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Anticoagulant Activity and Molecular Docking of 7,9-*bis*(4-Chlorophenyl)-6-methyl-1,4-dioxa-8-azaspiro[4.5]decane through Target Protein Thrombin

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The present study reported the synthesis and anticoagulant activity of spiro compounds, the potential compound was subjected to study their binding mode and interaction using bioinformatics tools. Among the five compounds tested, 7,9-*bis*(4-chlorophenyl)-6-methyl-1,4,8-triaza spiro[4.5]decane has prolonged activated partial thromboplastin time and prothrombin time as 22.7s and 18.5s at 25 μg mL^{-1}, respectively. Hence, the mandatory way study was carried out for this compound by using bioinformatics method. The interaction results shows that this compound binds in the active site of the target protein thrombin which is similar to that of existing inhibitor and it may also considered as an anticoagulant drug in future.

Keywords: Anticoagulant, Prothrombin, Spiro and Thrombin inhibitor.

INTRODUCTION

The asymmetric point of the molecules due to the chiral spiro carbon is one of the significant criterions for their biological activities. Spiro compounds have potential biological properties [1]. A lot of synthetic methodologies have been developed for constructing these spiro cycles; most of them prefer cyclo addition or condensation reactions [2-4].

The extrinsic and intrinsic coagulation schemes congregate at the activation of factor X to Xa. Activated factor X (fXa) play an important role in the conversion of prothrombin to thrombin, which creates blood clots by converting factor XI to XIa, VIII to VIIIa, V to Va, fibrinogen to fibrin and XIII to XIIIa. Thus, thrombin inhibition is a key mechanism in the coagulation cascade and is also an attractive target enzyme for the therapy of atrial fibrillation to avoid thromboembolism [5].

Edoxaban (**1**) is an anticoagulant drug which acts as a direct factor Xa inhibitor. It was developed by Daiichi Sankyo and approved in July 2011 in Japan for prevention of venous thromboembolisms (VTE) following lower-limb orthopedic surgery.

Rivaroxaban (**2**) is an oral anticoagulant invented and manufactured by Bayer [6,7], and marketed as Xarelto. It is the first orally active direct factor Xa inhibitor. Rivaroxaban is well absorbed from the gut and maximum inhibition of factor Xa occurs 4 h after a dose. Dabigatran (**3**) (Pradaxa) is an oral anticoagulant from the class of the direct thrombin inhibitors. It has been studied for various clinical indications and in some cases it offers an alternative to warfarin as the preferred orally administered anticoagulant (blood thinner) [8,9].

Warfarin (**4**) used for preventing thrombosis as well as embolism and inhibition of metastasis [10-13]. It is widely being used to prevent strokes. Warfarin, as an anticoagulant, suppresses the formation of calcium dependent clotting factors II, VII, IX and X. It diminishes the regeneration of vitamin K and vitamin K hydroquinone in the tissues that inhibits the vitamin K-dependent carboxylation activity of the glutamyl carboxylase enzyme. Inhibition of carboxylation stops the clotting factors to be active [14-16].

Betrixaban (**5**) is an anticoagulant drug which acts as a direct factor Xa inhibitor [17]. It is potent, orally active and highly selective for factor Xa. Betrixaban has undergone human clinical trials for prevention of embolism after knee surgery [18] and prevention of stroke following atrial fibrillation [19].

Heparin (**6**) is a highly sulfated glycosaminoglycan, widely used as an injectable anticoagulant and has the highest negative charge density of any known biological molecule [20]. It can also be used to form an inner anticoagulant surface on various experimental and medical devices such as test tubes and renal dialysis machines. Although it is used principally in medicine for anticoagulation, its true physiological role in the body remains unclear, because blood anticoagulation is

achieved mostly by heparansulfate proteoglycans derived from endothelial cells [21]. Few important anticoagulant drug structures are shown in Fig. 1.

EXPERIMENTAL

All the chemicals and reagents used in the present study were of laboratory grade and purchased from Sigma Aldrich.

Methods: The melting points were determined in open capillaries and are uncorrected. IR spectra were recorded on AVATAR 330 FT-IR Thermo Nicolet spectrometer in KBr pellets. Elemental analysis was performed on an Elemental Vario EL III (C, H, N and O & Cl) analyzer. The purity of the compounds was checked by thin layer chromatography (TLC) using silica gel plates. ^{1}H NMR spectra were recorded on a Bruker AMX-300 NMR spectrometer operating at 300.03 MHz for ^{1}H with the following spectral parameters; acquisition time = around 3.0 s, number of scans = 100 and spectral width = 10,330 Hz. Proton decoupled ^{13}C NMR spectra were recorded on a Bruker AMX-300 NMR spectrometer operating at 75.07 MHz for ^{13}C with the following spectral parameters; acquisition time around 0.5 s; number of scans = 1000; spectral width = 30,000 Hz. All NMR measurements were made in 5 mm NMR tubes using solutions made by dissolving about 10 mg of the material in 0.5 mL of DMSO-d_6.

Anticoagulant activity

Preparation of plasma: Blood was collected from individual healthy donor through vein puncture without bleeding or thrombosis and then it was mixed with 3.8 % tri sodium citrate at 9:1 ratio. Further it was centrifuged for 20 min and the plasma was stored at -40 °C until use.

Activated partial thromboplastin time (APTT): For activated partial thromboplastin time assay, citrated normal human plasma (90 µL) was mixed with spiro compounds **7-11** (10 µL) in each concentration (25, 50, 100, 150 and 200 µg/mL), respectively and incubated for 1 min at 37 °C, followed by activated partial thromboplastin time reagent (100 µL) was added to the mixture and incubated for 5 min at 37 °C. Thereafter, the clotting was induced by adding 0.02 M calcium chloride (100 µL) and clotting time was recorded (Method followed by Pacific hemostasis kit).

Prothrombin time (PT): In prothrombin time, the citrated normal human plasma (90 µL) was mixed with 10 µL of spiro compounds **7-11** in each concentration (25, 50, 100, 150 and 200 µg/mL) and incubated for 10 min, respectively. Then, prothrombin time reagent (200 µL) pre-incubated for 10 min at 37 °C was added and clotting time was recorded (Method followed by Pacific hemostasis kit).

Preparation of compounds: The target spiro compounds **7-11** are prepared by Natarajan *et al.* [22].

Retrieval of 3D structure: The 3D structure of the protein was downloaded from Research Collaborator for Structural Bioinformatics (RCSB), Protein Databank (PDB, http://www.pdb.org). The PDB ID of the selected protein was found to be 3DA9. The Water molecules and ligands attached to the protein were removed by using Swiss PDB viewer.

Model quality assessment: Quality of the models was assessed by using Ramachandran plot. It can be used in two different ways. One is to show in theory which values, or conformations, of the ψ and φ angles are possible for an amino-acid residue in a protein. A second is to show the empirical distribution of data points observed in a single structure in usage for structure validation or else in a database of many structures. Either case is usually shown against outlines for the theoretically favoured regions. Here, the structural assessment was done by using SAVS server (http://nihserver.mbi.ucla.edu/SAVES) and it provides the values of Phi and Psi angle for the amino acids present in the target protein.

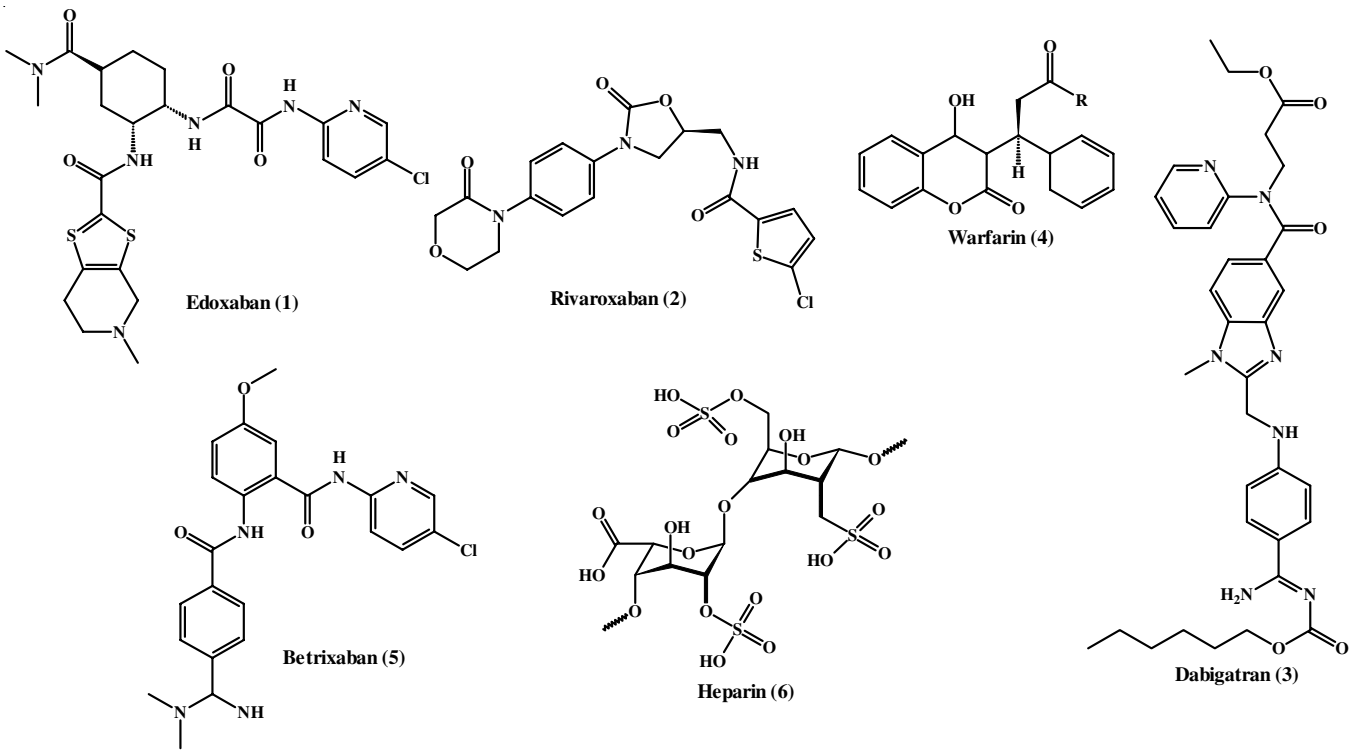


Fig. 1. Few important anticoagulant drugs structure

Selection of potential drug candidates: Based on the experimental results obtained from the analysis of activated partial thromboplastin time and prothrombin time, the compound 7,9-*bis*(4-chlorophenyl)-6-methyl-1,4-dioxa-8-azaspiro[4.5]decane was chosen, because this compound took prolonged time requirement for clotting among the studied five compounds.

Docking process: Protein-ligand docking is used to check the structure, position and orientation of a protein when it interacts with small molecules like ligands. The software 'Hex' is used to calculate the binding energy requirements of ligand with its receptors. In HEX, full rotation search mode is used and shape only correlation type for docking. The docking parameters employed are as follows:

Search mode: Full rotation; Correlation type: Shape only; Radial filter: None; Post processing: None; Grid dimension: 0.6; Solution: 500, samples: 642; Receptor range: 180, samples: 642; Ligand range: 180, samples: 128; Twist range: 360; Distance range: 40; Scan step: 1.0; Sub step: 2.

Protein ligand interaction analysis: By using ligand tool, the interactions of the PDB inhibitor, β-phenyl-D-phenyl-alanyl-N-propyl-L-prolinamide and the synthesized compound 7,9-*bis*(4-chlorophenyl)-6-methyl-1,4-dioxa-8-azaspiro-[4.5]decane with the target protein thrombin were analyzed.

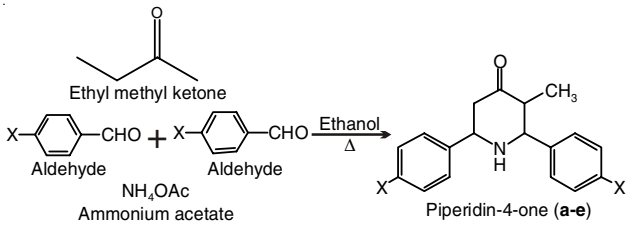
RESULTS AND DISCUSSION

The synthesis of piperidine-4-one derivatives were shown in **Scheme-I** and the synthesis of spiro derivatives were shown in **Scheme-II** (Fig. 2). Many synthetic approaches [23] have been reported for the preparation of cyclopropane such as intramolecular cyclization, addition of carbenes to olefins and Michael initiated ring closure [24-30]. Spiro triazoles and their derivatives can be conveniently synthesized from aldehyde/ ketone thiosemicarbazones and also from substituted thio-semicarbazides by cyclization using suitable reagents. Earlier $FeCl_3 \cdot 6H_2O$ [31], H_2O_2 [32] and *m*-CPBA [33] were used to effect cyclization of steroidal and non-steroidal ketone thiosemicar-bazones into corresponding 1,2,4-triazolidin-3-thiones.

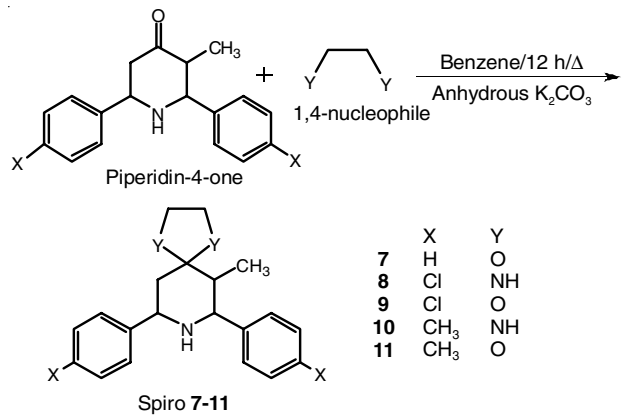
Here we report a simple and effective procedure for condensation reaction without catalyst and anticoagulant activities of the following compounds (**7-11**). 6-Methyl-7,9-diphenyl-1,4-dioxa-8-aza spiro[4.5]decane (**7**), 7,9-*bis*(4-chlorophenyl)-6-methyl-1,4,8-triaza spiro[4.5]decane (**8**), 7,9-*bis*(4-chlorophenyl)-6-methyl-1,4-dioxa-8-aza spiro[4.5]decane (**9**), 7,9-*bis*(4-methoxyphenyl)-6-methyl-1,4,8-triazaspiro[4.5] decane (**10**) and 7,9-*bis*(4-methoxyphenyl)-6-methyl-1,4-dioxa-8-aza spiro[4.5]decane (**11**). The molecular structure of the spiro compounds are given in Fig. 3.

Activated partial thromboplastin time and prothrombin time analysis: Compounds were evaluated for their anti-coagulant activity according to their PDBID:3DA9 values and for anti-coagulant activity *in vitro* by the CT2 values of their prothrombin times (PT). The CT2 value was defined as the concentration required double the clotting time. In addition, oral anticoagulant activity was evaluated by the ability of compounds to prolong prothrombin time following oral administration in human being.

The activated partial thromboplastin time and prothrombin time of synthesized spiro compounds are presented in Tables 1 and 2.



Scheme-I: General reaction scheme for synthesis of piperidine-4-one (**a-e**) compounds



Scheme-II: General reaction scheme for synthesis of spiro (**7-11**) compounds

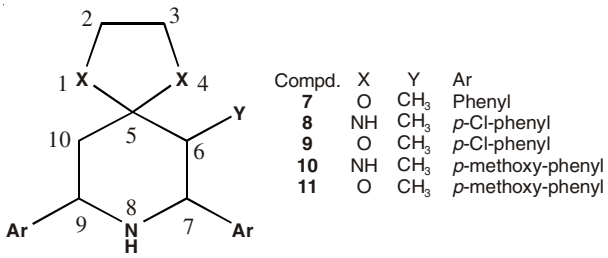


Fig. 2. General templates for synthesized compounds (**7-11**)

TABLE-1
ACTIVATED PARTIAL THROMBOPLASTIN TIME DATA OF SPIRO COMPOUNDS WITH DIFFERENT CONCENTRATIONS

Conc. (μg/mL)	Compounds				
	7	**8**	**9**	**10**	**11**
25	20.2s	18.3s	22.7s	20.4s	20.6s
50	31.4s	24.0s	31.8s	36.9s	34.6s
100	43.7s	41.0s	49.3	46.4s	46.8s
150	55.8s	53.1s	61.1s	68.7s	60.9s
200	99.3s	89.9s	110.6s	91.1s	109.2s

Retrieval of target protein: The target protein thrombin with its inhibitor (PDBID:3DA9) was retrieved from the Protein Databank. The structural analysis using Rasmol software shows that the structure consists of 10 helices, 26 strands and 30 turns. The target protein structure shows in Fig. 6.

TABLE-2
PROTHROMBIN TIME DATA OF SPIRO COMPOUNDS WITH DIFFERENT CONCENTRATION

Conc. (μg/mL)	Compounds				
	7	**8**	**9**	**10**	**11**
25	18.6s	15.4s	18.5s	14.4s	17.9s
50	29.3s	21.6s	26.4s	29.6s	29.1s
100	36.7s	31.8s	38.3s	40.7s	40.0s
150	48.0s	42.2s	54.2s	56.0s	53.1s
200	63.0s	81.0s	97.2s	90.1s	98.3s

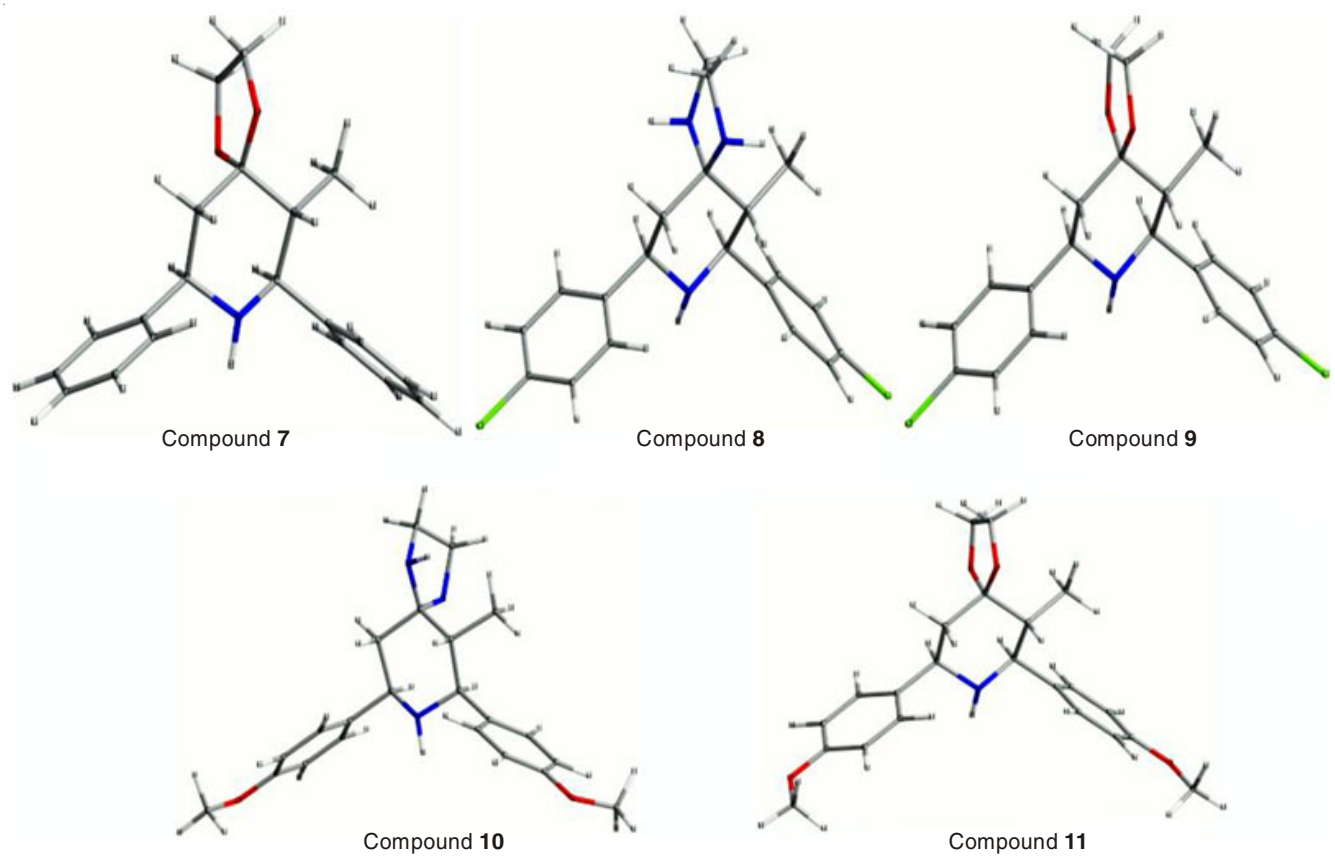


Fig. 3. Molecular structure of the spiro compounds

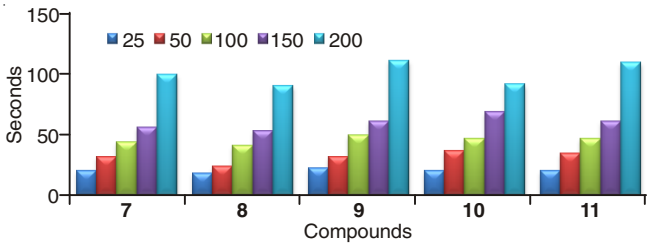


Fig. 4. Activated partial thromboplastin time data of spiro compounds with different concentrations

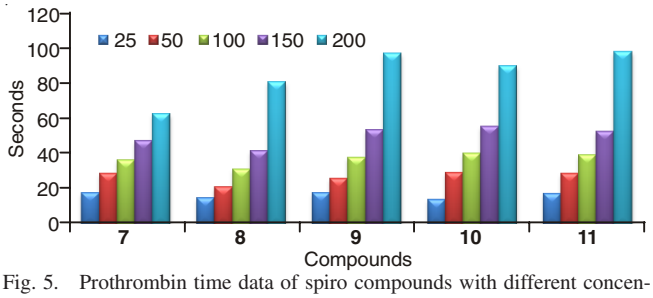


Fig. 5. Prothrombin time data of spiro compounds with different concentration

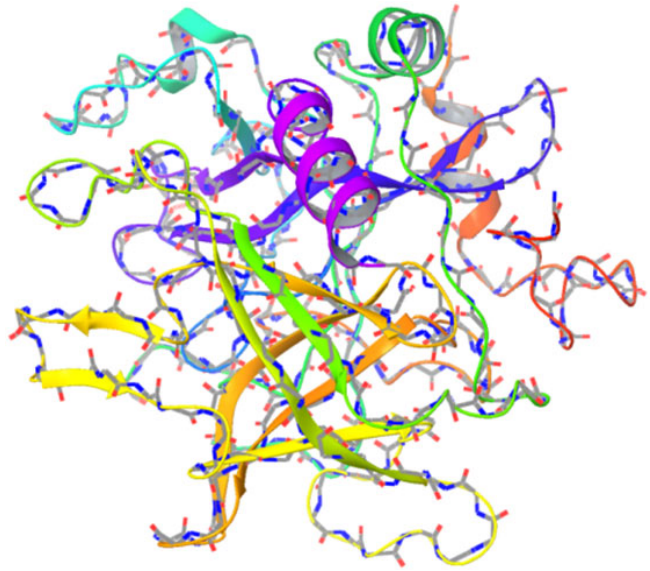


Fig. 6. Target protein

Energy minimization: The process of energy minimization is very important for docking and this was done with the help of SWISS PDB Viewer software and the values for bonds, angles, torsion, improper, non-bonded, electro statistics, constraints and the total are given in Table-3. The computations were done *in vacuo* with the GROMOS 96, 43B1 parameters set without reaction field. The results shows that the total energy for thrombin is -12365.702 kJ/mol and this energy were minimized to -16334.184 kJ/mol. The energy minimization values are shown in Table-3.

Tertiary structure analysis: The PDB structure was validated using Ramachandiran plot from SAVS Server. The results showed that the modeled structure contains 85.2 % of amino acids are present in most favoured regions which show that the retrieved structure has good quality. Ramachandran plot for 3DA9 protein was given in Fig. 7.

Structure of ligand: The structure of ligand done by using open babel software the. cdx molecular file for this chemical compound was converted in to pdb file. The ligand structure are shown in Fig. 8.

TABLE-3
ENERGY MINIMIZATION REPORT

Protein name	Energy minimization	Bonds	Angles	Torsion	Improper	Non-bonded	Electro-statics	Cons-traints	Total
Thrombin in complex with inhibitor	Before	935.29	1537.42	1599.07	290.81	-8821.03	-7907.27	0.000	-12365.70
	After	181.66	1027.22	1392.13	248.68	-10194.1	-8989.72	0.000	-16334.18

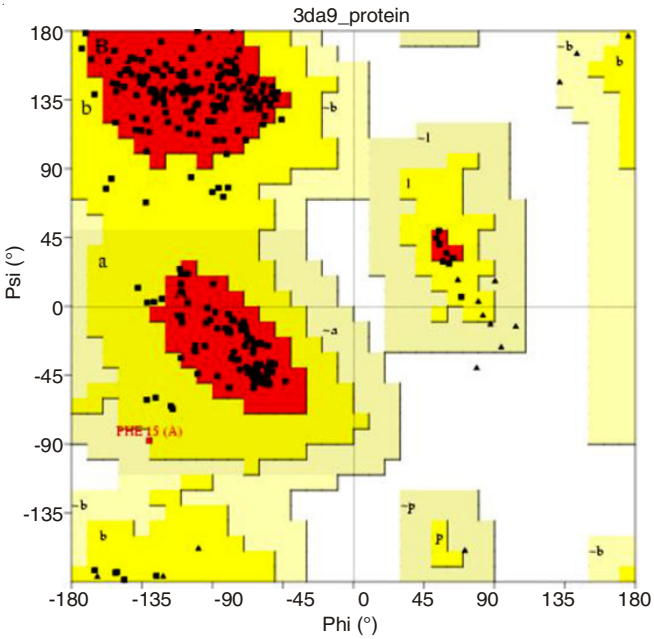


Fig. 7. Ramachandran plot

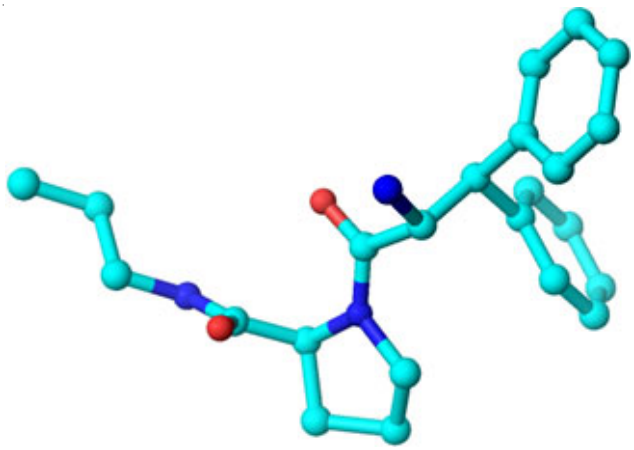


Fig. 8. 3D-Ligand structure of thrombin inhibitor

Docking: Docking is the process of binding of receptor with its ligand. The thrombin protein was docked with the compound 7,9-*bis*(4-chlorophenyl)-6-methyl-1,4-dioxa-8-aza spiro[4.5]decane using HEX software. The requirement of binding energy E of thrombin protein lies between -263.23 and -102.38 kJ/mol for molecular docking. The molecule before and after docking are given in Figs. 9 and 10, respectively.

Analysis of interactions: Interactions of the compound 7,9-*bis*(4-chlorophenyl)-6-methyl-1,4-dioxa-8-aza spiro[4.5]-decane for their binding mode analysis of thrombin with its inhibitor, we noticed that the inhibitor produces hydrophobic interactions with the active sites residues of His(79), Tyr(83), Trp(86), Glu(130), Asn(131), Leu(132), Ile(209), Cys(231),

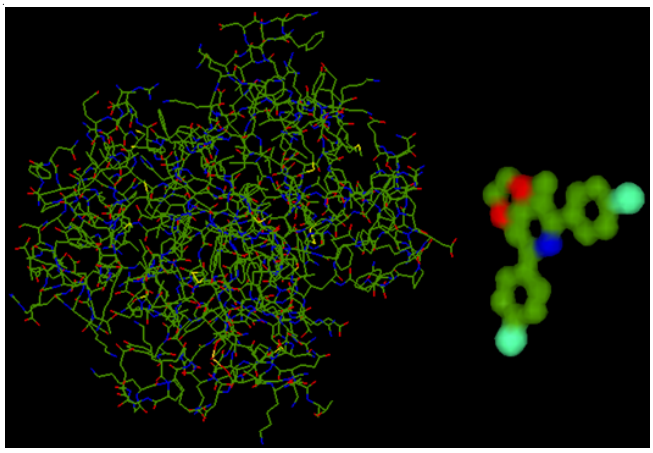


Fig. 9. Before docking of 7,9-*bis*(4-chlorophenyl)-6-methyl-1,4-dioxa-8-azaspiro[4.5]decane (**9**) with thrombin

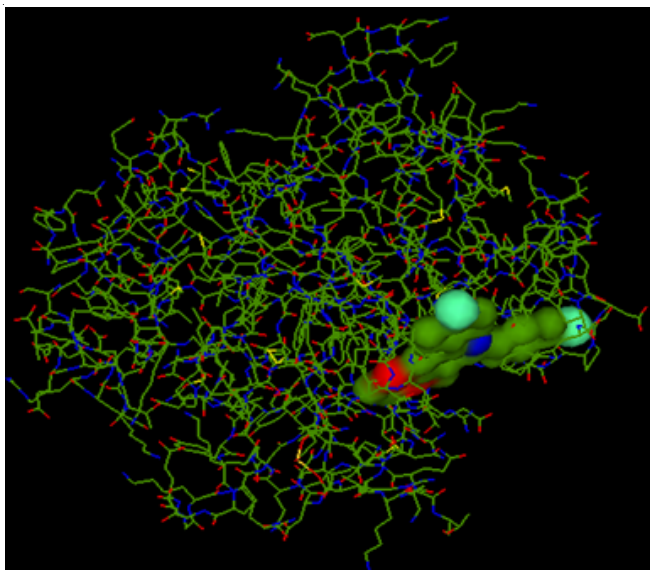


Fig. 10. After docking of 7,9-*bis*(4-chlorophenyl)-6-methyl-1,4-dioxa-8-azaspiro[4.5]decane (**9**) with thrombin

Ser(235), Val(255), Ser(256), Trp(257), Gly(258), Glu(259). Similarly, the 7,9-*bis*(4-chlorophenyl)-6-methyl-1,4-dioxa-8-azaspiro[4.5]decane also produces hydrophobic interactions with the same active sites and also it produces π-π interactions with the residues His(79), Tyr(83).The binding mode analysis of β-phenyl-D-phenylalanyl-N-propyl-L-prolinamide and 7,9-*bis*(4-chlorophenyl)-6-methyl-1,4-dioxa-8-aza spiro[4.5]decane were shown in Figs. 11 and 12, respectively.

Conclusion

In the present study cyclo condensation method was reported for the synthesis of spiro compounds and are evaluated for their anticoagulant activity. Among the five compounds, compound 9 was identified as potent anticoagulant (25 µg

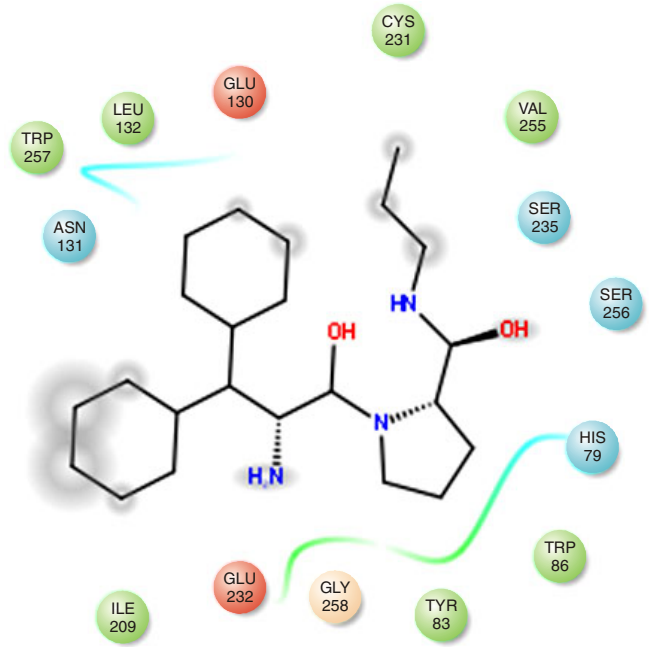


Fig. 11. Interactions of the PDB inhibitor β-phenyl-D-phenylalanyl-N-propyl-L-prolinamide

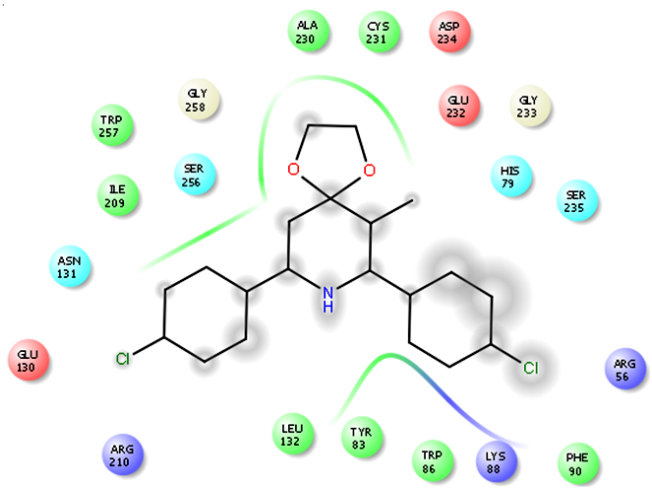


Fig. 12. Binding mode analysis of 7,9-*bis*(4-chlorophenyl)-6-methyl-1,4-dioxa-8-aza spiro[4.5]decane

mL^{-1}) against activated partial thromboplastin time and prothrombin time assay. The binding mode analysis of 7,9-*bis*(4-chlorophenyl)-6-methyl-1,4-dioxa-8-aza spiro-[4.5]-decane revealed that compound produces hydrophobic interactions with the same active sites and also it produces π-π interactions with the residues His(79), Tyr(83).

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