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Original Article

2D QSAR, design, in-silico studies, synthesis, characterization and in-vitro evaluation of some novel 1,2,4 triazole as the effective anti-alzheimer's agents inhibiting acetylcholinesterase enzyme

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ABSTRACT

Background: Alzheimer's disease, characterized by cognitive decline, involves acetylcholinesterase (AChE) in acetylcholine degradation. Designing novel 1,2,4-triazole derivatives as AChE inhibitors may provide effective, safer treatments, overcoming limitations of existing therapies.
Objective: The ligands are designed based on pharmacophoric features and docked against a specific target ACHE inhibitor. Performed 2D QSAR using QSARINs. The ligands are optimized based on drug likeness properties and toxicity profile. The optimized leads are synthesized, characterize and evaluate for Anti-alzheimer's activity against acetylcholinesterase enzyme.
Methods: Docking and 2D QSAR experiments were used to generate a number of new 1,2,4 triazole derivatives, around 160 compounds were designed by using Chemsketch. Based on docking scores and QSAR studies, the best 5 compounds were synthesized using a variety of chemical processes. Spectroscopic methods were used to confirm the compounds' structures and also used to describe the compounds. The enzyme inhibition assay was used to assess their anti-alzheimer's activity against acetylcholinesterase enzyme.
Results: When the synthesized compounds were tested for in-vitro anti-alzheimer's activity, they showed an IC50 value between 19 to 23µg/ml, which was nearly as strong as the current standard medications (Rivastigmine). According to these results, PD35, PD65, and PD72 had the strongest inhibitory effects on acetylcholinesterase enzyme.
Conclusion: The present study demonstrates the potential of 1,2,4 triazole derivatives as anti-alzheimer's agents against acetylcholinesterase enzyme. Further optimization and in-vivo evaluation are necessary to translate these findings into clinical applications.

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Introduction

Alzheimer's is a slowly worsening brain disease that negatively affects the brain and nerve cells by greatly reducing the ability to repair nerve cells. People with Alzheimer's disease (AD) often experience dementia and problems with thinking, which are common in both developed and developing countries. Many factors play an important role in the chain of events that cause nerve inflammation, dementia, and the advancement of AD. Currently, available medications only help with symptoms and do not provide a complete cure, leading researchers to look for new targets and treatments. Notably, nanomedicines born from the development of nanotechnology are being widely studied for the treatment of AD [1].

Treatment of Alzheimer's Disease

Cholinesterase inhibitors

Right now, there are a few drugs approved by the FDA that are used to manage thinking issues in people with symptomatic AD. Cholinesterase inhibitors help improve low levels of acetylcholine by reducing its breakdown. They are used for mild to moderate AD and mainly differ in how reversible or irreversible their effects are and how they work in the body. The medications include Tacrine, donepezil, rivastigmine, and Rivastigmine [2,3].

Target

- **Enzyme Name:** Acetylcholinesterase
- **Classification**: Type B Carboxylesterase enzyme
- **Type:** Protein
- **Chains**: A, B
- **Organism:** Homosapien
- **Functional Category:** Hydrolase/Hydrolase inhibitor
- **PDB ID:** 4EY7
- **Resolution:** 2.35 A^0

Structure

The enzyme Acetylcholinesterase exists as a homomeric assembly and is encoded by a single gene located at chromosome 7q22 with alternative mRNA processing, which is responsible for producing three catalytic subunits [4].

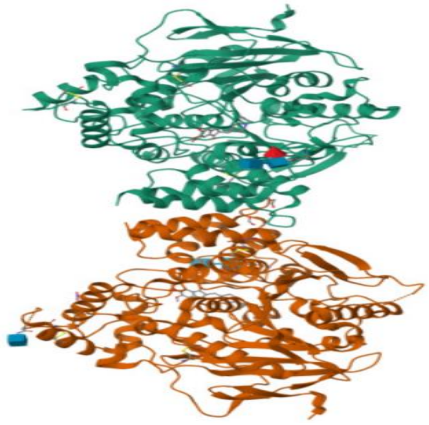


Figure 1: Recombinant acetylcholinesterase enzyme.

Mechanism of acetylcholinesterase

- AChE is made in nerve cells, muscle cells, and some blood-forming cells located at the neuromuscular junctions of skeletal muscles.
- Its main job is to break down acetylcholine into choline and acetate to stop cholinergic signalling.
- The non-catalytic role of AChE is to speed up the build-up of amyloid beta (Aβ) peptide and help create Aβ plaques.
- Further research showed that blocking acetylcholinesterase led to better thinking ability and behavior in people.
- Therefore, it is one of the main treatment methods for Alzheimer's disease (AD). Reversible AChE inhibitors (AChEIs) attach either to the catalytic site (CAS) or the peripheral site (PAS), while dimeric inhibitors attach to both sides at the same time.
- The main effect of AChEIs is to raise the level of acetylcholine (ACh) by blocking AChE. AChEIs that attach to PAS also prevent Aβ amyloid and fibril formation. They also increase the solubility of Amyloid Precursor Protein (APP) [5].

Importance of Heterocyclic Rings

Heterocyclic systems are very important because they have proven useful in medicinal chemistry. Heterocyclic compounds are ring-shaped groups of atoms that contain at least one different atom. The most common different atoms are nitrogen, oxygen, and sulphur, but rings with other different atoms like phosphorus, iron, magnesium, selenium, etc., are also found [6].

Scaffold Moiety

- 1,2,4-Triazole is a five-membered, π-excessive, aromatic nitrogen-containing cycle made of two

carbon atoms and three nitrogen atoms located at the 1-, 2-, and 4-positions of the ring. All the atoms in 1,2,4-triazoles are sp2 hybridized and have 6π electrons spread across the ring, which gives it its aromatic quality [7].

- The 1,2,4-triazole ring has become an important heterocyclic base in medicinal chemistry. It has a wide range of biological effects, including antimicrobial, anticancer, antiepileptic, anti-inflammatory, antiviral, antihypertensive, and sedative-hypnotic properties etc. [8].

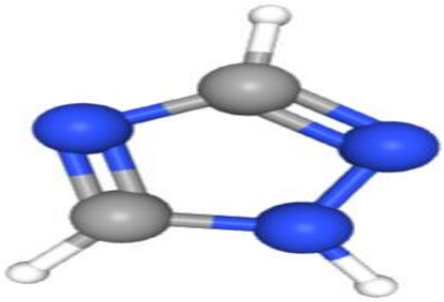


Figure 2: Ball and stick model of 1,2,4 triazole

Materials and Method

Drug design

Generation of 2D QSAR equation

Data Collection

160 compounds of 1,2,4 triazole derivatives and their anti-Alzheimer's activity against the acetylcholinesterase enzyme were collected from several research articles. The anti-Alzheimer activity of the compounds was recorded as IC50 (nm) and changed to PIC50 (nm) for this study.

Geometric Optimization

The 2D shapes of the molecules were created using Chem Sketch software, converted to 3D, and improved using Chem Draw 3D software.

Molecular Descriptors Calculation

Data Division

The genetic algorithm was used to split the data into a training set and a test set through QSARIN software. The training set (70%) was used to build the model, while the test set (30%) was used to check the model.

Model Building and Validation

The genetic algorithm (GA) method was used to select the best descriptors to create models based on a large number of descriptors. The MLR model was made using the ordinary least squares (OLS) method.

Internal Validation

The model was validated by OECD principles, which state that the model should have a clear endpoint, a defined applicability domain, a clear algorithm, and suitable measures of reliability and predictability with a systematic explanation.

Cross Validation

For cross validation (CV), the Q2LOO criteria were used by repeatedly removing one compound from the dataset while building the model with the other compounds. The following factors were assessed to evaluate the model's quality: R2: higher values reflect better model quality, Q2LOO: high values should equal R2, R2-Q2LOO: lower values indicate model stability, RMSE: values should be low and close to the training dataset and other prediction methods. Another approach for cross validation, called Leaving Many Out (LMO), enables the study of compounds by excluding many compounds. The stability of the model was determined based on the computed values of R2 and Q2 (LMO), with their averages being close to the R2 and Q2LOO values of the model.

y-Scrambling

The y-scrambling method was used to check that the created model was not due to random correlation. The responses were mixed to eliminate any correlation with the descriptors, resulting in a significant drop in model performance. For a high-quality model, the R2 and Q2 values and their averages should be lower than the model values.

External Validation

The created model was then evaluated based on various measures such as RMSE external, Q2-F1, Q2-F2, Q2-F3, r2M, Δr2m, and CCC.

Applicability Domain

The applicability domain was checked to ensure the model's reliability within the chemical area it was created. Leverage methods were used, and the

William's plot was drawn between the standardized residuals and leverages [9].

Selection of target

The Protein Data Bank is a database that contains three-dimensional structural information about large biological molecules like proteins, nucleic acids, and complex assemblies. The goals for treating Alzheimer's include targets like Acetylcholinesterase, N methyl-D-aspartate receptor, Amyloid-β protein, Tau protein, Beta secretase, Glycogen Synthase Kinase (GSK3), Phosphodiesterase, Cyclooxygenase, and Szeto-Schiller peptide. Human trials help us to understand the possible risks and benefits of these new methods. In this study, Acetylcholinesterase (AChE) was chosen as a target for treating Alzheimer's. It is an important target for anti-Alzheimer drugs. Blocking AChE stops the breakdown of Acetylcholine and the creation of β-Amyloid fibrils. This makes it a more precise platform for further drug development. Some effective PDB enzyme targets with lower resolution were selected along with the results. The best PDB target (4EY7) with a resolution of 2.3509 Å was used in this study [10].

Pharmacophore modelling

A Pharmacophore is described as "a collection of structural features in a molecule that a receptor site recognizes and is responsible for that molecule's biological activity." Based on the knowledge of existing inhibitors, how they interact with the protein, and the common characteristics needed for biological activity, new ACHE inhibitors are created.

Creation of virtual library

A library of nearly 160 new lead molecules as strong AChE inhibitors was made using the understanding of how the ligand binds to the protein and the shared pharmacophoric features important for the biological activity of a molecule. Chemical features such as Hydrogen bond acceptor (HBA), Hydrogen bond donor (HBD), and aromatic ring features were used to filter the database.

Creation of a virtual library of ligands and novelty checking

The ligands were drawn using the ACD Chem Sketch program based on the required pharmacophoric features. The Zinc15 and PubChem databases were used to check the novelty of the compounds. The designed compounds were considered novel because there was no information available in the ZINC® database [11].

***In-silico* drug likeness screening**

Drug likeness is a general concept shown by the molecular properties that affect the absorption, distribution, metabolism, excretion, and toxicity (ADMET) of a compound. Drug likeness, based on the structures and properties of existing drugs and drug candidates, is often used to eliminate unwanted compounds in the early stages of drug discovery. The key properties include hydrophobicity, electronic distribution, hydrogen bonding characteristics, molecular size, and flexibility, plus the presence of various pharmacophoric features that affect the behavior of molecules in a living organism, including bioavailability, protein affinity, and others. Drug likeness screening of the designed ligands was performed using online software tools Molinspiration and Osiris Property Explorer [12].

Molinspiration®

Molinspiration is an online software tool to assess the in silico pharmacokinetic properties of ligands based on the Lipinski rule of five. It was used to predict molecular properties like molecular weight, Log P, Total Polar Surface Area, the number of hydrogen bond acceptors and donors, the number of atoms, and the number of rotatable bonds, among others. It also estimates the bioactivity score of ligands [13].

ADMET properties: Osiris Property Explorer

Osiris Property Explorer is an online cheminformatics tool used to evaluate the toxicity potential of designed molecular compounds. The virtual toxicity results are color-coded either in green or red. Green indicates that the molecules are safe and non-toxic, while red means the molecules are toxic and can have negative effects such as Tumorigenicity, Mutagenicity, Irritant effects, and Reproductive effects. The in silico toxicity of the designed compounds was determined by drawing the structures in the online tool [14].

Molecular docking study

Molecular docking is typically used to examine the orientation and interaction between protein and ligand. In this study, Autodock Tools 4.2(1.5.6) software was utilized to predict the binding energy of the ligands.

Preparation of target

Protein co-crystallized ligands, cofactors, and water molecules were taken out from the crystal structure using Molegro Molecular Viewer. The protein chain

was exported from Molegro Molecular Viewer in .pdb format and saved in a project work folder - destination folder. The designed molecules PD35, PD65, and PD72 showed strong attraction to the target enzyme Acetylcholinesterase and also had good internal and external checks in QSAR studies, suitable drug characteristics, and no toxicity in the computer tools. Therefore, they were chosen for laboratory synthesis.

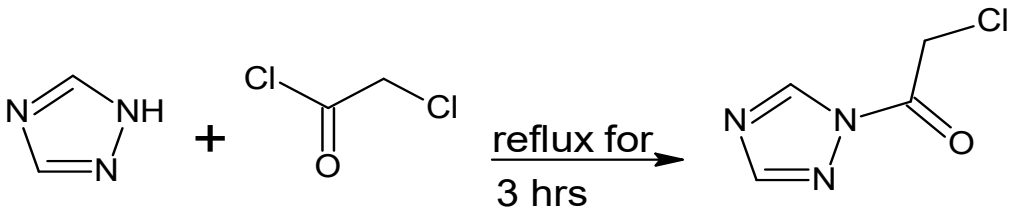
Synthetic Method

The created compounds have suitable drug characteristics and no toxicity as shown by computer methods. They also show strong attraction to the target enzymes ACHE and displayed good biological activity in QSAR studies. As a result, they were selected for laboratory synthesis.

Synthetic Scheme

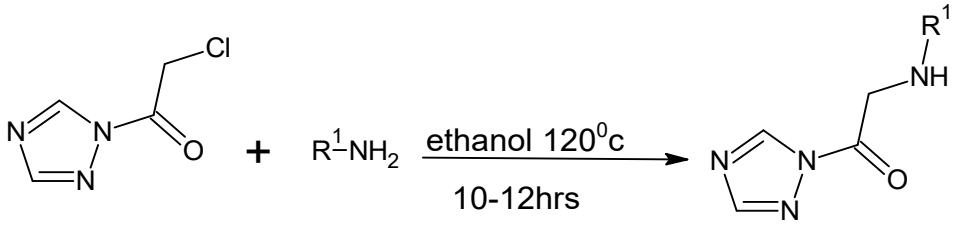
Scheme 1:

Step 1: Synthesis of 1 substituted 1,2,4 triazole derivatives.



A mixture of 1,2,4 triazole (0.5 mol) and chloroacetylchloride (2.5 mol) are combined in a 500ml round bottom flask and refluxed for 3 hours with stirring. At the completion of the reaction excess acid chloride is distilled off at atmospheric pressure. The hot (80^0c) mixture is poured into 250ml of hexane with vigorus stirring. The filtered product is filtered under suction, washed with 50ml of hexane. The precipitated product is recrystallised with ethanol [15].

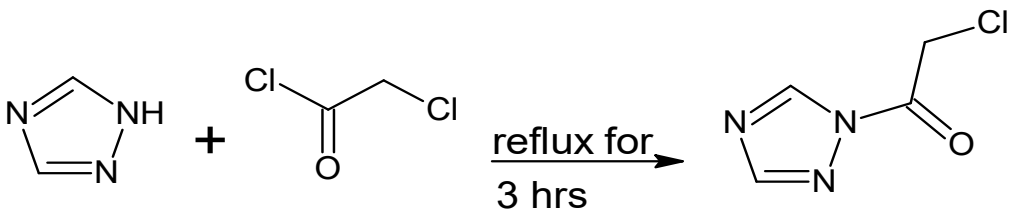
Step 2: Synthesis of amine substituted 1,2,4 triazole compound



To the compound from step1 (1.5 mmol) and the appropriate amine (1.8 mmol) was stirred under reflux in ethanol at 120°C for 10–16 h. After completion of the reaction, the solvent was evaporated under pressure. The dried residue was recrystallized from ethanol [16].

Scheme 2:

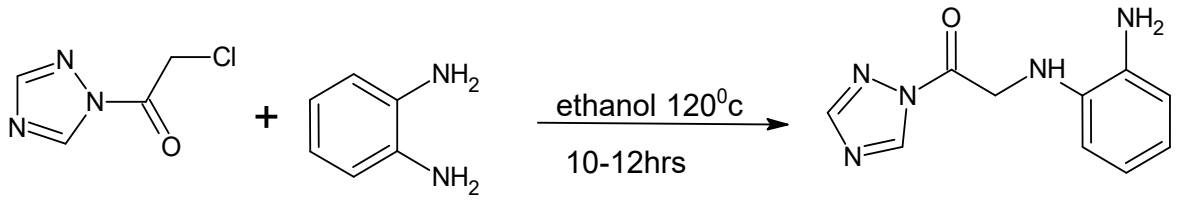
Step 1: Synthesis of 1 substituted 1,2,4 triazole derivatives.



A mixture of 1,2,4 triazole (0.5 mol) and chloroacetylchloride (2.5 mol) are combined in a 500ml round bottom flask and refluxed for 3 hours with stirring. At the completion of the reaction excess acid chloride is distilled off at atmospheric pressure. The hot (80^0c) mixture is poured into 250ml of hexane with

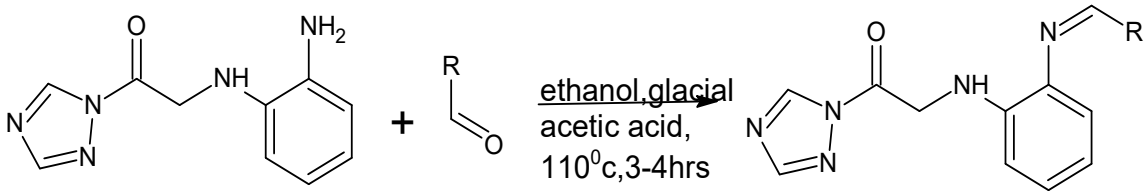
vigorus stirring. The filtered product is filtered under suction, washed with 50ml of hexane. The precipitated product is recrystallised with ethanol.

Step 2: Synthesis of o phenylenediamine substituted 1,2,4 triazole.



To the compound from step1 (1.5 mmol) and the O phenylenediamine (1.8 mmol) was stirred under reflux in ethanol at 120°C for 10–16 h. After completion of the reaction, the solvent was evaporated under pressure. The dried residue was recrystallized from ethanol [17].

Step 3: Synthesis of Schiff base



An equal amount (0.01 mole) of aromatic primary amine and chosen aldehyde were dissolved in 30 ml of ethanol, in a round-bottom flask. Then, 1.5 ml of glacial acetic acid was added to create a mild acidic environment. The mixture was heated under reflux for 3-6 hours at 110°C, and the reaction was tracked using TLC. On above the crushed ice, the resultant solution was poured. The solid that formed was filtered, dried, and recrystallized with ethanol [18].

Characterization

The purity of the made compounds was checked using the TLC method and by finding the precise melting point. Thin-layer chromatography used to observe the reaction's development. TLC was done using aluminum plates coated with Silica gel 60F 254 (E-Merck); the mobile phase was a mix of methanol and chloroform in a 9:1 ratio. Spots were seen using a UV light chamber. All the made compounds were characterized by the following methods:

UV Spectroscopy

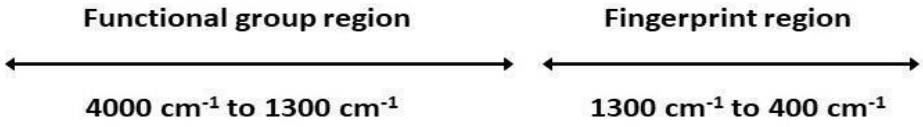
UV Spectroscopy is used to get the absorbance spectra of the compounds. These spectra can help identify compounds and measure the concentrations of colored compounds. When a molecule absorbs light at various wavelengths, the result is an absorption spectrum. A graph of absorbance versus wavelength is all that constitutes an absorption spectrum. The wavelength in the absorption spectrum with the maximum absorbance is known as the λmax. UV spectra were recorded using a UV-1900 Series UV-visible spectrometer.

IR Spectroscopy

IR spectroscopy focuses on the infrared area of the electromagnetic spectrum, which involves light with longer wavelengths and lower frequencies than visible light. Infrared Spectroscopy mainly analyzes how a molecule interacts with Infrared Light. The main purpose of infrared spectroscopy is to find the functional groups of molecules, important in both organic and inorganic chemistry. IR spectra were recorded using an ABB MB3000-PH FT-IR Spectrometer.

Regions of the infrared spectrum

Most bands that show functional groups are found between 4000 cm-1 and 1300 cm-1; these bands can be identified and used to determine the functional group of an unknown compound. Unique bands for each molecule, like a fingerprint, are found in the fingerprint region, from 1300 cm-1 to 400 cm-1. The sole purpose of these bands is to compare the spectra of various substances.



Nuclear Magnetic Resonance (NMR) Spectroscopy

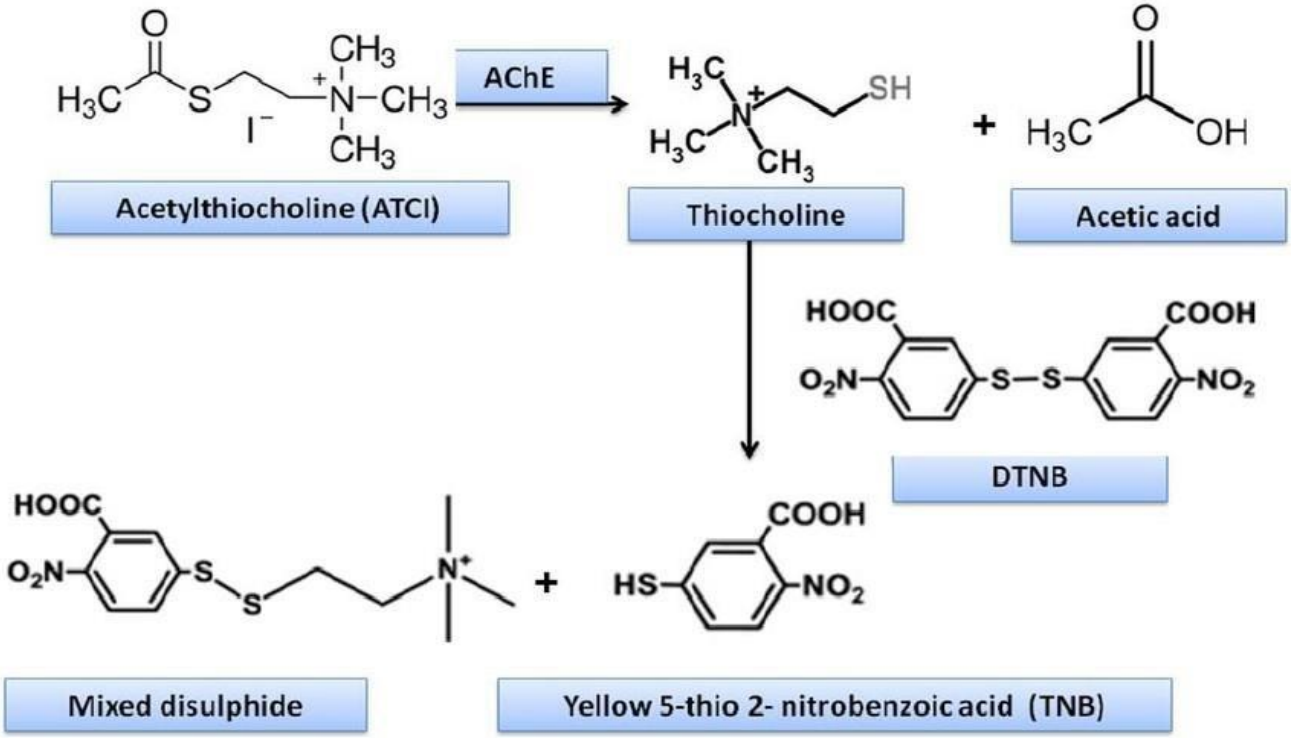
Nuclear Magnetic Resonance (NMR) spectroscopy is a method used to find out what is in a sample and how pure it is, as well as to understand its molecular structure. NMR is the strongest method for getting structural details about a compound. 1H NMR and 13C NMR spectroscopies are used to analyze all organic compounds. Proton NMR Spectroscopy helps to examine the number of similar protons and their surroundings, which helps determine the structure of a molecule. The NMR spectra were recorded on a BRUKER Topspin Advance 400 MHZ NMR Spectrometer using Deuterated DMSO as a solvent. The NMR effect is based on the fact that the nuclei of atoms have magnetic properties that can provide chemical information. The chemical shift of a certain nucleus can be linked to its chemical surroundings. The scalar coupling (or J-coupling) shows an indirect connection between individual nuclei, which is influenced by electrons in a chemical bond. Under suitable circumstances, the area of resonance relates to the number of nuclei that cause it [19].

Mass Spectrometry

Mass spectrometry is an analysis method used to find out the molecular structure and weight of the substance being studied. In this method, the compound being examined is hit with a beam of electrons, creating ionic fragments of the original molecule. The relative amount of the fragment ion produced depends on how stable the ion is and how reactive it is. The resulting charged particles are then sorted based on their masses. A mass spectrum is a record of information about various masses created and their relative quantities [20].

In vitro evaluation of anti-Alzheimer's activity

The blocking of AChE activity was measured using a 96-well microplate reader based on Ellman's method. The enzyme breaks down the alternative substrate Acetylthiocholine (ATCI) into thiocholine and acetic acid. Thiocholine then reacts with 5,5'-dithiol-bis-(2-nitrobenzoic acid) (DTNB). This reaction creates 5-thio-2-nitrobenzoate, which has a yellow color due to the movement of electrons to the sulfur atom. The color strength of the product is measured at 405 nm, and it is linked to the enzyme's activity.



Procedure

The AChE inhibition of the tested compounds was measured using a slightly changed version of Ellman's method. Rivastigmine served as a reference compound in the concentration range of 1.56-500 µg/ml. The compounds being tested were dissolved in DMSO to achieve a final concentration range of 10–5000 µg/ml in the combined solution. In brief, 0.3 mL of 100 mM sodium phosphate buffer at pH 8, 0.3 mL of the sample, and 0.3 mL of AChE solution with 0.54 U/mL were mixed in a 3 mL cuvette and let to sit for 15 minutes at 37 °C. After that, 0.3 mL of an acetylcholine iodide solution (15 mM, mixed in water) and 1.5 mL of 3 mM DTNB were added. The absorbance at 405 nm was measured. A control reaction was done using the same amount of DMSO instead of the tested solutions. The percentage of AChE inhibition was calculated based on the absorbance values as follows [21]:

$$\% \text{ Inhibition} = (1 - At/A_0) \times 100$$

Where, A_0 = absorbance of the control.
At = absorbance of the tested compound.

Result and Discussion

QSAR design

To create 2D-QSAR models for acetylcholinesterase enzyme inhibitors, we studied the structural features of drugs that contain 1,2,4 triazole. We can make several QSAR models using the 70 compounds. In this process, variables are gradually added and removed from the equation. First, the variable that is looked at for removal has the lowest partial correlation with the dependent variable; if it meets the removal criteria, it is taken out. After removing the first variable, the next variable with the lowest partial correlation coefficient that is still in the equation is considered for removal. This process continues until each variable kept leads to a clear improvement in R2. The goal is to create prediction models using a smaller set of relevant descriptors while following all QSAR methods for the anti-Alzheimer's activity of various 1,2,4 triazole derivatives against the acetylcholinesterase enzyme. One QSAR model was selected based on its statistical reliability. The factors that determine the strength of the QSAR model are predictability (external predictive ability) and robustness (cross-validated performance). For the QSAR analysis, three models were selected based on these criteria.

Model 1:
BA=7.2257-1.2849(SaasN)+0.1278(minHBint4)-0.0066(ToPoPSA)
Model 2:
BA=7.2792-1.2788(SaasN)+0.1403(minHBint4)-0.0071(ToPoPSA)
Model 3:
BA=7.6534-1.2504(SaaSN)-.2992(Lipnskifailure)-0.0074(ToPoPSA)

With a squared correlation value (R2) of 0.9227, Model 1 was shown to be a well-fitting model. Its strength or stability was confirmed through internal validation, as shown by the Q2LMO score of 0.9016 and the R2adj of 0.9164, which indicate how easy it is to add new molecular descriptors. The model does not suffer from overfitting, as shown by the LOF value of 0.1308. A low Kxx value also indicates a weak connection between the descriptors and confirms that there are no unnecessary descriptors. The R2Yscr values of 0.0761 indicate that the developed model was not just a coincidence. The created model has strong predictive ability, as demonstrated by the Q2-F1, Q2-F2, and Q2-F3 values being over 0.85 and the CCC value being above 0.85. Each training and test compound was placed within the applicable area of the William plot. Every compound had a leverage value that was lower than the warning leverage. This indicates a good chance that the observed and predicted values will match up.

Statistical criteria

Statistical criteria for the developed model are shown in table no.6, which provides a collection of different statistical features for the created QSAR predictive models.

Table 1: statistical criteria for the developed model

Statistical parameters	Threshold value	Model 1	Model 2	Model 3
Fitting criteria				
R2	>0.6	0.9227	0.9208	0.9190
S	<0.3	0.3249	0.3423	0.3464

R2adj	>0.6	0.9164	0.9144	0.9124
R2- R2adj	<0.3	0.0063	0.0064	0.0066
LOF	<0.3	0.1308	0.1451	0.1486
RMSEtr	Better <0.3	0.3087	0.3252	0.3291
CCCtr	>0.85	0.9598	0.9588	0.9578
F	Higher than the theoretical value	147.1505	143.4856	139.8472
Internal validation criteria				
Q2loo	>0.5	0.9065	0.9052	0.9052
R2-Q2LOO	<0.3	0.0162	0.0157	0.0138
Q2LMO	>0.5	0.9016	0.9008	0.9027
RMSEcv	<0.3	0.3394	0.3559	0.3559
R2Yscr	<R2[smallest is better]	0.0761	0.0747	0.0740
Q2Yscr	<Q2[smallest is better]	-0.1381	-0.1414	-0.1381
CCCcv	>0.85	0.9514	0.9508	0.9506
External validation criteria				
R2ext	>0.6	0.9257	0.9172	0.9370
RMSEext	<0.3	0.3603	0.2613	0.2595
Q2-F1	>0.7	0.9193	0.9256	0.9266
Q2-F2	>0.7	0.9159	0.9142	0.9153
Q2-F3	>0.7	0.9239	0.9489	0.9496
CCCext	>0.85	0.9587	0.9576	0.9609
r2m	>0.5	0.8910	0.8790	0.8902
Δr2m	<0.2	0.9952	0.9950	0.9957
K1	0.85<K (or) K1<1.15	0.9952	0.9950	0.9957
K	0.85<K (or) K1<1.15	0.9999	0.9999	0.9992

The physicochemical properties of designed compounds were predicted using the PaDEL descriptor. Their Anti-Alzheimer's activity against acetylcholinesterase enzyme was predicted using the generated QSAR models. When compared to the most active chemical in the original data set, some of the newly created compounds showed substantial inhibitory activity. The most active compound in the original data set had an IC50 value of 0.9. Its pIC50 value was found to be 6.044312.

Table 2: Predicted Pic50 of the designed compounds.

Name	SaasN	minHBint4	TopoPSA	Pic50
PD35	1.233906	0	72.7	5.160434
PD65	1.209514	0	59.81	5.27685
PD72	0	0	16.77	7.115018

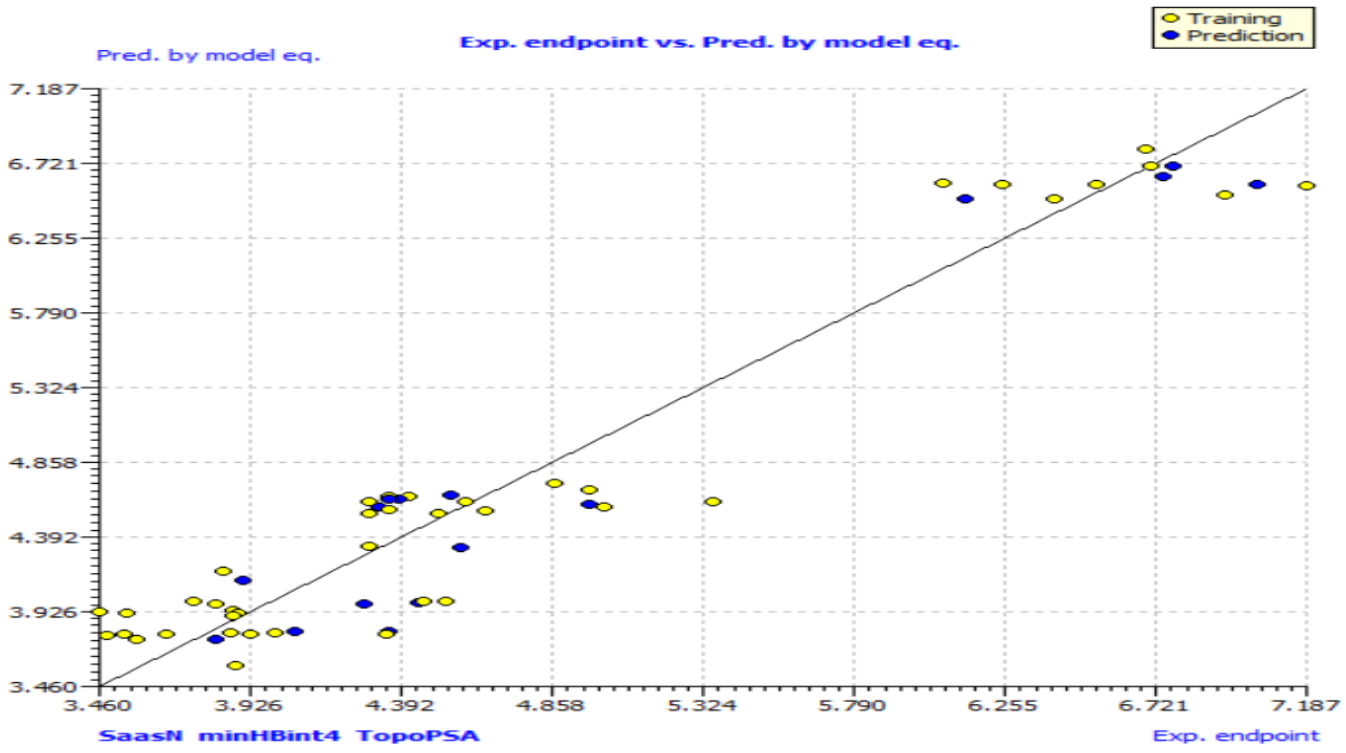


Figure 3: Plot of Standardized residuals against experimental activities of the compounds.

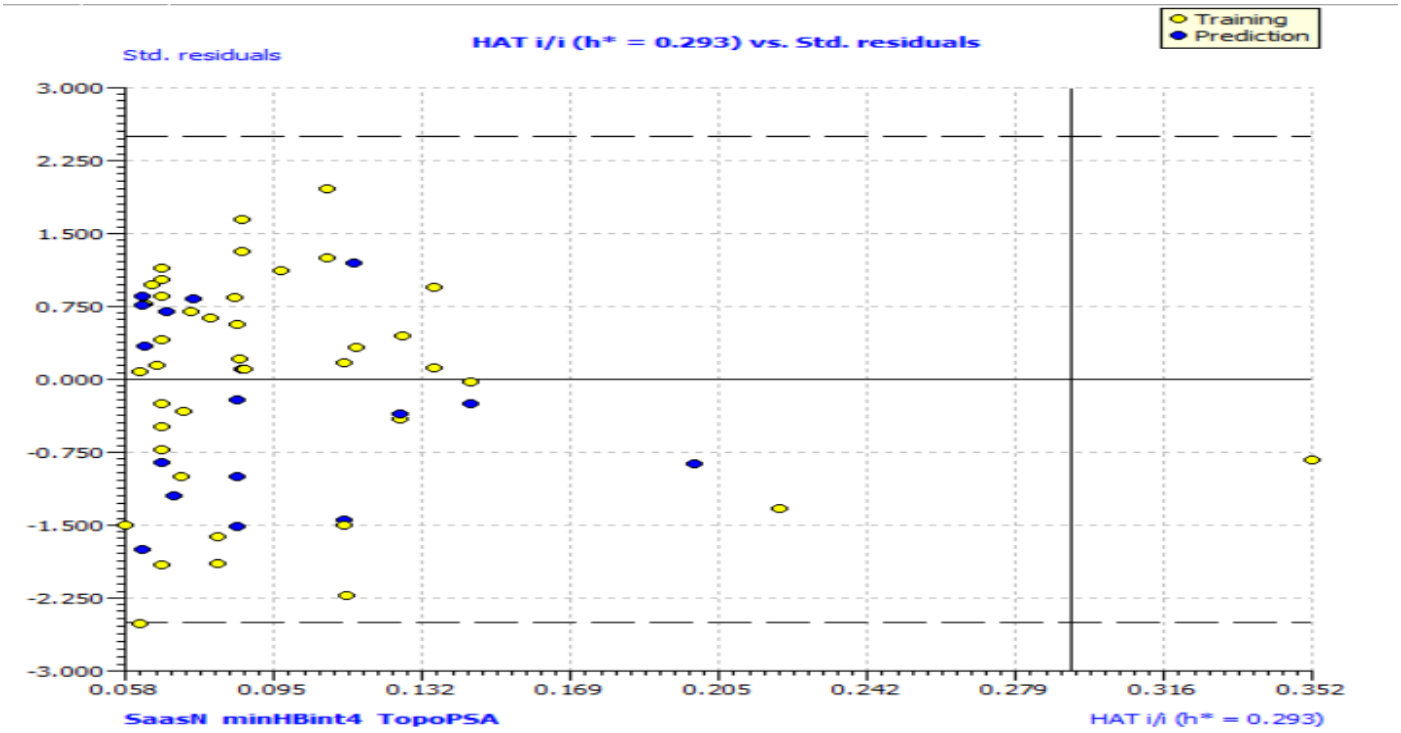


Figure 4: William's plot of the best model. the dashed lines are the cut-off 3_ and the warning value of hat (h*, 0.293).

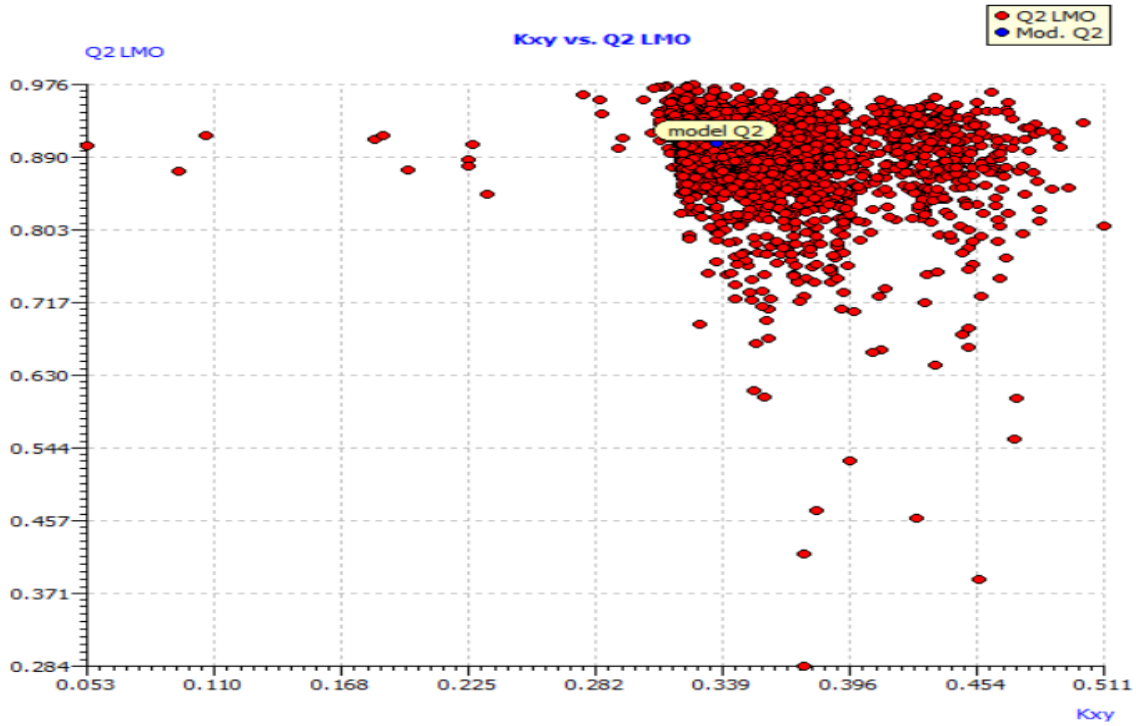


Figure 5: Scatter plot of visualization of a dataset of LMO validation.

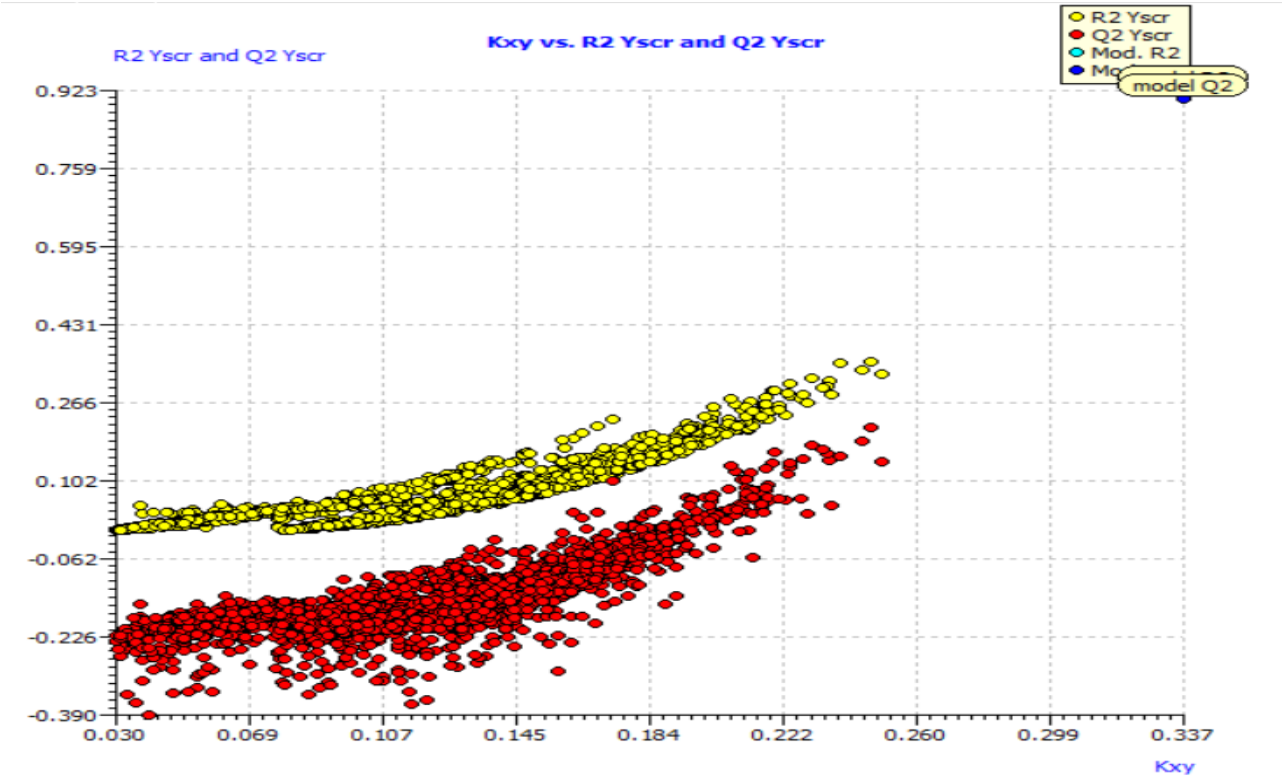
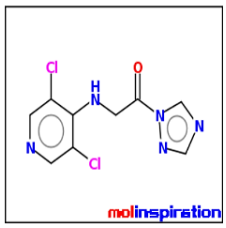
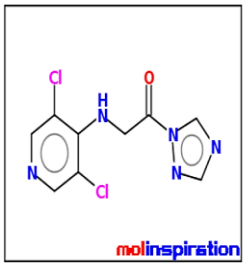
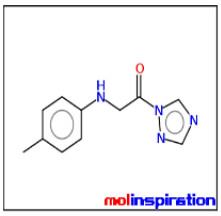
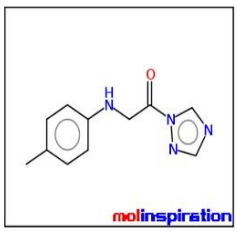
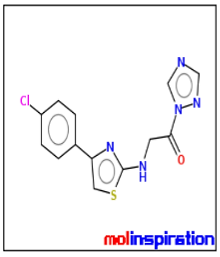
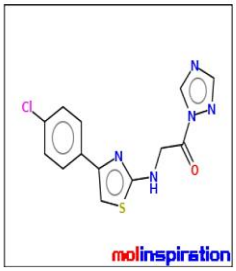


Figure 6: Layout of Y-scramble method.

In-silico drug-likeness property

In silico drug-likeness properties software is used to predict the molecular physiochemical properties and to calculate the bioactivity score to find out whether the drug is orally bioavailable.

Table 3: *In-silico* drug likeliness property of the top scored compounds.

Sample code	Molecular properties	Bioactive properties
PD35	miSMILES: O=C(CNc1c(Cl)cncc1Cl)n2cncn2  Molinspiration property engine v2022.08 miLogP 1.18 TPSA 72.71 natoms 17 MW 272.10 nON 6 nOHNH 1 nviolations 0 nrotb 3 volume 203.61 Get data as text (for copy / paste).	miSMILES: O=C(CNc1c(Cl)cncc1Cl)n2cncn2  Molinspiration bioactivity score v2022.08 GPCR ligand -0.18 Ion channel modulator -0.44 Kinase inhibitor -0.19 Nuclear receptor ligand -0.85 Protease inhibitor -0.79 Enzyme inhibitor 0.03 Get data as text (for copy / paste). Get 3D geometry BETA
PD65	miSMILES: Cc2ccc(NCC(=O)n1cncn1)cc2  Molinspiration property engine v2022.08 miLogP 1.27 TPSA 59.82 natoms 16 MW 216.24 nON 5 nOHNH 1 nviolations 0 nrotb 3 volume 197.25 Get data as text (for copy / paste).	miSMILES: Cc2ccc(NCC(=O)n1cncn1)cc2  Molinspiration bioactivity score v2022.08 GPCR ligand -0.44 Ion channel modulator -0.63 Kinase inhibitor -0.54 Nuclear receptor ligand -1.07 Protease inhibitor -0.84 Enzyme inhibitor -0.34 Get data as text (for copy / paste). Get 3D geometry BETA
PD72	miSMILES: O=C(CNc2nc(c1ccc(Cl)cc1)cs2)n3cncn3  Molinspiration property engine v2022.08 miLogP 2.23 TPSA 72.71 natoms 21 MW 319.78 nON 6 nOHNH 1 nviolations 0 nrotb 4 volume 252.19 Get data as text (for copy / paste).	miSMILES: O=C(CNc2nc(c1ccc(Cl)cc1)cs2)n3cncn3  Molinspiration bioactivity score v2022.08 GPCR ligand -0.14 Ion channel modulator -0.56 Kinase inhibitor -0.11 Nuclear receptor ligand -1.01 Protease inhibitor -0.55 Enzyme inhibitor -0.19 Get data as text (for copy / paste). Get 3D geometry BETA

Osiris toxicity explorer®

Osiris Toxicity Explorer® software is used to predict whether the designed molecules are toxic or not. The green color indicates that the molecules are safe and nontoxic and the red color indicates that the molecules are toxic.

Table 4: *In-silico* drug toxicity by Osiris toxicity explorer.

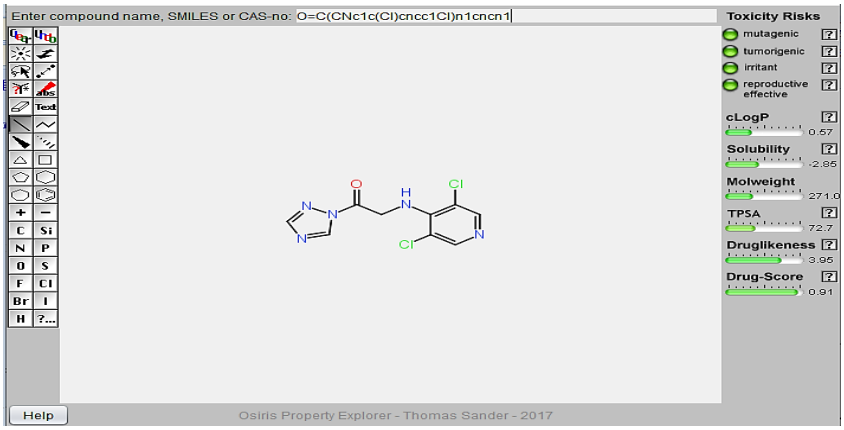
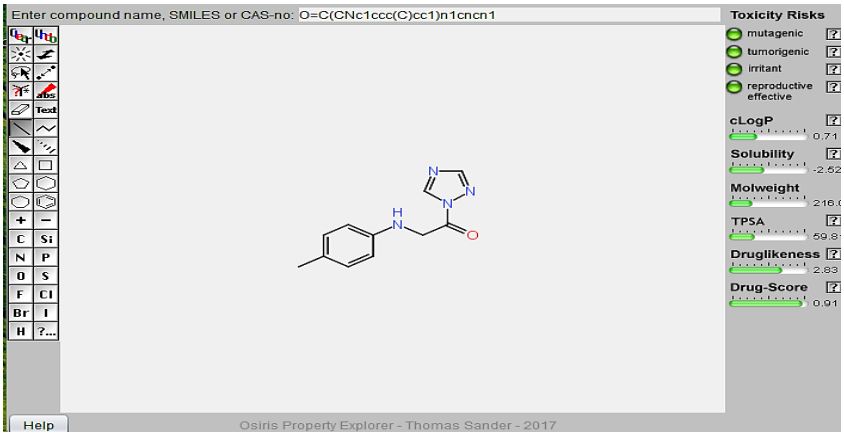
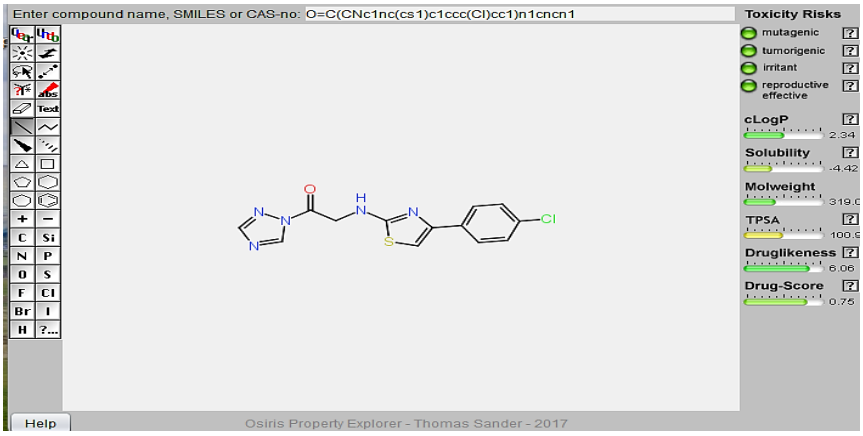
Sample code	Toxicity prediction
PD35	
PD65	
PD72	

Table 5: Physicochemical and ADMET properties.

Physicochemical and ADMET properties	PD35	PD65	PD72
Mutagenic	No	No	No
Tumorigenic	No	No	No
Irritant	No	No	No
Reproductive effective	No	No	No
LogP	0.57	1.27	2.23
Solubility	-2.85	-2.52	-4.42
Molecular weight	271g/mol	216g/mol	319g/mol
Blood-Brain Barrier	Yes	yes	Yes
HBA	6	5	6
HBD	1	1	3
No rotatable bonds	3	4	3

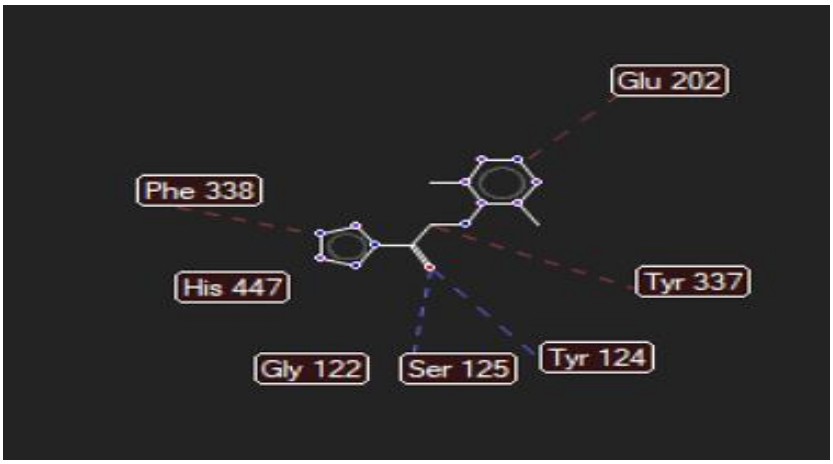
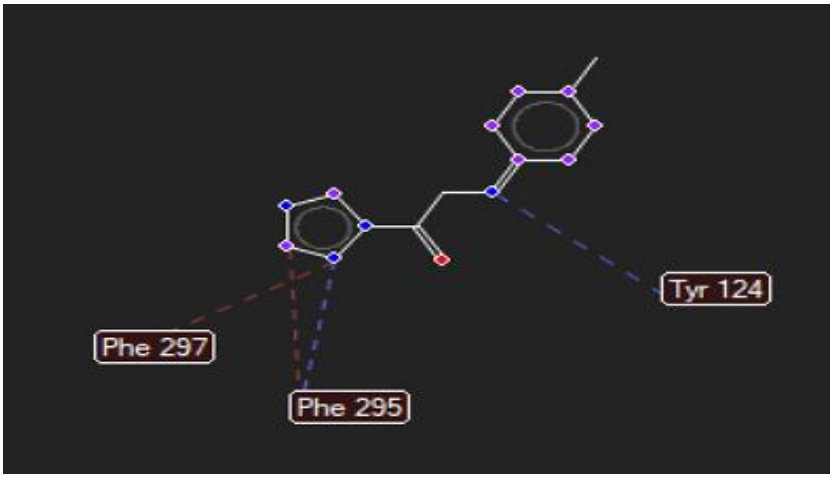
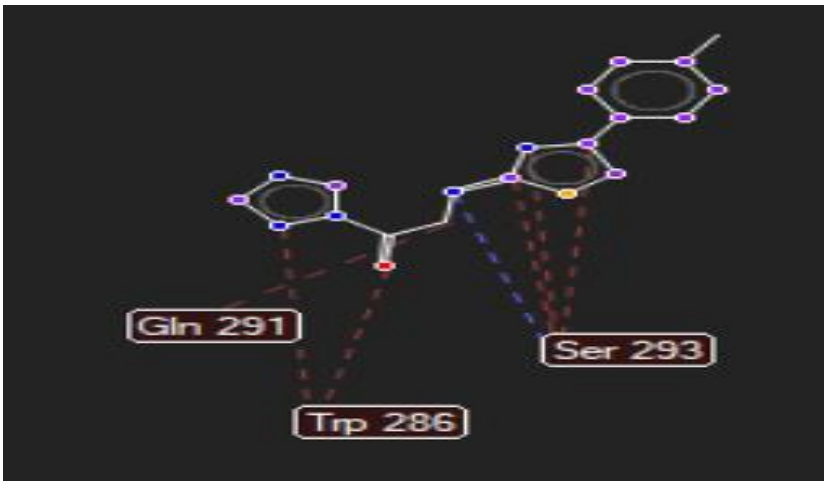
Docking studies

The docking study is intended to determine the optimum position for the ligand (benzimidazole derivative) within the receptor-binding site (4EY7) using the (PDB) protein databank at a resolution of 2.35Å. The docked ligands interacted effectively with the receptor's active sites, with binding affinities varying from -6.91 to -8.97 kcal/mol. They all exhibit significant hydrogen bonding and hydrophobic interactions with the protein's amino acids. Results can be assessed with the Discovery Studio 2021 Client program. Visual analysis is crucial when assessing the program's performance.

Table 6: Docking scores of the top scored compounds.

S. No	Sample Code	Binding Energy
1	PD35	-7.88
2	PD65	-7.47
3	PD72	-8.97

Table 7: Binding interaction of the top scored compounds.

Ligand	Binding Interaction
PD35	
PD65	
PD72	

Synthesis

For the selected 3 compounds the synthetic scheme was developed. Then they were synthesized using appropriate procedures and purified by recrystallization. The melting point and TLC were performed to check purity.

Characterization

UV Spectrum of PD35

Table 8: Wavelength and absorbance of PD35.

S. No.	Wavelength (nm).	Absorbance
1	354.0	-0.004
2	278.5	0.100
3	248.5	0.366
4	211.0	1.252

λmax value of the compound PD 35 was found to be 248.5nm

IR Spectrum of PD35

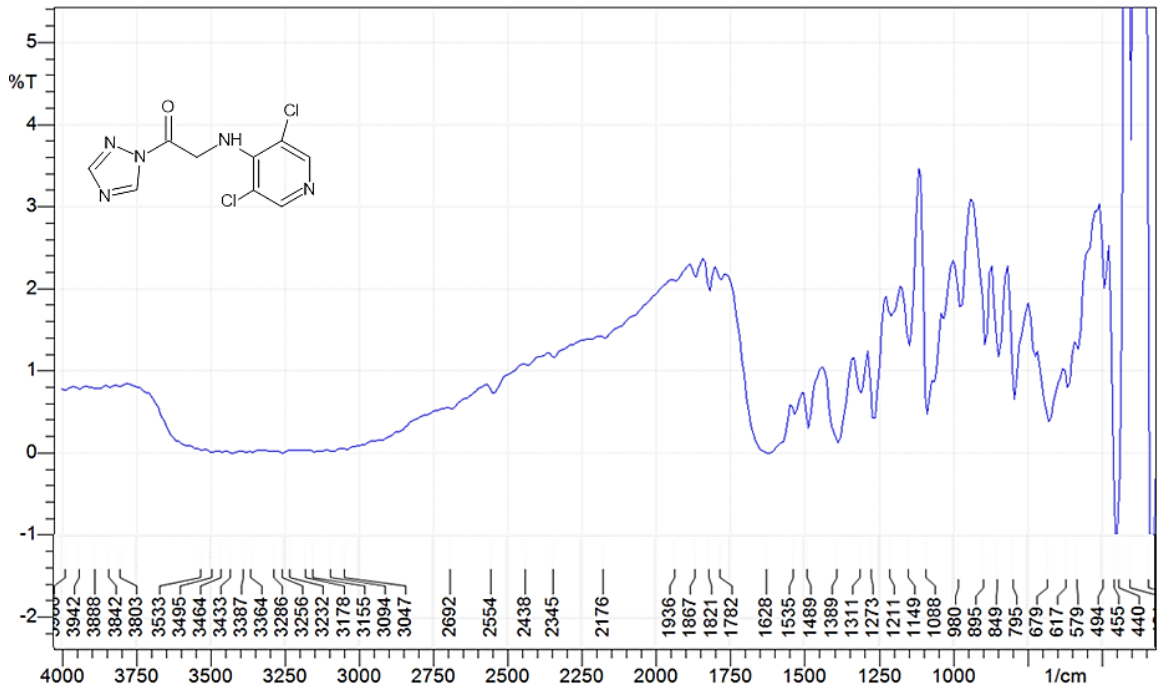


Figure 7: IR interpretation of compound PD35

Table 9: IR interpretation of compound PD35.

S. No	Wavelength (Cm-1)	Functional Group
1	1535	C=C
2	1489	C=N
3	1628	C=O
4	3433	NH
5	795	C-Cl

NMR Spectrum of compound PD35

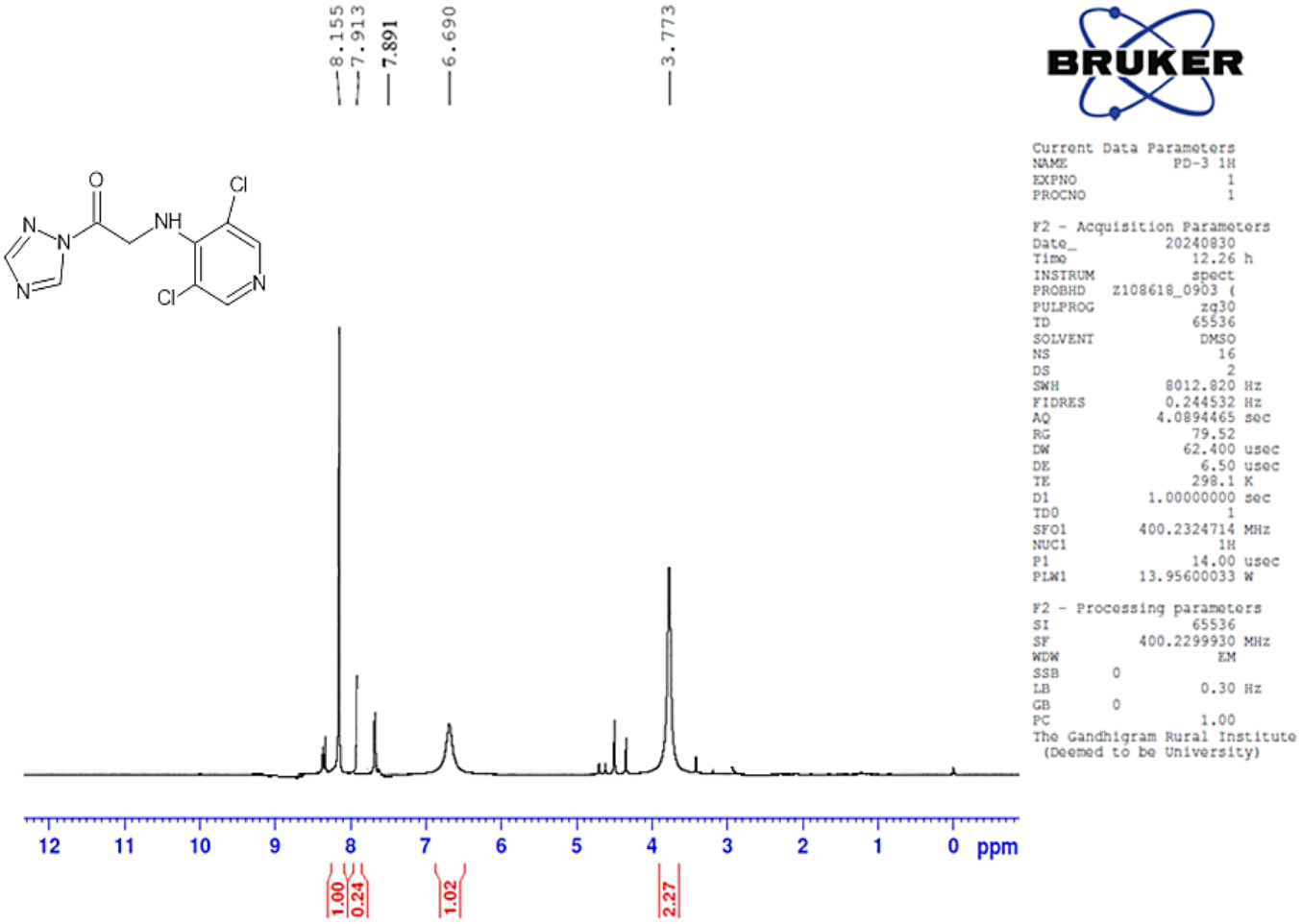


Figure 8: NMR spectra of compound PD 35.

Table 10: NMR interpretation of compound PD35.

S. No	Delta Value (Ppm)	Nature of Proton	Nature of Peak	Number of Hydrogens
1	6.690	NH	singlet	1
2	7.891	Aromatic (C-H) 1,2,4 triazole	singlet	2
3	8.155	Aromatic (C-H) pyridine	singlet	2
4	3.773	methylene	singlet	2

Mass Spectrum of Compound PD35

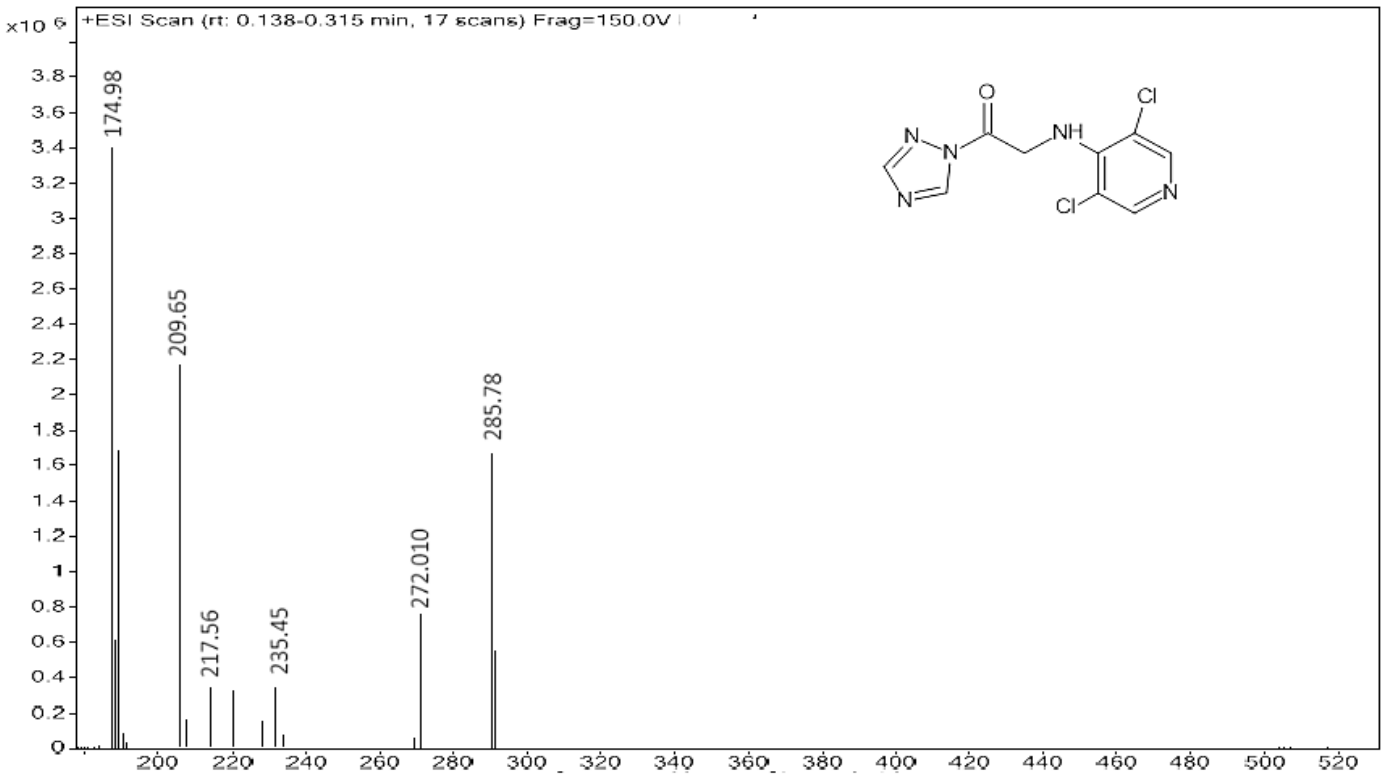


Figure 9: Mass spectrum of the compound PD 35.

The molecular weight of the compound was found to be PD35 272.01

UV Spectrum of the Compound PD65

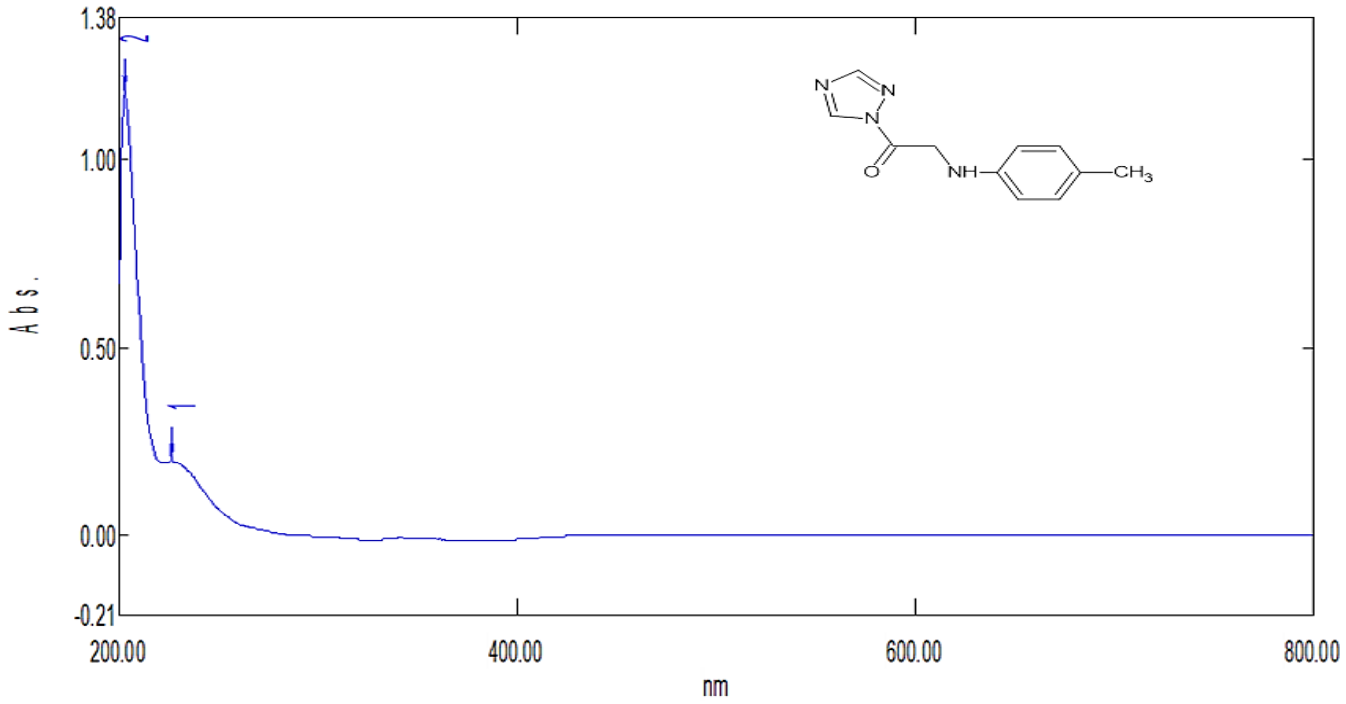


Figure 10: UV spectrum of the compound PD65.

Table 11: UV spectrum of the compound PD65

S. No.	Wavelength(nm)	Absorbance
1	226.5	0.198
2	203.0	1.180

λmax value of the compound PD 65 was found to be 226.5

IR spectrum of the compound PD65

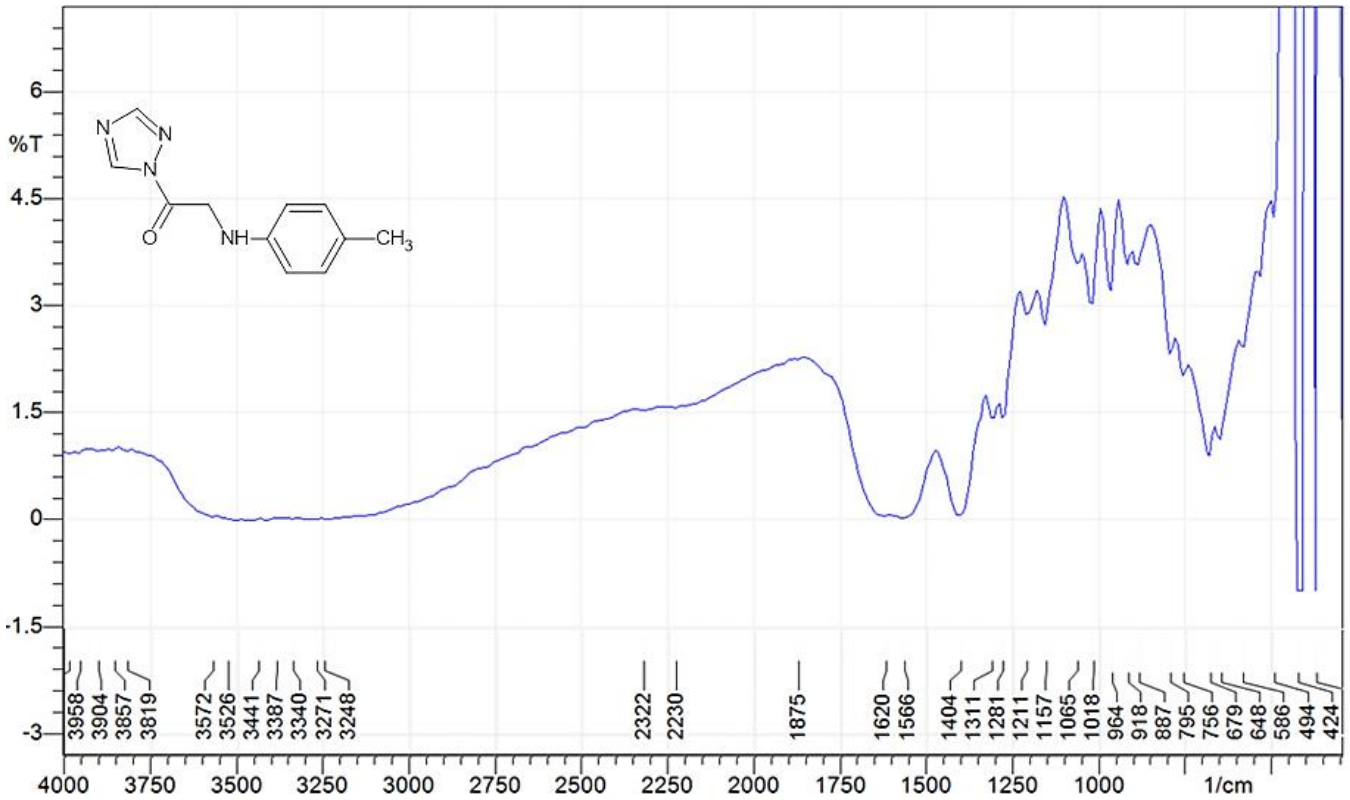


Figure 11: IR spectrum of the compound PD65.

Table 12: IR interpretation of the compound PD65.

S. No	Wavelength (Cm-1)	Functional Group
1	1620	C=C
2	1566	C=N
3	1620	C=O
4	3441	NH (amine)
5	1404	CH3(C-H)

NMR spectrum of the compound PD65

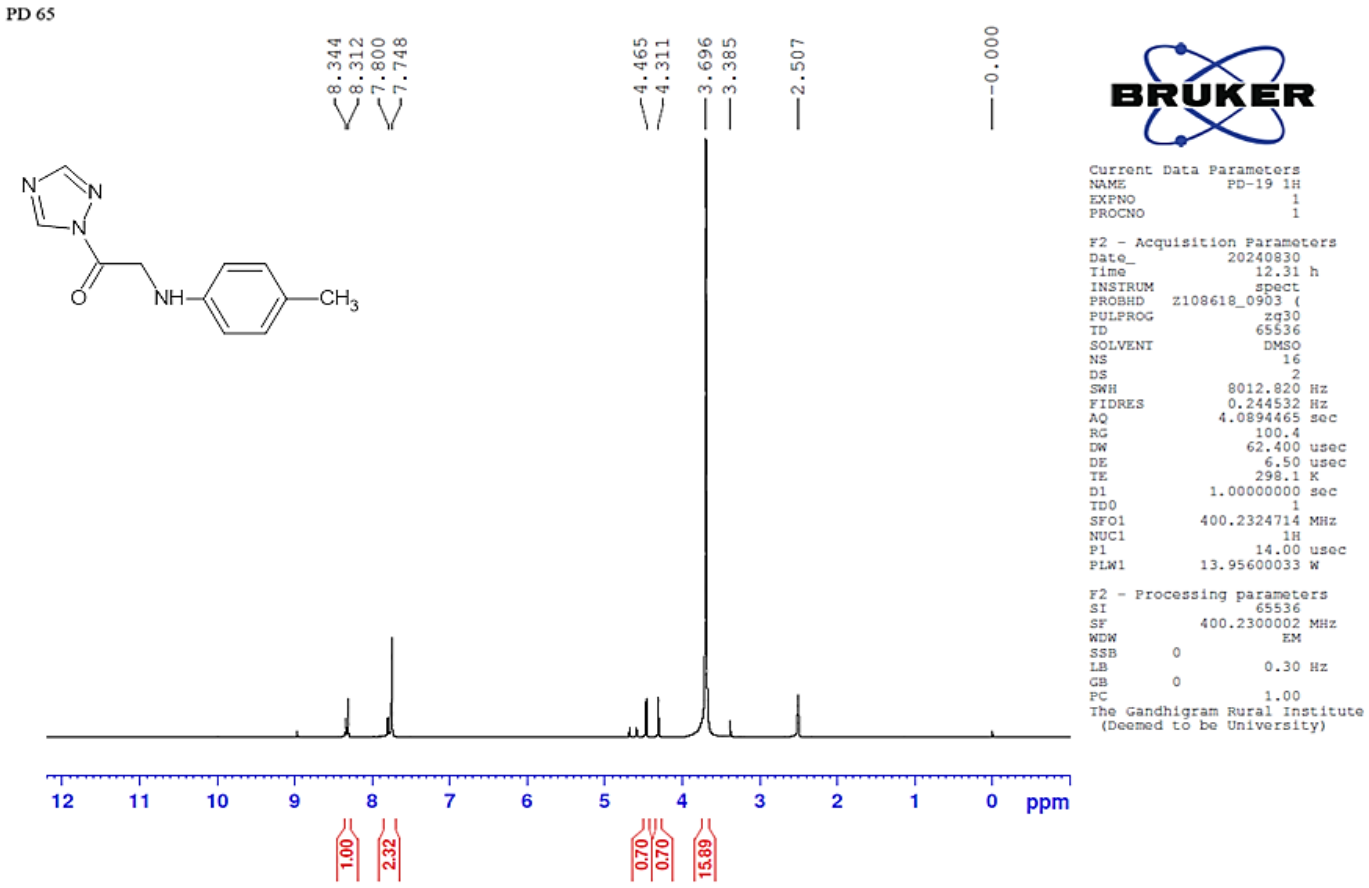


Figure 12: NMR spectrum of the compound PD65.

Table 13: NMR interpretation of the compound PD65.

S. No	Delta Value (Ppm)	Nature of Proton	Nature of Peak	Number of Protons
1	8.334	Aromatic C-H	Singlet	2
2	4.465	NH	Singlet	1
3	4.311	CH2	Singlet	2
4	7.7-7.800	Aromatic C-H	Doublet	4
5	2.507	CH3	singlet	3

MASS spectrum of the compound PD65

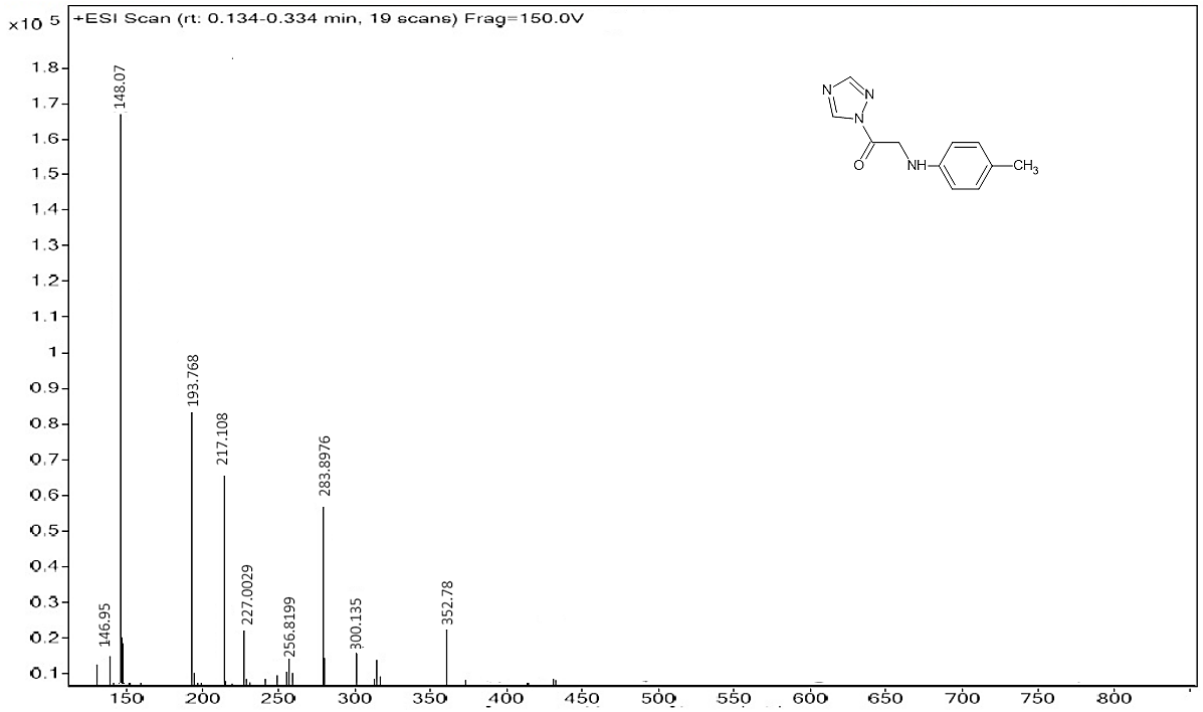


Figure 13: Mass spectrum of the compound PD65.

The molecular weight of the compound was found to be 217.108

UV spectrum of the compound PD 72

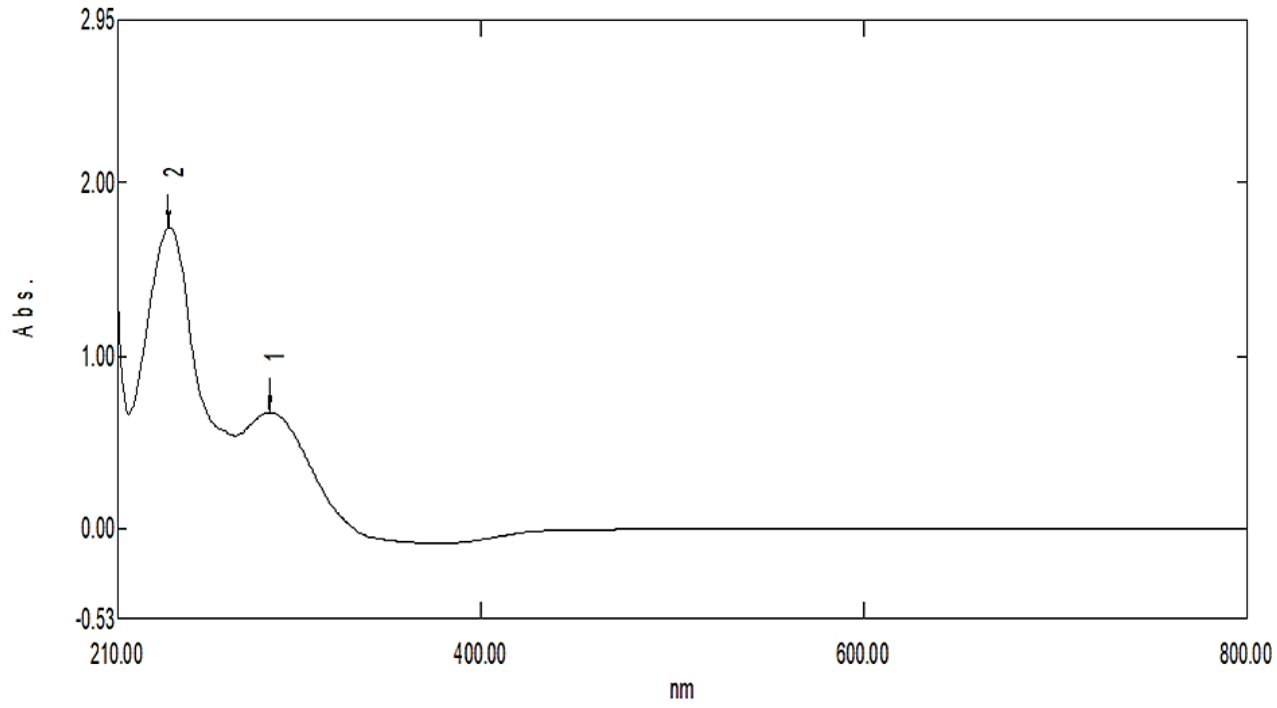


Figure 14: UV spectrum of the compound PD72.

Table 14: UV spectrum of the compound PD72.

S. No.	Wavelength (nm)	Absorbance
1	289.5	0.673
2	236.5	1.744

λmax value of the compound PD 35 was found to be 0.673

IR Spectrum of the Compound PD72

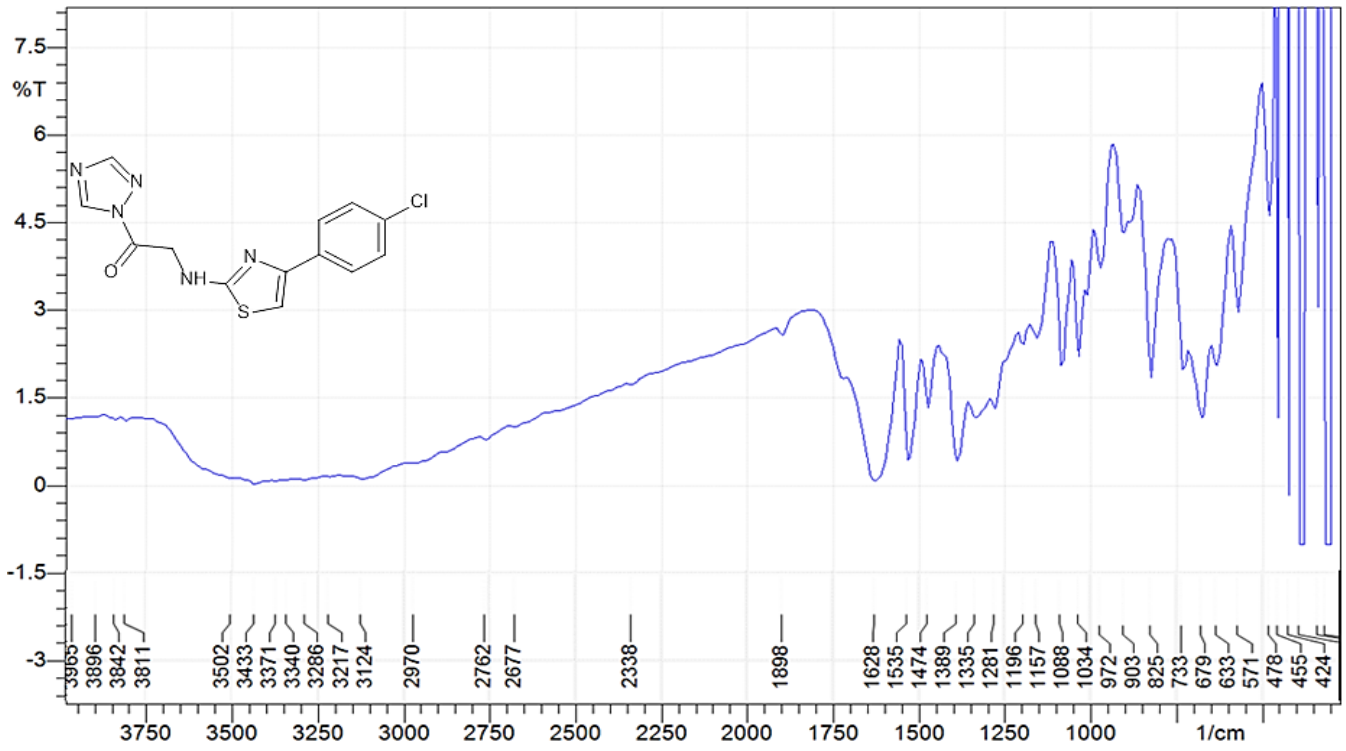


Figure 15: IR spectrum of the compound PD72.

Table 15: IR interpretation of compound PD72.

S. No.	Wave Number (Nm)	Functional Group
1	1474	C=C
2	3124	C-H
3	1535	C=N
4	1628	C=O
5	3433	NH(AMINE)
6	733	C-Cl
7	825	C-S

NMR Spectrum of the Compound PD72

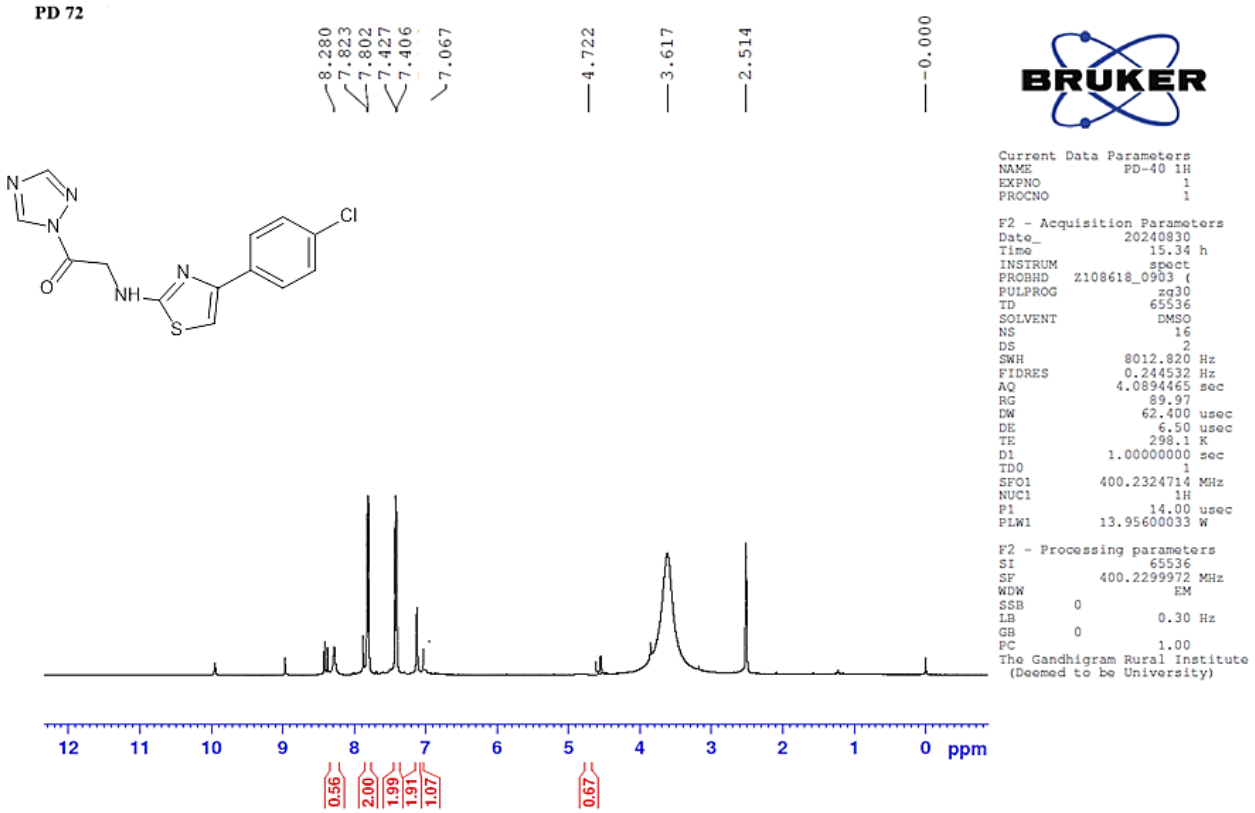


Figure 16: NMR spectrum of the compound PD 72.

Table 16: NMR interpretation of the compound PD72.

S. No	Delta Value (Ppm)	Nature of Proton	Nature of Peak	Number of Proton
1	7.4-7.8	Aromatic (C-H) benzene	Doublet	4
2	4.7	N-H	singlet	1
3	7.06	Aromatic (C-H) thiazole	singlet	1
4	8.28	Aromatic (C-H)1,2,4 triazole	singlet	2
5	3.6	methylene	singlet	2

Mass Spectrum of the Compound PD72

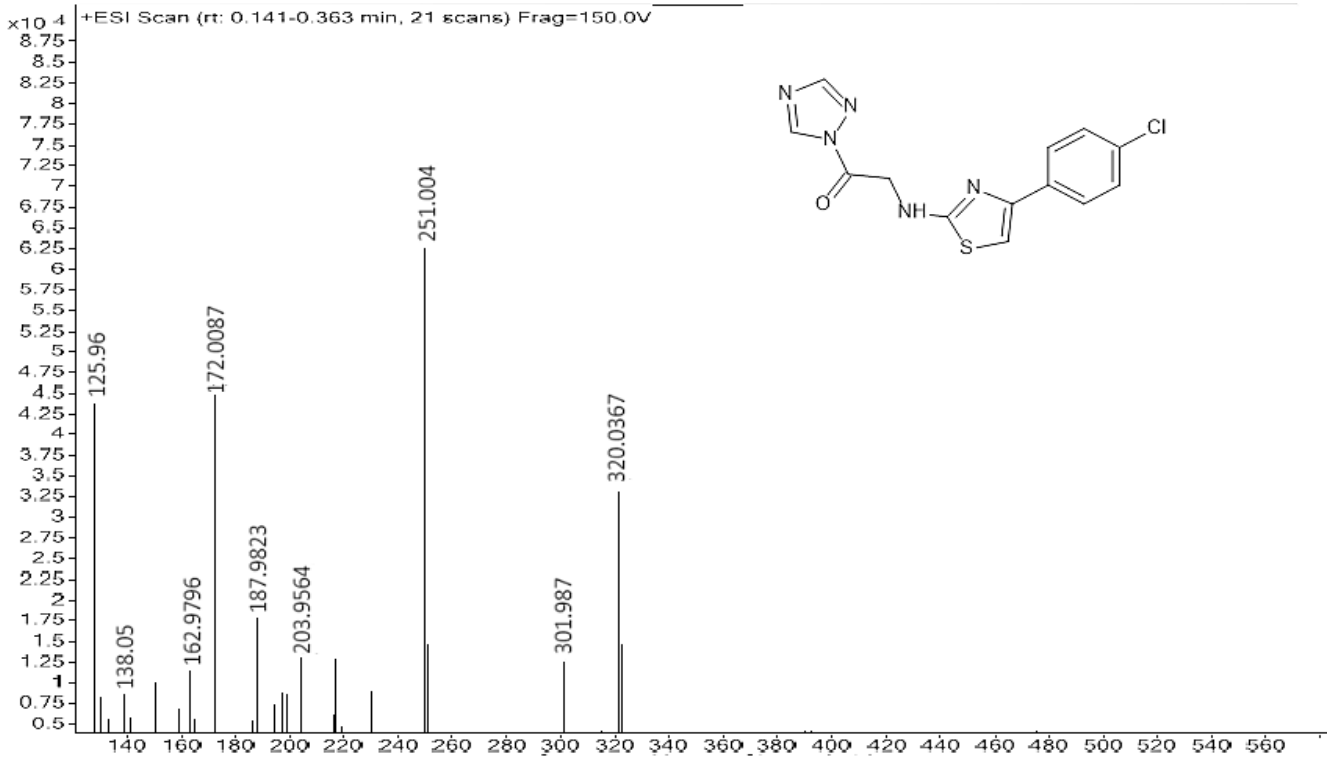


Figure 17: Mass spectrum of the compound PD72.

The molecular weight of the compound was found to be 320.0367

In-vitro evaluation of anti-alzheimer's activity

The % cell viability and IC50 value of compounds PD35, PD65, PD72 and the standard Rivastigmine against Acetylcholinesterase enzyme have been determined by Ellman's Assay method.

Table 18: Percentage inhibition standard on Acetylcholinesterase enzyme.

S. No.	Standard Concentration (µg/mL)	Inhibition percentage (%)
1	2	41.79
2	4	64.07
3	8	68.93
4	16	73.56
5	32	75.92
6	64	82.71
7	128	88.69
8	256	93.39
9	IC50	1.67

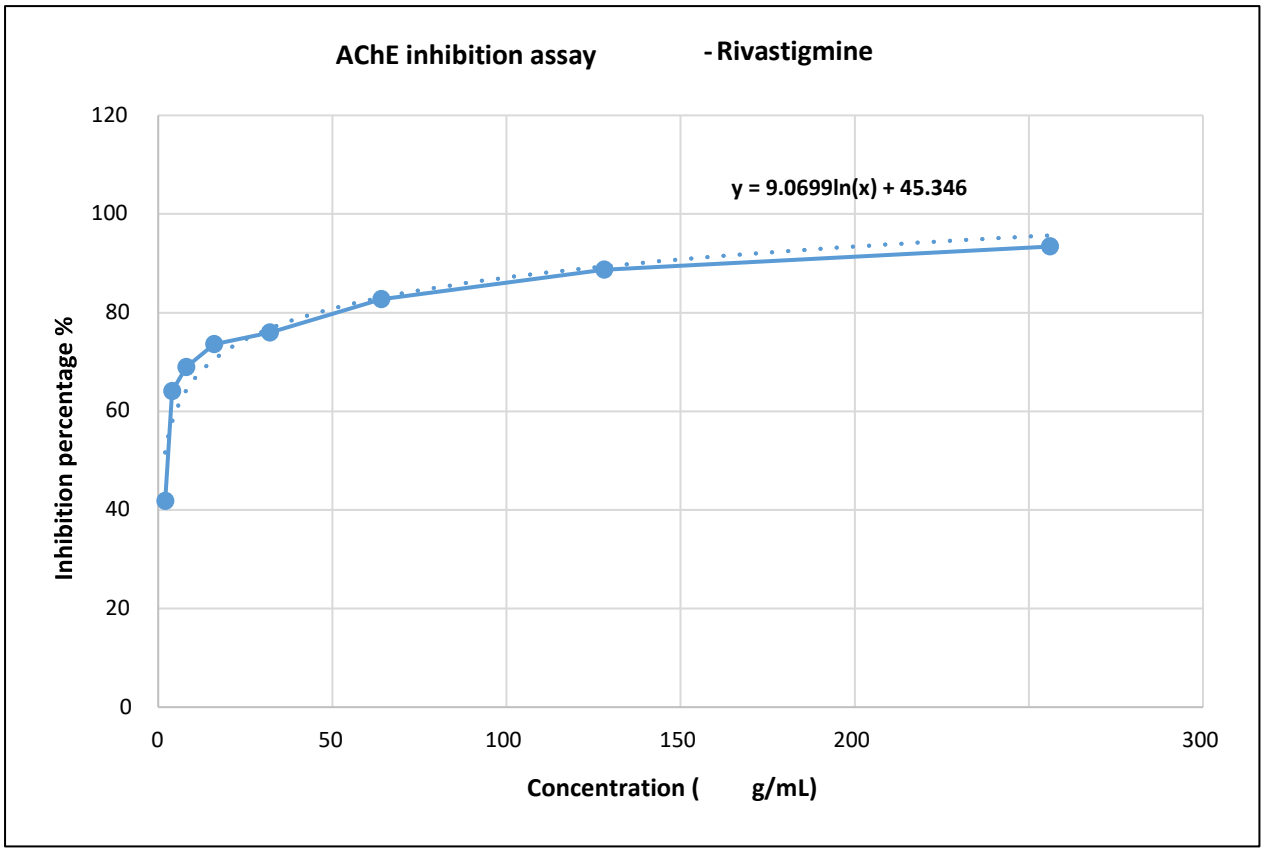


Figure 18: Graphical representation of AChE Inhibition assay-Rivastigmine.

Table 19: Percentage inhibition of PD35 on Acetylcholinesterase enzyme.

S. No.	PD-35 Concentration (µg/mL)	Inhibition percentage %
1	2	1.82
2	4	20.32
3	8	32.48
4	16	42.70
5	32	54.38
6	64	66.12
7	128	76.88
8	256	89.14
9	IC50	25.42

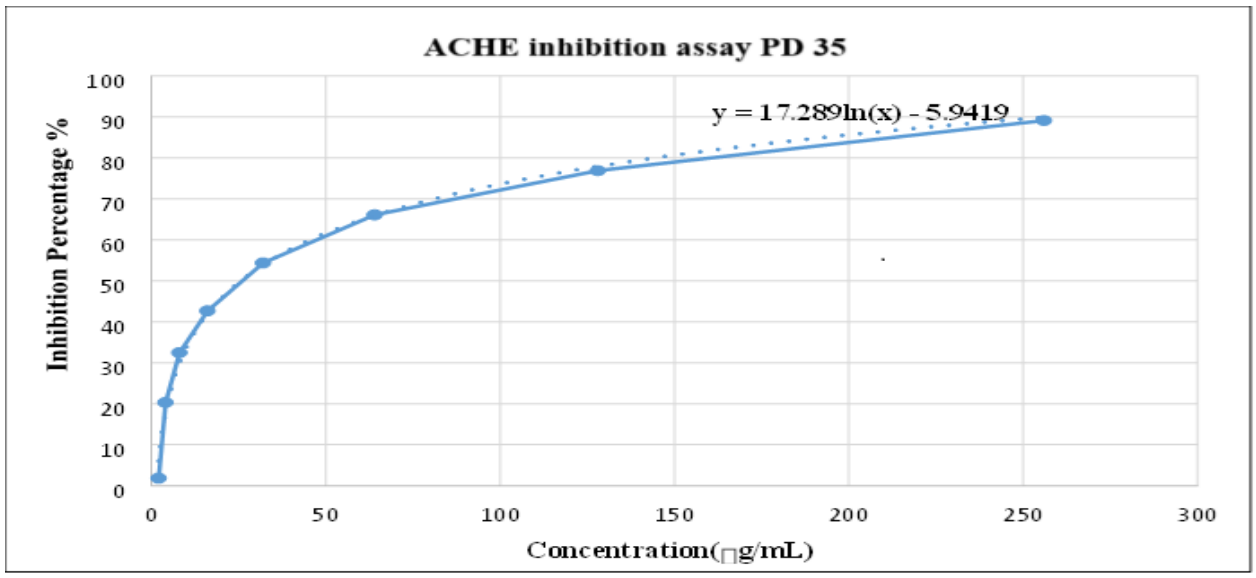


Figure 19: Graphical Representation of AChE Inhibition assay-PD35.

Table 20: Percentage inhibition of PD65 on Acetylcholinesterase enzyme.

S. No.	PD-65Concentration (µg/mL)	Inhibition percentage %
1	2	3.92
2	4	12.13
3	8	37.04
4	16	46.75
5	32	60.94
6	64	74.19
7	128	87.35
8	256	91.14
9	IC50	20.71

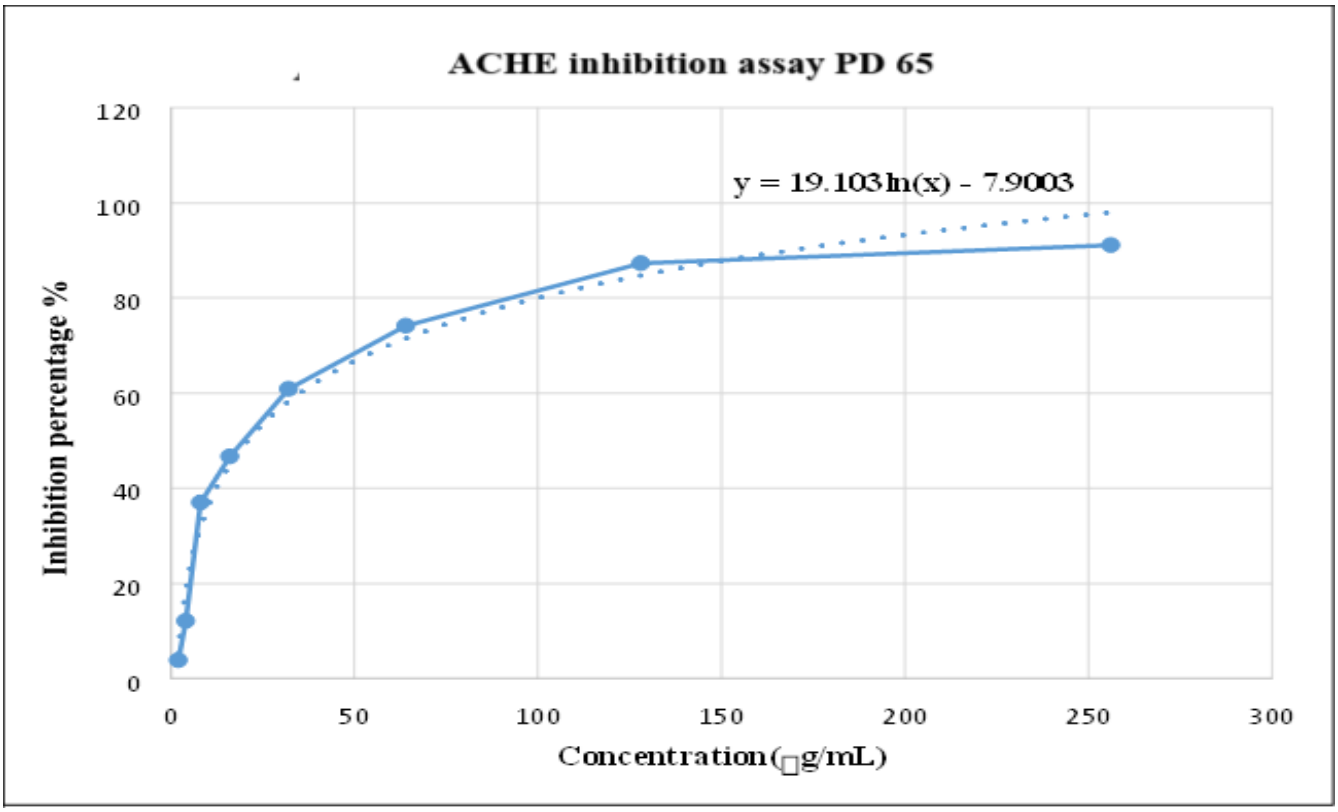


Figure 20: Graphical Representation of AChE Inhibition assay-PD65.

Table 21: Percentage inhibition of PD72 on Acetylcholinesterase enzyme.

S.NO.	PD-72 Concentration (µg/mL)	Inhibition percentage %
1	2	6.72
2	4	20.09
3	8	27.56
4	16	38.37
5	32	49.27
6	64	59.67
7	128	77.45
8	256	81.71
9	IC50	19.06

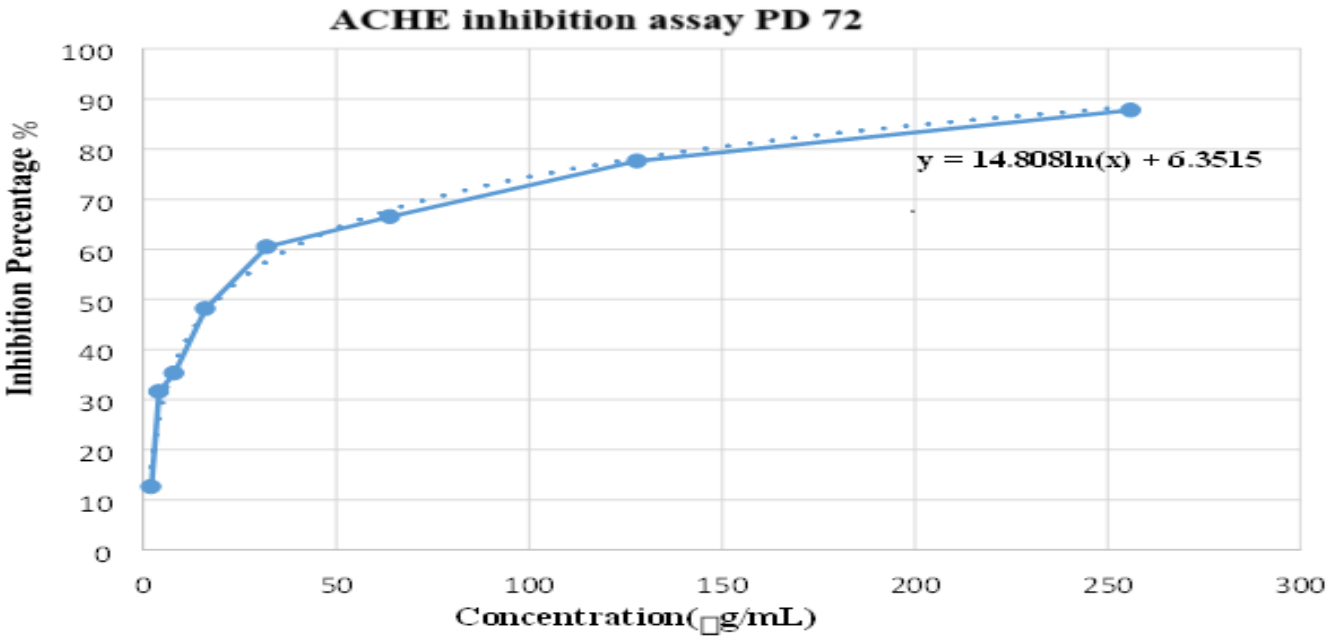


Figure 21: Graphical Representation of ACHE Inhibition assay-PD72.

Table 22: IC50 values of top-scored compounds.

S. No.	Sample	IC50 VALUE (mg/mL)
1	Standard (Rivastigamine)	1.67
2	PD-35	25.42
3	PD-65	20.71
4	PD-72	19.06

Summary

Computational drug discovery aims to produce a novel molecule that potentially prevents or treats a disease or infection. Alzheimer's is the most fatal neurodegenerative disorder, according to WHO reports. Therefore, the current study aimed to design and synthesize some newer anti-Alzheimer agents. Based on the literature review, Acetylcholinesterase was chosen as the effective target for anti-Alzheimer therapy. Reviewing the literature and with the knowledge of available inhibitors and their interaction with enzymes, a scaffold library has been constructed with 159 newly designed ligands. The QSAR model for Acetylcholinesterase enzyme was able to show stability and predictive power, confirmed by internal and external validation. The ligands were subjected to docking studies using the software Auto dock 4.2 (1.5.6) to find better AChE inhibitors. The ligands were further optimized by performing drug-likeness screening such as Lipinski rule of five and prediction of good ADMET properties using online tools like Molinspiration and in silico toxicity study using Osiris property explorer. All the designed ligands were found to be non-toxic and can be orally bioavailable.

Based on a high docking score and synthetic feasibility, five leads were selected for synthesis. All five leads were synthesized, and the purity of the molecules was assessed by TLC and melting point. Characterization of the compounds was carried out by analytical technique using UV Spectroscopy, IR Spectroscopy, 1H NMR Spectroscopy, and Mass Spectroscopy. The Compound PD35, PD65, PD72 with optimum docking scores were selected for further in vitro studies. In vitro Acetylcholinesterase inhibition assay was performed

and inhibitory concentration was determined. The IC50 value was found to be 25.42µg, 20.71µg, and 19.06 µg which was found to be almost potent with the existing standard Rivastigmine.

Conclusion

The present study gives insights into various heterocycles like 1,2,4 triazole, benzimidazole, piperidine, and piperazine as potent anti-Alzheimer agents inhibiting the AChE enzyme. The QSAR model linking the inhibitory activity of the Acetylcholinesterase enzyme and two 2D descriptors provides the best statistical parameters, which led us to treat a molecular docking to predict the probable interactions between the newly designed 160 ligands and the amino acids forming the skeleton of the receptor. The QSAR model for Acetylcholinesterase enzyme was able to show stability and predictive power, confirmed by internal and external validation. The 160 compounds were docked against AChE using Auto dock 4.2 (1.5.6). The synthesized 3 compounds PD35, PD65 and PD72 were found to possess drug-likeness, obey Lipinski's rule of 5, and are non-toxic. A series of 2-substituted benzimidazole derivatives were synthesized and their structures were elucidated by spectral data. All the synthesized compounds were screened for their In vitro anti-Alzheimer's activity and most of them had significant effects. It was found to exhibit 25.42µg, 20.71µg, and 19.06µg which is almost as potent that of standard Rivastigmine.

Author Contributions

All authors contributed equally to this research. All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

Funding

The authors did not receive any fundings from any private or government sources.

Ethical Approvals

This study does not involve experiments on animals or human subjects.

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