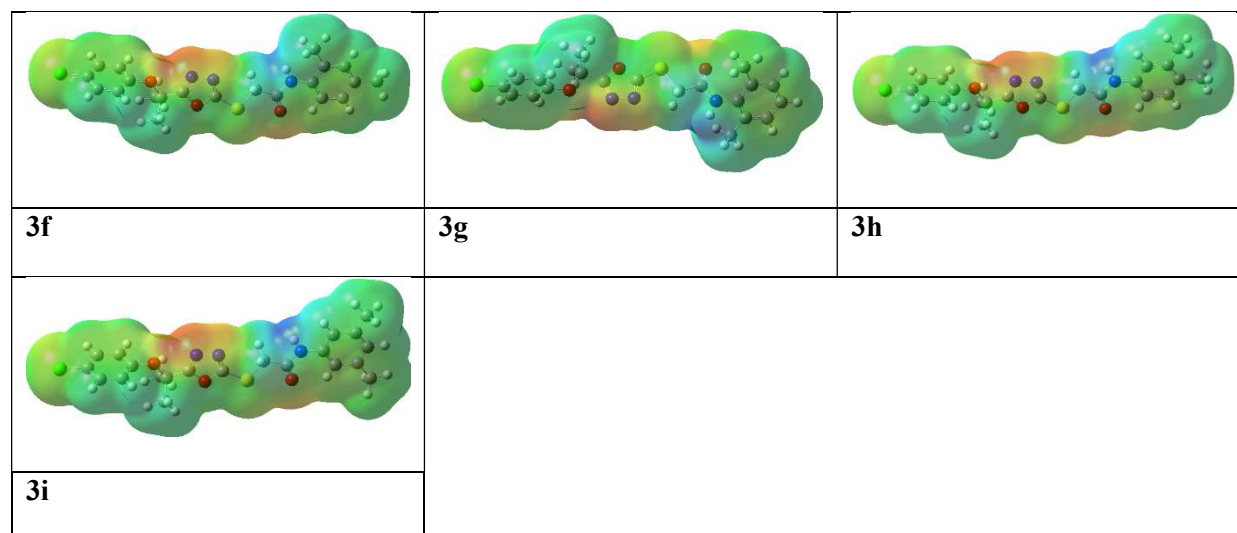


Figure 7. The molecular electrostatic potentialsurfaces of the oxadiazoles derivatives

4. Experimental Method

4.1. Chemistry

Chemicals were purchased from Sigma Aldrich and Alfa Aesar. Melting points were taken on Griffin and George melting point apparatus using open capillary tube method and are reported as uncorrected. Infrared spectra were recorded in KBr on a Jasco-320-A spectrophotometer. ¹H-NMR signals were recorded on AVANCE AV-300 MHz, AVANCE AV-400 MHz or AVANCE AV-500 MHz, while ¹³C-NMR spectra were taken on a Bruker AVANCE 125 MHz spectrometer. EIMS signals were recorded on JEOL MS 600H-1 spectrometer.

4.1.1. 5-[2-(4-Chlorophenoxy)propan-2-yl]-1,3,4-oxadiazole-2-thiol (**1**) [24] and aromatic *N*-substituted-2-bromoacetamides (**2a-2i**) were synthesized by already reported methods with slight modifications [12]

4.1.2. General procedure for the synthesis of *N*-substituted 5-{[2-(4-chlorophenoxy)propan-2-yl]-1,3,4-oxadiazol-2-ylthio}acetamides (3a-3i)

A mixture of 5-[2-(4-Chlorophenoxy)propan-2-yl]-1,3,4-oxadiazole-2-thiol (**1**) (0.271g; 1mmol) and lithium hydride (0.004g; 2mmol) was dissolved in DMF (10ml) and the contents were subjected to ultrasound irradiations for 15 minutes followed by addition of *N*-substituted-2-bromoacetamide (1mmol) with further irradiation till completion of the reaction as indicated by TLC (Ethylacetate : n-Hexane, 1:4). Reaction times for different *N*-substituted 5-{[2-(4-chlorophenoxy)propan-2-yl]-1,3,4-oxadiazol-2-ylthio}acetamides varies from 1-1.5 hours. After completion, the reaction mixture was poured on crushed ice. The precipitates were filtered, washed with distilled water and dried to afford *N*-substituted 5-{[2-(4-chlorophenoxy)propan-2-yl]-1,3,4-oxadiazol-2-ylthio}acetamide. The products were recrystallized from 30% ethanol.

4.1.3. 2-{5-[2-(4-Chlorophenoxy)propan-2-yl]-1,3,4-oxadiazol-2-ylthio}-*N*-phenylacetamide (**3a**)

The compound **3a** was obtained from the reaction of 5-[2-(4-chlorophenoxy)propan-2-yl]-1,3,4-oxadiazol-2-thiol **1** (0.271 g, 1mmol) with of 2-bromo *N*-phenylacetamide (0.213 g, 1 mmol) after 60 minutes. Shining white fluffy amorphous flakes; yield: 75 % (0.302 g); m.p. 84-86 °C; IR (KBr, ν_{\max} , cm^{-1}): 3138 (N-H str.), 3000 (C-H aromatic str.), 1671 (C=O amide str.), 1552 (C=C aromatic ring str.); $^1\text{H-NMR}$ (500 MHz, DMSO-d_6): δ (ppm) 10.42 (s, 1H, NH), 7.57 (d, $J = 8$ Hz, 2H, H-3' & H-5'), 7.33 (t, $J = 7.8$ Hz, 2H, H-2'' & H-6''), 7.28 (d, $J = 8.5$ Hz, 2H, H-3'' & H-5''), 7.08 (t, $J = 7.3$ Hz, 1H, H-4''), 6.75 (d, $J = 9$ Hz, 2H, H-2' & 6'), 4.33 (s, 2H, S- CH_2 -CO), 1.71 (s, 6H, C(CH_3) $_2$); $^{13}\text{C-NMR}$ (500 MHz, DMSO-d_6 , δ /ppm) : 168.85 (C=O), 165.17 (C-2), 164.90 (C-5), 153.29 (C-1'), 139.10 (C-1''), 129.93 (C-4'), 129.76 (C-3' & C-5'), 129.32 (C-3'' & C-5''), 128.56 (C-4''), 124.18 (C-2'' & C-6''), 119.62 (C-2' & C-6'), 75.99 (C(CH_3) $_2$), 37.32 (S- CH_2 -CO), 25.70 (C(CH_3) $_2$); EIMS: m/z 403 [M^+], 405 [$\text{M}^+ + 2$].

4.1.4. 2-{5-[2-(4-Chlorophenoxy)propan-2-yl]-1,3,4-oxadiazole-2-ylthio}-*N*-(2-methylphenyl)acetamide (3b)

The compound **3b** was synthesized by the reaction of 5-[2-(4-chlorophenoxy)propan-2-yl]-1,3,4-oxadiazol-2-thiol **1** (0.271 g, 1mmol) with 2-bromo-*N*-(2-methylphenyl)acetamide (0.227 g, 1 mmol) after a period of 1 hour and 20 minutes. Light yellow color powder; yield: 87 % (0.362g); m.p. 140-142 °C; IR (KBr, ν_{\max} , cm^{-1}): 3272 (N-H, str.), 2965 (C-H, str. of aromatic ring), 1644 (C=O amide str.), 1383 (C=C, aromatic str.); $^1\text{H-NMR}$ (500 MHz, DMSO- d_6): δ (ppm) 10.48 (s, 1H, NH), 7.38-7.32 (m, 5H, H-3', H-5', H-4'', H-5'' & H-6''), 7.22 (d, $J = 8$ Hz, 1H, H-3''), 6.94 (d, $J = 9$ Hz, 2H, H-2' & H-6'), 4.26 (d, $J = 17.5$ Hz, 1H, Ha), 4.19 (d, $J = 17.5$ Hz, 1H, Hb), 2.18 (s, 3H, Ar-CH₃), 1.47, 1.46 (s, C(CH₃)₂); $^{13}\text{C-NMR}$ (500 MHz, DMSO- d_6 , δ /ppm) : 171.80 (C=O), 169.58 (C-2), 164.64 (C-5), 154.10 (C-1'), 136.48 (C-1''), 134.57 (C-2''), 131.26 (C-3''), 129.71 (C-5''), 129.46 (C-3' & C-5'), 129.15 (C-4'), 127.31 (C-4''), 126.40 (C-6''), 121.91 (C-2' & C-6'), 80.83 (C(CH₃)₂), 33.08 (S-CH₂-CO), 25.47, 25.44 (C(CH₃)₂), 17.56 (Ar-CH₃); EIMS: m/z 417 [M^+], 419 [$\text{M}^+ + 2$].

4.1.5. 2-{5-[2-(4-Chlorophenoxy)propan-2-yl]-1,3,4-oxadiazole-2-ylthio}-*N*-(3-methylphenyl)acetamide (3c).

The compound **3c** was achieved from the reaction of 5-[2-(4-chlorophenoxy) propan-2-yl]-1,3,4-oxadiazol-2-thiol **1** (0.271 g, 1mmol) with 2-bromo-*N*-(3-methylphenyl)acetamide (0.227 g, 1 mmol) after a period of 1 hour and 15 minutes. Lemon yellow color powder; yield: 78 % (0.325 g); m.p. 71-72 °C ; IR (KBr, ν_{\max} , cm^{-1}): 3155 (N-H, str.), 2989 (C-H, str. of aromatic ring), 1655 (C=O amide str.), 1488 (C=C, aromatic str.); $^1\text{H-NMR}$ (500 MHz, CDCl₃): δ (ppm) 8.93 (s, 1H, NH), 7.35 (s, 1H, H-2''), 7.27 (d, $J = 8.5$ Hz, 1H, 6''), 7.19 (t, $J = 7.8$ Hz, 1H,

5"), 7.11-7.10 (m, 2H, H-3' & H-5'), 6.93 (d, $J = 7.5$ Hz, 1H, H-4"), 6.65-6.63 (m, 2H, H-2' & H-6'), 3.97 (s, 2H, S-CH₂-CO), 2.32 (s, 3H, Ar-CH₃), 1.77 (s, 6H, C(CH₃)₂); ¹³C-NMR (400 MHz, DMSO-d₆, δ /ppm): 169.88 (C=O), 166.17 (C-2), 165.02 (C-5), 152.84 (C-1'), 139.04 (C-3"), 137.43 (C-1"), 129.41 (C-3' & C-5'), 128.87 (C-5"), 125.61 (C-4'), 122.98 (C-2', C-6' & C-6"), 120.46 (C-4"), 116.99 (C-2"), 75.55 (C(CH₃)₂), 36.23 (S-CH₂-CO), 25.86 (C(CH₃)₂), 21.44 (Ar-CH₃); EIMS: m/z 417 [M⁺], 419 [M⁺+2].

4.1.6. 2-{5-[2-(4-Chlorophenoxy)propan-2-yl]-1,3,4-oxadiazole-2-ylthio}-N-(4-methylphenyl)acetamide (3d).

The compound **3d** was obtained from the reaction of 5-[2-(4-chlorophenoxy)propan-2-yl]-1,3,4-oxadiazol-2-thiol **1** (0.271 g, 1 mmol) with 2-bromo-N-(4-methylphenyl)acetamide (0.227 g, 1 mmol) after 60 minutes. Dirty white color powder; yield: 84 % (0.350 g); m.p. 68-70 °C; IR (KBr, ν_{\max} , cm⁻¹): 3313 (N-H, stretching), 2979 (C-H, str. of aromatic ring), 1676 (C=O amide str.), 1483 (C=C, aromatic str.); ¹H-NMR (500 MHz, DMSO-d₆): δ (ppm) 10.33 (s, 1H, NH), 7.46 (d, $J = 8$ Hz, 2H, H-3' & H-5), 7.27 (d, $J = 9$ Hz, 2H, H-2' & H-6'), 7.12 (d, $J = 8$ Hz, 2H, H-2" & H-6"), 6.75 (d, $J = 9$ Hz, 2H, H-3" & H-5"), 4.31 (s, 2H, S-CH₂-CO), 2.25 (s, 3H, Ar-CH₃), 1.71 (s, 6H, C(CH₃)₂); ¹³C-NMR (500 MHz, DMSO-d₆, δ /ppm): 168.84 (C=O), 164.91 (C-2), 164.90 (C-5), 153.29 (C-1'), 136.60 (C-4"), 133.13 (C-1"), 129.75 (C-3' & C-5'), 129.68 (C-3" & C-5"), 128.57 (C-4'), 124.18 (C-2" & C-6"), 119.64 (C-2' & C-6'), 75.98 (C(CH₃)₂), 37.31 (S-CH₂-CO), 25.70 (C(CH₃)₂), 20.91 (Ar-CH₃); EIMS: m/z 417 [M⁺], 419 [M⁺+2].

4.1.7. 2-{5-[2-(4-Chlorophenoxy)propan-2-yl]-1,3,4-oxadiazol-2-ylthio}-N-(2,3-dimethylphenyl)acetamide (3e).

The compound **3e** was obtained from the reaction of 5-[2-(4-chlorophenoxy)propan-2-yl]-1,3,4-oxadiazol-2-thiol **1** (0.271 g, 1 mmol) with 2-bromo-*N*-(2,3-dimethylphenyl)acetamide (0.241 g, 1 mmol) after 1 hour and 30 minutes. Creamy white color powder; yield: 86 % (0.371 g); m.p. 186-188 °C ; IR (KBr, ν_{\max} , cm^{-1}): 3300 (N-H, stretching), 2952 (C-H, str. of aromatic ring), 1645 (C=O amide str.), 1392 (C=C, aromatic str.); ^1H - NMR (500 MHz, DMSO- d_6): δ (ppm) 10.45 (s, 1H, NH), 7.34 (d, $J = 9$ Hz, 2H, H-3' & H-5'), 7.27 (d, $J = 7.5$ Hz, 1H, H-6''), 7.21 (t, $J = 7.6$ Hz, 1H, H-5''), 7.04 (d, $J = 8$ Hz, 1H, H-4''), 6.94 (d, $J = 9$ Hz, 2H, H-2' & H-6'), 4.26 (d, $J = 17.5$ Hz, 1H, *Ha*), 4.18 (d, $J = 7$ Hz, 1H, *Hb*), 2.31 (s, 3H, Ar-CH₃), 2.03 (s, 3H, Ar-CH₃), 1.47, 1.46 (s, 6H, C(CH₃)₂); ^{13}C -NMR (500 MHz, DMSO- d_6 , δ /ppm): 171.87 (C=O), 169.49 (C-2), 164.72 (C-5), 154.07 (C-1'), 138.23 (C-6''), 134.94 (C-1''), 134.58 (C-3''), 130.93 (C-4'), 129.45 (C-3' & C-5'), 126.68 (C-2''), 126.60 (C-4''), 126.38 (C-5''), 121.90 (C-2' & C-6'), 80.82 (C(CH₃)₂), 33.03 (S-CH₂-CO), 25.45 (C(CH₃)₂) 20.36 (Ar-CH₃), 14.22 (Ar-CH₃); EIMS: m/z 431 [M^+], 433 [M^{+2}].

4.1.8. 2-{5-[2-(4-Chlorophenoxy)propan-2-yl]-1,3,4-oxadiazole-2-ylthio}-*N*-(2,4-dimethylphenyl)acetamide (**3f**).

The compound **3f** was obtained from the reaction of 5-[2-(4-chlorophenoxy)propan-2-yl]-1,3,4-oxadiazol-2-thiol **1** (0.271 g, 1 mmol) with 2-bromo-*N*-(2,4-dimethylphenyl)acetamide (0.241 g, 1 mmol) after 1 hour and 20 minutes. Golden yellow color powder; yield: 81% (0.349g); m.p. 78-80 °C ; IR (KBr, ν_{\max} , cm^{-1}): 3260 (N-H, stretching), 2980 (C-H, str. of aromatic ring), 1632 (C=O amide str.), 1381 (C=C, aromatic str.); ^1H - NMR (CDCl₃, 500 MHz): δ (ppm) 8.59 (s, 1H, NH), 7.69 (d, $J = 8$ Hz, 1H, H-6''), 7.11-7.09 (m, 2H, H-3' & H-5'), 6.98-6.97 (m, 2H, H-3'' & H-5''), 6.65-6.63 (m, 2H, H-2' & H-6'), 4.01 (s, 2H, S-CH₂-CO), 2.27 (s, 3H, Ar-CH₃), 2.16 (s, 3H, Ar-CH₃), 1.77 (s, 6H, C(CH₃)₂); ^{13}C -NMR (500 MHz, CDCl₃, δ /ppm):

169.93 (C=O), 166.13 (C-2), 165.32 (C-5), 152.87 (C-1'), 135.19 (C-4''), 132.85 (C-2''), 131.24 (C-1''), 129.39 (C-3' & C-5'), 129.17 (C-3''), 127.23 (C-5''), 122.83 (C-2', C-6' & C-6''), 122.66 (C-4'), 75.51 (C(CH₃)₂), 35.97 (S-CH₂-CO), 25.88 (C(CH₃)₂), 20.85 (Ar-CH₃), 17.85 (Ar-CH₃); EIMS: m/z 431 [M⁺], 433 [M⁺+2].

4.1.9. 2-{5-[2-(4-Chlorophenoxy)propan-2-yl]-1,3,4-oxadiazole-2-ylthio}-N-(2,6-dimethylphenyl)acetamide (3g).

The compound **3g** was obtained from the reaction of 5-[2-(4-chlorophenoxy)propan-2-yl]-1,3,4-oxadiazol-2-thiol **1** (0.271 g, 1 mmol) with 2-bromo-N-(2,6-dimethylphenyl)acetamide (0.241 g, 1 mmol) after 1 hour. Creamy white color powder; yield: 89 % (0.383 g); m.p. 98-100 °C ; IR (KBr, v_{max}, cm⁻¹): 3330 (N-H, stretching), 2958 (C-H, str. of aromatic ring), 1661 (C=O amide str.), 1482 (C=C, aromatic str.); ¹H-NMR (500 MHz, DMSO-d₆): δ(ppm) 9.71 (s, 1H, NH), 7.26 (d, *J* = 9 Hz, 2H, H-3' & H-5'), 7.09-7.06 (m, 3H, H- 3'', H-4'' & H-5''), 6.76 (d, *J* = 9 Hz, 2H, H-2' & H- 6'), 4.34 (s, 2H, S-CH₂-CO), 2.13 (s, 6H, Ar-2CH₃), 1.73 (s, 6H, C(CH₃)₂); ¹³C-NMR (500 MHz, DMSO-d₆, δ /ppm) : 168.9 (C=O), 164.98 (C-2), 164.94 (C-5), 153.31 (C-1'), 135.60 (C-2'' & C-6''), 135.01 (C-1''), 129.76 (C-3' & C-5'), 128.54 (C-4''), 128.18 (C-3'' & C-5''), 127.14 (C-4'), 124.15 (C-2' & C-6'), 75.98 (C(CH₃)₂), 36.25 (S-CH₂-CO), 25.75 (C(CH₃)₂), 18.47 (Ar-2CH₃); EIMS: m/z 431 [M⁺], 433 [M⁺+2].

4.1.10. 2-{5-[2-(4-Chlorophenoxy)propan-2-yl]-1,3,4-oxadiazole-2-ylthio}-N-(3,4-dimethylphenyl)acetamide (3h)

The compound **3h** was obtained from the reaction of 5-[2-(4-chlorophenoxy)propan-2-yl]-1,3,4-oxadiazol-2-thiol **1** (0.271 g, 1 mmol) with 2-bromo-N-(3,4-dimethylphenyl)acetamide (0.241 g, 1 mmol) after 1 hour and 10 minutes. Off-white color powder; yield: 91 % (0.392 g); m.p. 80-82 °C ; IR (KBr, v_{max}, cm⁻¹): 3357 (N-H, stretching), 2904 (C-H, str. of aromatic ring),

1640 (C=O amide str.), 1380 (C=C, aromatic str.); ¹H-NMR (300 MHz, DMSO-d₆): δ(ppm) 10.22 (s, 1H, NH), 7.33 (s, 1H, H-2''), 7.26-7.22 (m, 2H, H-5'' & H-6''), 7.04 (d, *J* = 8 Hz, 2H, H-3' & H-5'), 6.75-6.71 (m, 2H, H-2', & H-6'), 4.27 (s, 2H, S-CH₂-CO), 2.16 (d, *J* = 4.5 Hz, 6H, Ar-2CH₃), 1.69 (s, 6H, C(CH₃)₂); ¹³C-NMR (300 MHz, DMSO-d₆, δ /ppm) : 168.37 (C=O), 164.38 (C-2), 164.35 (C-5), 152.79 (C-1'), 136.42 (C-3''), 136.32 (C-1''), 131.47 (C-4''), 129.63 (C-5''), 129.24 (C-3' & C-5'), 128.08 (C-4'), 123.74 (C-2' & C-6'), 120.39 (C-2''), 116.72 (C-6''), 75.51 (C(CH₃)₂), 36.79 (S-CH₂-CO), 25.22 (C(CH₃)₂), 19.75 (Ar-CH₃), 18.75 (Ar-CH₃); EIMS: m/z 431 [M⁺], 433 [M⁺+2].

4.1.11. 2-{5-[2-(4-Chlorophenoxy)propan-2-yl]-1,3,4-oxadiazole-2-ylthio}-*N*-(3,5-dimethylphenyl)acetamide (3i)

The compound **3i** was obtained from the reaction of 5-[2-(4-chlorophenoxy)propan-2-yl]-1,3,4-oxadiazol-2-thiol **1** (0.271 g, 1 mmol) with 2-bromo-*N*-(3,5-dimethylphenyl)acetamide (0.241 g, 1 mmol) after 1 hour and 30 minutes. Off-white colour powder; yield: 85 % (0.366 g); m.p. 122-124 °C ; IR (KBr, ν_{max}, cm⁻¹): 3299 (N-H, stretching), 2995 (C-H, str. of aromatic ring), 1680 (C=O amide str.), 1580 (C=C, aromatic str.); ¹H-NMR (500 MHz, DMSO-d₆): δ(ppm) 10.27 (s, 1H, NH), 7.27 (d, *J* = 8.5 Hz, 2H, H-3' & H-5'), 7.20 (s, 2H, H-2'' & H-6''), 6.75 (d, *J* = 8.5 Hz, 2H, H-2' & H-6'), 6.72 (s, 1H, H-4''), 4.29 (s, 2H, S-CH₂-CO), 2.23 (s, 6H, Ar-2CH₃), 1.70 (s, 6H, C(CH₃)₂); ¹³C-NMR (500 MHz, DMSO-d₆, δ /ppm) : 168.3 (C=O), 165.03 (C-2), 164.90 (C-5), 153.27 (C-1'), 138.96 (C-1''), 138.29 (C-3'' & C-5''), 129.73 (C-3' & C-5'), 128.59 (C-4''), 125.69 (C-4'), 124.27 (C-2'' & C-6''), 11.39 (C-2' & C-6'), 76.00 (C(CH₃)₂), 37.31 (S-CH₂-CO), 25.68 (C(CH₃)₂), 21.53 (Ar-2CH₃); EIMS: m/z 431 [M⁺], 433 [M⁺+2].

4.2. Biological Assay

4.2.1. Sample solution preparation

A 100 mg each of each synthetic compound was mixed in 10 ml distilled water and shaken vigorously on a vortex mixer and stirred overnight. The resulting solution was then filtered by using 0.22 μm syringe filter. A 100 μl of this aqueous preparation was added to the pre-weighed eppendorf tube containing the clots to check thrombolytic activity.

4.2.2. *In vitro* clot lysis activity assay

Experiments for clot lysis were carried as reported earlier [25]. Briefly, venous blood was drawn from the healthy volunteers without a history of anti-coagulant therapy or oral contraceptive. 0.5 ml was used in pre-weighed sterile eppendorf tubes and incubated at 37 °C for 45 minutes. After clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight. 100 μl of tested compounds filtrate was added separately. Streptokinase (SK) 15, 00,000 I.U (Square Pharmaceuticals Ltd.) used as positive control (100 μl) while sterile distilled water (100 μl) was used as negative control. All the tubes were then incubated at 37 °C for 90 minutes and observed for clotlysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clotlysis was expressed as percentage of clot lysis [26]. The experiment was repeated three times with the blood samples of the 12 volunteers. Following formula was used to determine the percentage of clot lysis and results are represented in table 2.

Percentage of clot lysis (%) = $[\text{Initial clot weight} - \text{Final clot weight} / \text{Initial weight of clot}] \times 100$

4.2.3. *In vivo* anti-coagulation activity

4.2.3.1. *Experimental Animals*

Sprague Dawley (SD) rats (100-120 g) were used to determine the *in vivo* anti-coagulation effects of tested compounds and kept under control temperature (25 ± 5 °C) and humidity (50 ± 10 %) in animal house, with free access of pathogen and autoclave tap water for 24 hours. Experiments were approved by Institutional Ethical Committee, University of the Punjab, Lahore and international ethical guideline was also followed for the care of laboratory animals to provide them healthy environment.

4.2.3.2. *In vivo* Clotting Time (CT) determination

Blood CT determination was used to evaluate the anti-coagulant activity. Previously established method with slight modifications was used to estimate the CT [7,8]. Seventy two rats were divided into twelve groups of 6 rats each. The first group was given 0.5% carboxy methyl cellulose (CMC) orally and served as negative control. The second groups were designated as positive control, received 500 IU/ kg unfractionated heparin orally. The group 3-12 was given the tested compounds suspended in 0.5% CMC and given to the rats orally at a dose of 25 mg/kg body weight. A drop of blood was drawn from the tail of each rat on clean dry glass slide. One end of a capillary tube was dipped into blood drop without pressure. Three quarter length of capillary tube was filled with blood and CT was measure at the regular interval of 30 seconds which was represented in figure 1.

4.3. Molecular docking studies

In-silico analysis of the newly designed 1,3,4-oxadiazoles derivatives against F-Xa protein were carried out. The crystal structure of F-Xa protein was retrieved from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB ID 1NFY). The

experimental (*in vivo*) studies were carried out against Factor Xa (Ec: 3.4.21.6) from Sprague Dawley rats and docking study against human F-Xa (PDB ID: 1NFY at 2.1 Å resolution).

4.3.1. Preparation of target F-Xa and compounds for docking

The coordinate files were subjected to Discovery Studio 4.5 Visualizer for pre-docking receptor preparation by removing water molecules and adding hydrogen atoms. Ligands **1**, **RPR200095**, **3a-3i** were docked with F-Xa (1NFY) by *Patch Dock* (<http://bioinfo3d.cs.tau.ac.il/PatchDock/>) to find the docking transformations that produce good molecular shape complementarity based on shape complementarity principles [27]. The input files include the receptor protein and ligand in PDB format.

Patch Dock offers multiple solutions and the “solution 1” was selected as it surrounded the most crucial residues as binding pocket for docking analyses assigned in crystal structure of F-Xa target site (1NFY) [28]. The docked structures were examined by using Discovery Studio 4.5 Visualizer and Chimera 1.9.

4.3.2. Docking analysis

The binding affinities of the docked ligands were evaluated as scores and Atomic Contact Energy (ACE) of the docked complexes. The hydrogen bonding and hydrophobic interactions of each ligand was assessed within binding pocket of receptor protein. The conformation of the ligands which illustrated the highest biological activities is showed in Table 4 and Figure 2-5 with their favorable contacts in the binding pockets. To get qualitative evaluation and to recognize molecular basis of the calculated biological activities, the docked complexes of ligands **1**, **RPR200095**, **3a-3i** were investigated.

4.4. Computational methodology

In this study, all the computational calculations (including representation of Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO) in the checkpoint files) were performed by Gaussian 09 software [29] with Becke's three parameter hybrid exchange functional [30] and Lee-Yang-Parr correlation functionals (B3LYP) [31,32]. The geometry of all the structures were optimized using B3LYP/6-31G** basis set. To ensure that the optimized geometry actually corresponds to the equilibrium (minimum energy) structure, the harmonic vibrational frequency analysis was also performed at same basic set level to detect any imaginary frequency. The Gauss view software package was used to visualize the computed structures including HOMO, LUMO and Molecular electrostatic potential (MEP) representations.

3. Conclusion

Newly synthesized 5-[2-(4-chlorophenoxy)propan-2-yl]-1,3,4-oxadiazol-2-thiol derivatives (**3a-3i**) were tested for clot lysis on the human blood of twelve volunteers (table 2). Besides, these compounds were tested for their anti-coagulant effects by investigating the CT values in mice (figure 1). Our docking experiments have shown that ligands (**3a, 3e, 3i, 3d & 3h**) have showed very good inhibitory potential against F-Xa and show a higher docking score than control ligand **RPR200095** (table 3). In *silico* results are good in agreement with anti-coagulant activity of the newly synthesized tested compounds (**3a, 3e, 3i, 3d and 3h**) even better than standard drug unfractionated heparin. Our experimental and computational results suggest that these compounds have potent anti-thrombotic potential and might be used as effective anti-coagulant agents.

Consent

Volunteers have given oral consent for the participation in research.

Supporting Information

¹H-NMR spectra, ¹³C-NMR spectra and EI-MS of compounds **3a-3i** are provided.

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Conflicts of Interest

The authors declare no conflict of interest.

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