5. DISCUSSION

AD a progressive neurodegenerative disorder results in cognitive impairment in elders. AD is mainly characterised by deposition of Aβ plaques extracellularly and *p*-tau intracellularly that ultimately result in loss of cholinergic neurons of the hippocampal region of brain [4, 88]. According to the cholinergic hypothesis, the AD pathogenesis is responsible for the rapid decline of cholinergic neurotransmission which store ACh in the brain regions [3]. The AChE and BuChE enzymes act upon ACh to hydrolyse it in the synaptic cleft into choline and acetate. During the early stage of AD, AChE plays a vital role. However, as the disease progresses there occurs depletion of cholinergic neurons and BuChE plays a major role in ACh degradation [94].

The amyloid hypothesis states that AD progression is attributed to the accumulation of self induced and/or AChE-induced aggregation of toxic forms of Aβ peptides which originate from the cleavage of amyloid precursor protein (APP) by β-secretase (BACE) and γ-secretase enzymes [95].

Many studies have pointed a link between the cholinergic and amyloid hypotheses [50, 96]. AChE induces Aβ formation that ultimately leads to the development of highly toxic AChE-Aβ complexes [97, 98]. This multifactorial AD pathogenesis demands development of therapies that possess both anti-ChE as well as anti-Aβ aggregatory activities. The Pharmaceutical Chemistry Laboratory synthesized some isoalloxazine, triazinoindole-3-thiol, indolequinoxaline, iminoquinazoline-4-one and diarylthiazol-benzylpiperdine derivatives which were taken up for preliminary screening for ChE inhibitory and $A\beta_{1-42}$ anti-aggregatory activities. The test compounds showed good cholinesterase inhibitory and $A\beta_{1-42}$ anti-aggregatory activities in the *in vitro* studies. Further, the detailed pharmacological assessment of neuroprotective potential of the lead compounds [**13, 17, 70** and **131** (as assessed from the *in vitro* experiments)] was performed using *in vivo* Alzheimer's rodent models.

The most common approach adopted for the treatment of AD-like condition is to inhibit ChE enzyme activity which leads to increased levels of brain ACh. Anti-ChE potential of the selected compounds (**13, 17, 70** and **131**) was demonstrated *in vivo* using scopolamine-induced amnesic mice model. This model is well documented to study the anti-ChE effect of test compounds [61]. In the present study, scopolamine-treated control animals showed spatial

learning and memory impairment as assessed by MWM test. A significant rise in ELT and decreased count of platform area crossings were observed in scopolamine-treated animals in MWM test suggesting impairment in learning ability. However, treatment with **13, 17, 70** and **131** significantly reduced the ELT and increased the count of platform area crossings in mice as compared to the scopolamine-treated control animals. Thus, compounds (**13, 17, 70** and **131**) have the potential to improve the spatial learning ability which was altered by scopolamine.

Furthermore, scopolamine-treated control mice showed increased AChE and BuChE activities in brain homogenates which were significantly abrogated by treatment with **13, 17, 70** and **131** similar to the standard drug i.e. donepezil. Previous studies have reported that increased oxidative stress is closely associated with cognitive impairment as observed in scopolamine-treated mice [61]. In the present study, scopolamine-treated control animals showed significant elevation in MDA levels and significant reduction in CAT levels suggesting increased oxidative burden. Test compounds (**13, 17, 70** and **131**) significantly reversed the altered oxidative stress parameters. These results revealed the ability of **13, 17, 70** and **131** to improve memory impairment which could be attributed to their anti-ChE and antioxidant effects.

Primary rat hippocampal neuronal culture is widely used to determine the *in vitro* neuroprotective potential of test compounds [87, 99]. Compounds (**13, 17** and **70**) did not show any toxicity in hippocampal neurons even up to 40 μM concentration. $A\beta_{1-42}$ plaques are the most toxic form of amyloid peptide having strong association with AD pathogenesis [95]. The mechanism behind the $A\beta_{1-42}$ toxicity involves alteration of intracellular calcium, production of free radicals, phosphorylation of tau protein and activation of caspase cascade, all together lead to apoptotic cell death [84, 100]. A compound possessing antioxidant, free radical scavenging and antiapoptotic activities could protect cells against the $A\beta_{1-42}$ toxicity [87, 99]. The current study revealed that $A\beta_{1-42}$ insult caused significant cytotoxicity in the rat primary hippocampal neurons. Pre-treatment with **13, 17** and **70** showed significant neuroprotection against $A\beta_{1-42}$ toxicity in the hippocampal neurons as evidenced by significant increase in cell viability which was assessed utilizing MTT assay. ROS scavenging potential reflects antioxidant profile of the compounds. Increased oxidative stress elevates ROS generation in a variety of pathological conditions [85]. $A\beta_{1-42}$ insult increases oxidative stress in AD pathogenesis [86]. Free radical scavenging ability of the test compounds was determined using DCFH-DA assay. $A\beta_{1-42}$ insult

resulted in generation of ROS in the hippocampal neurons which was significantly abrogated by **13, 17, 70** and **131**. In the Hoechst staining assay, **13, 17** and **70** significantly reduced the number of fragmented and/or apoptotic nuclei suggesting antiapoptotic potential of the test compounds. In the flow cytometric assessment, **13, 17, 70** and **131** significantly attenuated the percentage of $A\beta_{1-42}$-induced early apoptotic cells. Moreover, caspase-3 activation is a key process during apoptosis progression [84]. ICC was performed to determine the expression of cleaved caspase-3 protein. $A\beta_{1-42}$ toxicity increased the mean fluorescence intensity of cleaved caspase-3 expression and the number of cleaved caspase-3 positive cells in rat hippocampal culture suggesting induction of apoptosis through caspase cascade. **13, 17** and **70** significantly reduced the mean fluorescence intensity of cleaved caspase-3 expression and the number of cleaved caspase-3 positive cells similar to the reference drug i.e. donepezil, confirming their antiapoptotic potential.

To replicate the AD pathogenesis, transgenic animal models need to be used ideally. However, non-transgenic animal models have also been extensively utilized to reproduce AD-like pathology. $A\beta_{1-42}$-induced Alzheimer's rat model is widely accepted for this purpose. In this model, AD-like pathogenesis was induced using injection/infusion of $A\beta_{1-42}$ into the hippocampal region of the rodent brain. $A\beta_{1-42}$ injection degrades cholinergic neurons in the hippocampal region [59, 87]. Furthermore, $A\beta_{1-42}$ forms a complex with AChE (AChE-$A\beta_{1-42}$ complex) which is a more toxic form than the Aβ peptide alone [97, 98]. Alzheimer's rat model was developed through stereotaxic injection of $A\beta_{1-42}$ in the hippocampal region of brain. Y maze test was adopted for the assessment of immediate working memory. $A\beta_{1-42}$ treatment reduced "spontaneous alteration" arm entries in rats. However, treatment with compounds (**13, 17, 70** and **131**) significantly normalized the immediate working memory impaired by $A\beta_{1-42}$, confirming anti-amnesic potential of the test compounds. The result denotes the neuroprotective effect of the test compounds (**13, 17, 70** and **131**) in $A\beta_{1-42}$-induced Alzheimer's rat model.

As discussed earlier, two main pathological hallmarks of AD are $A\beta_{1-42}$ and *p*-tau [88]. A number of studies have shown that $A\beta_{1-42}$ toxicity is mediated via increased phosphorylation of tau protein which affects multiple signalling cascades including MAPK, PI3K/AKT, NF-*k*B and Wnt pathways [100, 101]. ICV injection of $A\beta_{1-42}$ peptide elevated the $A\beta_{1-42}$ and *p*-tau protein levels in the hippocampal region of the rat brain as evidenced by immunoblot analysis. However, treatment with **13, 17, 70** and **131** significantly attenuated the $A\beta_{1-42}$ and *p*-tau levels similar to

donepezil. Hence, regulation of the key pathological biomarkers of AD by **13, 17, 70** and **131** revealed their anti-AD potentials. As discussed earlier, a key event in apoptosis is the activation of caspase-3 protein [84]. Significant evidence depicts that caspase-3 is either partially or totally responsible for the proteolytic cleavage of many key proteins, including poly(ADP-ribose) polymerase-1 (PARP). PARP is a nuclear-DNA binding protein [102, 103] which is involved in different cellular events including DNA repair [104]. Hyperactivation of PARP is involved in various neurodegenerative disorders including AD [105]. Aβ peptide induces the PARP activity in the hippocampal region of adult rats [106, 107]. Cleavage of PARP is followed by the activation of caspase-3 which subsequently results in apoptosis and ultimately neuronal cell death. Thus, activated caspase-3 and cleaved PARP are the two pathological hallmarks of apoptosis [108]. Antiapoptotic potential of the test compounds (**13, 17, 70** and **131**) was further verified in the hippocampal region of $A\beta_{1-42}$ injected rat brains. Western blot analysis revealed significant rise in cleaved caspase-3 and cleaved PARP levels in $A\beta_{1-42}$-treated control rat brains. Supporting the previous results, **13, 17, 70** and **131** significantly reduced the levels of cleaved caspase-3 as well as cleaved PARP in AD rat brains. These results further revealed antiapoptotic potential of the test compounds (**13, 17, 70** and **131**).

The canonical Wnt/β-catenin signalling pathway is important for survival and development of nervous system. The two key components of Wnt/β-catenin signalling pathway are GSK-3 and β-catenin which regulate neurogenesis. The canonical Wnt/β-catenin signalling pathway appears to be altered or involved in the pathogenesis of AD. Previous studies have reported altered expression of key components of the canonical Wnt signalling pathway (i.e. GSK-3 and β-catenin) in the pathogenesis of neurodegeneration in AD [89, 90]. GSK-3β gets inactivated upon phosphorylation at Ser9 followed by accumulation of cytoplasmic β-catenin [109]. β-Catenin is a critical component in the downstream of the Wnt signalling pathway which works as an intracellular signal transducer to regulate gene transcription, that can promote cell survival [110]. Thus, the Wnt pathway-mediated regulation of neurogenesis involves the transcription of β-catenin target genes. It was determined that the expression of the transcription factor neuroD1 is regulated by Wnt/β-catenin signalling activation [44]. A β-catenin target gene-neuroD1 has shown a vital role in the generation of granular cell and olfactory neurons in the embryonic and adult brain [43]. $A\beta_{1-42}$ mediated neurotoxicity is associated with reduced levels of *p*-GSK-3 and β-catenin in the hippocampal neurons [87, 91]. It is also reported that $A\beta_{1-42}$

caused altered neurogenesis through abrogation of transcription factor neuroD1 in embryonic hippocampal progenitors [92] and in AD rat brains [93]. Moreover, the AD patients showed reduced levels of β-catenin and neuroD1 indicating altered Wnt/β-catenin signalling pathway [111]. Lithium and rosiglitazone repair memory impairment and reduce astrocyte and microglia activation induced by amyloid insult by stabilizing Wnt components [112]. Curcumin-loaded nanoparticles have induced neurogenesis in AD rat brains through modulating Wnt/β-catenin signalling pathway [93]. Huperzine A (an AChE inhibitor) activates Wnt/β-catenin signalling pathway by abrogating amyloid burden in AD brains [113]. Previous studies have also reported altered expression of Wnt components in AChE-Aβ mediated neurotoxicity [114]. IBU-PO, an anti-ChE and anti-inflammatory compound repaired the impaired functioning of Wnt signalling pathway induced by $A\beta_{1-42}$ [115]. The said findings indicate that neuroprotective potential of ChE inhibitors is attributed to the stabilization of Wnt/β catenin signalling pathway. In the present study, **13, 17** and **70** treatment significantly increased *p*-GSK-3 activity, and stabilized the β-catenin protein and the transcription factor neuroD1 in the hippocampal regions of $A\beta_{1-42}$-induced Alzheimer's rat brain. These findings revealed the potential of **13, 17** and **70** to activate the canonical Wnt/β-catenin signalling pathway which was impaired by $A\beta_{1-42}$ toxicity.

The results indicated a therapeutic potential of the novel test compounds (**13, 17** and **70**) for the treatment of AD through activation of the canonical Wnt/β-catenin signalling pathway. Moreover, the selected test compounds **(13, 17**, **70** and **131)** were found to be nontoxic and well tolerated up to 2000 mg/kg, p.o. dose. Pharmacokinetic analysis revealed that compound **(131)** exhibited good oral absorption, and was eliminated at a relatively moderate rate compared to the absorption phase.

In conclusion, the present study has revealed neuroprotective potential of the novel isoalloxazine, triazinoindole-3-thiol, indolequinoxaline, iminoquinazoline-4-one and diarylthiazol-benzylpiperdine derivatives (**13, 17, 70** and **131**) against $A\beta_{1-42}$-induced toxicity. Compounds **(13, 17, 70** and **131)** have the ability to interact with different AD targets as they have shown significant multiple effects including anti-ChE, anti-Aβ aggregatory, neuroprotective, ROS scavenging, antioxidant and antiapoptotic activities in different *in vitro* and *in vivo* experimental models. All put together, the beneficial effects of the novel isoalloxazine, triazinoindole-3-thiol, indolequinoxaline, iminoquinazoline-4-one and

diarylthiazole-benzylpiperdine hybrid molecules have qualified them as potential lead candidates to be developed as new drugs for the treatment of AD, and the most promising multi-target-directed derivative **(131)** amongst them could be considered as a potential drug molecule for further development.

5. DISCUSSION

Selective serotonin reuptake inhibitors (SSRIs) affect the synaptic serotonin levels through their action on diverse serotonin (5-HT) receptor subtypes (upwards of 14 separate receptors). The antidepressant activity of SSRIs is likely mediated by one or greater number of these receptors, yet it is impossible that every one of the 14 subtypes play a similar role. Also, the unwanted adverse effects of SSRIs are likely to be attributed to the activation of one or more number of these receptors, which may be unique and different from those that produce antidepressant activity. At the onset of treatment, indirect activation of 5-HT$_{2C}$ receptors is responsible for the anxiogenic effects of SSRIs and their inhibition affect sleep, sexual behaviour and appetite. SSRIs like nefazodone or mirtazapine, act as direct antagonists of 5-HT$_{2C}$ receptors. There are several other evidences indicating that majority of SSRIs mediate antidepressant-like effect through 5-HT$_{2C}$ receptor antagonism. These observations suggest that 5-HT$_{2C}$ receptor blockade could be an important strategy for treating depressive and anxious states [31, 70, 71].

A wide exhibit of behavioural changes associated with 5-HT$_{2C}$ receptor agonism is truly interesting. WAY-163909, a 5-HT$_{2C}$ receptor agonist, produces antipsychotic-like and anorectic effects [72, 73]. The capability of 5-HT$_{2C}$ receptor antagonists to attenuate the effects of WAY-163909 is consistent with the preposition that WAY-163909 produces its effects via activation of the 5-HT$_{2C}$ receptor. 5-HT receptors are abundantly expressed in brain and that may, to some extent, clarify various properties of 5-HT$_{2C}$ receptors. As it has long been realized that 5-HT has a wide variety of physiological functions, a widespread distribution of the 5-HT$_{2C}$ receptors may permit their activation to produce a number of effects of 5-HT.

5-HT$_2$ receptor family comprises of three subunits: 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C.}$ They exhibit 46-50% overall sequence identity due to their homologous nature. 5-HT$_{2C}$ receptors play a pivotal role in the regulation of anxiety, depression, food intake, penile erection etc. Therefore researchers have put great emphasis on finding of potent and selective 5-HT$_{2C}$ receptor modulators devoid of hallucinogenic and cardiac side effects associated with 5-HT$_{2A}$ and 5-HT$_{2B}$ receptor respectively. Recent evidences on vabicaserine [74] and lorcaserin [27] supports the above arguments.

The present study finds some potent compounds showing selectivity towards the 5-HT$_{2C}$ receptors. From the series, test compounds (**1-8, 11, 17, 18, 22-26, 30-33, 36, 38** and **39**) were found to be inactive in the *in vitro* isolated tissue experiments suggesting their lack of affinity for the 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors. Thus such compounds would be either inactive or selective 5-HT$_{2C}$ receptor modulators. The proposed hypothesis was checked using different *in vivo* murine models. The compounds were first checked in the despair swim test, a model for the assessment of the depression. From the listed compounds, **1, 6-8, 18, 24,** and **30** have shown significant rise in the immobility time as compared to the saline-treated control group which might be due to their 5-HT$_{2C}$ receptor activity. These compounds have shown depressogenic response similar to the standard, i.e. *m*-CPP. There is a controversial effect of 5-HT$_{2C}$ receptor agonists on the depression. Rajkumar *et al* [39] have demostrated the ability of *m*-CPP to induce depressogenic behaviour in rodents while Moreau *et al* [75] have revealed antidepressant effect of 5-HT$_{2C}$ agonists RO-600175 and RO-600332. Previous studies revealed that 5-HT$_{2C}$ receptors have influence on the firing of monoamine neurotransmitters. They negatively regulate firing of DA and 5-HT neurons in the dorsal raphe nucleus (DRN) and ventral tegmental area (VTA), respectively [38, 69, 76, 77]. Present study revealed that the test compounds significantly decreased the DA and 5-HT levels in the rat brain similar to that of *m*-CPP [57]. This supports the above finding showing that 5-HT$_{2C}$ receptor agonists mediate depressogic effect.

The compounds **1, 6-8, 18, 24,** and **30** were further evaluated for their anxiogenic effect on the anxiety model. Elevated plus maze test was adopted for the assessment of anxiety. They significantly reduce the exploration as well as time spent in open arm showing anxiogenic response similar to *m*-CPP. Anxiety is mainly regulated by amygdala region of the brain having high number of 5-HT$_{2C}$ receptor expression. Previous reports have demonstrated that activation of amygdala by 5-HT$_{2C}$ receptor agonist is strongly associated with a state of anxiety [61]. So, antagonism of 5-HT$_{2C}$ receptor might be beneficial for the treatment of anxiety [52, 62].

Compounds **1, 6-8, 18, 24,** and **30** were further assessed for the 5-HT$_{2C}$ receptor mediated hypophagic response. They significantly attenuated the food intake which was reversed by RS-102221, a selective 5-HT$_{2C}$ receptor antagonist, confirming their 5-HT$_{2C}$ receptor selectivity. Tecott *et al* [63] have demonstrated that 5-HT$_{2C}$ receptor knockout mice develop obesity and such animals remain hyperphagic throughout their life. Pro-opiomelanocortin (POMC) neurons

predominantly express 5-HT$_{2C}$ receptor mRNA where its agonism lead to increased production of α-msh (melanocyte stimulating hormone) which ultimately enhances MC$_4$ receptor signalling [64-66], resulting in reduced food intake. 5-HT$_{2C}$ receptor agonists are also able to increase satiety that results in reduced food intake [65, 78].

Compounds (**1, 6-8, 18, 24,** and **30**) were also evaluated for their effect on 5-HT$_{2C}$ receptor mediated penile erection along with RS-102221. *m*-CPP induces penile erection and excessive grooming with increased levels of oxytocin, prolactin and corticosterone. Paraventricular nucleus is believed to control these behavioural and neuroendocrine responses [68]. RO 60-0175, a selective 5-HT$_{2C}$ receptor agonist, mimics *m*-CPP-induced penile erection [79]. SB-200646 and SB-206553, potent 5-HT$_{2C}$ receptor antagonists [80, 81], reverse this effect. 5-HT$_{2C}$ receptors at the lumbosacral level are strongly associated with the supraspinal serotonergic control of erection [82].

As discussed earlier, 5-HT$_{2C}$ receptors negatively regulate the firing of monoamine neurotransmitters in the dorsal raphe nucleus (DRN) and ventral tegmental area (VTA) [38, 69, 76, 77]. In the present study, the test compounds significantly reduced the DA and 5-HT levels in the rat brain similar to that of *m*-CPP which further supported the above findings [57]. Thus, the results obtained in this study indicated that the compounds (**1, 6-8, 18, 24,** and **30**) were potential 5-HT$_{2C}$ agonists that could be evaluated further pre-clinically.

5. DISCUSSION

Excitotoxicity plays a pivotal role in the onset and development of neurodegeneration making the neuronal cells vulnerable to damage and extermination. Among the factors which are responsible for the onset of excitotoxicity, glutamatergic neurotransmission has the highest impact [75, 76]. NMDAR, a glutamate receptor subtype, is predominantly associated with high conducting calcium channels which are blocked voltage-dependently by magnesium in resting conditions. NMDAR-mediated calcium conduction activates a number of calcium dependant enzymes affecting various cellular components which maintain neuronal cell viability. The physiological role of NMDAR seems to be related to synaptic plasticity, neuronal outgrowth and survival, learning, memory and behaviour [73, 77, 78]. However, overactivation of NMDAR triggers excessive calcium influx, initiating a series of cytoplasmic and nuclear processes which ultimately result in neuronal cell death [79, 80]. Therefore, a ligand capable of attenuating NMDAR overactivation could be a useful tool for the management of excitotoxicity. NMDAR seems to have several binding sites including the agonist binding sites, ion channel pore binding sites and several allosteric binding sites [73, 78]. Among the agonist binding sites, glutamate predominantly binds to the glutamate binding site of the receptor playing a major role in NMDAR-mediated excitotoxicity [52, 72]. Another agonist binding site of NMDAR is glycine binding site where a co-agonist glycine preferentially binds to it to activate the receptor. For complete activation of NMDAR, both of the agonist binding sites must be occupied with the respective agonists which together lead to excitotoxicity. Therefore, a ligand capable of blocking either of the binding sites could overcome NMDAR-mediated excitotoxicity [7, 53, 73, 74].

In the present study, a series of benzazepine derivatives synthesized in the Pharmaceutical Chemistry Laboratory with a core structure of 3-methyltetrahydro-3*H*-benzazepin-2-one were evaluated for NMDAR antagonist activity. NMDA preferentially binds to the glutamate binding site of the NMDAR which at higher concentrations triggers excitotoxic neuronal cell death [52, 72]. Among the series, most of the compounds demonstrated significant neuroprotection against NMDA-induced excitotoxicity in SH-SY5Y cells at 10 µM concentration. Compounds (**3** and **10)** were found to be the most effective neuroprotective agents against NMDA-mediated excitotoxicity in the series. Considering the most potent compound (**10)** as a representative lead, molecular docking studies were performed to determine its

preferential binding affinity for both of the NMDAR agonist binding sites. The docking results showed that the compound (**10)** has relatively higher affinity for the glycine binding site as compared to the glutamate binding site of NMDAR. Excitotoxicity is the key pathophysiological process in the development of AD. In the early stages of AD, glycine antagonists and low affinity channel blockers of the NMDAR have so far been used successfully to treat the disease and are of interest to the researchers [81, 82]. Therefore, it is logical to assume that compounds acting as NMDAR antagonists at the glycine site could be efficiently used to treat neurodegenerative conditions [83]. Previously, benzazepine [84, 85], quinolinone [86] and quinoxalinedione [87] like heterocyclic compounds have been developed as NMDAR antagonists acting at the glycine site. NMDAR antagonist activity at glycine site has also been established for some benzazepine derivatives like 8-methyl-3-hydroxy-2,5-dihydro-1*H*-benzazepine-2,5-dione (**a,** Fig. 11) and 8-chloro-3-hydroxy-2,5-dihydro-1*H*-benzazepine-2,5-dione (**b,** Fig. 11) [88]. Moreover, there is a report of some benzazepine derivatives antagonising the NMDAR at the glycine site in submicromolar potency which was equal to or greater than those of other bicyclic antagonists, including kynurenic acid, indole-2-carboxylic acid, 7-chloro-4-hydroxy-3-(3-(thiophen-3-yloxy)phenyl)quinolin-2(1*H*)-one (**c,** Fig. 11) and quinoxaline-2,3-dione (**d,** Fig. 11) [89]. These findings further supported our results indicating potential usage of the synthesized benzazepines as NMDAR antagonists having a preferential binding to the glycine site of the receptor.

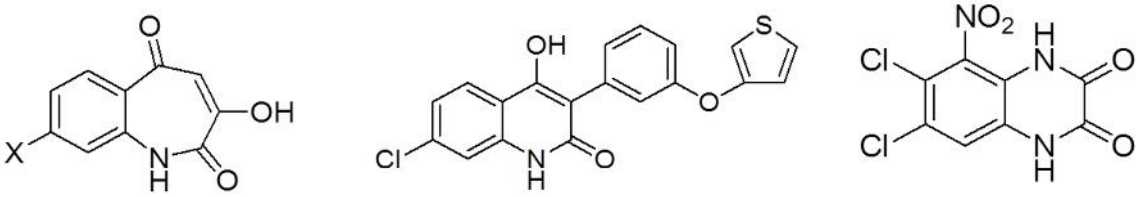

Fig. 11: Some reported NMDAR antagonists.

Aβ, a key pathological marker of AD also mediates toxicity, at least in part through glutamate excitotoxicity. Previously, it was reported that direct treatment of the cultured hippocampal neurons with Aβ triggered sustained calcium influx through NMDAR. This influx

was attenuated by NMDAR antagonists [49, 56, 57]. Aβ also mediated neurodegeneration in the adult rat brain, in part, through NMDAR overactivation [54, 90]. Moreover, transgenic mice exhibiting high levels of Aβ, demonstrated increased vulnerability to excitotoxicity [91, 92]. Aβ peptides bind to glutamate and glycine recognition sites of NMDAR to cause excitotoxicity [57, 93]. So, to avoid excitotoxic damage efficiently, a ligand should also possess Aβ aggregation inhibitory property. To assess the multi-target-directed biological activity of the test compounds exhibiting potent NMDAR antagonist activity, they were evaluated for $A\beta_{1-42}$ aggregation inhibitory activity also, using ThT and CR binding assays. Compounds (**3** and **10**) demonstrated significant inhibition of $A\beta_{1-42}$ aggregation at 10 μM concentration in both the assays. The results revealed NMDAR antagonistic and $A\beta_{1-42}$ aggregation inhibitory potentials of the test benzazepines (**3** and **10**) substantiating their beneficial role in excitotoxicity.

Oxidative stress also plays a major role in the pathogenesis of neurodegenerative conditions. The relationship between oxidative stress and neuronal cell death caused by excitotoxicity has been extensively investigated. Oxidative stress alters the structures of proteins, lipids and nucleic acids, and opens the mitochondrial permeability transition pores causing further stimulation to ROS production and release of proapoptotic factors [17, 94]. Previous reports indicate that during the NMDAR overactivation, ROS are produced by NADPH oxidase action in postsynaptic neurons [95]. Elevated ROS generation and down regulated antioxidant defense mechanisms jointly cause neuronal cell death in various neurodegenerative conditions including AD [17]. In AD, Aβ causes neurotoxicity through alteration in intracellular calcium levels, production of free radicals, phosphorylation of tau protein and activation of caspase cascade, which all together lead to apoptotic cell death. The Aβ-mediated neurotoxicity could be attenuated by free radical scavengers such as vitamine E [18, 59, 60, 63, 67]. Thus, a ligand exhibiting free radical scavenging, antioxidant and antiapoptotic properties could protect the cells efficiently against the Aβ-induced excitotoxic damage. In the present study, the neuroprotective potential of the two promising benzazepines (**3** and **10**) was further assessed using primary rat hippocampal neuronal culture. The compounds (**3** and **10**) did not show any toxicity in hippocampal neurons even up to 40 μM concentrations while $A\beta_{1-42}$ imparted significant toxicity to the hippocampal neurons, which was significantly attenuated by pre-treatment of the cells with **3** and **10** in the MTT assay. Supporting the previous reports, $A\beta_{1-42}$ insult to the cells significantly elevated the ROS generation and caused apoptotic cell death [18,

33, 59]. Treatment with **3** and **10** significantly abrogated $A\beta_{1-42}$-induced ROS generation in DCFH-DA assay. Additionally, **3** and **10** significantly reduced the number of apoptotic nuclei in Hoechst staining assay and attenuated the rate of apoptosis in annexine V-FITC and PI staining assay performed using flow cytometry. Compounds (**3** and **10**) also attenuated the expression of cleaved caspase-3 protein in the ICC experiment. Put together, these results revealed the neuroprotective, free radical scavenging, antioxidant and antiapoptotic potential of the test compounds (**3** and **10**) against $A\beta_{1-42}$ insult.

Possible *in vivo* BBB permeation of the test compounds was evaluated using PAMPA-BBB assay. The calculated permeability values (P_e) indicated that **3** and **10** could cross the BBB by passive diffusion. To assess the *in vivo* neuroprotective potential of the potent benzazepines, ICV rat model of $A\beta_{1-42}$-induced toxicity was adopted. Previously, it has been demonstrated that direct application of Aβ in the hippocampal region of the rodent brain hampered learning and memory [33, 44, 65]. In the MWM test, $A\beta_{1-42}$-injected animals showed significant rise in ELT and reduced platform area crossings suggesting altered spatial learning and memory. In the Y maze test, $A\beta_{1-42}$-treated rats exhibited significantly reduced "spontaneous alterations" which revealed impairment of immediate working memory in the animals. However, treatment of the animals with **3** and **10** significantly improved learning and memory in both of the behavioural experimental models. To determine the *in vivo* antioxidant potential of the test compounds, MDA and CAT levels were determined in the hippocampal region of rat brains which underwent ICV injection of $A\beta_{1-42}$. Supporting the previous reports [33], significantly increased MDA levels and reduced CAT levels were observed in the $A\beta_{1-42}$-treated control group suggesting elevated oxidative stress. However, **3** and **10** significantly normalized the altered oxidative stress parameters which further confirmed their antioxidant potential. It has been reported that Aβ-mediated excitotoxicity is strongly associated with the elevated levels of excitatory neurotransmitters in the brain that could be attenuated by NMDAR antagonists [44, 49]. Compounds (**3** and **10**) significantly attenuated glutamate and glycine levels in the hippocampal region of the rat brain that were elevated after $A\beta_{1-42}$ toxicity, substantiating further the previous findings.

As discussed earlier, Aβ mediates toxicity via increased phosphorylation of tau protein which affects multiple signaling cascades including MAPK, PI3K/AKT, NF-*k*B and Wnt

pathways [67, 68]. In the present study, immunoblot analysis revealed that ICV injection of $A\beta_{1-42}$ significantly increased $A\beta_{1-42}$ and *p*-tau levels in the hippocampal region of the rat brain. However, treatment of the animals with compounds (**3** and **10**) significantly attenuated $A\beta_{1-42}$ and *p*-tau levels similar to memantine. Thus, it can be inferred that **3** and **10** have the potential to attenuate $A\beta_{1-42}$ toxicity through abrogating $A\beta_{1-42}$-induced tau phosphorylation. As discussed earlier, excitotoxicity is a key pathogenic process in apoptosis which is characterized mainly by the activation of the caspase cascade [63, 67]. Caspase-3 activation is a key event in apoptosis, which is responsible for the proteolytic cleavage of many proteins, including poly (ADP-ribose) polymerase-1 (PARP). PARP plays a vital role in DNA repair [96, 97]. Cleavage of PARP is observed in different neurodegenerative conditions including AD [98]. Aβ peptides induce the PARP activity in the hippocampal region of adult rats [99, 100]. During apoptosis, cleavage of PARP is followed by the activation of caspase-3 which ultimately leads to neuronal cell death. Thus, activated-caspase-3 and cleaved PARP are the two important pathological hallmarks of apoptosis [101]. In the present study, the *in vivo* antiapoptotic potential of compounds (**3** and **10**) was evaluated using Western blot analysis. $A\beta_{1-42}$ toxicity caused caspase-3 activation and PARP cleavage in the hippocampal region of rat brain which were attenuated significantly by treatment of the animals with **3** and **10**, further confirming their neuroprotective and antiapoptotic potentials.

After demonstrating $A\beta_{1-42}$-induced toxicity via phosphorylation of tau protein, activation of tau kinases was evaluated subsequently. Previous reports have demonstrated that Aβ triggers tau phosphorylation through a mechanism which involved activation of various tau kinases including MAPK and GSK-3β [49, 69-71]. In the present study, $A\beta_{1-42}$-induced tau phosphorylation significantly elevated the levels of MAPK, i.e. *p*-ERK1/2 in the hippocampal region of rat brain. Previous reports suggest that Aβ production is attributed mainly to the reduced *p*-GSK-3α activity [102], while tau phosphorylation is due to reduced *p*-GSK-3β activity [103]. In the present study, $A\beta_{1-42}$ toxicity significantly reduced the levels of *p*-GSK-3α and *p*-GSK-3β in the hippocampal region of rat brain which further supported the previous findings. However, treatment of the animals with **3** and **10** significantly attenuated *p*-ERK-1/2 levels and increased *p*-GSK-3 levels. Thus, the results together revealed the therapeutic potential of the compounds (**3** and **10**) to attenuate $A\beta_{1-42}$-induced tau phosphorylation by abrogating the activation of tau kinases.

In conclusion, the present study revealed the multi-target-directed potential of benzazepine derivatives against excitotoxicity as they have demonstrated efficient NMDAR antagonist, $A\beta_{1-42}$ aggregation inhibitory, neuroprotective, free radical scavenging, antioxidant and antiapoptotic activities in different *in vitro* and *in vivo* experiments.

5. DISCUSSION

The D_1 receptors are expressed abundantly in different parts of basal ganglia in rodents, monkeys and humans [40, 41]. There are multiple behavioural and biochemical evidences which indicate involvement of the D_1 receptors in the functioning of the rat basal ganglia. However, the role(s) of D_1 receptors in functioning of the basal ganglia in monkeys and humans has yet to be fully understood [42-44]. SKF-38393, a highly used experimental D_1 agonist, fails to produce beneficial effects in the Parkinsonian pathology either in MPTP-treated primates or in people with idiopathic Parkinsonism [45, 46]. A series of isochromans having potent, specific and long-acting D_1 receptor agonistic activities have been accounted biologically [47, 48]. A-77636 ((1*R*, 3*S*) 3-(l'-adamantyl)-1-aminomethyl-3,4-dihydro-5,6-dihydroxy-l*H*-2-benzopyran hydrochloride) is a D_1 receptor-specific isochroman with *in vitro* and *in vivo* D_1 agonistic activity. Previous studies have demonstrated beneficial effects of A-77636 in marmosets, treated with the neurotoxin MPTP, supporting its anti-Parkinsonian effect [49]. There are several other reports which display that D_1 receptors play an important role in the working of the extrapyramidal nervous system in primates.

Involvement of D_1 receptors in the functioning of the extrapyramidal nervous system is in agreement with several observations. D_1 receptors have high abundance in several sites inside the basal ganglia, most notably the substantia nigra pars reticulata and caudate putamen [2, 50, 51]. Moreover, the formation of a second messenger (cyclic-AMP) was potentiated by D_1 agonists in the substantia nigra and the caudate-putamen [52].

Upon intoxication of striato-nigral dopaminergic neurons with 6-OHDA, D_1 receptors (not D_2 receptors) get stimulated causing increased utilization of glucose in the substantia nigra pars reticulata and the entopeduncular nucleus [53-55]. Similarly, *L*-DOPA, the most widely used therapeutic agent for Parkinson's disease, increases glucose utilization in 6-OHDA-induced Parkinson's experimental model [56]. Additionally, D_1 agonist A-68930, an isochroman, has demonstrated increased levels of 2-deoxyglucose in substantia nigra [48]. These observations clearly demonstrated that D_1 receptor played an important role in the physiology of the extrapyramidal nervous system. Thus, the D_1 receptor is in focus for rational designing of anti-Parkinsonian drugs.

A series of benzazepine derivatives were synthesized to assess their potential as D_1 agonists. The synthesized compounds were evaluated for their D_1 agonistic potential using isolated rat superior mesenteric artery strip preparation. Among the series of benzazepines, compounds (**4, 16** and **19**) were found to be potent D_1 agonists in the *in vitro* isolated tissue experiments. Compound (**16**) was selected for further assessment against 6-OHDA-induced injury in human SH-SY5Y neuroblastoma cell lines. The compound (**16**) showed significant neuroprotection in the cultured cells against 6-OHDA toxicity through D_1 agonism.

Previously it was shown that, dinapsoline (DNS) treatment caused vigorous contralateral turnings in rats which underwent unilateral 6-OHDA lesioning in the medial forebrain bundle. The DNS-induced rotational behaviour is attributed to the *in vivo* D_1 receptor stimulation. The rotations induced by DNS were fully blocked by selective D_1 receptor antagonist SCH-23390 while no influence was seen on rotations caused by D_2-selective antagonist raclopride [27]. To mimic the 6-OHDA-induced unilateral rotational behaviour in rodents, 6-OHDA is injected unilaterally into the substantia nigra, or the striatum and the medial forebrain bundle. This leads to damage of dopaminergic terminals and neurons along with the loss of striatal DA. As a result, reflective functional dopaminergic supersensitivity develops on the lesioned side. When treated with direct-acting DA receptor agonists, unilaterally lesioned (by 6-OHDA) rats turn contralaterally (far from the side of injury) due to increased sensitivity of the postsynaptic DA receptors on the lesioned side [57]. Compound (**16**), which was found to be a potent D_1 agonist in the preliminary *in vitro* studies, was further evaluated using unilaterally 6-OHDA-lesioned Parkinson's rat model. Compound (**16**) significantly increased the number of contralateral rotations in 6-OHDA unilaterally lesioned rats. This showed that compound (**16**) is having affinity for D_1 receptors and the rotations were due to D_1 receptor stimulation.

6-OHDA is a neurotoxin that offers a functional animal model of PD by inducing the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and injures the nerve endings in the striatum [58, 59]. According to the previous reports, 6-OHDA lesioning leads to degeneration of dopaminergic neurons which results in significant neurochemical changes such as decreased DA content and reduced TH immunoreactivity [26]. In the present study, 6-OHDA significantly decreased ipsilateral striatal DA levels and reduced TH expression in ipsilateral SNpc region which indicate degeneration of dopaminergic neurons. Treatment with

the compound (**16)** significantly increased the DA level and the expression of TH in 6-OHDA-induced Parkinson's rat brain. This showed that compound (**16**) possessed neuroprotective effect against 6-OHDA-induced Parkinson's rat model.

Apoptosis is an important biological process through which defective cells are disposed off during cell development because of injury. In various neurodegenerative conditions, apoptosis has been found to cause neuronal cell death [60]. PD is strongly associated with the progressive degeneration of dopaminergic neurons by apoptosis of the nigrostriatal neurons. Furthermore, mitochondrial or Fas pathway mediates caspase-3 activation upon 6-OHDA intoxication [61]. Although various mechanisms have been involved in the progression of PD, it has been revealed that oxidative stress plays a crucial role in the progression of neurodegeneration in PD [16, 62].

Antioxidant defence mechanisms have been recognized in various cells to control the harmful effects of oxidative stress. Among the different antioxidants present in the brain, the GSH is especially critical in controlling cell redox state which is essential for peroxide removal from the brain [63, 64]. Additionally, MDA plays an important role in scavenging ROS, including hydroxyl radicals [65]. Hence, MDA (a non-radical product), plays an essential role against the alterations induced by unilateral icv injection of 6-OHDA. After getting significant results in behavioural studies, the compound (**16**) was further evaluated for its potential antioxidative effects. The compound (**16**) significantly and positively modified the oxidative parameters viz. GSH, MDA, SOD and catalase. Treatment with the compound (**16**) significantly increased GSH, SOD and catalase levels while it (**16**) significantly decreased MDA levels in 6-OHDA-induced Parkinson's rat brain. These results proved that compound (**16**) also possessed antioxidant property.

The caspase family of enzymes is one of the most important apoptotic activators. Caspase-3 is known to play an essential role in the final regulated pathway of apoptosis. Fas (the death receptors present on the cell surface) mediated pathway is solely controlled by caspases. In this pathway, binding of a ligand to the death receptor leads to accumulation of a series of proteins, which leads to activation of pro-caspase-8 [66, 67]. During the caspase cascade, caspase-8 activates caspase-3, which further activates other caspases that cleave different substrates. Caspase-3 sets free caspase-dependent endonuclease in the cytoplasm which then

consequently penetrates into the nucleus, where it slices DNA into oligonucleosomal fragments [68]. In an alternate pathway of apoptosis, mitochondrial dysfunction occurs during apoptosis leading to the release of cytochrome c from the mitochondria into cytosol, where it attaches to the apoptotic protease activating factor 1(Apaf-1) [69, 70]. This complex activates the apoptosome followed by recruitment and activation of the inactive pro-caspase-9 which then activates effector caspases and triggers a cascade of events leading to apoptosis [71, 72]. Therefore, 6-OHDA mediated caspase-3 activation might be due to the after-effects of either the mitochondrial- or Fas-pathways [61, 73]. In agreement with the above reports, it was planned to evaluate whether compound (**16**) has any effect on caspase-3 levels. For this purpose, the animals treated with the test compound (**16**) were sacrificed and the brain sections were utilized for immunohistochemical analysis of cleaved caspase-3. Confocal microscopy images demonstrated that 6-OHDA significantly increased cleaved caspase-3 expression in substantia nigra region. Compound (**16**) significantly decreased cleaved caspase-3 expression in substantia nigra region of rat brains with 6-OHDA-induced Parkinson's disease. This validated the earlier observations that compound (**16**) demonstrated neuroprotective activity through D_1 agonism and the antioxidant mechanism shown by **16** may be responsible in part for its neuroprotective effect. As there are very few D_1 agonist medications available to treat PD, compound (**16**) could be a potential lead for development of a drug candidate to treat PD.

5. DISCUSSION

DA receptors are mainly classified in D_1- (D_1 and D_5) and D_2- (D_2, D_3 and D_4) like family of receptors. Amongst them, D_2-like family receptors play a pivotal role in the pathogenesis of various neuropsychiatric conditions. Most of the currently available antipsychotic drugs target mainly the D_2 receptors [27]. Haloperidol, having beneficial effects in regulation of positive symptoms of schizophrenia, is a classical D_2 receptor antagonist [5, 28]. The major drawback of classical antipsychotic drug therapy is the manifestation of extrapyramidal (motor and endocrine) side effects which could be overcome by the use of selective D_3 receptor antagonists [10, 15]. The beneficial effects of targeting D_3 receptors are attributed mainly to their atypical distribution in different regions of brain [7, 29]. As compared to D_2, D_3 receptors are fewer in number but have predominant abundance in the limbic region of striatum, nucleus accumbens and islands of Calleja, which are the active centers for regulation of emotion, memory and motor function [11].

Earlier reports have demonstrated selective D_3 receptor antagonist potential of some benzazepine derivatives [10, 30]. Antipsychotic potential of benzazepine derivatives has also been revealed in different rodent models of psychosis which further supported their suitability as a therapeutic alternative in neuropsychiatric diseases [8, 9]. In the present study, DA receptor modulating properties of a series of benzazepine derivatives have been evaluated *in vitro* using isolated rat superior mesenteric artery strips. Amongst the compounds so evaluated, **8** and **15** were found to be the most potent and relatively more selective D_3 antagonists. Compounds **(8** and **15)** significantly attenuated 7-OH-PBZI (selective D_3 agonist) mediated relaxation of pre-constricted mesenteric arterial strips. Compounds **(8** and **15)**-mediated D_3 antagonist activity was evidenced by their pA_2 values of 7.68±0.15 and 8.12±0.34 respectively. **8** and **15** have shown least effect on A-77636 (selective D_1 agonist) or bromocriptine (preferential D_2 agonist) mediated relaxation suggesting their relatively selective D_3 antagonist activity. After determining D_3 receptor antagonist activity in the *in vitro* evaluation, compounds **(8** and **15)** were further assessed in different behavioural rodent models for antipsychotic activity. Apomorphine (0.5 mg/kg, s.c.) induced significant stereotype behaviour in the treated animals as compared to the vehicle-treated control animals. However, **8** and **15** attenuated apomorphine-induced stereotype behaviour in rats. Apomorphine-induced stereotype behaviour is attributed to the stimulation of striatal DA receptors and therefore this model is used for screening of DA receptor agonists

and/or antagonists to determine their striatal DA receptor activity [31]. The ability of compounds **(8** and **15)** to attenuate apomorphine-induced stereotype behaviour suggests their DA receptor antagonist activity.

Antagonism of DA receptor mediated locomotor activity is a useful tool to determine antipsychotic potential of a compound [32]. In the present study, compounds **(8** and **15)** significantly attenuated spontaneous locomotor activity as compared to the vehicle-treated control group which indicated antipsychotic effect of **8** and **15**. Supporting previous reports, Clozapine, a D_4 receptor antagonist, also attenuated spontaneous locomotion in rodents [33]. Some reports also state that preferential D_3 antagonists do not block DA receptor mediated locomotion [34, 35], rather they promote hyperlocomotor activity [36]. However, selective D_3 antagonists such as S18126 and PD152255 have exhibited significant attenuation of the DA receptor mediated locomotor activity [37]. The reason behind this discrepancy might be attributed to the difference in receptor selectivity and/or procedural differences. Thus, the precise role of D_3 receptor agonist/antagonist in locomotion is still a subject of investigation using more potent and selective ligands acting as D_3 agonist/antagonist.

8-OH-DPAT mediated hypothermia is attributed to the activation of postsynaptic D_3 receptors [38]. Hence, to evaluate the postsynaptic D_3 receptor antagonist activity of the test compounds, 8-OH-DPAT-induced hypothermia rodent model was used. 8-OH-DPAT (0.2 mg/kg, s.c.), a selective D_3 agonist, induced significant hypothermia in rats as compared to the vehicle-treated control animals. However, pre-treatment of the animals with compounds **(8** and **15)** significantly attenuated 8-OH-DPAT-induced hypothermia in a dose dependant manner. There are evidences which have demonstrated attenuation of 8-OH-DPAT-induced hypothermia using selective D_3 antagonists, which further supported the present finding [15]. In contrast to this, clozapine has distinct hypothermic effect [39]. Treatment with clozapine elicited significant hypothermia as compared to the vehicle-treated control animals. Thus, clozapine potentiated 7-OH-DPAT-induced hypothermia. Clozapine is a potent D_4 antagonist which potentiates 8-OH-DPAT-induced hypothermia instead of attenuating it, suggesting that D_4 receptor blockade is inadequate to abrogate 8-OH-DPAT-induced hypothermia. The results further revealed selectivity in D_3 antagonist activity of **8** and **15**, as they significantly attenuated selective D_3 agonist induced hypothermia.

It has been reported that blockade of striatal D_2 receptors leads to induction of extrapyramidal side effects such as catalepsy and rota rod ataxia [40]. However, preferential D_3 or D_4 receptor antagonists are least prone to induce extrapyramidal side effects [15, 26]. Therefore, to rule out D_2 antagonist activity for the test compounds, the ability of the compounds **(8** and **15)** to induce catalepsy and rota rod ataxia was assessed. It was found that **8** and **15** did not induce catalepsy or rota rod ataxia at moderate doses (5 and 10 mg/kg, p.o.). However, at the same doses, **8** and **15** have significantly attenuated apomorphine-induced stereotype behaviour. At a relatively higher dose (20 mg/kg, p.o.) the test compounds **(8** and **15)** induced significant catalepsy as well as rota rod ataxia compared to the vehicle-treated control animals which might be attributed to their D_2 antagonist activity at higher concentrations. In contrast to this, clozapine significantly induced catalepsy and rota rod ataxia even at lower doses (5 and 10 mg/kg, p.o.). Thus, the results revealed that compounds **(8** and **15)** are devoid of D_2 antagonist activity at relatively lower doses which further point out towards the antipsychotic potential of the compounds **(8** and **15)** with low incidence of extrapyramidal side effects.

Finally, the DA receptor antagonist activity of **8** and **15** was assessed by determining striatal DA levels following apomorphine-induced stereotype behaviour rat model. Apomorphine administration significantly elevated striatal DA levels in rat brains as compared to the vehicle-treated control group. However, pre-treatment of the animals with compounds **(8** and **15)** significantly attenuated apomorphine-induced striatal DA levels. The ability of **8** and **15** to normalize striatal DA levels is attributed to their DA receptor antagonist activity. Previous reports have demonstrated that selective D_3 antagonists normalized elevated brain DA level which further supported the present finding [34, 35]. Moreover, compounds **(8** and **15)** did not produce any observable behavioural side effects and were relatively safe in acute lethality test in mice (LD_{50}≥300mg/kg, p.o.).

Most of the currently available antipsychotic drugs target D_2 receptors. However, selective blockade of D_2 receptor develops extrapyramidal side effects which are less common with preferential D_3 antagonists. The beneficial effects of D_3 blockade is mainly attributed to the atypical distribution of D_3 receptors in the brain. As compared to other D_2 family members, the D_3 receptors are few in numbers but have predominant abundance in the brain regions which are considered as the centres for emotional, memory and motor functions. Thus, selective D_3

antagonists could be useful to treat neuropsychiatric conditions without producing extrapyramidal side effects. In the present study, novel benzazepine derivatives (**8** and **15)** were found to be potent and preferentially selective D_3 receptor antagonists that have significant antipsychotic activity with low incidence of extrapyramidal side effects. However, further studies are needed to determine the selectivity of compounds **(8** and **15)** towards several other receptor subtypes for their complete pharmacological profiling.