Design, Synthesis and Biological Evaluation of Disubstituted 1,3,4 Thiadiazoles Derivatives as Potential Candidates For The Management of Cognitive Decline

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ABSTRACT

Acetylcholinesterase has been a promising target for the development of potential treatments for cognitive deterioration. The deleterious effect of oxidative stress on the learning and memory paradigms of an individual has also been well documented. In view of this, the present study demonstrates the design, synthesis, and pharmacological evaluation of 2, 5 disubstituted 1,3,4 thiadiazole derivatives. Twenty-two molecules (**28-50**) were designed and the molecular docking study performed to envisage the binding mode of the designed compounds also suggested that these compounds bind appreciably to the amino acids present in the active site of the recombinant human acetylcholinesterase (rhAChE). Based upon docking analysis, Five novel hybrids (**28, 29, 30, 32, and 36**) have been synthesized by employing suitable synthetic procedures and characterized by various spectral and elemental techniques. Further, these synthesized compounds were studied in the pharmacokinetic study and evaluated against behavioral alterations using step-down passive avoidance and escape learning protocol at a dose of 0.5 mg/kg with reference to the standard, donepezil. All the synthesized compounds were evaluated for their *in vitro* acetylcholinesterase (AChE) inhibition at five different concentrations using mice brain homogenate as the source of the enzyme. Biochemical estimation of markers of oxidative stress has also been carried out to assess the role of synthesized molecules on oxidative damage induced by ascorbic acid. Compounds **28** and **36** established a significant acetylcholinesterase inhibition activity. These compounds also decreased ascorbic acid-induced oxidative stress, thus serving as promising leads for the amelioration of oxidative stress-induced cognitive decline. The results indicated that these compounds could be investigated further as AChE and oxidative stress inhibitors for the treatment of cognitive dysfunction.

Keywords Cognitive Decline; Acetylcholinesterase;1,3, 4 Thiadiazole; Biochemical; Docking; Pharmacological Activity

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disease of the central nervous system correlated with the progressive loss of cognition and memory [1]. Cognitive dysfunction has become a major public health concern because of its increasing ubiquity, chronicity, caregiver burden, and high personal and financial costs of care[2]. Alzheimer's disease is an irreversible, progressive brain disease that slowly destroys memory and cognition, thinking skills, and eventually, even the ability to carry out the simplest tasks. The advancement in research, exploring the biological mechanisms underlying memory and learning has opened many avenues for the discovery of pharmacological treatments for Alzheimer's disease. It is a progressive disorder that affects the ability to perform daily activities, doing judgments, learn, and understand through thought, experience, and senses[3]. It involves processes such as knowledge, attention, working memory, judgment and evaluation, reasoning and computation, problem-solving and decision-making, comprehension and production of language, etc [4].

The therapeutic strategies for Alzheimer's disease have been directed to two main targets: reduced cholinergic neurotransmission and aggregation of β- amyloid proteins which destroy the structural proteins of the neurons. Since acetylcholine (ACh) is a neurotransmitter associated with learning and memory, treatment approaches have been focused on the compensation of deficit cholinergic neurotransmission in the CNS. The formation of ACh from choline and acetyl Co-A is influenced by the enzyme cholinesterase which is stored in the presynaptic part of the neuronal cells. The secretion of the neurotransmitterin the synapse is modulated by the action potential

and opening and closing of the calcium channels. The hydrolysis of free ACh into choline and acetic acid is mediated by the action of the enzyme acetylcholinesterase (AChE) present in the synaptic cleft. AChE is one of the most crucial enzymes of the family serine hydrolyses involved in the hydrolytic cleavage of ACh, depleting the levels of ACh implicated in memory and learning [5]. Hence, the cholinergic hypothesis for neurodegenerative disorders suggests that the debility in the neurons containing ACh, has a notable contribution in the Alzheimer's disease associated with old age. Cholinesterase inhibitors (ChEIs) are therefore used to reduce cholinergic deficiency.

The past several decades have witnessed the development of several cognition enhancers, but the discovery and development of several potential ACh have paved the way for a better therapeutic and treatment approach toward neurodegenerative disorder[6].

Diverse pharmacophoric scaffolds have been employed in the designing of AChEIs. Acetylcholinesterase inhibitors like physostigmine, rivastigmine, tacrine, and Donepezil have already been approved by the U.S. Food and Drug Administration (FDA) for the treatment of neurodegenerative disorders like AD[7].

Heterocyclic moieties have been employed in the designing and development of potent molecules for a broad range of pharmacological disorders. Thiadiazole are such heterocyclic units that find their incorporation in various compounds for the treatment of a diverse variety of diseased conditions. A literature survey has revealed promising applications of thiadiazole scaffolds in the designing of molecules as AChEIs for Alzheimer's disease[8,9]. 1,3,4-Thiadiazole has been incorporated in medicinally important agents which have been approved for the treatment of various diseased conditions. Thiadiazole-containing compounds possess a wide variety of pharmacological activities like anti-Alzheimer's, anti-inflammatory,anticonvulsant, anticancer, antioxidant, antimalarial, antiviral, anthelmintic, and other activities[10–13] .Based on the literature review, we have selected 2,5 di-substituted thiadiazole moiety as a potential candidate for anti- Alzheimer activity[14]. The research work will provide novel chemical entities that can be used in the treatment of Alzheimer's disease. Despite the currently available treatment options, continual research is being carried out in order to find safer, cheaper, and more efficient alternatives.

2. Results and Discussion

2.1 Design

In silico techniques have been mostly used to design novel compounds. As reported in the literature, Thiadiazole and heterocyclic rings may contribute to cognitive dysfunction as they interact with the catalytic anionic site (CAS) and peripheral active site (PAS) of AChE respectively through hydrophobic, van der Waals, and π-π interactions. It has been established that substitution at the second position of the thiadiazole ring affects the potency and affinity of the ligands toward AChE and hence their pharmacological effects[15,16]. Also, the effect of the substitution of peripheral binding moieties at the fifth position of the thiadiazole ring has been investigated.

Based on the design, it was predicted that these molecules would have similar interactions to donepezil complexed with AChE protein. The electron-withdrawing substitution was supposed to interact with the CAS and PAS regions of AChE protein via π -π stacking, whereas the amino group of the thiadiazole ring was supposed to have hydrogen bonding interactions similar to donepezil in the AChE mid-gorge region. All of the designed analogs were then subjected to docking simulations to see how they interacted with the essential amino acids of the AChE protein to achieve the desired pharmacological effect.

2.2 Docking Studies

The most recent X-ray crystallographic record of rhAChE (**PDB ID: 4EY7**) co-crystallized with donepezil has revealed that it extends itself covering the intact span of the enzymatic active site gorge. The idiosyncratic orientation of donepezil in the active site gorge permits it to reach out from the catalytic anionic site at the bottom near Trp86 to the peripheral anionic site at the top near Trp286. Since donepezil is a selective AChE

inhibitor, it was used as a reference ligand for more apparent, specific, and reliable designing of the molecules for selective AChE inhibition. It was used as a reference ligand to design more obvious, specific, and reliable molecules for selective AChE inhibition. To investigate the preferred binding mode of the designed molecules, a docking study was performed on the enzyme AChE (rhAChE with donepezil as the reference ligand) [17–19].

Docking studies of the designed molecules on rhAChE using the V life module based on the Bio force field revealed significant binding interactions between the molecules and amino acid residues at both the catalytic and peripheral sites of the AChE enzyme. All of the designed molecules were docked into the active site of the enzyme AChE and demonstrated significant interactions with the key amino acid residues throughout the entire active site region. Docking interactions of designed molecules (**28–50**) are depicted in 2D and 3D diagrams in Table 1. The majority of these ligands exhibited significant hydrogen bonding and aromatic π- π stacking interactions with the essential amino acid residues present on the active site gorge of AChE. This demonstrated the favorable position of the ligand in the AChE enzyme's binding pocket. In all designed molecules **28-50**, the amino functional group of thiadiazole interacted via hydrogen bonding with PHE295 in the middle gorge of the protein. Most of the compounds **28, 29, 30, 32,** and **36** exhibited strong aromatic interactions with TRP86 that covered the catalytic binding gorge. Compounds **28, 29, 30, 32,** and **36** have established the aromatic π- π stacking interaction with TRP286. Compounds **28, 29, 30, 32,** and **36** also exhibited cationic interactions with HIS447, whereas compounds **28, 29, 30, 32,** and **36** exhibited hydrogen bonding interactions with amino acids TYR341 and TYR124, respectively. The results of the docking study's analysis of binding interactions were consistent with the design pattern of the molecules shown in **Fig 1-Fig 5**. The dock score of designed compounds (**28-50**) have been shown in **Table 1.**

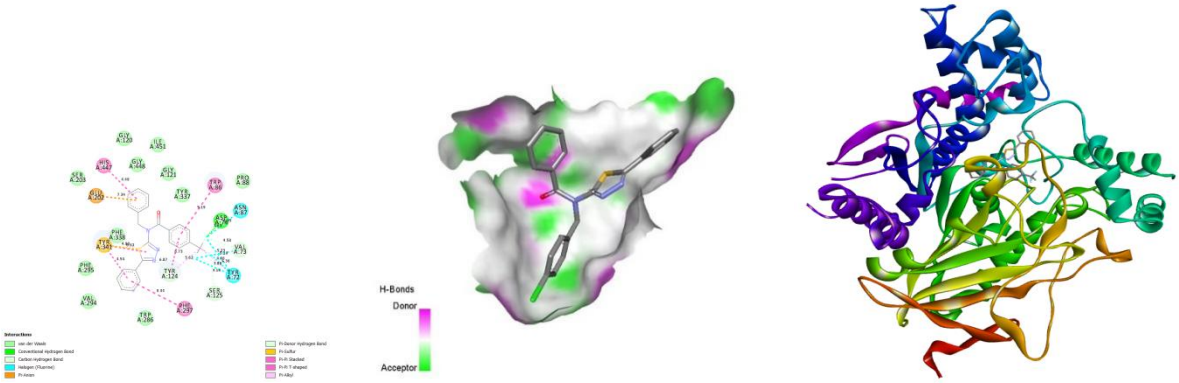


Figure 1: a) Binding site interactions of the compound 2**8** with the enzyme 4EY7. (**a**) 2D docking pose of ligand **28**; Dotted green line shows the H-bond interaction and dotted pink line shows the π-π interaction (**b)** Molecular docking image of compound 2**8** (AA-28) into the active sites of rhAChE. **c**) pink area indicates the H bond donor region and green area indicates the H bond acceptor region.

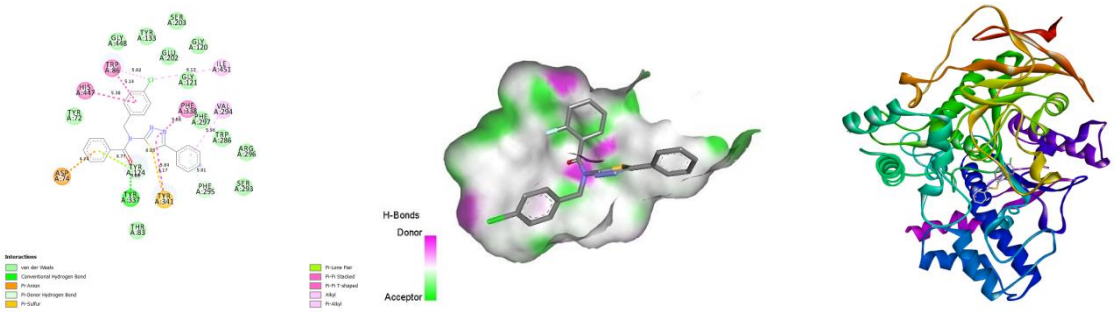


Figure 2 a) Binding site interactions of compound **29** with the enzyme 4EY7. (**a**) 2D docking pose of ligand **29**; Dotted green line shows the H-bond interaction and dottted pink line shows the π-π interaction (**b)** Molecular docking image of compound **29** (**AA-29**) into the active sites of rhAChE. **c**) pink area indicates the H bond donor region and green area indicate the H bond acceptor region.

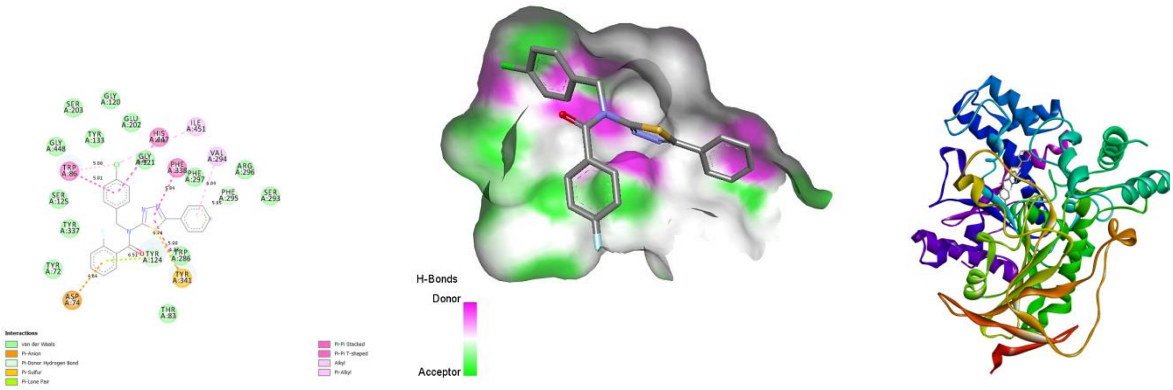


Figure 3 a) Binding site interactions of the compound **30** with the enzyme 4EY7. (**a**) 2D docking pose of ligand **30**; Dotted green line shows the H-bond interaction and dotted pink line shows the π-π interaction (**b)** Molecular docking image of compound **30** (**AA-30**) into the active sites of rhAChE. **c**) pink area indicates the H bond donor region and green area indicate the H bond acceptor region.

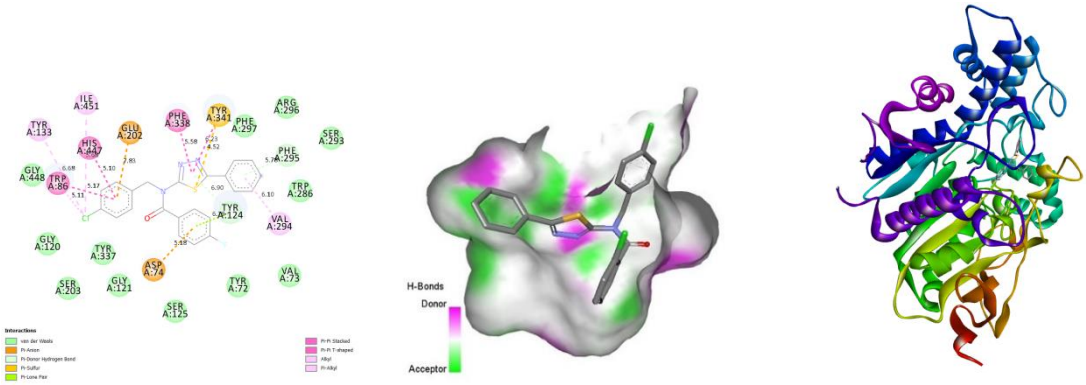


Figure 4 a) Binding site interactions of compound **32** with the enzyme 4EY7. (**a**) 2D docking pose of ligand **32**; Dotted green line shows the H-bond interaction and dotted pink line shows the π-π interaction (**b)** Molecular docking image of compound **32** (**AA-32**) into the active sites of rhAChE. **c**) pink area indicates the H bond donor region and green area indicates the H bond acceptor region.

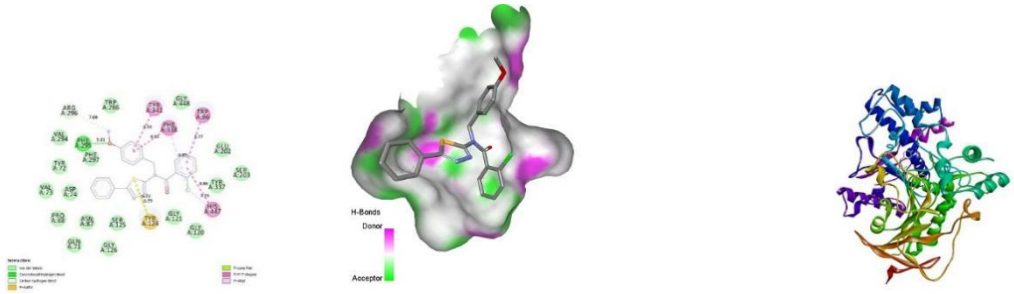


Figure 5: **a)** Binding site interactions of compound **36** with the enzyme 4EY7. (**a**) 2D docking pose of ligand **36**; Dotted green line shows the H-bond interaction and dotted pink line shows the π-π interaction (**b)** Molecular docking image of compound **36** (**AA-36**) into the active sites of rhAChE. **c**) pink area indicates the H bond donor region and green area indicates the H bond acceptor region.

Table 1: Dock score of Designed compounds

Compound	**DockScore (Kcal/mol)**	**Compound**	**Dock Score (Kcal/mol)**
COMP28 (AA28)	-11.97	**COMP40**	-9.06
COMP29 (AA29)	-11.68	**COMP41**	-11.08
COMP30 (AA30)	-11.75	**COMP42**	-10.61
COMP31	-9.03	**COMP43**	-10.48
COMP32 (AA32)	-11.80	**COMP44**	-9.76
COMP33	-10.79	**COMP45**	-9.12
COMP34	-11.47	**COMP46**	-9.06
COMP35	-11.2	**COMP47**	-9.26
COMP36 (AA36)	-12.16	**COMP48**	-9.55
COMP37	-11.18	**COMP49**	-10.44
COMP38	-11.13	**COMP50**	-11.43
COMP39	-9.78	**DONEPEZIL**	-11.75

2.3 Chemistry

Based on the comprehensive delineation of the structural and functional aspects of the enzyme AChE, role of oxidative stress in dementia, and enhanced information about 1,3,4-thiadiazoles, novel synthetic analogs have been designed, synthesized, and evaluated for their propitious role in the attenuation of cognitive decline.

2.3.1 Synthesis

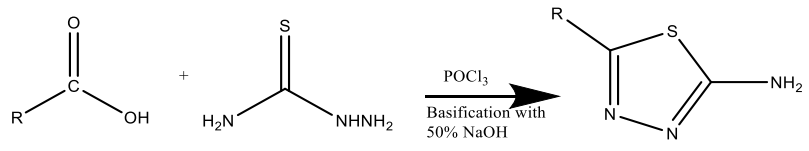
A comprehensive literature search has revealed wide panoramic strategies for the synthesis of 1,3,4-thiadiazoles. This heterocycle can be synthesized from a variety of substrates by employing different reaction conditions (discussed in the experimental section). In the present study,the parent 1,3,4-thiadiazoles have been successfully synthesized according to the method described in the literature report.

The synthetic pathway for the synthesis of compounds has been depicted in **Scheme 1**. The key intermediate 5-phenyl-1,3,4-thiadiazoles 2-amines **(2)** were synthesized according to the reported method summarized in the proceeding text[20–22].

The compound 5-phenyl-1,3,4-thiadiazoles 2-amines **(2)** was prepared by benzoic acid **(1)** which was cyclized with thiosemicarbazides in the presence of phosphorus oxychloride ($POCl_3$). The completion of the reaction was monitored by TLC. After completion of the reaction, the reaction mixture was further basified with 50% sodium hydroxide solution to a pH of 8-9, and the precipitates obtained were filtered and recrystallized from ethanol. Subsequently, the synthesized 2,5-substituted 1,3,4-thiadiazoles **(2)** were refluxed with a substituted aromatic aldehyde **(3a-3b)** in ethanolic solution and glacial acetic acid to give condensed Schiff base derivatives (**4a-4b**) **Scheme 2**. These Schiff derivatives were further undergone reduction with sodium borohydride to yield the 2,5-disubstituted 1,3,4-thiadiazol-2-benzyl amine derivatives **(5a-5b) Scheme 3**. The structures of the synthesized compounds were established using spectral techniques (IR, ^{1}H NMR and ^{13}C NMR). The infrared red spectrum of these compounds exhibited a strong band at ~3300-3200 cm^{-1} corresponding to the N-H stretching vibrations. The aromatic nucleus in the structural skeleton of these compounds exhibited C-H stretching bands in the range of 3100-3000 cm^{-1}. The C=N stretch for compounds (**2)** has been observed at ~ 1630 cm^{-1} in the IR spectra. Skeletal vibrations corresponding to C=C stretching within the aromatic ring appeared in the region of 1572.9-1569.8 cm^{-1}. Strong absorption bands appearing in the region 723.1-704.5 cm^{-1} have been attributed to out-of-plane (oop) ring bending.

A broad exchangeable singlet corresponding to the two protons of the NH_2 group appeared at varied positions within the range of δ 7-7.30 ppm in the proton NMR spectra of all the compounds (**2)**. The structural confirmation of 5-phenyl-1,3,4-thiadiazol-2-amine **(2)** was carried out using various spectral techniques. The Infrared spectrum of these compounds **4a-4b** exhibited a strong band at ~1603.6-1620.6 cm-^{1} corresponding to the C=N stretching vibrations.

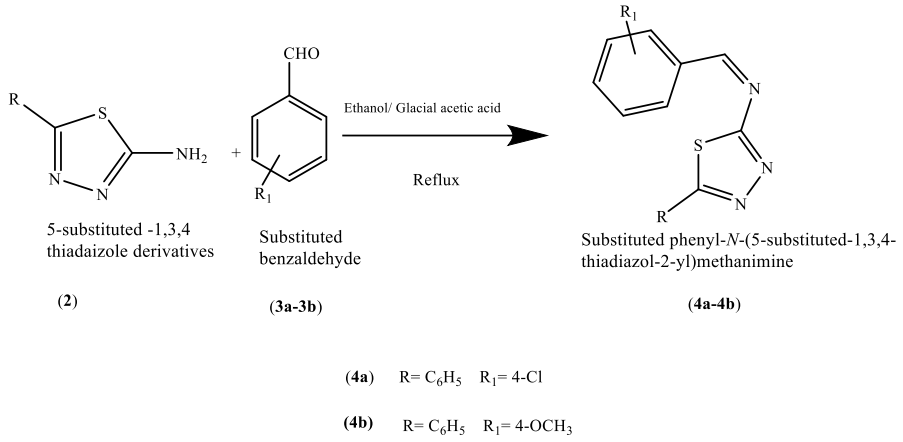
The structural confirmation of compound **5a-5b** was carried out using various spectral techniques. The infrared spectrum of these compounds exhibited a strong band at ~3500-3300 cm^{-1} corresponding to the N-H stretching vibrations. The aromatic nucleus in the structural skeleton of these compounds exhibited C-H stretching bands in the range of 3100-3000 $cm^{-1.}$ A prominent bending vibration of the chloro group-containing compound(**5a)** was exhibited at ~ 688.8 cm-1. A prominent stretching vibration of the C-O-C group-containing compound(**5b)** was exhibited at ~1213.4 cm-1[1].



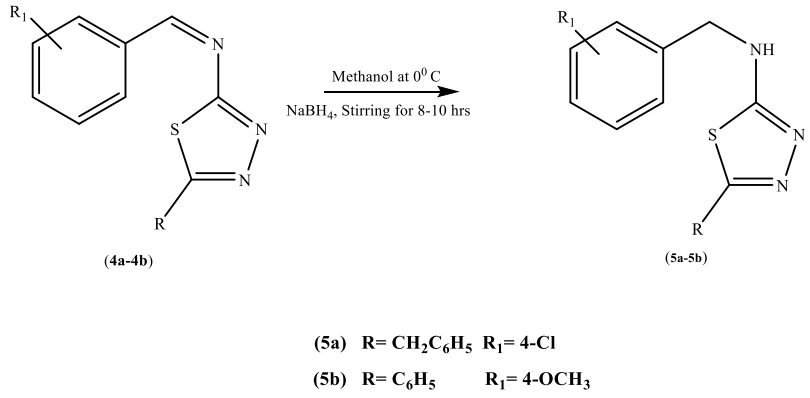
(1) **(2)**

(2) **R= C_6H_5**

Scheme 1: Reagents and Reaction Conditions: (a) $POCl_3$, Reflux (b)NaOH, Reflux



Scheme 2: Reagents and Reaction Conditions: (a) Glacial acetic acid and Ethanol, Reflux



Scheme 3: Reagents and Reaction Conditions: Methanol & $NaBH_4$, Stirring

A broad exchangeable singlet corresponding to the two protons of the N*H*$_2$ group appeared at varied positions within the range of δ 4.35-5.30 ppm in the proton NMR spectra of all the compounds **5a-5b**. The aromatic proton appeared at 7.54-7.71 ppm in the proton NMR spectra of compounds **5a-5b.**

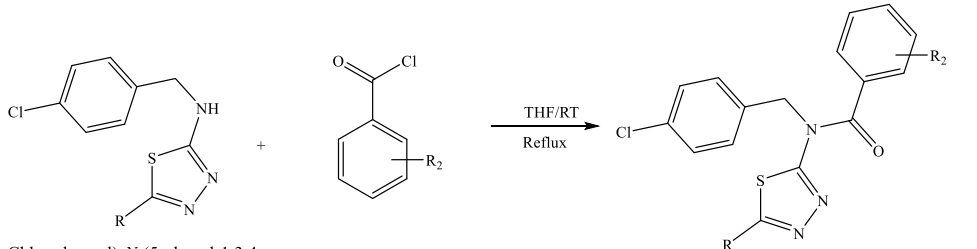
2.3.2 Synthesis of Disubstituted Amide Derivatives of 2,5-Substituted 1,3,4-Thiadiazoles

According to the literature, certain compounds with the nucleus 1,3,4-thiadiazole have significant AChE inhibitory activity. Based on the rational drug design extension strategy, the current study was designed to investigate the acetylcholinesterase inhibitory activity and antioxidant effects of thiadiazole benzylamine. The amide derivatives were created by acylating disubstituted thiadiazol-2-amines with various acid chlorides in a basic medium using the principles of the Schotten-Baumann reaction. Because it actively participates in hydrogen bonding at the target enzymatic site, the Amide group has been reported to be a promising linker. Due to the presence of aromatic amino

acids in the binding pocket of the enzyme AChE, the acid chlorides were selected so that they can furnish an aromatic π-system that can efficiently bind with the amino acid residues through π-π interactions.

2.3.3 Synthesis of *N*-(benzyl)-*N*-(phenyl-1,3,4-thiadiazol-2-yl)benzamide derivatives

Targeted compound *N-(4-chlorobenzyl)-5-phenyl-1,3,4-thiadiazol-2-amine* **(5a)** were further refluxed with substituted benzoyl chloride (**6a-6b**) in anhydrous tetrahydrofuran (THF), respectively to afford 2,5-disubsituted1,3,4-thiadiazol-*N*- benzyl-2-benzamide derivatives **28,29,30 & 32** in **Scheme 4.** The procedure yielded the final products in excellent yield ranging from 65.1, 62.1, 58.0 and 53.0 % [23].



1-(4-Chloro benzyl)-*N*-(5-phenyl-1,3,4 -thiadiazol-2-yl)methanimine

Subsitituted Benzoyl chloride

N- Chlorobenzyl- *N*-[5-subsituted-1,3,4 thiadiazole-2-yl)] subsituted benzamide

(**5a**) (**6a-d**) (**28-32**)

(**28**) R=C_6H_5 R_2=H

(**29**) R=C_6H_5 R_2= 2-F

(**30**) R=C_6H_5 R_2= 4-F

(**32**) R=C_6H_5 R_2= 2-Cl

Scheme 4: Reagents and Reaction Conditions: (a) THF and Room Temperature, Reflux

The structures of the target Compound **28, 29, 30** & **32** have been confirmed by various spectral and elemental techniques. In the IR spectrum of these compounds **28, 29, 30** & **32** strong bands at approximately 3000 and 2900 cm-1 confirmed the presence of aromatic and aliphatic C- H stretching. The C=N, C…C ring stretch was observed at a frequency ranging from 1572.3-1400 cm-1. A characteristic sharp peak was visible at ~1517.4-1440.4cm-1 for the C-N and ~ 1673.6 -1666.1 cm^{-1} for C=O vibrational stretching of the teritary amide group present in the compounds **28, 29, 30** & **32**.

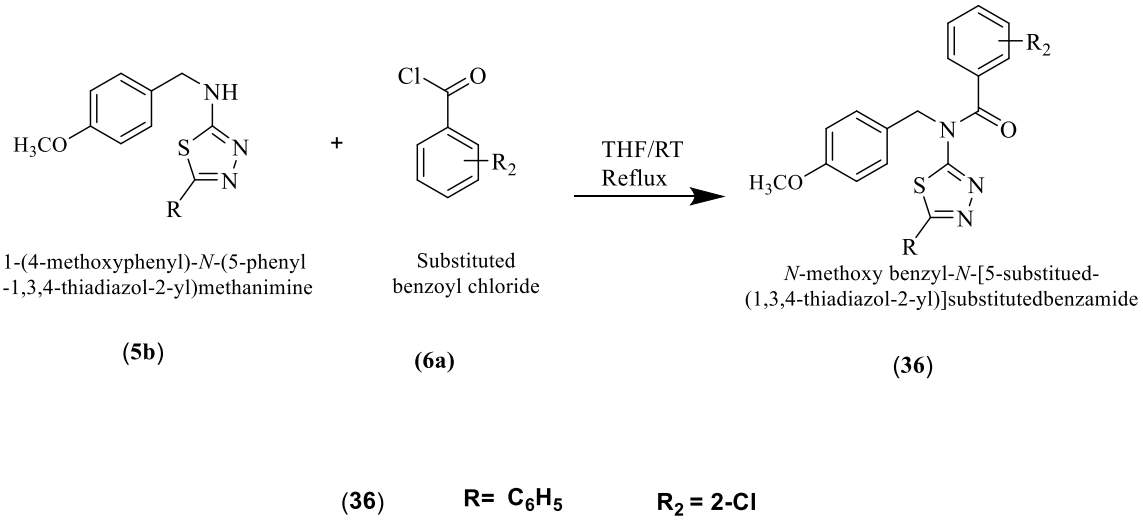
The proton NMR spectrum of compounds **28, 29, 30** & **32** further confirmed its structure. The disappearance of the broad peak for protons of the amino group of 2- amino-1,3,4-thiadiazole further substantiated the formation of the target compounds **28, 29, 30** & **32.** Additionally, it exhibited a singlet of two protons at δ 5.01 ppm corresponding to the methylene group due to an anisotropic effect. Aromatic protons in all the compounds resonated at their usual chemical shift (~ δ 7.30 to 8.03 ppm).

The 13C NMR spectra of these compounds **28, 29, 30** & **32** have further confirmed the structure of the synthesized compounds. The quaternary carbon (bearing the thione group) of the thiadiazole ring in all the compounds of the series was observed in the range of ~ δ163.4 - 174.1 ppm in the 13C NMR spectra. The other carbonyl carbon bearing the amide group functionality) of the thiadiazole ring exhibited a low-intensity signal in the range of ~ δ 169.6-171.8 ppm. While the aromatic carbons appeared at δ115.6-135.4 ppm in all the analogs compounds **28, 29, 30** & **32**. The prominent peak of the carbon of the methyl -*C*H2 group has appeared at δ 49.9 ppm of all the target compounds. In this spectrum of compounds **28, 29, 30 & 32** the presence of chloro-substituted carbon appeared at

δ 132.3 ppm. In this spectrum of compounds **29 and 30**, the presence of the carbon-containing fluoro substituents has appeared at δ159.3- and 166.3 ppm. *p*-fluoro substituents has more downward to chemical shift as compared to ortho fluoro substituents due to stearic hinderance. In this spectrum of compound **32**, two chloro-substituted carbon was appeared at δ 132.3 &134.6 ppm which confirmed the target compound. Prominent molecular ion peaks $[M+H]^+$ at *m/z* 406.8.09, 426.5 424.6 & 441.6 further substantiated the proposed structures of compounds **28, 29, 30, and 32** respectively.

2.3.4 **Synthesis of *N-(4methoxybenzyl) -N-(substituted phenyl)-1,3,4-thiadiazol-2-yl)benzamide (*36)**

Compound *N-(4methoxybenzyl)-5-phenyl-1,3,4-thiadiazol-2-amine (***5b***)* were further refluxed with 2 chloro benzoyl chloride (**6b**) in anhydrous tetrahydrofuran (THF), respectively to afford 2,5-disubsituted1,3,4-thiadiazol-*N*- benzyl-2-benzamide derivatives **(36) Scheme 5.** The procedure yielded the final products in excellent yield from 65.1%[24].



1-(4-methoxyphenyl)-*N*-(5-phenyl -1,3,4-thiadiazol-2-yl)methanimine

(**5b**)

Substituted benzoyl chloride

(6a)

N-methoxy benzyl-*N*-[5-substitued-(1,3,4-thiadiazol-2-yl)]substitutedbenzamide

(**36**)

(**36**) **R= C_6H_5** **R_2 = 2-Cl**

Scheme 5: Reagents and Reaction Conditions: (a) THF and Room Temperature, Reflux

The structures of the target compound **36** have been confirmed by various spectral and elemental techniques. In the IR spectrum of these compounds **36**, strong bands at approximately 3000 and 2900 cm-1 confirmed the presence of aromatic and aliphatic C- H stretching. The C=N, C-C ring stretch was observed at a frequency ranging from 1615-1400 cm-1. A characteristic sharp peak was visible at ~1569.1 cm-1 for the C-N and ~ 1673.6 cm^{-1} for C=O vibrational stretching of the teritary amide group present in the target compounds **36.**

The proton NMR spectrum of compound **36** further confirmed its structure. The disappearance of the broad peak for protons of the amino group of 2- amino-1,3,4-thiadiazole further substantiated the formation of the target compounds **36.** Additionally, it exhibited a singlet of two protons at δ 5.01 ppm corresponding to the methyl group. Aromatic protons in all the compounds resonated at their usual chemical shift (~ δ 8.03 to 6.87 ppm). The prominent peak of the methoxy group (-OCH_3) appeared at δ 3.83 ppm which confirmed the target compound.

The ^{13}C NMR spectra of these compounds **36** have further confirmed the structure of the synthesized compounds. The quaternary carbon (bearing the thione group) of the thiadiazole ring in all the compounds of the series was observed in the range of ~ δ163.4- 174.1 ppm in the ^{13}C NMR spectra. The other carbonyl carbon bearing the amide group functionality) of the thiadiazole ring exhibited a low-intensity signal in the range of ~ δ 169.6 ppm. While the aromatic carbons appeared at ~ δ 114.1-133.5 ppm in the analogs **36**. In the ^{13}C NMR spectrum of compound **36** presence of the chloro-substituted carbon appeared at 134.6 ppm. The prominent peak of the methoxy group -OCH_3 group has appeared at δ 55.8 ppm which confirmed the target compound **36**. Prominent molecular ion peaks $[M+H]^+$at *m/z* 435.08 further substantiated the proposed structures of compounds **36** respectively.

2.4 Prediction of ADME Properties of the Synthesized Compounds

The development of an orally active drug bestows several advantages in delivering drugs to the patients such as safety, good patient compliance, ease of ingestion, pain avoidance, and versatility to accommodate various types of drugs. In the drug discovery and development pipeline,the prediction of the ADME properties of a molecule seems to be of great significance. Lipinski's rule of five can be used to predict a molecule's drug-like properties and help it become an orally active therapeutic agent. When the molecules do not violate Lipinski's rule of five, a molecule is predicted to be orally active. This rule takes into account parameters like log P, molecular weight, the number of hydrogen bond acceptors, and the number of hydrogen bond donors. Log P determines the octanol-water partition coefficient of the molecule, i.e. whether the molecule is lipophilic or hydrophilic, indicating brain or peripheral targeting. This rule states that the Log P of a drug-like molecule should be 5. The molecule's molecular weight should be 500. The calculation of topological polar surface area (TPSA) is an important molecule parameter. It is calculated as the sum of the contributions of O- and N-polar fragments. It multiplies the number of hydrogen bond acceptors and donors in the molecule. TPSA predicts drug absorption, including bioavailability and blood-brain penetration ability [25,26]The ADME properties were predicted using the Molinspiration cheminformatics online property calculation toolkit, and all synthesized compounds violated Lipinski's rule of five. As a result, all other conjugates are assumed to be druggable and capable of being developed as orally active drug candidates. All the calculated parameters are listed in **Table 2.**

Table 2: **Predicted pharmacokinetic parameters of the synthesized compounds**

Sr.No	Comp	Log P	TPSA	nAtom	MW	nON	nOHNH	nViolation	nRotb
1.	**28**	5.79	46.09	28	405.91	4	0	1	5
2.	**29**	5.90	46.09	29	423.90	4	0	1	5
3.	**30**	5.95	46.09	29	423.90	4	0	1	5
4.	**32**	6.42	46.09	29	440.36	4	0	1	5
5.	**36**	5.79	55.33	30	435.94	5	0	1	6

LogP: predicted logarithm of partition coefficient of the compound between n-octanol and water, TPSA: topological polar surface area, nAtom: number of atoms, MW: molecular weight, nON acceptors: number of hydrogen bond acceptors, nOHNH donors: number of hydrogen bond donors, nViolation: number of violation(s) from Lipinski's rule of five, n-ROTB: number of rotatable bonds.

2.5 PHARMACOLOGICAL STUDIES

2.5.1 In-Vitro Studies

2.5.1.1 ***Assay of Acetylcholinesterase***

The inhibitory potential of the synthesized molecules against acetylcholinesterase has been assessed *in vitro* according to the method of Ellman *et al*.[27]. The supernatant obtained from the decapitated brains of fresh mice has been used as the source of the enzyme AChE. The percentage inhibition of the synthesized compounds and the reference standard, donepezil was calculated at five different concentrations (10, 25, 50, 75 and 100 µM). The percentage inhibition of acetylcholinesterase activity by the newly synthesized compounds has been tabulated in **Table 3.**

Amongst the series of novel conjugates, compounds **36**, **28,** and **32** exhibited promising inhibitory potential with percentage inhibition values of 63 ± 3.0, 54 ± 2.3 and 51 ± 0.64 respectively at a concentration of 100 µM in comparison to the reference drug donepezil exhibiting percentage inhibition of 88.72 ± 1.71 at the same concentration against the activity of enzyme AChE. Compounds **36 & 28** with chloro substitution demonstrated significant AChE inhibition with percentage inhibition values 63 ± 3.0, 54 ± 2.3 respectively. Derivatives **36, 28** and **32** with phenyl substitution also displayed encouraging results in terms of AChE inhibition. The most promising compounds of the series, compound **36, 28** and **22** possessed chloro group substitution. Hence, the incorporation of an electron-withdrawing group and electron donating group at the fourth position of the phenyl ring (substituted at the fifth position of the thiadiazole ring) afforded compounds that were found to be highly active in terms of AChE inhibitory potential.

Table 3: Percentage inhibition of acetylcholinesterase activity of the synthesized compounds.

Compound	**Inhibition of acetylcholinesterase activity (in %) (Mean ± SEM)**				
	10µm	**25µm**	**50µm**	**75µm**	**100µm**
28	37±2.1	38±1.4	42±1.4	44±1.6	54 ± 2.3
29	12 ± 2.0	13 ± 1.7	14 ± 1.9	15 ± 1.8	14 ± 1.6
30	22 ± 2.0	24 ± 1.7	28 ± 1.0	30 ± 2.1	34 ± 2.9
32	26 ± 2.4	28 ± 2.1	33 ± 2.3	34 ± 2.1	51 ± 0.64
36	52 ± 2.3	52 ± 3.3	54 ± 3.4	55 ± 2.5	63 ± 3.0
Donepezil	75 ± 3.2	76 ± 3.2	77 ± 3.2	84 ± 2.5	88 ± 1.7

2.5.1.2 **DPPH (1,1-diphenyl-2-picrylhydrazyl) Scavenging Activity**

DPPH scavenging activity of the synthesized compounds (**28, 29, 30, 32 & 36**) was examined using DPPH method [28,29]. Standard ascorbic acid was used. Five different concentrations for each standard ascorbic acid and the synthesized compound were prepared at 10, 25, 50, 75, and 100 µM in DMSO (0.1%), 1 mL of this solution was mixed with 2 mL of DPPH solution (0.5 Mm). The above mixture was shaken well and kept aside for settling at room temperature for 30 min. The absorbance was measured using a spectrophotometer at 517 nm against blank solutions of the synthesized compounds at 5 different concentrations 10, 25, 50, 75, and 100 µM with 0.1% DMSO without DPPH. The reaction mixture which showed lower absorbance possessed higher antiradical activity. The experiment was performed in triplicates. The results of the DPPH scavenging activity assay were expressed as percentage inhibition.

% Scavenged DPPH = ((Absorbance of control – Absorbance of sample)/Absorbance of control)) x 100

Table 4: DPPH radical scavenging activity of the synthesized compounds.

Compound	**Scavenging of DPPH (in %) (Mean ± SEM)**				
	10µm	**25µm**	**50µm**	**75µm**	**100µm**
28	19 ± 0.92	31 ± 1.2	40 ± 1.5	49 ± 1.7	60 ± 2.9
29	8.5 ± 0.52	12 ± 1.2	13 ± 1.2	16 ± 0.99	18 ± 1.1
30	11 ± 1.0	24 ± 1.8	23 ± 1.5	26 ± 1.4	31 ± 1.2
32	14 ± 1.2	14 ± 1.2	27 ± 1.4	39 ± 2.3	51 ± 1.3
36	26 ± 1.2	37 ± 1.6	46 ± 1.5	56 ± 1.5	73 ± 2.0
Ascorbic acid	31 ± 1.1	48 ± 0.87	62 ± 1.5	76 ± 1.6	87 ± 0.67

2.5.1.3 **Scavenging of Hydrogen Peroxide**

The synthesized compounds **(28, 29, 30, 32 & 36)** were tested for their ability to scavenge H_2O_2 spectrophotometrically[30–32] A 4mM solution of H_2O_2 was prepared at room temperature in phosphate-buffered saline. Using a molar extinction coefficient for H_2O_2 of 81 mol/cm the concentration of H_2O_2 was determined spectrophotometrically. The synthesized compounds were dissolved in DMSO (0.1%) and then added to the H_2O_2 solution at a final concentration of 0, 10, 25, 50, 75, and 100 µM at 20 °C. H_2O_2 absorbance was measured after 10 mins at 230 nm using PERKIN ELMER UV/VIS spectrophotometer against blank solutions containing

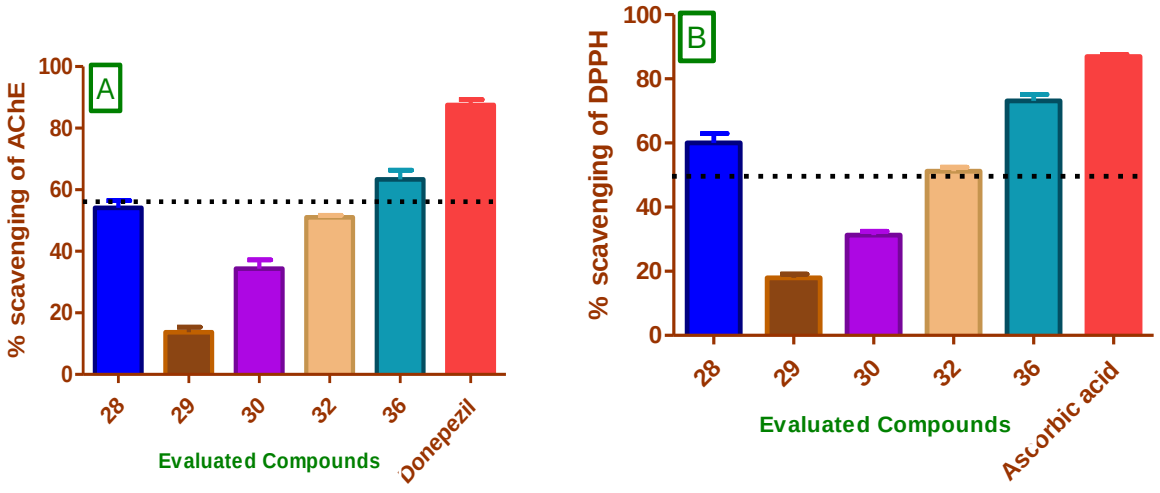
synthesized compounds at concentrations of 10, 25, 50, 75, and 100 µM in PBS without H_2O_2. All the experiments were performed in triplicate. The results of the H_2O_2 scavenging activity assay were expressed as percentage inhibition.

% Scavenged H_2O_2 = ((Absorbance of control – Absorbance of sample)/Absorbance of control)) x 100

Table 5: H_2O_2 radical scavenging activity of the synthesized compounds.

Compound	**Scavenging of H_2O_2 (in %) (Mean ± SEM)**				
	10µm	**25µm**	**50µm**	**75µm**	**100µm**
28	18 ± 1.2	31 ± 1.0	47 ± 1.6	49 ± 1.0	64 ± 1.8
29	7.7±0.72	13 ± 1.2	18 ± 1.4	24 ± 1.8	29 ± 1.2
30	12± 0.69	22 ± 1.1	37 ± 1.8	39 ± 1.0	44±0.55
32	15 ± 1.0	26± 0.97	36 ± 1.5	43 ± 1.2	57 ± 1.2
36	24± 0.39	37 ± 1.2	48 ± 1.6	54 ± 1.0	74 ± 2.6
Ascorbic acid	38 ± 1.7	53 ± 1.4	70 ± 1.2	82 ± 1.2	90 ± 1.2

Based on the initial *in vitro* screening assays (inhibition of acetylcholinesterase activity and scavenging of DPPH and scavenging of H_2O_2), with more than 50% at the tested concentration of 100 µM **(Fig. 6)** a total of three compounds **28, 32 & 36** were identified. All these selected compounds were promoted for their *in vivo* behavioral memory assessment, *ex vivo* assay of acetylcholinesterase, and evaluation of oxidative stress biomarkers determination.



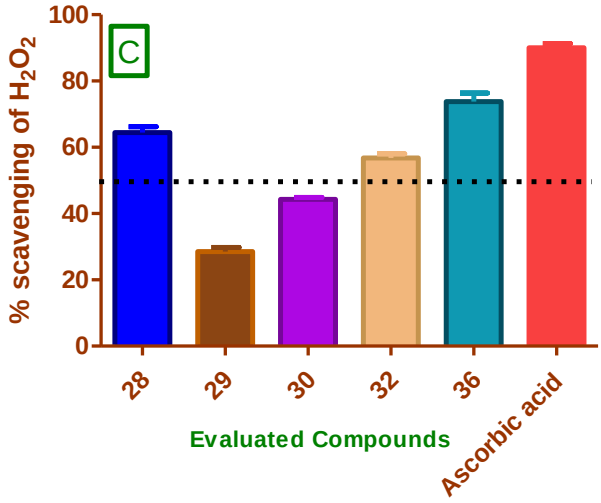


Figure 6: Screening of all the synthesised Compounds for inhibition of acetylcholinesterase activity (A), scavenging of DPPH activity (B) and scavenging of H_2O_2 activity (C) at 100 µM concentration. The data is presented as mean ± SD of three individual experiments performed in triplicate. The dashed lines are representing 50% inhibition or scavenging value; the compound with over 50% inhibition or scavenging value in all the *in vitro* assays were selected for further analyses.

2.5.2 In-Vivo Studies

2.5.2.1 Behavioral Studies for Assessment of Memory

On the basis of *in-vitro* studies three synthesized compounds (**28, 32, and 36**) were selected for further in-vivo studies[29,33].These compounds were analyzed for their memory improvement activity using step-down passive avoidance and escape learning procedure. Donepezil was used as a standard drug. The step-down latency was significantly shorter in the scopolamine-treated group (10 ± 1.0 s) when compared with the control group (149 ± 3.2 s). The short step-down latency induced by the scopolamine was reversed by the standard drug donepezil (98 ± 2.9 s). Among the tested synthesized compounds, all the treated groups showed a significant increase in step-down latency ($p < 0.001$), as compared to scopolamine **Table 6**.

The study highlighted the effect of the newly synthesized compounds on step-down passive avoidance and escape learning which is largely focused on short-term and aversive memory. The escape latency in the scopolamine group was significantly ($p < 0.001$) higher (35 ± 2.4 s) as compared to the control group (22 ± 2.2 s). The longer escape latency time induced by the scopolamine was significantly reversed by the treatment with the standard drug donepezil (20 ± 2.2^{a} s) ($p < 0.001$). Among the tested derivatives, compounds **36** showed a significant reduction in escape latency time in reaching the shock-free safe zone with 20±1.8 s. whereas step-down latency induced by the scopolamine-treated group was significantly shorter than that of the vehicle-treated control mice group, amongst these compounds. There was a significant increase in the number of mistakes made by the scopolamine group ($p < 0.001$), when compared with the control group. Treatment with the selected synthesized compounds **28, 32,** and **36** caused a significant decrease in the number of mistakes made when compared with the scopolamine group ($p < 0.001$). The analysis of the memory parameters clearly indicated that compounds **36 & 28** exhibited significant prowess in the amelioration of scopolamine-induced memory impairment. This compound may be considerably better in terms of efficacy than the standard donepezil. **Table 6, Figure 7-9.**

Table 6: Memory parameters (latency and the number of mistakes) of the selected synthesized compounds in step- down passive avoidance and escape learning protocol.

Groups	Memory parameter (s) (Mean ± SD)				
	Basal SDL	**SDL**	**Basal EL**	**EL**	**No. of mistakes**
Control	6.5±0.76	149±3.2	39±2.5	22±2.2	3.7±0.49
Scopolamine (SCO)	6.7±0.71	10±1.0$^{\alpha}$	38±1.9	35±2.4$^{\alpha}$	16 ± 1.4
SCO + 28	6.5±0.76	83 ± 2.3^{a}	39±2.1	23±1.7^{b}	5.8±0.40
SCO + 32	6.5±0.56	55 ± 2.5^{a}	39±2.8	25±1.6^{c}	6.3±0.80
SCO + 36	6.7±0.67	100±2.9^{a}	40±1.5	20±1.8^{a}	5.3±0.49
SCO + Donepezil	7.2±0.60	98± 2.9^{a}	38±1.9	20±2.2^{a}	5.7±0.67

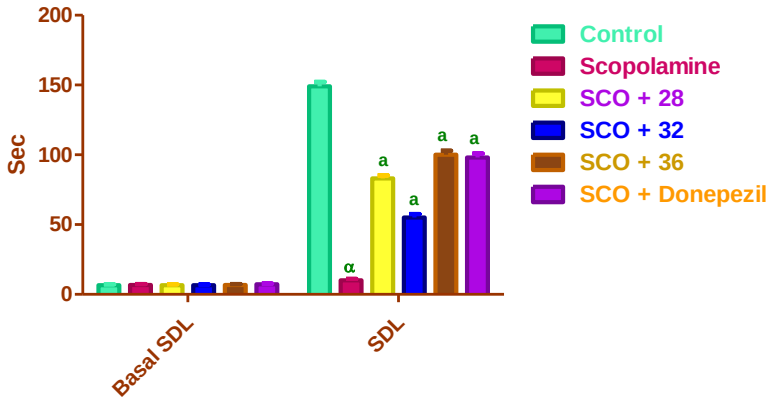


Figure 7: Screening of synthesized compounds for Basal latency and Step-down latency

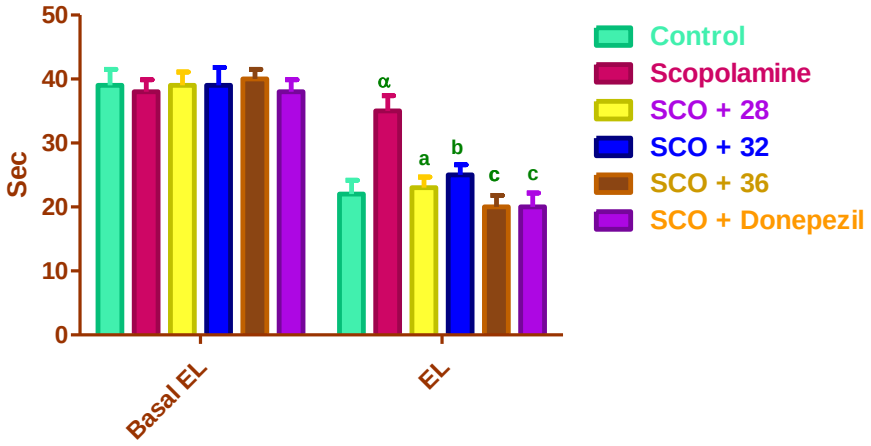


Figure 8: Screening of synthesized compounds for Basal latency and Escape latency

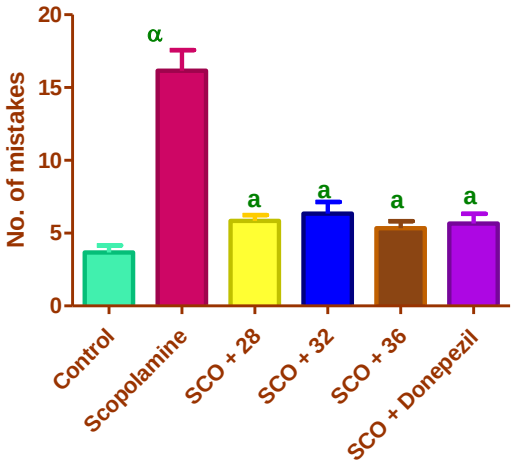


Figure 9: Screening of synthesized compounds for no of mistakes.Data were expressed as mean±SEM, 5 rats in each group, and analyzed by ANOVA followed by Tukey's post hoc test. $^{\alpha}p < 0.001$, when compared to the normal control group; $^{a}p < 0.001$ when compared to Scopolamine group.

2.6. Ex-vivo studies and estimation of the biomarkers of oxidative stress

2.6.1. Assay of acetylcholinesterase:

The three selected compounds **28, 29, 30, 32 & 36** were also subjected to *ex vivo* studies to investigate the brain AChE inhibition abilities using the Ellman method [27,34]All the above three compounds were administered to the mice at a dose of 0.5 mg/kg body weight. The animals used in *in vivo* studies were sacrificed by cervical dislocation after the completion of behavioral assessment and the brain AChE activity (nmoles of substrate hydrolyzed/ minute/ mg protein) was measured versus the control, scopolamine or donepezil as enlisted in **Table 7**.

The graphical representation of the acetylcholinesterase inhibitory profile of the synthesized compounds (**28, 32** & **36**) in comparison to the control, standard donepezil and the inducer scopolamine is illustrated in **Figure 10.** The results were in concordance with the behavioural studies. A significant increase in acetylcholinesterase activity in scopolamine-treated mice (31 ± 1.6 nmoles of substrate hydrolyzed/ min/mg protein) as compared to the control (18 ± 1.0 nmoles of substrate hydrolyzed/min/mg protein) and donepezil 20 ± 1.0[a] nmoles of substrate hydrolyzed/min/mg protein) treated mice have been observed. This increase in acetylcholinesterase activity has been correlated with diminished cholinergic transmission due to depleted acetylcholine levels. Compounds **28 & 36** displayed AChE activity 23 ± 0.72, 18.1 ± 0.91 nmoles of substrate hydrolyzed/min/ mg protein respectively. Hence this analog (**28, 36**) exhibited even better AChE inhibition as compared to the standard drug donepezil. Moreover, increased AChE level induced by scopolamine was significantly reversed by the standard drug donepezil and all tested derivatives. This implies that the memory-improving effects of the synthesized analogs (**28, 32** and **36**) might be attributed to the inhibition of AChE leading to elevated levels of ACh in the brain. The results of the biochemical estimation reinforced the results of the behavioral assessment by step down passive avoidance and escape learning, thus suggesting a plausible explanation of the mechanism of action of these synthesized compounds by acetylcholinesterase inhibition.

Table 7: *Ex vivo* AChE activity (nmoles of substrate hydrolysed/min/mg protein) of the compounds **28,32 and 36**

Groups	**Acetylcholinesterase activity**
Control	18 ± 1.0
Scopolamine	31 ± 1.6[α]
SCO + 28	23 ±0.72[a]
SCO + 32	26 ±0.90[c]
SCO + 36	18 ± 0.91[a]
SCO + Donepezil	20 ± 1.0[a]

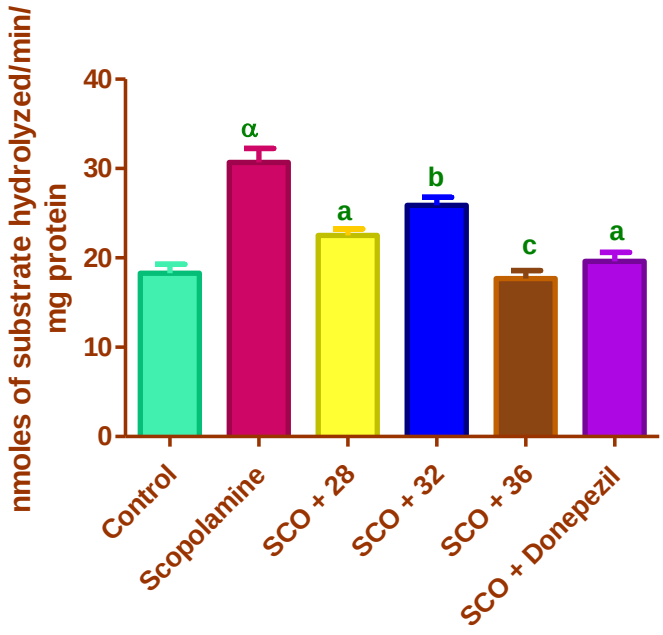


Figure 10: Effect of selected synthesized compounds on acetylcholinesterase activity .Data were expressed as mean±SEM, 5 rats in each group, and analyzed by ANOVA followed by Tukey's post hoc test. $^{\alpha}p < 0.001$, when compared to the normal control group; $^{a}p < 0.001$, $^{b}p < 0.01$, $^{c}p < 0.05$ when compared to Scopolamine group.

The results of the acetylcholinesterase inhibitory profile were in accordance with the behavioral studies. A significant increase in acetylcholinesterase activity was seen in scopolamine group ($p < 0.001$) when compared to the control group. Treatment with compounds **28 & 36** and donepezil showed a significant decrease in acetylcholinesterase activity ($p < 0.001$), whereas treatment with compound **32** resultant only moderate and mild significant decrease ($p < 0.01$ and $p < 0.05$).

2.7 Computational Study

2.7.1 Prediction of ADME Properties

In the drug discovery and development pipeline, the prediction of ADME properties of a molecule seems to be of great significance. Lipinski's rule of five can be used to predict the drug-like properties of a molecule and develop it as an orally active therapeutic agent. A molecule is predicted to be orally active when there is no violation of Lipinski's rule of five by the molecules. This rule encompasses the parameters such as log P, molecular weight, number of hydrogen bond acceptors, and number of hydrogen bond donors. Log P determines the octanol–water partition coefficient of the molecule *i.e.* the lipophilic or hydrophilic nature of the molecule indicative of brain or peripheral targeting. According to this rule, the Log P of a drug-like molecule should be ≤ 5. The molecular weight of the molecule should be < 500. The calculation of topological polar surface area (TPSA) is an important parameter of the molecule. It is calculated as a sum of O- and N- polar[26].

3.0 **Experimental**

3.1 Instrumentations and Chemicals

All reactions were carried out in oven dried-glassware's were appropriately monitored. All reagents were commercially available and were acquired from Sigma Aldrich (India), Loba Chemie, Mumbai, India, HiMedia Laboratories Pvt. Ltd., Mumbai, India, Qualigens fine chemicals, Sisco research laboratories Pvt. Ltd, Mumbai, and Spectrochem Pvt. Ltd. Mumbai. The progress of the chemical reactions were monitored by thin-layer chromatography on silica gel 60-F254 plates acquired from E. Merck (Darmstadt, Germany). The reaction products were purified by the method of recrystallization by using an appropriate solvent. Melting points were determined on the Veego melting point apparatus and were uncorrected. IR spectra were recorded on Perkin Elmer spectrum version 10.03.08. The synthesized compounds were analyzed by NMR on Bruker Avance II 400 NMR spectrometer. ^{1}H and ^{13}CNMR spectra were recorded as solutions in DMSO d6 (Aldrich) and $CDCl_3$ (Aldrich). Chemical shifts (d) are given in parts per million (ppm).

3.2 *Chemistry*

3.2.1 **5-Phenyl-1,3,4-thiadiazol-2-amine (2)**

A mixture of benzoic acid (**1**) (5.0 g, 40.94 mmol), thiosemicarbazide (3.67 g, 40.26 mmol) and phosphorus oxychloride (9.25 mL) was refluxed at 120°C for 12 h under continuous stirring. The reaction mixture was cooled to room temperature and was refluxed further for 2 h after the addition of water (55 mL). After cooling, the mixture was basified to pH 9 by dropwise addition of 50% sodium hydroxide solution. The solid so obtained was filtered, dried, and recrystallized from ethanol to afford **2** (5.65 g, 78%), **m.p.** 220-221°C (Lit 224-225 °C)[23].

Spectral Analysis

FT-IRν_{max} (KBr): 3276.58 (*N-H* stretch), 3086.61, 1631.67 (*C*=N stretch), 1514.43, 1468.73, 1263.15, 1136.64, 1058.25, 759.26 and 688.87 cm^{-1}.

^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 4.18 (s, 2H, -CH_2-), 6.95 (br s, 2H, -NH_2) and 7.28 (m, 5H, Ar*H*) ppm.

3.2.3 **Synthesis of *N*-(5-phenyl-1,3,4-thiadiazol-2-yl)-1-(4-chlorophenyl)methanimine (4a)**

A mixture of 4-chloro benzaldehyde (**3a**) (11.8 ml) and 2-amino-5-phenyl-1,3,4-thiadiazole (**2**) (1.41 mmol, 0.400 g) in absolute methanol (5.0 ml) and few drops of glacial acetic acid was refluxed for 26 h. The reaction mixture was cooled to room temperature and the suspension was then stirred for 30 min at room temperature after the addition of 10.0 ml of distilled water. The solid so obtained was filtered, dried, and recrystallized from ethyl acetate to afford **4a** (0.360g, 90%) (mp226-228 ^{0}C) [28].

Spectral Analysis

FT-IRν_{max} (KBr): 3086.61, 1603.67 (*C*=N stretch), 1516.43, 1468.73, 1263.15, 1136.64, 1058.25, 759.26 and 688.87 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ 7.54.87 (s, 2H, Ar*H*), 7.61 (m, 3H, Ar*H*), 7.61 (d, 1H, J_o = 7.72 Hz, Ar*H*), 7.75 (d, 1H, J_o = 7.72 Hz, Ar*H*), 7.99 (m, 2H, Ar*H*), 9.12 (s, 1H, *N*=*C*H) ppm.

3.2.4 **Synthesis of 1-[4-methoxy phenyl-*N*-(5-phenyl-1,3,4-thiadiazol-2-yl)]methanimine (4b)**

A mixture of 4- methoxy benzaldehyde (**3b**) (11.8 ml) and 2-amino-5-phenyl-1,3,4-thiadiazole (**2**) (1.41 mmol, 0.400 g) in absolute methanol (5.0 ml) and few drops of glacial acetic acid was refluxed for 26 h. The reaction mixture was cooled to room temperature and the suspension was then stirred for 30 min at room temperature after the addition of 10.0 ml of distilled water. The solid so obtained was filtered, dried, and recrystallized from ethyl acetate to afford **4b** (0.360g, 90%) (mp220-222 ^{0}C) [28].

Spectral analysis:

FT-IRν_{max} (KBr): 3086.61, 1603.67 (*C=N* stretch), 1516.43, 1468.73, 1263.15, 1136.64, 1058.25, 759.26 and 688.87 cm^{-1}.

3.2.5 Synthesis of *N-(4-chlorobenzyl)-5-phenyl-1,3,4-thiadiazol-2-amine (**5a**)*

Solid $NaBH_4$ (15.67 mmol, 0.593 g) was slowly added to the compound (**4a**) (1.00 mmol, 0.297 g) in methanol (10.0 ml), and the resulting mixture was then continued for 1 h at room temperature Afterward, 20.0 ml of distilled water was added, as well as 2 M HCl, until pH 2 was reached, and the formed precipitate was stirred for 30 more minutes at the room temperature. After cooling the flask in a refrigerator for 1.5 h, the precipitate was filtrated, dried and recrystallized from EtOH to afford **5a** (0.245g, 82.4%) **m.p.** 216-218 °C [28].

Spectral Analysis

FT-IRν_{max} (KBr): 3576.58 (*N-H* stretch), 3086.61, 1603.67, 1516.43, 1468.73, 1263.15, 1136.64, 1058.25, 759.26 and 688.87 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ 7.54.87 (s, 2H, Ar*H*), 7.61 (m, 3H, Ar*H*), 4.35 (d, 2H, J_o = 5.4 Hz, NH_2), 7.75 (d, 1H, J_o = 7.72 Hz, Ar*H*), 7.77 (m, 2H, Ar*H*), 9.12 (s, 1H, N=*C*H) ppm.

3.2.6 Synthesis of *N-(4-methoxybenzyl)-5-phenyl -1,3,4-thiadiazol-2-amine (**5b**)*

Solid $NaBH_4$ (15.67 mmol, 0.593 g) was slowly added to the compound (**4b**) (1.00 mmol, 0.297 g) in methanol (10.0 ml), and resulting mixture was then continued for 1 h at room temperature Afterward, 20.0 ml of distilled water was added, as well as 2 M HCl, until pH 2 was reached, and the formed precipitate was stirred for 30 more minutes at the room temperature. After cooling the flask in a refrigerator for 1.5 h, the precipitate was filtrated, dried, and recrystallized from EtOH to afford **5b** (0.245g, 82.4%) **m.p.** 216-218 °C [23].

Spectral Analysis

FT-IRν_{max} (KBr): 3576.58 (*N-H* stretch), 3086.61 (aromatic -*CH* Stretch), 1603.67 (*C*=N Stretch), 1516.43, 1468.73, 1263.15, 1136.64 (C-O-C Stretch) 1058.25, 759.26 and 688.87 cm^{-1}.

3.2.7 **Synthesis of** *N-(4-chlorobenzyl)-N-(5-phenyl-1,3,4-thiadiazol-2-yl) benzamide **(28) (AA-28)***

N-(4-chlorobenzyl)-5phenyl-1,3,4-thiadiazol-2-amine **(5a)** (0.5 g, 2.82 mmol) was dissolved in anhydrous tetrahydrofuran (30 mL) and aq NaOH (50%) was added in catalytic amount (60 mL) to the solution with continuous stirring. Subsequently, benzoyl chloride (**6a**) (0.49 mL, 3.4 mmol) was added dropwise to the reaction mixture and the contents were refluxed at 70 °C for 10 h. The completion of the reaction was ascertained by TLC. The slurry formed was filtered and the solvent was removed under reduced pressure to obtain a solid residue. It was then recrystallized from ethanol to afford (2**8**) **AA -28** as a yellow crystalline solid (0.67 g, 67%), **m.p.** 241-243 °C[22].

Spectral and elemental analyses:

FT-IRν_{max} (KBr): 3104.3 (Aromatic CH), 2933.07 (Aliphatic-CH), 1666.6 (C=O, amide), 1572.8, 1371.08(C-N Stretch), 1118.1, 1028.4 (Ar-Cl) 723.2 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ 5.01 (s, 2H, -CH_2), 7.30 (m, 2H, Ar*H*), 7.32 (m 2H, Ar*H*), 7.37 (m, 2H, Ar*H*), 7.41 (m, 1H, Ar*H*), 7.51 (m, 2H, Ar*H*), 7.63 (m, 2H, Ar*H*), 7.70 (m, 1H, Ar*H*), 8.03 (m, 2H, Ar*H*) ppm.

^{13}C NMR ($CDCl_3$ + DMSO-d_6): δ 49. 9(*C*H_2), 127.5 (2X Ar*C)*, 128.6 (2X Ar*C*), 128.7 (Ar*C*), 128.8 (2×Ar*C*), 129.2 (2×Ar*C*), 129.3 (2×Ar*C*), 130.9 (2 X Ar*C*), 132.1(Ar*C*), 132.3(Ar*C*q-Cl), 133.5 (Ar*C*q), 134.4 (Ar*C*q), 135.6 (Ar*C*q), 163.4 (*C*q, thiadiazole), 171.8(Ar*C*=O) 174.1 (*C*q, thiadiazole) ppm.

ESI-MS: m/z 406.08 $[M+1]^+$

3.2.8 **Synthesis of** *N-(4-chlorobenzyl) -N-(5-phenyl-1,3,4-thiadiazol-2-yl)-2-fluorobenzamide **(29) (AA-29)***

*N-4-chlorobenzyl-5-phenyl-1,3,4-thiadiazol-2-amine **(5a)*** (0.5 g, 2.82 mmol) was dissolved in anhydrous tetrahydrofuran (30 mL) and aq NaOH (50%) was added in catalytic amount (60 mL) to the solution with continuous stirring. Subsequently, 2-fluoro benzoyl chloride (**6b**) (0.49 mL, 3.4 mmol) was added dropwise to the reaction mixture and the contents were refluxed at 70 °C for 10 h. The completion of the reaction was ascertained by TLC. The slurry formed was filtered and the solvent was removed under reduced pressure to obtain a solid residue. It was then recrystallized from ethanol to afford **(29) AA-29** as a yellow crystalline solid (0.66 g, 66.0%), **m.p.** 196-198 °C[35].

Spectral Analysis

FT-IRν_{max} (KBr): 3104.9 (Aromatic CH), 2981.9 (Aliphatic-CH), 1666.6 (C=O, amide), 1572.9, 1464.08(C-N Stretch), 1192.1, 1114.5 (Ar-F) 723.2(Ar-Cl) cm^{-1}.

^{1}H NMR ($CDCl_3$): δ 5.01 (s, 2H, -CH_2), 7.32 (m, 2H, Ar*H*), 7.37 (m 2H, Ar*H*), 7.40 (m, 1H, Ar*H*), 7.41 (m, 1H, Ar*H*), 7.42 (m, 1H, Ar*H*), 7.51 (m, 2H, Ar*H*), 8.0 (m, 1H, Ar*H*), 8.01 (m, 1H, Ar*H*), and 8.03 (m, 2H, Ar*H*) ppm.

^{13}C NMR ($CDCl_3$ + DMSO-d_6): δ 49. 9(*C*H_2), 115.6 (*ArC*), 125.1 (Ar*C)*, 126.0 (*ArC*), 128.6 (2X Ar*C*), 128.7 (Ar*C*), 129.2 (2×Ar*C*), 129.3 (2×Ar*C*), 130.9 (2 X Ar*C*), 131.3(Ar*C*), 132.3(Ar*C*q-Cl), 133.5 (Ar*C*q), 134.2 (Ar*C*q),163.4 (*C*q, thiadiazole), 169.6 (Ar*C*=O) 174.1 (*C*q, thiadiazole) ppm.

ESI-MS: m/z 424.1 $[M+1]^+$ 425.06 $[M+2]^+$

3.2.10 **Synthesis of** *N-4-chlorobenzyl-N-4-fluoro-(5-phenyl-1,3,4-thiadiazol-2-yl)-benzamide **(30) (AA-30)***

*N-4-chlorobenzyl-5-phenyl-1,3,4-thiadiazol-2-amine **(5a)*** (0.5 g, 2.82 mmol) was dissolved in anhydrous tetrahydrofuran (30 mL) and aq NaOH (50%) was added in catalytic amount (60 mL) to the solution with continuous stirring. Subsequently, 4-fluoro benzoyl chloride (**6c**) (0.49 mL, 3.4 mmol) was added dropwise to the reaction mixture and the contents were refluxed at 70 °C for 10 h. The completion of the reaction was ascertained by TLC. The slurry formed was filtered and the solvent was removed under reduced pressure to obtain a solid residue. It was then recrystallized from ethanol to afford **30 (AA-30)** as a brown crystalline solid (0.48 g, 48.7%), **m.p.** 208-210 °C[35].

Spectral Analysis

FT-IRν_{max} (KBr): 3101.1 (Aromatic CH), 2981.9 (Aliphatic-CH), 1666.6 (C=O, amide), 1572.9, 1461.1(C-N Stretch), 1192.1, 1114.5 (Ar-F) 723.2 (Ar-Cl) cm^{-1}.

^{1}H NMR ($CDCl_3$): δ 5.01 (s, 2H, -CH_2), 7.32 (m, 2H, Ar*H*), 7.37 (m 2H, Ar*H*), 7.41 (m, 1H, Ar*H*), 7.42 (m, 2H, Ar*H*), 7.51 (m, 2H, Ar*H*), 8.03 (m, 2H, Ar*H*), and 8.12 (m, 2H, Ar*H*) ppm.

^{13}C NMR ($CDCl_3$ + DMSO-d_6): δ 49. 9(*C*H_2), 115.6 (2X *ArC*),128.6 (2X Ar*C*), 128.7 (Ar*C*), 129.1 (2 X Ar*C*), 129.2 (2×Ar*C*), 129.3 (2×Ar*C*), 130.9 (2 X Ar*C*), 131.2(Ar*C*), 132.3(Ar*C*q-Cl), 133.5 (Ar*C*q), 134.2 (Ar*C*q),163.4 (*C*q, thiadiazole), 166.3(Ar*C*q-F), 171.8 (Ar*C*=O) 174.1 (*C*q, thiadiazole) ppm.

ESI-MS: m/z 424.01 $[M+1]^+$

3.2.11 **Synthesis of** *N-(4-chlorobenzyl)-N-2-chloro-(5-phenyl-1,3,4-thiadiazol-2-yl)-benzamide (32)* ***(AA-32)***

N-(4-chlorobenzyl)-5-phenyl-1,3,4-thiadiazol-2-amine ***(5a)*** (0.5 g, 2.82 mmol) was dissolved in anhydrous tetrahydrofuran (30 mL) and aq NaOH (50%) was added in catalytic amount (60 mL) to the solution with continuous stirring. Subsequently, 2-chloro benzoyl chloride (**6d**) (0.49 mL, 3.4 mmol) was added dropwise to the reaction mixture and the contents were refluxed at 70 °C for 10 h. The completion of the reaction was ascertained by TLC. The slurry formed was filtered and the solvent was removed under reduced pressure to obtain a solid residue. It was then recrystallized from ethanol to afford (**32**) (**AA-32)** as a light brown crystalline solid (0.56 g, 56%), **m.p.** 218-221°C [35].

Spectral Analysis

FT-IRν_{max} (KBr): 3101.1 (Aromatic CH), 2929.9 (Aliphatic-CH), 1673.6 (C=O, amide), 1517.0, 1423.1(C-N Stretch), 1174.1, 1088.5 (Ar-Cl) 711.2 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ 5.01 (s, 2H, -CH_2), 7.32 (m, 3H, Ar*H*), 7.37 (m 2H, Ar*H*), 7.41 (m, 1H, Ar*H*), 7.43 (m, 1H, Ar*H*), 7.51 (m, 3H, Ar*H*) and 8.03 (m, 2H, Ar*H*) ppm.

^{13}C NMR ($CDCl_3$ + DMSO-d_6): δ 49. 9(*C*H_2), 126.9 (*ArC*),128.6 (2X Ar*C*), 128.7 (Ar*C*), 128.9 (Ar*C*), 129.2 (2×Ar*C*), 129.3 (2×Ar*C*), 130.1 (Ar*C*), 130.9 (2 X Ar*C*), 132.3(Ar*C*q-Cl), 132.3 (Ar*C*q), 133.5 (2 X Ar*C*q), 134.2 (Ar*C*q), 134.6(Ar*C*q-Cl),163.4 (*C*q, thiadiazole), 1696.0 (Ar*C*=O) 174.1 (*C*q, thiadiazole) ppm.

ESI-MS: m/z 440.67 $[M+1]^+$

Synthesis of *N-(4-methoxybenzyl)-N-2-chloro-(5-phenyl-1,3,4-thiadiazol-2-yl)-benzamide* ***(36) (AA-36)***

N-(4-methoxybenzyl)-5-phenyl-1,3,4-thiadiazol-2-amine **(5b)** (0.5 g, 2.82 mmol) was dissolved in anhydrous tetrahydrofuran (30 mL) and aq NaOH (50%) was added in catalytic amount (60 mL) to the solution with continuous stirring. Subsequently, 2-chloro benzoyl chloride (**6b**) (0.49 mL, 3.4 mmol) was added dropwise to the reaction mixture and the contents were refluxed at 70 °C for 10 h. The completion of the reaction was ascertained by TLC. The slurry formed was filtered and the solvent was removed under reduced pressure to obtain a solid residue. It was then recrystallized from ethanol to afford (**36**) **AA-36** as a pale yellow crystalline solid (0.67g, 67.7%), **m.p.** 249-252 °C [24].

Spectral Analysis

FT-IRν_{max} (KBr): 3071.1 (Aromatic CH), 2929.7 (Aliphatic-CH), 1673.6 (C=O, amide), 1569.0, 1453.1(C-N Stretch), 1285.1(C-O-C Stretch), 1189.1, 1084.5 (Ar-Cl) 767.2 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ 3.83 (s.3H -OCH_3), 5.01 (s, 2H, -CH_2), 6.87 (m. 2H, Ar*H*)7.25 (m, 2H, Ar*H*), 7.43 (m 2H, Ar*H*), 7.41 (m, 1H, Ar*H*), 7.43 (m, 1H, Ar*H*), 7.51 (m, 3H, Ar*H*) and 8.03 (m, 2H, Ar*H*) ppm.

^{13}C NMR ($CDCl_3$ + DMSO-d_6): δ 49. 9(*C*H_2), 55.8 (-*OC*H_3). 114.6 (2X *ArC*), 126.9 (Ar*C*),128.4 (2Ar*C*), 128.9 (Ar*C*), 129.2 (2 X Ar*C*), 130.1 (Ar*C*), 130.5 (2 X Ar*C*), 130.9 (2 X Ar*C*), 132.3(Ar*C*q-), 133.5 (Ar*C*), 133.5 (Ar*C*q), 134.6(Ar*C*q-*Cl*), 158.6 (Ar*C*q-O*C*H_3), 163.4 (*C*q, thiadiazole), 169.6 (Ar*C*=O) 174.1 (*C*q, thiadiazole) ppm.

ESI-MS: m/z 435.08$[M+1]^{+,}$ 437.27 $[M+2]^{+}$

4.0 ***Pharmacological Evaluation***

4.1 Animals:

Swiss Albino mice (Balb/c strain, 25-30 g) were purchased from CSIR IHBT Palampur HP, India. All the research protocols were approved by the Institutional Animal Ethical Committee (CPCSEA/LIPH/2022/16). The treatment and maintenance of animals were conducted in accordance with the CPCSEA guidelines. Animals were housed 6 per cage with food and water ad libitum and maintained at a constant temperature (25 ±1 ^{o}C) and humidity (55 ± 10%) under a 12 h light/dark cycle. All the behavioral tasks were performed between 10.00 h and 16.00 h.

4.1.1 In-vitro Acetylcholinesterase Inhibition

Acetylcholinesterase inhibition was determined by following the protocol laid by Ellman et al. [27,34] by using mouse brain homogenate as the source of the enzyme. Whole mouse brains of Balb/C strain (25–40 g) were decapitated and rinsed with ice-cold normal saline solution and homogenized in a glass Teflon homogenizer (REMI MOTORS, India) containing 50 vol of sodium phosphate buffer (pH 8.0, 0.1 M) and centrifuged (Research centrifuge, REMI, R- 24) at 10,000 rpm for 20 min at 40^0C to prepare the supernatant. The obtained supernatant was used as the source of enzyme for the assay. Stock solutions of the synthesized compounds and their five different concentrations level (1, 5, 10, 25, and 50 ml) were prepared. Subsequently, a liquor from various concentrations was mixed with phosphate buffer (pH 8.0, 3.0 ml), 100 ml of buffered Ellman's reagent, 100 ml of acetylthiocholine iodide solution, and 50 ml of supernatant and the change in optical density (OD) was measured immediately at 412 nm for 2 min using PERKIN ELMER UV/VIS spectrophotometer. Donepezil (1) was used as the reference standard. The AChE activity was calculated using the formula: AChE degree = (0.05 change in optical density)/mg protein. Additionally, the concentration of each compound required to inhibit acetylcholinesterase activity by 50% (IC_{50}) was also calculated. The protein estimation was carried out using the Biuret method using a Biuret reagent prepared by mixing sodium potassium tartrate (4.5 g) in 40 ml of 0.2 N NaOH, cupric sulfate pentahydrate and potassium iodide (0.5 g) and made up the volume 100 ml with 0.2 N NaOH solution [17]. The working solution was prepared by mixing 50 ml of supernatant, 2.9 ml of normal saline, and 3 ml of Biuret reagent and incubated at room temperature for 10 min and the optical density was measured at 540 nm in the UV–VIS spectrophotometer.

4.1.2 **DPPH (1,1-diphenyl-2-picrylhydrazyl) Scavenging Activity**

Antiradical activities of all the synthesized compounds (**28, 29, 30, 32 & 36)** were examined using DPPH (1,1-diphenyl-2-picrylhydrazyl) method. Ascorbic acid was used as the standard. 1 mL solution of each compound at different concentrations (10, 25, 50, 75, and 100 μM) in DMSO (0.1%) was mixed with 2 mL of DPPH solution (0.5 mM). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. The absorbance was measured at 517 nm against blank solutions containing synthesized compounds (10, 25, 50, 75, and 100 μM) in 0.1% DMSO without DPPH using a spectrophotometer. The higher antiradical activity was observed for the reaction mixture which showed lower absorbance. The experiment was performed in triplicates. The antiradical activity was calculated as a percentage according to the following formula.

% Scavenged DPPH = ((Absorbance of control – Absorbance of sample)/Absorbance of control)) x 100

4.1.3 **Scavenging of Hydrogen Peroxide**

The ability of all the synthesized compounds (**28, 29, 30, 32,** and **36**) to scavenge H_2O_2 was determined spectrophotometrically [30,31]A 4mM solution of H_2O_2 was prepared at room temperature in phosphate-buffered saline. Using a molar extinction coefficient for H_2O_2 of 81 mol/cm the concentration of H_2O_2 was determined spectrophotometrically. The synthesized compounds were dissolved in DMSO (0.1%) and then added to the H_2O_2 solution at a final concentration of 0, 10, 25, 50, 75, and 100 µM at 20 °C. H_2O_2 absorbance was measured after 10 mins at 230 nm using PERKIN ELMER UV/VIS spectrophotometer against blank solutions containing synthesized compounds at concentrations of 10, 25, 50, 75, and 100 µM in PBS without H_2O_2. All the experiments were performed in triplicate. The results of the H_2O_2 scavenging activity assay were expressed as percentage inhibition.

% Scavenged H_2O_2 = ((Absorbance of control – Absorbance of sample)/Absorbance of control)) x 100

4.2 In vivo Behavioral Studies

4.2.1 Step Down Passive Avoidance and Escape Learning Method

Swiss [33]Albino mice (Balb/c strain, 25-30 g) were purchased from CSIR IHBT Palampur HP, India (40 mice). All the research protocols were approved by the Institutional Animal Ethical Committee (CPCSEA/LIPH/2022/16). The treatment and maintenance of animals were conducted in accordance with the CPCSEA guidelines. Animals were housed 6 per cage with food and water ad libitum and maintained at a constant temperature (25 ±1ºC) and humidity (55 ± 10%) under a 12 h light/dark cycle. All the behavioral tasks were performed between 10.00 h and 16.00 h [33].

Three (based on in vitro studies) synthesized compounds (**28, 32,** and **36**) were evaluated for their effects on behavioral alterations using step down passive avoidance test. Donepezil (0.5 mg/kg) was used as a standard drug. Scopolamine (2 mg/kg i.p.) was used to induce cognitive impairment in mice. All the test compounds and the standard were suspended in 1% tween-80 solution and administered intraperitoneally (i.p.) to the animals. The control group was not treated with any compound.

The passive avoidance test is based on negative reinforcement and is used in the assessment of short-term and aversive memory. The mice were divided into 8 groups with five mice in each. Immediately after training the solution containing either saline or scopolamine (2.0 mg/kg) was administered to all groups followed by the second injection of either of the vehicle (1% tween-80), donepezil, or the test compounds, after 5 min of the first injection. Escape learning and step-down passive avoidance test were carried out using a rodent memory evaluator, which consists of a fabricated box with an electric grid and a wooden platform placed centrally (shock-free zone). The experiment consists of three phases: (i) Familiarization: Each mouse was allowed to explore the box for 10 s by being placed on the wooden platform set in the center of the grid floor and returned to the home cage. (ii) Training: Each mouse was placed on the wooden platform in the center of the grid floor. When the mouse stepped down from the wooden platform and placed all its paws on the grid floor, the intermittent shock was delivered until the mice returned to the wooden platform for escaping from the electric shock [33]The step-down latency (SDL) and escape latency (EL) were measured, and the mice in a range of SDL, 3-15 s; EL, 15-60 s were used for the retention test. Immediately after the training test, the mice were treated with either scopolamine or standard drug or test compounds immediately after training test. (iii) Retention: Twenty-four hours after the training, each mouse was placed on a wooden platform, and the SDL was recorded, when the animal stayed on the platform or stepped down, until the cut-off time (180 s). The animal was placed on the grid floor in the corner of the inner box with its tail towards the platform, EL was recorded with the electric shock given to the animal. Additionally, the mistake mode was turned on and the number of mistakes (as soon as the mice touched the electric grid and felt the shock) made by the mice in 15 min was noted. The number of mistakes and latency data was analyzed and the significance was determined by applying one-way ANOVA followed by Tukey test.

4.3 Ex-vivo Studies and Estimation of the Biomarkers of Oxidative Stress

For *ex-vivo* studies, the animals used in *in vivo* studies were sacrificed by cervical dislocation after the completion of behavioral assessment. The brains were removed and were separately rinsed with ice-cold isotonic saline solution. A homogenate was prepared in 0.1 M phosphate
buffer (pH 7.4). The homogenate was centrifuged at 10000 rpm for 15 min and the supernatant obtained was separated and used for the biochemical assays[30].

4.3.1. Assay of Acetylcholinesterase

The cholinergic marker, acetylcholinesterase, was estimated in the whole brain according to the method of Ellman, and the results were expressed as nmoles of substrate hydrolyzed/min/mg protein[34].

4.4 Statistical Analysis

Results were expressed as mean ± SEM The statistical analysis was done using GraphPad Prism 5. The intergroup variation was measured by analysis of variance (ANOVA). Statistical significance was considered at $p < .001$.

Conclusion

A series of novel benzyl benzamide substituted 1,3,4 Thiadiazole derivatives has been designed, synthesized and evaluated for acetylcholinesterase inhibitory activity and the management of Alzheimer's disease. Based on molecular docking studies, it can be inferred that these active compounds displayed significant binding interactions with the binding pocket of the enzyme acetylcholinesterase. The potent compounds were synthesized which afforded significant percentage yields ranging from 50 to 60%. Based on the results of the behavioral and biochemical studies, it can be ascertained that compounds **28** & **36** displayed appreciable acetylcholinesterase inhibitory activity. The results of the *ex-vivo* AChE inhibition assay were also in agreement with the behavioral studies data. Thus, the study indicated that benzyl benzamide substituted 1, 3,4 thiadiazole derivatives can serve as a propitious scaffold for exhibiting significant activity in the management of Alzheimer's disease, and compounds **28 & 36** can serve as promising leads for any further pursuit in this area of research.

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Declaration

The authors have not declared any financial interests/personal relationships which may be considered potential competing interests.

References

[1] O.D.A.D. Giuliana Klencklen. What do we know about aging and spatial cognition? Reviews and perspectives, Ageing Res Rev. 11 (2012) 123–135.

[2] Olle Blomberg, Conceptions of Cognition for Cognitive Engineering, The International Journal of Aviation Psychology. 21 (2011) 85–104.

[3] B. Klimova, K.K.-J. of A. Biomedicine, 2016, Speech and language impairments in dementia, Elsevier. (n.d.). https://www.sciencedirect.com/science/article/pii/S1214021X15300387 (accessed April 2, 2023).

[4] K. Dassel, J. Butler, J.T.-E. Gerontology, 2020, Development and evaluation of Alzheimer's Disease and Related Dementias (ADRD) best care practices in long-term care online training program, Taylor & Francis. (n.d.). https://www.tandfonline.com/doi/abs/10.1080/03601277.2020.1717079 (accessed April 2, 2023).

[5] J. Bongaerts, D. De Bundel, D.M. of P. 2018, Sensitive targeted methods for brain metabolomic studies in microdialysis samples, Elsevier. (n.d.). https://www.sciencedirect.com/science/article/pii/S0731708518314407 (accessed April 2, 2023).

[6] J.S. Buckley, S.R. Salpeter, A Risk-Benefit Assessment of Dementia Medications: Systematic Review of the Evidence, Drugs Aging. 32 (2015) 453–467. https://doi.org/10.1007/S40266-015-0266-9.

[7] Y. Li, J. Geng, Y. Liu, S. Yu, G. Zhao, Thiadiazole-a Promising Structure in Medicinal Chemistry, Chem. Med. Chem. 8 (2013) 27–41. https://doi.org/10.1002/CMDC.201200355.

[8] N. Siddiqui, W. Ahsan, S. Alam, Thiadiazoles: Progress Report on Biological Activities Quality control tests of dosage forms View project M.pharm View project, (2009). https://www.researchgate.net/publication/266049200 (accessed April 3, 2023).

[9] J. Sandström, Recent Advances in the Chemistry of 1,3,4-Thiadiazoles, Adv Heterocycl Chem. 9 (1968) 165–209. https://doi.org/10.1016/S0065-2725(08)60373-6.

[10] Carbonic anhydrase inhibitors: anticonvulsant sulfonamides incorporating valproyl and other lipophilic moieties, ACS Publications. (n.d.). https://pubs.acs.org/doi/abs/10.1021/jm0109199 (accessed April 3, 2023).

[11] J. Goerdeler, J. Ohm, O. Tegtmeyer, Darstellung und Eigenschaften des 1.2.4- und des 1.3.4-Thiodiazols, Chem Ber. 89 (1956) 1534–1543. https://doi.org/10.1002/CBER.19560890624.

[12] P.W.-C. neuroscience & therapeutics, 2011, Acute drug administration in epilepsy: a review, Wiley Online Library. 17 (2011) 442–448. https://doi.org/10.1111/j.1755-5949.2010.00167.x.

[13] D. Hartwig, J.E.R. Nascimento, L. Bettanin, T.F.B. Aquino, R.G. Jacob, E.J. Lenardão, Deep Eutectic Solvents: An Alternative Medium for the Preparation of Organosulfur Compounds, Current Green Chemistry. 7 (2020) 179–200. https://doi.org/10.2174/2213346107999200616110434.

[14] A. Siwek, T. Plech, J. Stefańska, … P.S.-C.C.-A., 2014, Molecular properties prediction, docking studies and antimicrobial screening of 1, 3, 4-thiadiazole and S-triazole derivatives, Researchgate.Net. (2013). https://doi.org/10.2174/15734099113096660033.

[15] B. Sharma, A. Verma, S.P. journal of medicinal, 2013, Synthetic methods, chemistry, and the anticonvulsant activity of thiadiazoles, Downloads.Hindawi.Com. 2013 (2013) 16. https://doi.org/10.1155/2013/348948.

[16] F. Gualtieri, S. Dei, D. Manetti, M.R.- Farmaco, 1995, The medicinal chemistry of Alzheimer's and Alzheimer-like diseases with emphasis on the cholinergic hypothesis, Hero.Epa.Gov.https://hero.epa.gov/hero/index.cfm/reference/details/reference_id/2747595 (accessed April 3, 2023).

[17] 17. VLife MDS 4.6, version 4.6.28102016, Molecular, Design, Suite, Vlife, Sciences Technologies Pvt. Ltd. 2013.www.vlifesciences.com (accessed April 3, 2023).

[18] T.L. Rosenberry, X. Brazzolotto, I.R. MacDonald, M. Wandhammer, M. Trovaslet-Leroy, S. Darvesh, F. Nachon, Comparison of the Binding of Reversible Inhibitors to Human Butyrylcholinesterase and Acetylcholinesterase: A Crystallographic, Kinetic and Calorimetric Study, Molecules 2017, Vol. 22, Page 2098. 22 (2017) 2098. https://doi.org/10.3390/MOLECULES22122098.

[19] 109. Guo, Y.; Yang, H.; Huang, Z.; Tian, S.; Li., C+Q. B., Du, C.; Chen, C.; Liu, C.; Sun, C. Design, Synthesis and Evaluation of Acetylcholinesterase and Butyrylcholinesterase Dual-Target Inhibitors Against Alzheimer's Diseases. Molecules, 2020, 25(2), 489. (accessed April 3, 2023).

[20] S. Haider, M. Alam, H.H.-E. journal of medicinal chemistry, 2015, 1, 3, 4-Thiadiazoles: A potent multi targeted pharmacological scaffold, Elsevier. https://www.sciencedirect.com/science/article/pii/S0223523414011441 (accessed April 3, 2023).

[21] Y. Hu, C.Y. Li, X.M. Wang, Y.H. Yang, H.L. Zhu, 1,3,4-Thiadiazole: Synthesis, reactions, and applications in medicinal, agricultural, and materials chemistry, Chem Rev. 114 (2014)5572–5610. https://doi.org/10.1021/CR400131U/ASSET/IMAGES/CR400131U.SOCIAL.JPEG_V03.

[22] M. Foxton, G. Gregory, 074,047 DM Rogers - US Patent 4, 1978, 7 2-(Aminocarbonylalkoxyimino) acetamido! derivatives of cephalosporin, Google Patents. (n.d.). https://patents.google.com/patent/US4074047A/en (accessed April 3, 2023).

[23] 24. Foxton, M. W.; Gregory, G. I.; Rogers, D. M.... - Google Scholar, (n.d.). https://scholar.google.com/scholar C.Foxton, C.M., W.B.Gregory , C.G., I.B., C.D., M. 2- Aminocarbonylalkoxyimino%29acetamido Derivatives of Cephalosporin.1978, (accessed April 3, 2023).

[24] A. Kulshreshtha, P. Piplani, Design, synthesis and pharmacological evaluation of carboxamide and carbothioamide derivatives of 1,3,4-thiadiazole as the inhibitors of acetylcholinesterase and oxi-piperazine) oxidative stress for the management of cognitive debility. Med. Chem. Res. 27(2018) 1800–1821. https://doi.org/10.1007/S00044-018-2193-X.

[25] C. da Silva, V. Campo, I.C.-J. of molecular, 2006, Molecular modeling, docking and ADMET studies applied to the design of a novel hybrid for treatment of Alzheimer's disease.https://www.sciencedirect.com/science/article/pii/S1093326305001701 (accessed April 3, 2023).

[26] A. Ajala, A. Uzairu, G.A. Shallangwa, S.E. Abechi, 2D QSAR, design, docking study and ADMET of some N-aryl derivatives concerning inhibitory activity against Alzheimer disease, Futur J Pharm Sci. 8 (2022). https://doi.org/10.1186/S43094-022-00420-W.

[27] 28. Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherst... - Google Scholar, (n.d.). https://scholar.google.com/scholar? C. Ellman, CG.L., B.Courtney, C.K.,D. Andres, B.Featherstone,C.R.+M.A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity. Biochem.Pharmacol.1961, 88-95.(accessed April 3, 2023).

[28] K. Jakovljević, M. Joksović, B. Botta, L.J. C.R., 2019, Novel 1, 3, 4-thiadiazole conjugates derived from protocatechuic acid: Synthesis, antioxidant activity, and computational and electrochemical studies. https://www.sciencedirect.com/science/article/pii/S1631074819300980 (accessed April 4, 2023).

[29] P. Guan, L. Wang, X. Hou, Y. Wan, W. Xu, … W.T.-B.& medicinal, 2014, Improved antiproliferative activity of 1, 3, 4-thiadiazole-containing histone deacetylase (HDAC) inhibitors by introduction of the heteroaromatic surface recognition motif. https://www.sciencedirect.com/science/article/pii/S0968089614006853 (accessed April 4, 2023).

[30] M.B.- Nature, 1958, Antioxidant determinations by the use of a stable free radical, Springer. https://link.springer.com/content/pdf/10.1038/1811199a0.pdf (accessed April 4, 2023).

[31] K. Kato, S. Terao, N. Shimamoto, M. Hirata, Studies on Scavengers of Active Oxygen Species. Synthesis and Biological Activity of 2-O-Alkylascorbic Acids, J Med Chem. 31 (1988) 793–798. https://doi.org/10.1021/JM00399A019.

[32] R. Kumar, B. Rajkapoor, P.P.-A.P. journal of tropical, 2012, Antioxidant activities of Indigofera cassioides Rottl. Ex. DC. using various in vitro assay models. https://www.sciencedirect.com/science/article/pii/S2221169112600197 (accessed April 4, 2023).

[33] T. Kameyama, T. Nabeshima, T.K. Pharmacological methods, 1986, Step-down-type passive avoidance-and escape-learning method: Suitability for experimental amnesia models. https://www.sciencedirect.com/science/article/pii/0160540286900276 (accessed April 4, 2023).

[34] G. Ellman, K. Courtney, V.A.J.-B., 1961, A new and rapid colorimetric determination of acetylcholinesterase activity. https://www.sciencedirect.com/science/article/pii/0006295261901459 (accessed April 3, 2023).

[35] L. Skulski, G. Palmer, M.C.-T. Letters, 1963, On the tautomerism of amides, Elsevier. (n.d.). https://www.sciencedirect.com/science/article/pii/S004040390190913X (accessed April 4, 2023).