Neurochemical Research (2021) 46:905–920
https://doi.org/10.1007/s11064-021-03229-w

ORIGINAL PAPER

Screening of Synthetic Isoxazolone Derivative Role in Alzheimer's Disease: Computational and Pharmacological Approach

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Received: 14 October 2020 / Revised: 16 December 2020 / Accepted: 2 January 2021 / Published online: 24 January 2021

Abstract

Alzheimer's disease (AD) is age-dependent neurological disorder with progressive loss of cognition and memory. This multifactorial disease is characterized by intracellular neurofibrillary tangles, beta amyloid plaques, neuroinflammation, and increased oxidative stress. The increased cellular manifestations of these markers play a critical role in neurodegeneration and pathogenesis of AD. Therefore, reducing neurodegeneration by decreasing one or more of these markers may provide a potential therapeutic roadmap for the treatment of AD. AD causes a devastating loss of cognition with no conclusive and effective treatment. Many synthetic compound containing isoxazolone nucleus have been reported as neuroprotective agents. The aim of this study was to explore the anti-Alzheimer's potential of a newly synthesized 3,4,5-trimethoxy isoxazolone derivative (TMI) that attenuated the beta amyloid (Aβ1-42) and tau protein levels in streptozotocin (STZ) induced Alzheimer's disease mouse model. Molecular analysis revealed increased beta amyloid (Aβ1-42) protein levels, increased tau protein levels, increased cellular oxidative stress and reduced antioxidant enzymes in STZ exposed mice brains. Furthermore, ELISA and PCR were used to validate the expression of Aβ1-42. Pre-treatment with TMI significantly improved the memory and cognitive behavior along with ameliorated levels of Aβ1-42 proteins. TMI treated mice further showed marked increase in GSH, CAT, SOD levels while decreased levels of acetylcholinesterase inhibitors (AChEI's) and MDA intermediate. The multidimensional nature of isoxazolone derivatives and its versatile affinity towards various targets highpoint its multistep targeting nature. These results indicated the neuroprotective potential of TMI which may be considered for the treatment of neurodegenerative disease specifically in AD.

Keywords Oxidative stress · Hippocampus · Beta amyloid · Isoxazolones · Antioxidant · AChEI's · Streptozotocin

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Abbreviations

GSH	Reduced Glutathione
SOD	Superoxide dismutase
CAT	Catalase
MDA	Malondialdehyde
TCA	Tricholoro acetic acid
DTNB	5, 5-Dithiobis-2-nitrobenzoic acid
TBA	Thiobarbituric acid
SEM	Standard error mean
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid

Introduction

Alzheimer's disease (AD) is age-dependent chronic neurodegenerative disease which is characterized by progressive loss of structure and function of neurons. This disease becomes more worsen with passage of time. [1]. According to survey report in 2012, 60–70% patients were found suffering with AD, worldwide [2]. AD has established linking with wide range of neurological problems such as cognitive impairment, short term memory loss, mood swing, behavioral changes, confusion and difficulty in walking and talking [3]. The statistical data suggests that the predicted rate and prevalence of AD is higher in developing countries as compared to developed countries [4]. Hippocampus plays a pivotal role in memory and learning aspects. Beta amyloid plaque and neurofibrillary tangles of tau proteins are reported to cause AD and are among the hallmarks of this neurological disease [5]. Hippocampus area of the brain is the first to be effected by this disease, resulting in loss of memory [6]. A marker of oxidative stress known as 8-hydroxy-2′-deoxyguanosine, was found intensified in the brain with aging and AD [7] that also serves as hypothetical basis of its involvement in the progression of AD [8]. For the last 20 years, the cholinergic hypothesis is well accepted for causing substantial neuronal damage. Based on post-mortem/ante-mortem studies, performed on brain tissue of experimental animals as well as the patients suffering from AD provided significant evidence that a change in release of acetylcholine (Ach) was reported that might be due to the reduction in transport of choline and expression of nicotinic & muscarinic receptors that plays a pivotal role in the progression of AD. Studies also provide the evidence that sex hormones (estrogen) also play a important contributing role in the progression of AD [9]. Reduction in ovarian functioning increased the risk factors of neuropsychiatric and neurodegenerative diseases [10, 11]. Therefore, this premise has since served as the basis for the majority of treatment strategies and drug development approaches for AD to date. The therapeutic front line medicines for the treatment of the AD are AchE inhibitors (Galantamine, donepezil, tacrine etc.) [12]. Although they don't provide the complete cure but cholinergic based therapies are pertinent to provide symptomatic relief and still it is thought to be one of the rational approaches for drug development and treatment of dementia and AD [13].

The aetiology and pathogenesis of AD is not well understood till date but oxidative stress is a major component [14]. The conditions like stress, depression, emotional disturbances and exposure to various drug molecules are evidential to cause elevation of reactive oxygen species (ROS) which ultimately cause tissue and neuronal damage [15, 16]. Furthermore, brain mostly uses glucose for energy, but in AD glucose metabolism is radically decreased probably due to oxidative damage that further contributes to impaired ATP biosynthesis. Subsequently, ATP-requiring processes for cognitive function are impaired and synaptic dysfunction and neuronal death result, following thinning of key brain areas [17]. The oxidative damage further contributes, at least in part, to variable enzymatic levels such as glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), acetylcholinesterase inhibitors (AChEI's) and malondialdehyde (MDA) in brain, probably owing to Neuropathological disorders [14, 18] [19].

Various factors are known that contribute in the pathogenesis of AD but dys-homeostasis between the production and clearance of amyloid beta (Aβ1-42 and other related peptides) is the most validated therapeutic target [20]. The accumulation of amyloid beta (Aβ) significantly enhances the progression of tau deposition. Furthermore, a mechanistic study proved that impaired clearance of amyloid beta (Aβ) is allied with the inheritance of ApoE4 [21, 22]. Alzheimer's disease shows initial appearance with hyper activation of acetylcholinesterase which further worsens with time as beta amyloid and tau pathology show attendance. The aggravated levels of these proteins are responsible for neurotoxicity [23].

Intracerebroventricular injection of streptozotocin (ICV-STZ) at sub-diabetogenic dose is commonly used method for the induction of cognitive impairment, oxidative stress, brain glucose metabolism impairment and cholinergic deficit. Studies showed that glucose hypo-metabolism produced by the ICV-STZ induced in monkeys was showed similar pattern to that of the AD patients at early stages [24]. Previous studies revealed that ICV-STZ induction model leads the tau and beta amyloid pathology leading cause of AD [25, 26].

Oxidative stress and neuroinflammation being the critical markers have a fundamental role in the pathophysiology of AD. These mediators aggravate the production of beta amyloid and ultimately lead to neurodegeneration in AD. Antioxidant substances play scavenging activities and remove free reactive oxygen species (ROS) [27]. The preventive strategies may have a tremendously successful role in the inhibition of neurological disorders. To date, no successful therapy is claimed to have curative role in such diseases and

likewise not a single therapy is available to completely suppress the progression of AD. The symptomatic treatments have been the only relying medication for AD since its discovery [28]. Isoxazole is an important nucleus containing five membered heterocycle ring with oxygen and nitrogen atom adjacent to each other. The importance of this nucleus is due to its broad spectrum regarding biological and therapeutic activities [29]. Isoxazole nucleus containing derivatives have been reported with promising multiple biological activities and low cytotoxicity that include anticancer, antimicrobial, anti-Alzheimer, anti-parkinson, antiviral and anticonvulsant activities [29–32]. Isoxazolones carry isoxazole nucleus and pre-treatment with these derivatives have been found to improve the cholinergic activities of brain that in turn enhance the intrinsic cognition activity. Furthermore, these derivatives were also reported to possess better anxiolytic actions [33]. The present study was designed to synthesize novel TMI to evaluate its effects on beta amyloid (Aβ1-42) and tau (τ) protein levels in brain that might have a significant effect on cellular protection in neurological diseases such as Alzheimer's disease. However they obtained results will further require detailed delineation to understand underlying mechanisms.

Materials and Methods

Drugs and Chemicals

Sodium chloride, Streptozotocin, potassium chloride, pyrogallol, sodium phosphate dibasic, sodium phosphate monobasic, chloroform, hydrogen peroxide, 5, 5′ -dithiobis-(2-nitrobenzoic acid) (DTNB) and sodium hydroxide and acetyl thiocholine iodide were purchased from Sigma-Aldrich (Germany). Piracetam injection was gifted by global pharmaceuticals (Pakistan). Magnesium chloride, calcium chloride, dextrose were purchased from Merck, (Germany). TBA was purchased from Applichem (USA) & Tri-chloro acetic acid was purchased from BDH (USA). All the chemicals purchased were of analytical grade and were used without further purification.

Synthesis

Synthesis of 4-Arylidene-3-Methylisoxazol-5(4H)-Ones (TMI)

The selected compound (TMI) was synthesized according to the scheme given in Fig. 1. Ethyl acetoacetate (0.012 M) was taken in a round bottom reaction flask and equimolar (0.012 M) amount of hydroxylamine hydrochloride was added after dissolving it in 10 mL ethanol. The solution was kept at room temperature overnight and next day it showed slight yellow tinge. To this solution, equimolar amount (0.012 M) of respective aldehyde was added and stirred for few minutes. The mixture was kept overnight at room temperature for completion of reaction. Product crystallized in the reaction mixture which was filtered and washed with little amount of ethanol. The crystals were dried in desiccator and then stored in an air tight container.

4(Z)-4-[(3,4,5-Trimethoxyphenyl) methylidene]-3-Methyl-1,2-Isoxazol-5(4H)-One (TMI)

Yield, 90.3%; m.p 1541° C; R_f value = 0.36 (ethyl acetate: Pet ether 2:1); ^{1}H-NMR (DMSO, δ ppm): 2.282 (s, 3H, CH_3), 3.321 (s, 3H, OCH_3) 3.859 (s, 3H, OCH_3), 7.210 (s, 1H, CH), 8.018 (s, 2H, ArH); ^{13}C-NMR (100 MHz DMSO) δ ppm: 167.88, 157.5, 151.44, 151.44, 138.23, 138.75, 128.23, 125.11, 105.65, 105.65, 59.90, 54.15, 54.15, 17; Anal. Calcd. for $C_{14}H_{15}NO_5$: C, 60.64; H, 5.45; N, 5.05; found: C, 60.85; H, 5.55; N, 5.14.

Molecular Docking

The selected ligand (TMI) was docked with selected AD proteins (acetylcholinesterase, β-amyloid and tau protein) in order to have insight of binding affinity toward therapeutic targets by using Auto dock Vina 1.1.2 [34]. Three dimensional X-ray crystallized structures of AChE (PDB ID: 1J06), β-Amyloid ((PDB ID: 2BEG) and tau (τ) protein (PDB ID: 5HF3) was obtained from RSCB protein data bank. TMI was sketched in ChemDraw ultra and energy minimized with Charmm27 forcefield utilizing MMFF94 partial

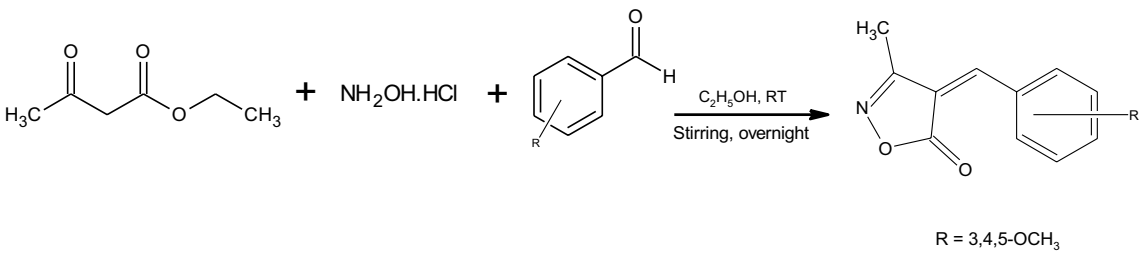


Fig. 1 Synthetic scheme of 3,4,5-trimethoxy isoxazolone derivative. R = 3,4,5-OCH_3

charge to relieve the geometrical constraints. The ligands and receptors were prepared and converted into PDBQT format using Auto dock tools. Compounds were assigned with Gasteiger charge and torsion count was inspected to ensure the preparation of flexible ligands. Protonation state of macromolecules was optimized to correct the ionization and tautomeric states of residues. The grid box was set to default size, grid point spacing and centered to binding pockets of macromolecules. The binding affinity of ligands to receptor was depicted in terms of binding energy (Kcal/mol) estimated by the scoring function of Auto dock Vina 1.1.2. Best binding pose, with lowest binding energy, was used to further investigate the complex orientation and interactions between the ligand and receptor by using the Accelrys discovery studio visualizer v17.2.

In-vitro Anti-oxidant Activity

Different concentrations of TMI ranging from 0.3125 to 0.5 mmol were prepared in DMSO. Different concentrations of Rutin and EDTA (0.3125-5 mmol) were used as a standard in DPPH assay and ferrous ion chelating activity respectively to perform the following assays.

DPPH Radical Scavenging Activity

Each concentration of isoxazolone derivative (1 mL) was added in DPPH solution (0.1 mM, 3 mL). Incubate the reaction mixture for 30 min in the dark room. After incubation absorbance of all the samples were measured at 517 nm. Blank contained the 1 mL DMSO instead of analyte. Different concentrations of rutin was used as a standard [35].

Reducing Power Assay

Phosphate buffer 2.5 mL (0.2 M, pH 6.6), K_3Fe (CN)6, and 2.5 mL (1%w/v) were added with 1 mL of each concentration of TMI. The resultant mixture was incubated at 50℃ for 20 min, followed by addition of 2.5 mL of tri-chloro acetic acid (10% w/v). The final mixture was centrifuged at 3000 rmp for 10 min. The upper layer was extracted and absorbance was measured at wavelength of 700 nm. Ascorbic acid was used as a standard [36].

Ferrous Ion Chelating Activityμ

Each concentration of Isoxazolone derivative (100 μL) was added to 50 μL of 2 mmol ferrous chloride and 200 μL of 5 mmol ferrozine solution. Resultant mixture was mixed thoroughly and incubated in dark at room temperature for 10 min. The absorbance was read at 562 nm. 100 μL ethylene diamine tri acetic acid (EDTA) were used as standard [37].

Experimental Animals

Albino mice (n = 60) of either sex, 8–10 weeks old were selected for experiment and has been taken from the animal house of Riphah International University. Animals were placed at 22 + 20° C having free access to food and water under a 12 h day and night cycle. Before 3 h of each experiment, animals were kept in laboratory to familiarize with lab conditions. Study was conducted after attaining permission by Research ethical committee of RIPS, Lahore, granted authorized no. of REC/RIPS-LHR/2017/015 governed under the ARRIVE guidelines, the National Centre for the Replacement, Refinement and Reduction of Animals in Research.

Study Design

At the start of experiment, a novel synthetic derivative (TMI) was synthesized and characterized in our lab. This synthesized compound was preliminary screened through molecular docking against selected targets and then through in vitro antioxidant assay. TMI was suspended in the 0.5% solution of carboxymethyl cellulose (CMC). For molecular analysis, Albino mice were divided into 6 groups each group having 10 mice (n = 10). Group I designated as the control group that was administered with CMC (carboxymethyl cellulose 0.5%), 1 mL/kg. Group II served as disease group that received streptozotocin (3 mg/kg). Group III served as reference group and received Piracetam (200 mg/kg). Group IV, V and group VI were treated with 10 mg/kg, 30 mg/kg and 100 mg/kg TMI, respectively.

Streptozotocin was injected i.c.v. on day 1st and day 3rd to all groups except control group while Piracetam (i.p) and different doses of TMI were given via oral route once daily for 14 days consecutively. The induction of Alzheimer's disease was assessed on the 1st day (14th day) by performing behavioral tests that included open field test, elevated plus maze test, passive avoidance test and Morris water maze test (MWM). The memory function was assessed on the next day (15th day). At the end of experiment the animals were killed using standard protocol and the hippocampus of mice brain were dissected out for oxidative stress studies, bio markers analysis and tissue Histopathological evaluation (n = 5/group).

Open Field Test

Open field test apparatus consisted of a white square box with 36 squares (10 × 10). Out of 36 squares four were red and rest were blue in color. The red squares are called central area and rest blue along the walls are called peripheral arena. On the 1st day (14th day) of treatment, each mice was placed in the red compartment of the box and time was

noted when mice leave the red compartment i.e., called the latency time (s). Along with this parameter, other parameters like Central Area Frequency and Peripheral Area Frequency were noted. The cut off time was 5 min to explore the open field apparatus for each mouse. The animals which did not move from the red compartment to periphery were excluded from the study. Test session was conducted on 2nd day (15th day) and all above parameters were noted and observed [38].

Passive Avoidance Test

A wooden box (27 × 27 × 27 cm) was used. This box consisted of four walls out of which three were wooden and one was made-up of plexiglas. The floor of the apparatus consisted of stainless steel grid. A wooden platform is present in the center of the apparatus. In this test each mouse was placed in top right corner of stainless steel platform. When the subject stepped down with its four paws on stainless steel grid platform, an electrical shock (20 V, A.C.) was produced and time was noted at which the mouse returned back to wooden platform. The training was conducted on the 1st day (14th day) of treatment with three different trials with the interval of 120 min. On the 2nd day (15th day), again the test was conducted and time was noted, taken by the mouse to reach to the gridded floor where there was no sensation of passive avoidance previously delivered. Increase in "Step down Latency" in each session of trials reflected the capacity of learning and sharp memory of mice [39].

Elevated Plus Maze Test

The apparatus consisted of four arms, two open (16 × 5 cm) and two close arms (16 × 5 × 12 cm). A central stage (5 × 5 cm) with the height of 25 cm is present. On 1st day (14th day) mouse was placed on open end of one arm facing away from the closed and open arm central square. Time was noted by which the mouse moved from the open arm to close arm and this time called as "Transfer Latency Time". Animal was allowed for 5 min to explore the maze and the behavior was noted such as number of entries in open arm and number of entries in closed arm. After 5 min of exploration of maze apparatus, animals were returned to their respective cages. On 2nd day (15th day), the above mentioned procedure was repeated and "Final Transfer Latency time" was noted [38].

Morris Water Maze Test

This apparatus consisted of a large circular pool with 59 cm and 45 cm in diameter from circumferences and bottom respectively. Water was filled up to 32 cm depth with temperature (24 ± 2° C). Pool was divided into the four different quadrants (N,S,E,W). A hidden platform was placed in the center of the one quadrant almost 1 cm below the water surface and different trials were given to each mouse by placing them at position north, west, east, and south facing to wall of pool. The time taken by each animal was noted to reach to platform called as "Transfer Latency time" and was permitted to stay on it for about 10 s. The trial was conducted for maximum 60 s after which the mice were restrained to their respective cages on 1st day (14th day). After trials given, the probe was removed and water was faded using the food grade nontoxic coloring agent. The experiment was conducted again to assess the retention memory of mice on 2nd day (15th day) [40].

Biochemical Estimation

Preparation of Tissue Homogenate Experimented animals were anesthetized by using halothane [41]. Halothane (2%) was delivered with oxygen for the induction of anesthesia. Brains were removed and hippocampus portion of brain tissue parts were dissected out. Homogenate was prepared in Phosphate buffer (pH 7.4) with respect to ratio of 1/10 (w/v). Homogenate was centrifuged at 600 × g for 10 min. Obtained supernatant was then further used for the analysis of certain bio markers like; GSH, MDA, CAT, SOD & AChEI's and neurotransmitters analysis.

Measurement of Reduced Glutathione Aliquot of tissue homogenate (1 mL) was added to 1 mL of (10% TCA), mixed well precipitation was formed at the bottom of the tube. In the mixture phosphate buffer (4 mL) (pH 8.4) and 0.5 mL of DTNB Ellman's reagent was added. Removed the supernatant and absorbance was checked at 412 nm approximately within 15 min. Following formula was used for the calculation of GSH values [42]:

$$GSH = Y - 0.00314 \div 0.034 \times DF/BT \times VU$$

where DF dilution factor, BT weight of the tissue and VU volume of aliquot.

Measurement of Malondialdehyde In this assay, oxidative stress and lipid damage mediated free radical levels were measured [43]. 1 mL of TBA 0.38% (w/w), 1 mL hydrochloric acid (0.25 M) and 1 mL of 15% (trichloro acetic acid) were mixed together and this solution was called as TBA reagent. 1 mL of aliquot of homogenized sample was added into prepared 3 mL of TBA reagent. Reaction mixture was incubated for about 15 min, then cooled in ice water bath and centrifuged for 10 min. Supernatant was collected and absorbance was measured at 512 nm. The already defined formula was used to calculate the concentration of MDA [42].

$$MDA = Abs \times 100 \times Vt/1.56 \times 105 \times Wt \times VU$$

where *Vt* total volume of the mixture, *Wt* weight of the hippocampus and *Vu* is volume of aliquot used.

Measurement of Catalase Supernatant (0.05 mL) of brain homogenate was added to phosphate buffer (1.95 mL, 50 mM, pH 7.0) along with 1 mL of 30 mM hydrogen peroxide. The absorbance of the solution was measured at 240 nm after 30 s and with intervals of 15 s. Already reported formula was used to estimate the catalase activity [42].

$$CAT\ levels = O.\frac{D}{E} \times vol\ of\ sample \times mg\ of\ protein$$

where O.D is the change in absorbance/minute, and E is the extinction coefficient of hydrogen peroxide having a value of 0.071 mmol cm − 1.

Lowery method was used for the measurement of protein contents with little modifications [44] while different concentrations of Bovine Serum Albumin (BSA) was plotted as standard curve. Protein contents were determined by below given equation.

$$Y = 0.00007571x + 0.0000476$$

Measurement of Superoxide Dismutase In a 0.1 mL of tissue homogenate, pyrogallol (0.1 mL) was added along with 2.8 mL of phosphate buffer (pH 7.4). The absorbance of the solution was measured at 325 nm [45]. Regression line equation was used to calculate SOD [46].

$$Y = 0.0095x + 0.1939$$

Acetyl Cholinesterase Assay 0.4 mL of tissue homogenate, 2.6 mL phosphate buffer 0.1 M (pH 8.0), and 100µL of DTNB (0.01 M) solution were mixed well and absorbance was measured at 412 nm. The initial reading of the mixture was noted. After that 20 µL of acetyl thiocholine iodide (1 mM) were added to the reaction mixture. Absorbance was noted for consecutive 10 min with the 2 min intervals at 412 nm. Calculation was performed by using the following formula [47].

$$R = 5.74 \times 10^{-4} \times A/C0$$

Dopamine Estimation

Tissue homogenate (0.2 mL), HCl 0.05 mL (0.4 M) and EDTA (0.1 mL) were mixed together followed by addition with 0.1 mL iodine solution (0.1 M) for oxidation. Reaction in solution was stopped subsequently after 2 min on addition of Na2SO3 (0.1 mL). Then after 1.5 min, acetic acid (0.1 mL) was added and solution was heated at 100° C for 6 min. After cooling of this solution (room temperature), the absorbance was checked at 370 nm [48]. Dopamine level in brain tissue was calculated by using below regression line.

$$Y = 0.0314X + 0.1067$$

Serotonin Estimation

TMI (0.2 mL) was added to O-phthaldialdehyde (OPT) reagent (0.25 mL). The solution was heated at 100° C for 10 min. The mixture was cooled to room temperature and the absorbance was checked at 420 nm. For blank reading absorbance of concentrated HCL (0.25 mL) without OPT was recorded [49]. Serotonin level in brain tissue was calculated by using below regression line;

$$Y = 0.0299X + 0.0918$$

ELISA Analysis

Beta amyloid 1–42 (Aβ1-42) (CAT #KHB3481) and tau proteins (CAT #LS-F6386-1) were estimated by using their respective ELISA kit. Aβ1-42 and tau protein were conjugated with horseradish peroxidase (HRP) complex. After conjugation, 3,3′,5,5′-Tetramethylbenzidine (TBM) agent was added to start reaction. The ongoing reaction was stopped by adding the sulfuric acid. Progressively the blue color of mixture changed to yellowish color. The change in absorbance was measured at 450 nm. The concentrations of the selected proteins were measured by using the regression line of standard proteins [50].

Polymerase Chain Reaction (PCR) and Analysis

Conventional PCR was used for the analysis of beta amyloid, and Tau gene expression in brain hippocampus of mice. Total RNA was isolated from brain hippocampus tissue by using the Triazole method. RNA was quantified by using the nanodrop 2000 (Thermofischer). Complementary DNA was synthesized using 1 µg according to the protocol of manufacturer kit (revertaid first strand cDNA synthesis kit, Thermofischer). cDNA product was diluted 50 times. 4 µl of the diluted cDNA was used for primer specific amplification of genes. Beta amyloid and tau protein gene expression were analyzed by using their specific primers; (5′ → 3′) forward primer: GTACCCACTGATGGCAACG, reverse primer AGGAACTTGCACTTGTCGG (Product size 350 bp) and forward primer: TCGACCTGAGCAAAGTGACC, reverse primer: TCATCGGCTAGTGTGGCAAG (product size 355 bp), respectively. Beta actin (5′ → 3′) forward primer:

GGAGATTACTGCTCTGGCTCC, reverse primer GTC GCCTTCACCGTTCCA (Product size 105 bp) was used as internal loading control. PCR amplification conditions were; 53℃ annealing temperature for 30 s and extension was 72℃ for 45 s in 36 cycles. Gene specific PCR product was verified by comparing product size against 100 bp ladder (ThermoFisher scientific) on 1.5% agarose gel.

Method of Quantification of Bands Intensity

Individual band density was measured using image J software V. 1.52a.

Histopathological Studies

Brains from mice (disease group, control group and treated groups) were fixed with formalin (10%,) encased in paraffin wax and then sliced into longitudinal section. Hematoxylin & eosin stain was used for staining. After fixation the slide histopathological observations were evaluated under microscope. 10X lens was used for magnification power.

Statistical Analysis

Data was analyzed as mean ± SEM, one way and two-way ANOVA (Bonferroni post hoc test) was used to analyze the data by using the Graph Pad Prism 5.01 version (USA). *$P < 0.05$ reflected as statistically significant, **$P < 0.01$ as moderate significant and ***$P < 0.001$ as highly significant.

Results

Chemistry

The scheme of the synthetic reaction for the preparation of isoxazolone derivative is described in Fig. 1

Molecular Docking

The compound was *in-silico* docked at the binding sites of receptors and conformational energies were estimated to address their inhibitory potential against key therapeutic targets in Alzheimer's disease. Compound was found to inhibit the target proteins with negative binding energy that showed their binding affinity toward selected targets (acetylcholinesterase, β-amyloid & tau proteins). The binding energies of this compound are tabulated for each target protein in Table 1. The best binding mode of this compound was extracted and visualized for their conformational alignment within binding pocket of target proteins.

The TMI shared the comparable conformational energy to bind with AChE. However, compound shared the binding pocket and attained conformational alignment that allowed their interactions with similar key residues (i.e. ASP131, MET85, LEU130 and VAL130) of AChE's active site (Fig. 2). The complex of TMI analogue was stabilized by the H-bonding with ASP131.

Table 1 The binding energy values after docking

Compounds	Analogues	Binding energy (Kcal/mol)		
		Acetyl cholinesterase (AChE)	β-Amyloid	Tau(τ)
1	TMI	− 8.0	− 7.4	− 6.9

In addition to AChE, this compound also showed the relative binding affinities towards β-amyloid protein. The similarity of conformational energy was consistent with the interaction pattern of this compound at the β-amyloid hotspot (Fig. 3).

Consistently, the binding affinity of TMI compound was almost similar for Tau protein. Although they were found to share the same binding pocket but the structural variations resulted in the different types of interactions with key residues at the active site of Tau protein. The TMI analogue was stabilized by the hydrogen bonding with SER27 and ARG6 in addition to one hydrophobic contact with PHE20 (Fig. 4).

Anti-oxidant Activities of TMI In Vitro Studies

Anti-oxidant activity of TMI was analyzed by using the DPPH, free radical scavenging and ferrous ion chelating assay method. Rutin, Ascorbic acid and EDTA was used as standard in different anti-oxidant methods respectively. IC_{50} value of TMI was 4.74 mmol/mL, 0.558 mmol/mL and 2.17 mmol/mL respectively. While IC_{50} of Rutin was 0.26 mmol/mL, for ascorbic acid IC_{50} value was calculated as 0.95 mmol/mL and for EDTA it was calculated as 0.3234 mmol/mL by using the regression line equation method [51].

Open Field Test

Transfer latency was decreased in all treatment groups except disease group as shown in Table 2. Disease group showed non-significant ($P > 0.05$) difference in transfer latency, central and peripheral area frequency. TMI at a dose group (30 mg/Kg), showed significant results ($P < 0.05$) while at dose level 100 mg/Kg, it showed moderate significant results in reducing the transfer latency and in increasing the central and peripheral area frequency as compared to disease group.

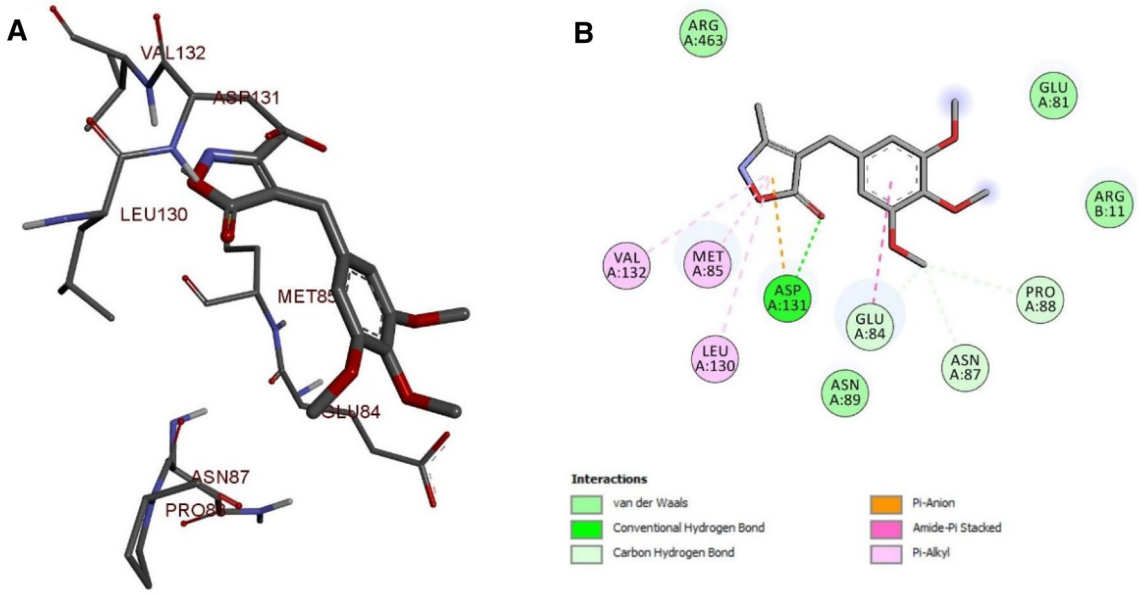


Fig. 2 The simulated pose of TMI docked at the active site of AChE; **a** The three dimensional (3D) conformational analysis of compound interacting at the active site. **b** The two dimensional (2D) interaction of compound with active site's key residues depicted as balls colored by type of interaction

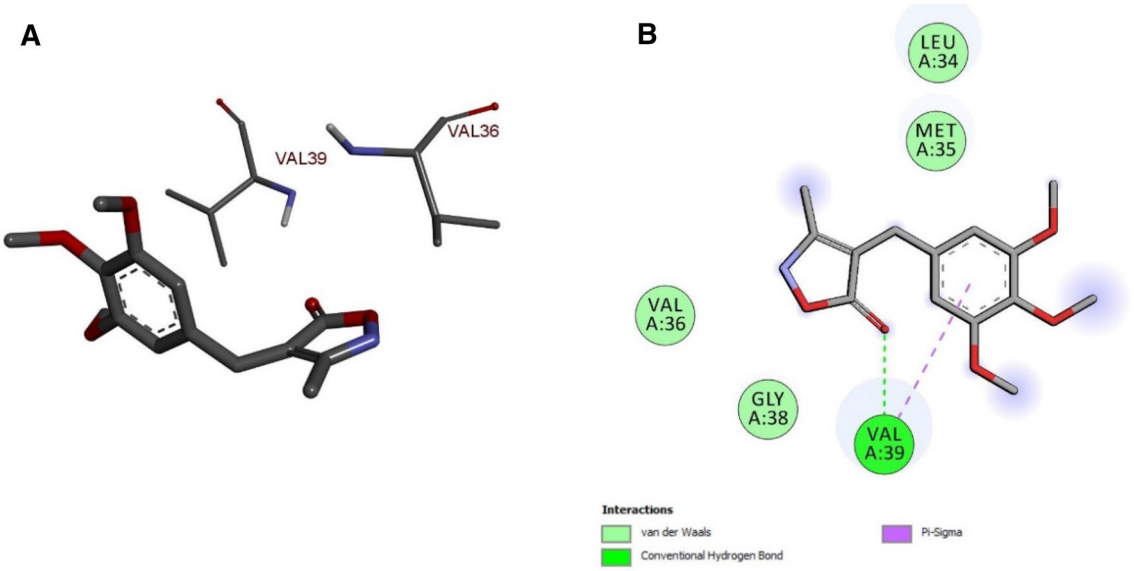


Fig. 3 The simulated pose TMI docked at the binding pocket of β-Amyloid. **a** The three dimensional (3D) conformational analysis of compound interacting at the active site. **b** The two dimensional (2D) interactions of compound with active site's key residues depicted as balls colored by type of interaction

Pre-treatment with TMI Increased the Step Down Latency in Mice

Disease group showed non-significant ($P > 0.05$) difference in "Transfer Latency". Dose group (10 mg/Kg) showed improvement in learning with significant results ($P < 0.001$), Dose group (30 mg/Kg) showed moderately significant result ($P < 0.001$) while dose group (100 mg/Kg) showed highly significant results ($P < 0.001$) in increasing the step down latency as compared to disease group (Table 3).

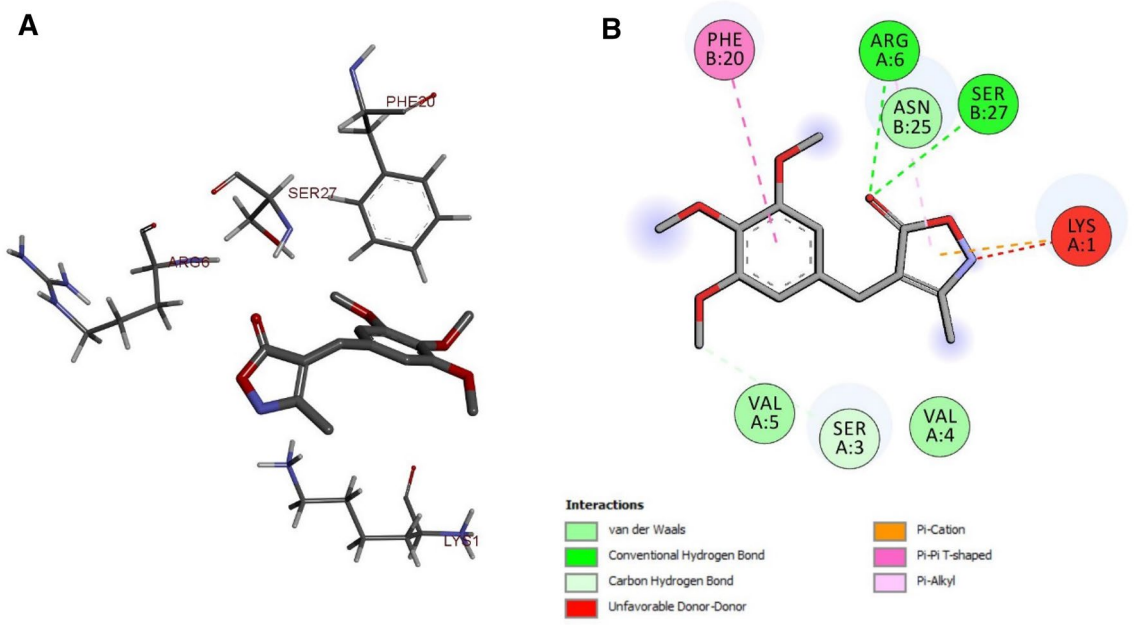


Fig. 4 The simulated pose of TMI docked at the binding pocket of Tau protein. **a** The three dimensional (3D) conformational analysis of compounds interacting at the active site. **b** The two dimensional (2D) interactions of compounds with active site's key residues depicted as balls colored by type of interaction

Table 2 Effect of TMI on Transfer latency and Central & Peripheral Area Frequency in Open Field Test

Treatment groups	1st day (14th day)			2nd day(15th day)		
	Transfer latency Time/s	Central area freq	Peripheral area freq	Transfer latency Time/s	Central area freq	Peripheral area freq
Open field test						
Control (1 mL/Kg)	7.6 ± 1.0	7 ± 0.7	14.2 ± 1.1	4.2 ± 0.6	11.2 ± 0.6	17.2 ± 1.3
Piracetam (200 mg/Kg)	6.4 ± 1.0	7.3 ± 0.9	15.9 ± 1.6	3.9 ± 0.5	8.5 ± 0.8	19.3 ± 2.0
STZ (3 mg/Kg)	9.9 ± 2.5	6.3 ± 0.4	8.8 ± 1.5	9.5 ± 0.5	2.7 ± 0.9	6.9 ± 1.8
TMI (10 mg/Kg)	7.3 ± 0.5*	2.3 ± 0.4	12.8 ± 1.5	5.4 ± 0.9	2.7 ± 0.7	8.3 ± 2.7
TMI (30 mg/Kg)	6.9 ± 1.2*	4.83 ± 0.8	11.2 ± 2.4	4.2 ± 0.3*	7.8 ± 0.4*	13.5 ± 3.2*
TMI (100mg/Kg)	5.1 ± 3.0*	4.3 ± 0.9	10.6 ± 1.4	2.9 ± 0.9**	10.3 ± 0.4**	15.5 ± 2.5**

Data presented as mean ± SEM, n = 10

*P < 0.05 & **P < 0.01 in comparison to disease group (STZ)

Elevated Plus Maze Test

In Table 4, disease group showed non-significant (P > 0.05) difference in transfer latency, open and close arm entries parameters. Dose group (30 mg/Kg) showed significant result (P < 0.05) while dose group (100 mg/Kg) showed moderate significant results (P < 0.01) in reducing the transfer latency and in increasing the open and close arm entries as compared to disease group.

Effect of TMI on Escape Latency

Disease group showed non-significant (P < 0.05) difference in transfer latency (Table 5). Dose group (10 mg/Kg) had showed improvement in learning but results were not statistically significant. Dose group (30 mg/Kg) showed significant result (P < 0.05) while dose group (100 mg/Kg) showed moderately significant results (P < 0.01) in reducing the transfer latency as compared to disease group.

Table 3 Influence of TMI on step down latency in passive avoidance test

Treatment groups	Step down latency (seconds)	
	1st day (14th day)	2nd day (15th day)
Passive avoidance test		
Control (1 mL/Kg)	6.2 ± 2.5	117.33 ± 1.0
Pirecetam (200 mg/Kg)	7.5 ± 2.4	218.7 ± 1.3***
STZ (3 mg/Kg)	29.1 ± 2.1	24.6 ± 3.5
TMI (10 mg/Kg)	31.5 ± 5.4	241.3 ± 3.8***
TMI (30 mg/Kg)	16.3 ± 4.7	226.8 ± 4.3***
TMI (100 mg/Kg)	11.3 ± 4.6	236.6 ± 1.9***

Data presented as mean ± SEM, n = 10

***P < 0.001 in comparison to disease group (STZ)

Endogenous Antioxidants Estimation in Mice Brain Tissues having Streptozotocin induced Alzheimer's disease

Different endogenous antioxidants in brain tissues such as GSH, MDA, CAT, SOD and AChEI's were measured to evaluate the antioxidant potential of TMI. Table 6 clearly shows the significant raised levels of GSH, CAT, SOD, AChEI's and significantly decreased MDA level in TMI pre-treated groups (100 mg/Kg) as compared to disease group.

Estimation of Dopamine and Serotonin Levels in Brain Tissue

Serotonin and dopamine play a significant role in the normal physiological function of brain. The levels of serotonin and dopamine were reduced in STZ exposed brains as compared to control group. While, pre-treatment with TMI significantly raised these neurotransmitter levels in dose dependent manner as compared to disease group. Figure 5 clearly indicates that TMI at dose 100 mg/kg showed highly significant results (Figs. 6, 7, 8 and 9).

ELISA Analysis

ELISA analysis of beta amyloid (Aβ1-42) and tau proteins showed that TMI significantly decrease the aggregates of beta amyloid and plaques of tau protein in the brain of treated mice.

mRNA Gene Expression Levels of Beta Amyloid (Aβ1-42) and Tau Protein

mRNA expression analysis by conventional PCR showed that TMI at higher doses significantly decreased mRNA expression of MAPT1 while a slight reduction in expression of beta amyloid mRNA was observed when compared with beta actin as internal control.

Table 5 Effect of TMI on escape latency in Morris Water Maze test

Treatment groups	1st day (14th day) Escape latency Time /s	2nd day (15th day) Escape latency Time /s
Morris Water Maze Test		
Control (1 mL/Kg)	8.0 ± 1.0	4.8 ± 0.7
Piracetam (200 mg/Kg)	9.3 ± 0.7	3.3 ± 0.6
STZ (3 mg/Kg)	18.7 ± 3.0	27.5 ± 5.6
TMI (10 mg/Kg)	19.6 ± 2.0	18.2 ± 1.8
TMI (30 mg/Kg)	25.2 ± 6.4	10.6 ± 3.9*
TMI (100 mg/Kg)	19. ± 0.9	5.7 ± 0.8**

Data presented as mean ± SEM, n = 10

*P < 0.05, **P < 0.01 in comparison to disease group (STZ)

Table 4 Effect of TMI on Transfer latency, Open & Close arm entries in Elevated plus Maze Test

Treatment groups	1st Day (14th day)			2nd Day (15th day)		
	Transfer latency Time/s	Open arm entries	Close arm entries	Transfer latency Time/s	Open arm entries	Close arm entries
Elevated Plus Maze Test						
Control (1 mL/Kg)	14.7 ± 3.8	1.7 ± 0.3	7.3 ± 1.2	10.2 ± 2.1	3 ± 0.3	11.6 ± 0.9
Piracetam (200 mg/Kg)	15.5 ± 3.6	0.8 ± 0.3	5.2 ± 1.5	12.83 ± 2	2.7 ± 0.3	9.2 ± 1.2
STZ (3 mg/Kg)	76.8 ± 7.9	0.9 ± 0.1	5 ± 1.1	102.8 ± 23.9	0.7 ± 0.3	3.9 ± 0.7
TMI (10 mg/Kg)	140.3 ± 4.2	0.8 ± 0.4	3 ± 1.1	109.8 ± 18.2	1 ± 0.3	4.5 ± 0.5
TMI (30 mg/Kg)	22.7 ± 0.7	1.7 ± 0.4	7.1 ± 1.2	17.6 ± 1.2*	2.83 ± 0.3*	7.7 ± 1.0*
TMI (100 mg/Kg)	14.6 ± 4.5	1.8 ± 0.3	1.7 ± 0.3	6.5 ± 2.6**	5.2 ± 0.2**	12.3 ± 0.3**

Data presented as Mean ± SEM, n = 10

*P < 0.05,**P < 0.01 in comparison to disease group (STZ)

Table 6 Effect of TMI on biomarkers used to identify the oxidative stress

Treatment Groups	Dose (mg/kg)	Glutathione (ug/mg of brain protein)	MDA (ug/mg of brain protein)	SOD (ug/mg of brain protein)	Catalase (ug/mg of brain protein)	AChEI's (ug/mg of brain protein)
Control	1 mL/Kg	25.0 ± 0.2	51.70 ± 0.4	26.22 ± 0.8	1.68 ± 0.05	38.01 ± 0.3
Piracetam	200	30.2 ± 0.6	48.30 ± 0.2	28.47 ± 0.6	2.40 ± 0.05	37.73 ± 0.2
STZ	3	10.5 ± 0.2	83.30 ± 0.9	14.61 ± 0.4	1.14 ± 0.01	41.94 ± 0.6
TMI	10	24.4 ± 0.2**	69.70 ± 0.5*	22.65 ± 0.3**	1.57 ± 0.09	35.84 ± 0.4
	30	28.0 ± 0.2**	58.90 ± 0.4**	27.37 ± 0.3**	1.92 ± 0.012	30.34 ± 0.9*
	100	32.9 ± 0.2***	43.9 ± 0.7***	30.34 ± 0.3***	2.21 ± 0.013*	25.30 ± 0.7**

Data presented as mean ± SEM, n = 5

*P < 0.05, **P < 0.01, & ***P < 0.001 in comparison to disease group (STZ)

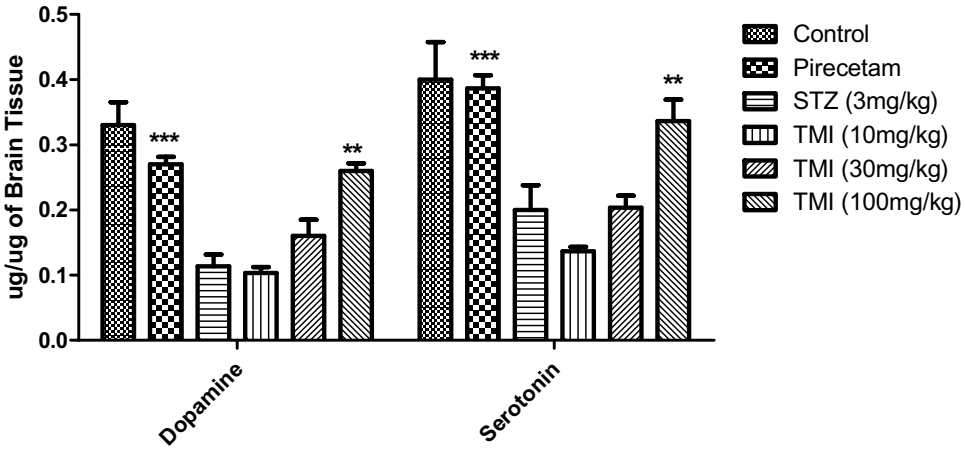


Fig. 5 Effect of TMI treatment on brain neurotransmitter levels. ***P < 0.001, **P < 0.01 was given when compared with disease group (STZ)

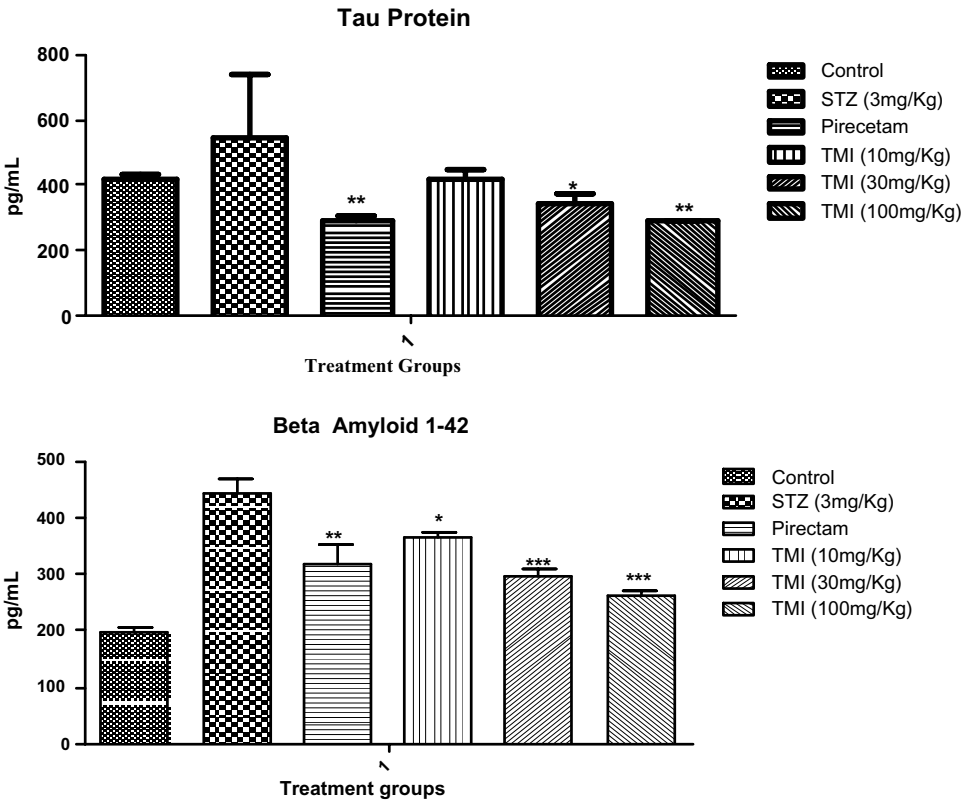
Band densities were measured using rectangle tool and base line corrections were made. First band densities were corrected for the internal control gene beta actin followed by relative band intensities vs control group. The gel images are representative of the three gels.

Histopathological Studies

In brain slide of disease group neurofibrillary tangles and senile plaques were observed along with that brain cells were not intact. However, standard group and control group showed no neurofibrillary tangles and senile plaques and their cells were intact. TMI group treated (10 mg/Kg) and TMI group treated (30 mg/Kg) showed neurofibrillary tangles in brain slides. The only difference observed was that, dose (10 mg/Kg) group showed less intact cells while dose (30 mg/Kg) group showed more intact cells. Brain slides of TMI treated group with dose (100 mg/Kg) showed intact cells as just like brain slides of control group and standard group. Hence histopathological slides reflected that TMI group (100 mg/kg) might have significant neuroprotective effect in similar manner as like standard (Piracetam).

Discussion

Alzheimer disease is one of the most commonly found neurodegenerative disorder which accounts for dementia. Plenty of evidence in the literature shows that oxidative stress plays major role in the pathogenesis of AD and its progression can be reversed by preventing the generation of free radicals through utilization of antioxidants [52]. AD occurs mostly in older people and as such no treatment is available to treat it successfully. Many herbal remedies are available for palliative treatment of the disease. Now the researchers are focusing on synthetic compounds to get a major breakthrough to treat AD successfully. For this reason TMI, a derivative of isoxazolone was investigated for its potential against AD [53]. STZ administered in lateral ventricles of mice can produce neurological, behavioral and metabolic disturbances which are similar to the human neurological



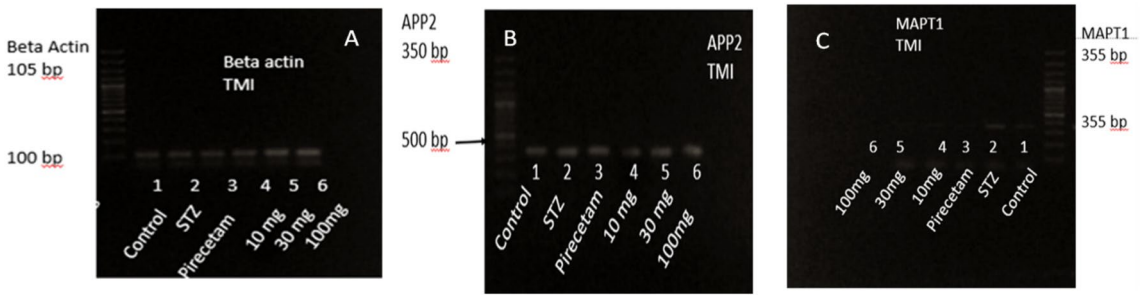


Fig. 6 Total Aβ 1–42 and tau levels in the mouse brain. N = 5, * P < 0.05, ** P < 0.01 and *** P < 0.001 Significance was given in comparison to the disease group (STZ)

Fig. 7 Resverse transcriptase-PCR based mRNA expression of MAPT1 and APP2 in hippocampuss: **a** Agarose gel electrophoresis of PCR product of beta actin (105 bp) as internal loading control, equal band intensities in **a** show equal loading of the cDNA in PCR reactions for APP2 and MAPT1. **b** agarose gel electrophoresis of pcr product of APP2 (350 bp). **c** agarose gel electrophoresis of pcr product of MAPT1 (355 bp) Aβ 1-42 and tau in the mouse brain

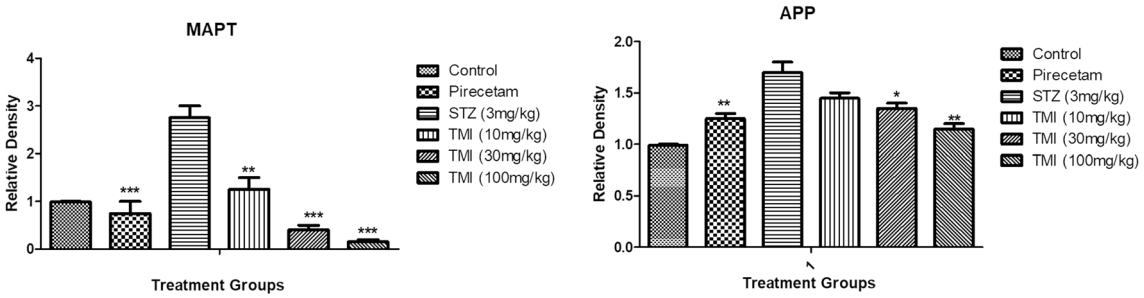


Fig. 8 Band intensity graphs of APP and MAPT mRNA expression in hippocampus of TMI treated mice by image J software. **P < 0.01, ***P < 0.001 when compared with disease group

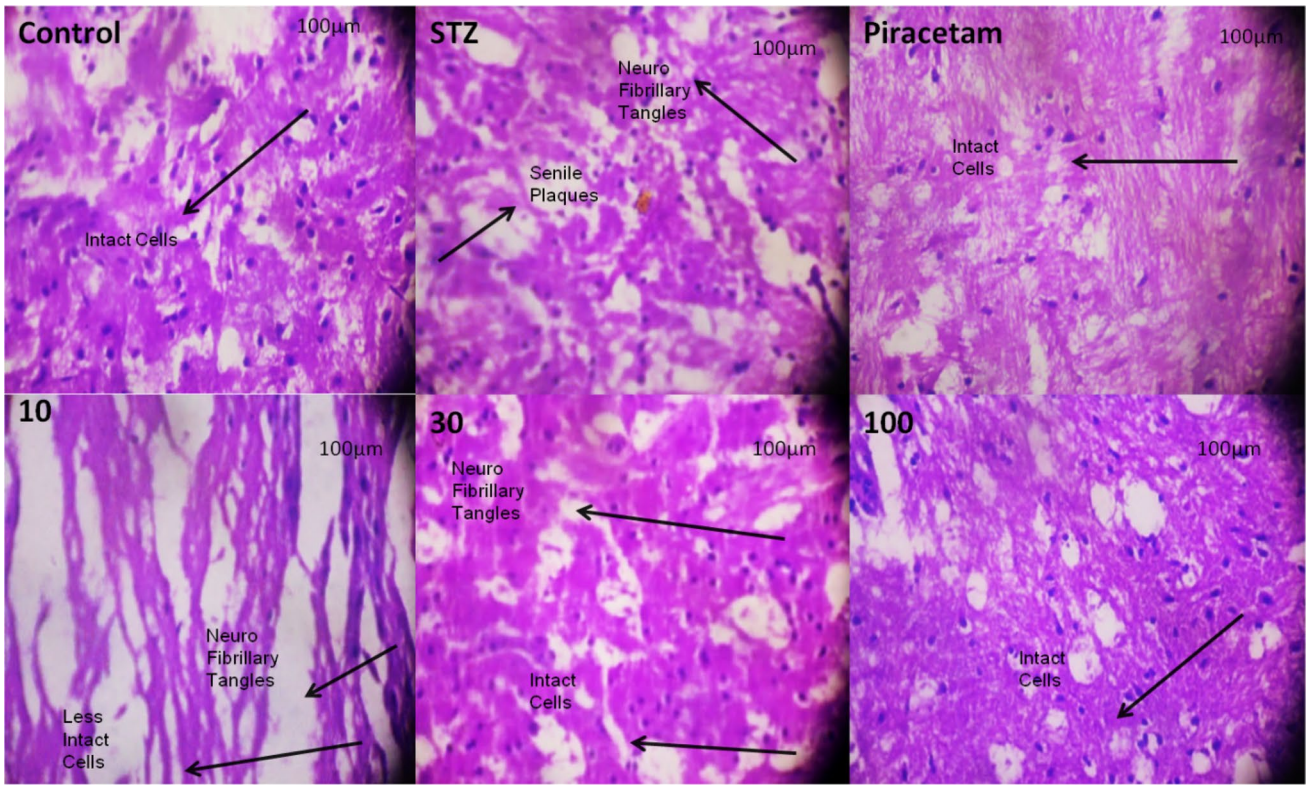


Fig. 9 Histopathological results. STZ exposed tissue slide clearly showing neurofibrillary tangles and senile plaques while TMI (100 mg/kg) showing significant intact cells

degenerative changes in AD [54, 55]. In mammalian cell ATP mediated energy production in mitochondria results in formation of free oxygen species which are known to be involved in the pathogenesis of stress, anxiety of neurons and decline in neurogenesis [56, 57]. GSH, CAT, SOD are endogenous antioxidant enzymes whereas MDA works to counteract the action of these enzymes [58]. The present study was focused on investigating the antioxidant activity and AD induced behavioral changes, biochemical markers and histopathological changes by the use of 3,4,5-trimethoxy isoxazolone derivative (TMI) in the mouse.

The *in-silico* molecular docking of compound was carried out to find out binding capability with AChE, β-amyloid and Tau protein, the potential targets for AD. The molecular docking is a reliable approach to computationally simulate and study the ligand-target complex formation, orientation and its interaction profile that aids the rational drug design. Our compound shared the comparable conformational energy or binding affinity and interactions profile for all targets. The docked complex of TMI revealed that it retained the favorable interactions with key residues of AChE active site. In addition to this, it efficiently interacted with the key hotspots of β-amyloid and Tau proteins which may prevent their pathological aggregations and further corroborated its therapeutic potential in Alzheimer's disease. Therefore, the favorable therapeutic profile of TMI analogue, to modulate the AChE [59]. Lower IC_{50} value accounts for greater antioxidant activity [60]. The antioxidant activity of under investigation compound was carried out using three different methods. In DPPH activity analysis IC_{50} value of our compound was 4.74 mmol/mL in comparison to Rutin's value of 0.26 mmol/mL. In Percent reducing power assay the IC_{50} value of test compound was 0.558 mmol/mL which is significantly lower than the value of standard ascorbic acid (0.95 mmol/mL). In Ferrous ion chelating assay the IC_{50} value of test compound was 2.17 mmol/mL as compared to the EDTA value (0.3234 mmol/mL). The dose selection was made on the basis of the in-vitro antioxidant IC_{50} values. The range of IC_{50} values lies between 0.5 and 4.00 mmol.

Memory are of different types i;e spatial, procedural, long term and episodic memory. Different behavioral paradigms were used for the analysis of the behavioral activity of the mice that co-relate with the memory retention. Water morris and elevated plus maze test was for the evaluation of spatial memory and passive avoidance and open field test were used for the assessment of long term memory [61]. Escape latency in water morris test and transfer latency in the open field test were significantly decreased in the animals treated with the TMI (100 mg/kg) dose. Step down latency was appreciably decreased in the mice treated with

the TMI. Behavioral paradigms results revealed a discemible improvement in the memory. TMI treated animals showed the remarkable improvement in the memory.

Glutathione (GSH) protects free radicals induced neurodegeneration in AD [62]. In present study GSH levels were significantly reduced in AD model whereas treatment with test compound dose dependently caused a significant increase in GSH brain levels. Contrarily to GSH levels, the malondialdehyde (MDA) levels are a scientifically prove to be elevated in AD due to excess of free radicals and oxygen species [63]. In our study MDA levels were decreased to a significant degree when compared with STZ-induced disease group, indicating the role of test compound in improving this biomarker. Superoxide Dismutase (SOD) a cell watch guard continuously regulates free radicals detoxifying chain. Its synthesis depends upon the cellular oxygenated reaction species and leads to the conversion of superoxide ions into H_2O_2, subsequently the increasing production of H_2O_2, SOD being a catalyst triggers endogenous antioxidant mechanisms [64]. The same was proved by our results meaning by that the disease group had markedly decreased SOD levels in comparison to control group while the levels of the enzyme dose dependently increased with test compound therapy. Catalase is an enzyme found in all living species, helps in neutralizing the hydrogen peroxide and its breakdown into molecules of water and oxygen and it further inhibits the biosynthesis of H_2O_2 [65]. The result of present study is in line with the above findings and catalase levels were increased significantly as compared to control and diseases group by test compound.

The deficiency of Acetylcholine was implicated in the brain of AD patients. Hence the researchers focused on preserving Ach in the brain to help to treat the disorder of neurodegeneration [66]. The present study results were found parallel to above statement showing decrease levels of Acetylcholinesterase enzyme in the mouse brain.

Two others neurotransmitters of brain dopamine and serotonin were measured in the present study. The levels of neurotransmitters were increased with the treatment by test compound revealing neuroprotective effect of the compound. Amyloid and tau protein play an important role in the pathogenesis of AD this has been proved in transgenic AD mice by the expression of mutant APP and beta amyloid aggregate lesions [67]. Our study showed reduction in aggregates of beta amyloid protein and tau neurofibrillary tangles in mice treated with test compound. Histopathological studies also confirmed the above findings that our compound showed defensive management of the AD [68]. The histopathological slides of control group had intact cells with no senile plaques and neurofibrillary tangles whereas the brain of disease group contained less intact cells and plenty of neurofibrillary tangles and senile plaques. Critical review of histopathological slides of the isoxazolone derivative-treated brains had greater number of intact brain cells and less senile plaques and less neurofibrillary tangles as compared to control/diseases groups. Although this study demonstrated the possible mechanism of the TMI role against Alzheimer disease however, estimation of more proteins through elisa and western blotting is the limitation of the study. Toxicity profiling is also the limitation of the study; however it is in progress in a project. Further studies are necessary for the molecular mechanism of the TMI.

Conclusion

Study concluded that different concentration of isoxazolone derivative (TMI) might have nootropic activity, antioxidant potential in dose dependent manner and has demonstrated highly significant effects on increasing spatial memory and learning. Higher doses of isoxazolone derivative (100 mg/kg) disclose more neuroprotective effect against streptozotocin induced Alzheimer in mice. Hence these isoxazolone derivative can be used for enhancing and improving the learning and spatial memory in neurodegenerative disease especially in Alzheimer disease. Much more extensive studies are mandatory to assess the active principle. Use of isoxazolone derivatives in Alzheimer's disease could prove valuable candidate as a drug that may prevent the generation of free oxygen radicals to induce Neuropathological disorders by scavenging them and helps in enhancing spatial memory and learning.

Acknowledgments Special thanks to Mr. Umer Farooq for providing financial support and technical guidance's in order to conduct study in a smooth way.

Author Contributions SMA, TA, M.I and FA had done all the related research work, studied and inspected the results and critically examined the important data. Designing of research protocol was conducted by NHK, BA & HN. Reliability of work was investigated by FA, US, MTK & SN along with proper guidance for publication. TI & NHK did the computational studies.

Funding Authors had no funding for this study.

Compliance with Ethical Standards

Conflict of interest The authors proclaim that research was accompanied in absence of any financial or even any commercial relationship that could be construed as a potential conflict of interest.

Consent for Publication All authors showed their consent for publication.

Ethical Approval Ethics approval and consent to participate: Study was conducted after attaining permission by Research ethical committee of RIPS, Lahore, granted authorized no. of REC/RIPS-LHR/2017/015 governed under the ARRIVE guidelines, the National Centre for the Replacement, Refinement and Reduction of Animals in Research.

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