Section-I

Chapter-4

RESULTS

4. RESULTS

4.1. Cholinesterase inhibition assay of triazine derivatives

3-Substituted 5,6-diaryl-1,2,4-triazines (TRZ-1-TRZ-22) were synthesized by Pharmaceutical Chemistry laboratory of the Department. The compounds were evaluated for AChE and BuChE inhibition (**Table 1**).

Compounds (**TRZ-15**, **TRZ-19** and **TRZ-20**) were found to be potent AChE and BuChE inhibitors amongst the 22 synthesized compounds (**Table 1**). **TRZ-15**, **TRZ-19** and **TRZ-20** provided ChE inhibition with superior potency and selectivity toward AChE; **TRZ-15** (AChE IC_{50} = 4.23 µM; BuChE IC_{50} = 13.3 µM), **TRZ-19** (AChE IC_{50} = 9.157 µM; BuChE IC_{50} = 67.19 µM) and **TRZ-20** (AChE IC_{50} = 5.79 µM; BuChE IC_{50} = 163.4 µM). Out of these three compounds, **TRZ-15** was found to be the most potent.

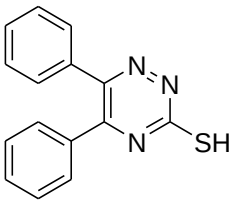
4.2. Cholinesterase inhibition assay of guanidine derivatives

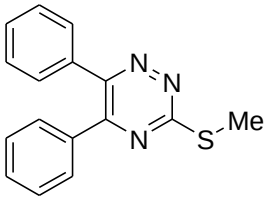
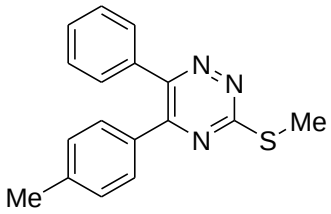
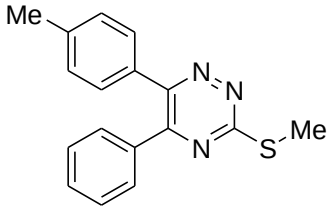
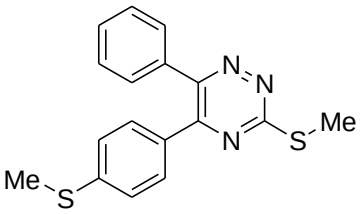
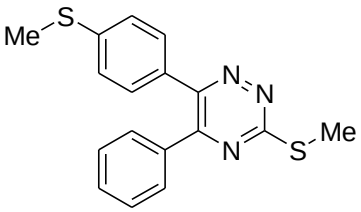
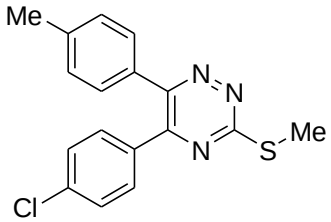
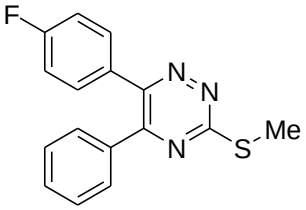
AChE and BuChE inhibitory activities were determined for quinazolne derivatives (**1a-6j**) possessing cyclic guanidine moiety supplied by Pharmaceutical Chemistry laboratory of the Department (**Table 2**).

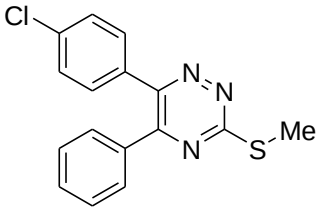
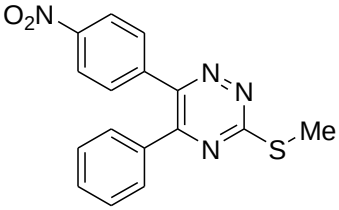
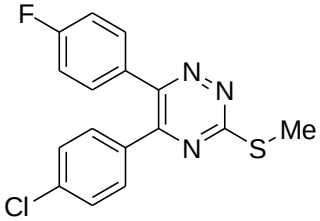
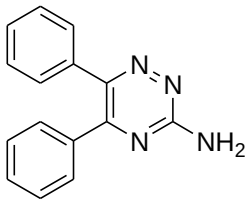
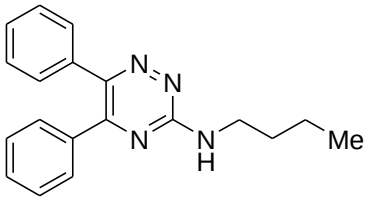
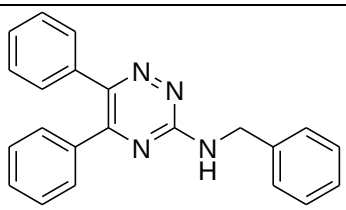
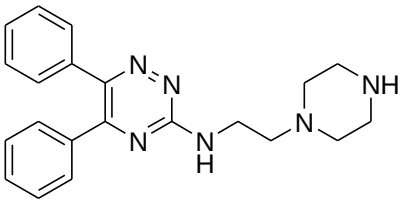
Compound (**3b**) emerged as the most potent AChE inhibitor amongst all of the synthesized compounds (**Table 2**). **3b** (AChE IC_{50} = 6.77 µM; BuChE IC_{50} = 22.13 µM) provided ChE inhibition with greater potency and selectivity toward AChE.

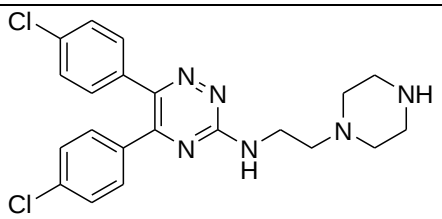
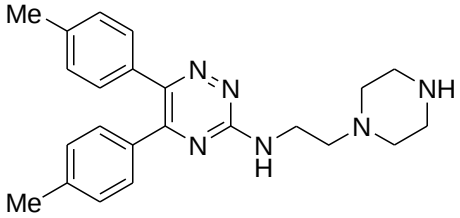
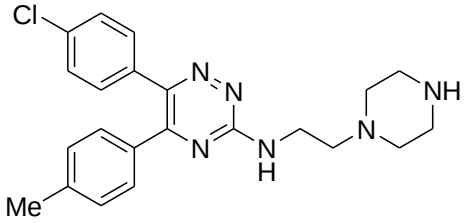
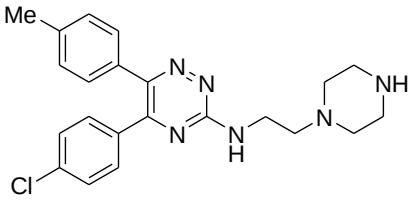
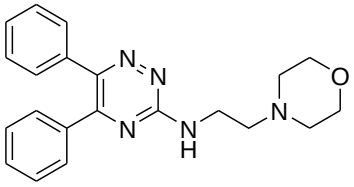
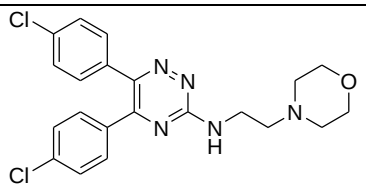
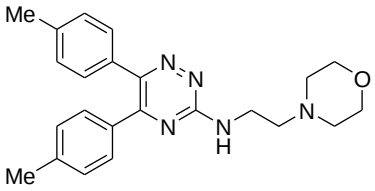
The most potential compounds from the two series (**TRZ-15**, **TRZ-19** and **TRZ-20** from triazine series and **3b** from the cyclic guanidine series) were further evaluated to assess their potential to be used in AD.

Table 1: AChE and BuChE inhibitory activities of the triazine derivatives

Sr. No	Code	Structure	IC_{50} (µM)*	
			AChE	BuChE
1	TRZ-1		14.57	238.9

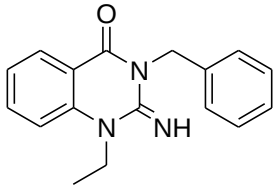
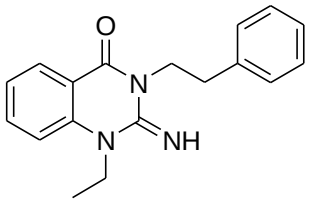
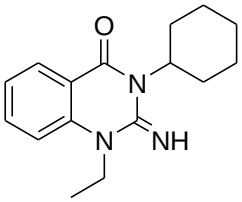
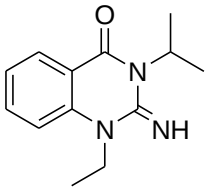
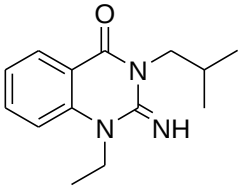
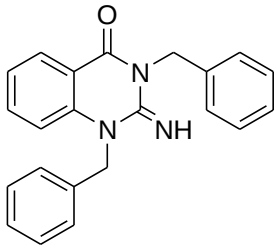
Sr. No	Code	Structure	IC_{50} (µM)*	
			AChE	BuChE
2	TRZ-2		1568	157
3	TRZ-3		>30000	116
4	TRZ-4		26282	29.97
5	TRZ-5		1364	116
6	TRZ-6		949	154.2
7	TRZ-7		641	225.5
8	TRZ-8		321.2	113.2

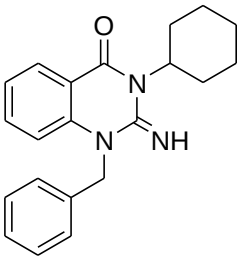
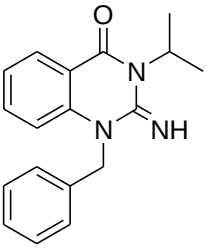
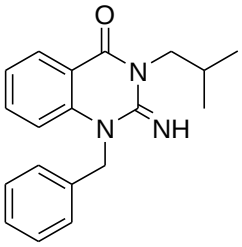
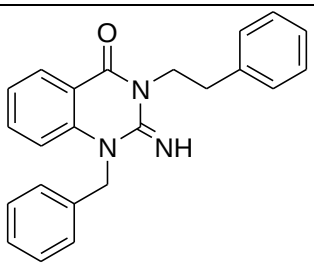
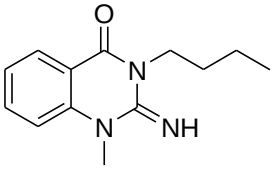
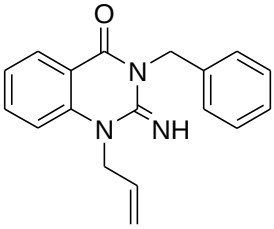
Sr. No	Code	Structure	IC_{50} (µM)*	
			AChE	BuChE
9	TRZ-9		422.1	NA
10	TRZ-10		249.5	1216
11	TRZ-11		223.9	NA
12	TRZ-12		102.5	250.2
13	TRZ-13		708.1	92.84
14	TRZ-14		107.2	9.53
15	**TRZ-15**		**4.23**	**13.3**

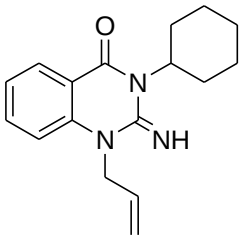
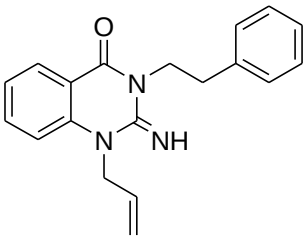
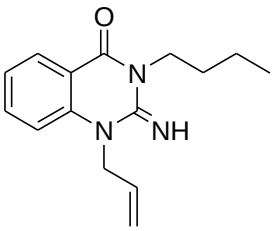
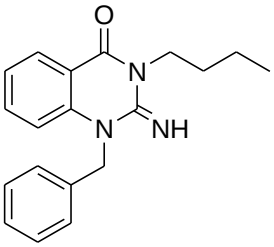
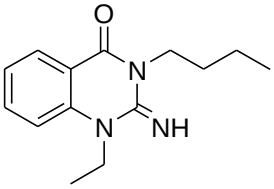
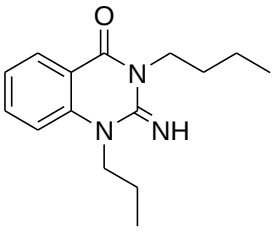
Sr. No	Code	Structure	IC_{50} (µM)*	
			AChE	**BuChE**
16	TRZ-16		12.53	57.25
17	TRZ-17		34.43	74.56
18	TRZ-18		10.14	103.1
19	**TRZ-19**		**9.16**	**67.19**
20	**TRZ-20**		**5.79**	**163.4**
21	TRZ-21		31.7	520.4
22	TRZ-22		47.32	190.6
23	-	Donepezil HCl	0.028	3.39
24	-	Tacrine HCl	0.079	0.0046

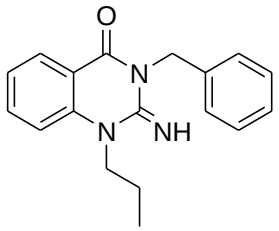
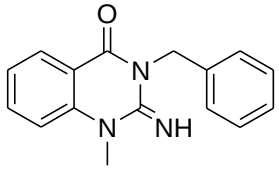
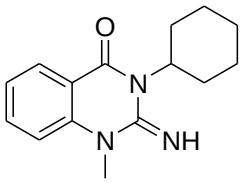
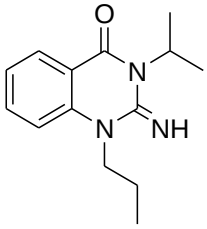
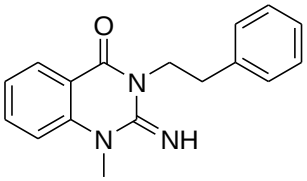
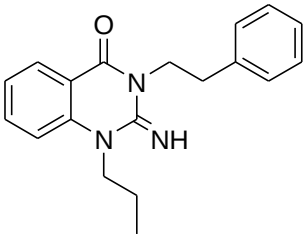
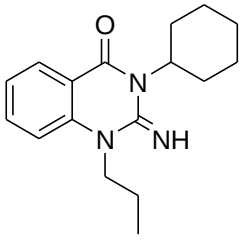
*The *in vitro* test compound concentration required to produce 50% inhibition of *h*AChE & equine BuChE.

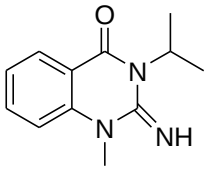
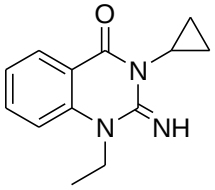
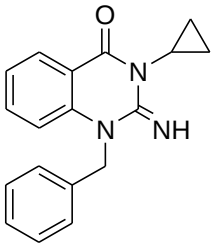
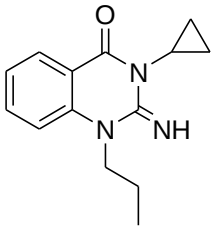
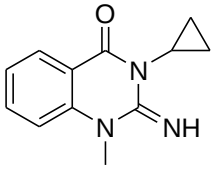
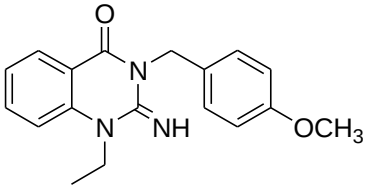
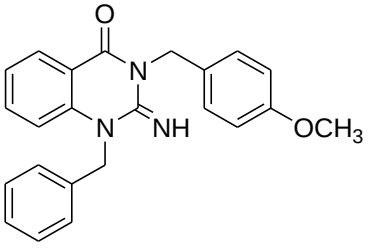
Table 2: AChE and BuChE inhibitory activities of the cyclic guanidine derivatives

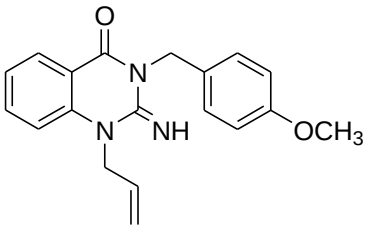
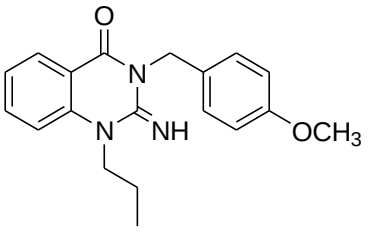
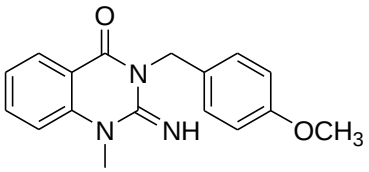
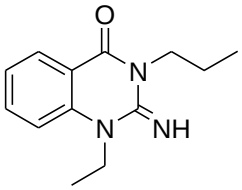
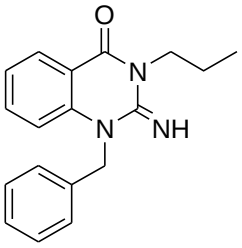
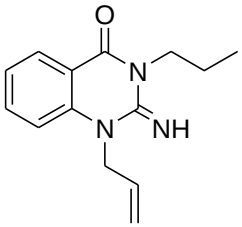
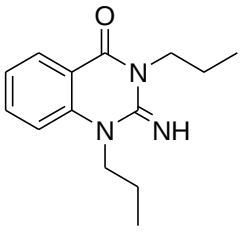
Sr. No	Code	Structure	IC_{50} (µM)*	
			AChE	**BuChE**
1	1a		10.25	>100
2	1b		9.150	>100
3	1c		9.148	>100
4	1d		9.143	>100
5	1e		9.721	>100
6	1f		>100	>100

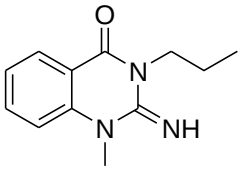
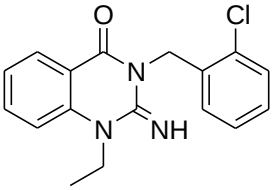
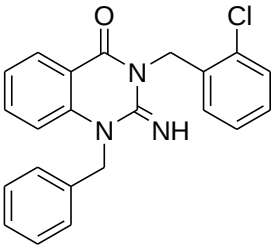
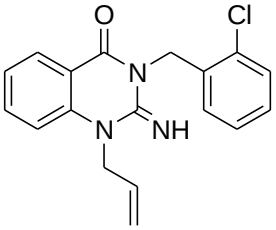
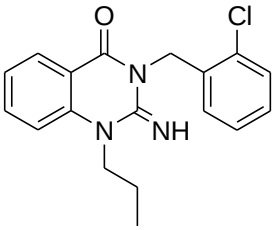
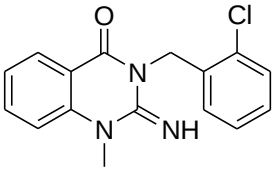
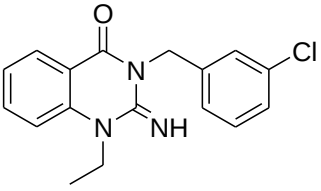
Sr. No	Code	Structure	IC_{50} (µM)*	
			AChE	BuChE
7	1g		9.467	>100
8	1h		>100	>100
9	1i		49.70	>100
10	1j		41.87	>100
11	1k		11.02	>100
12	1l		9.10	>100

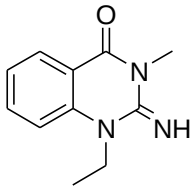
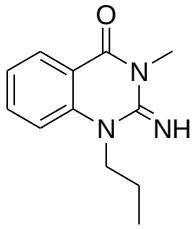
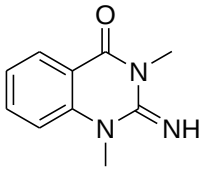
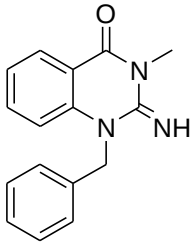
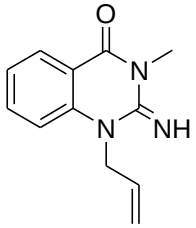
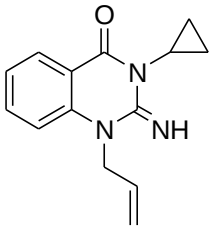
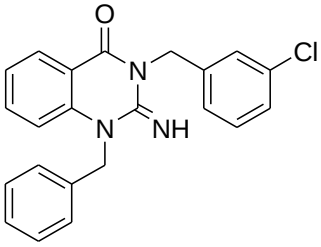
Sr. No	Code	Structure	IC_{50} (µM)*	
			AChE	BuChE
13	2a		39.13	>100
14	2b		45.71	>100
15	2c		16.43	>100
16	2d		12.93	>100
17	2e		>100	>100
18	2f		20.09	>100

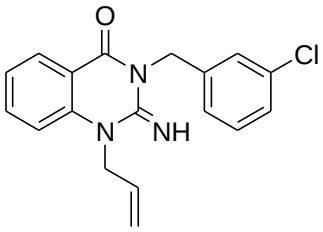
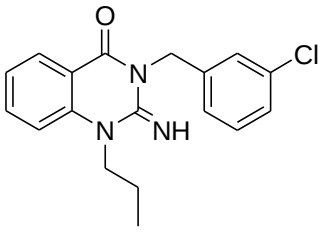
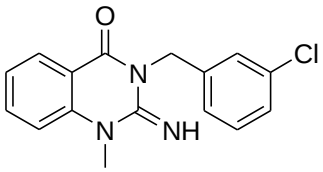
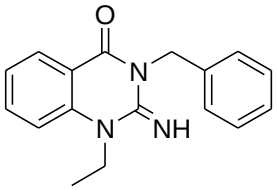
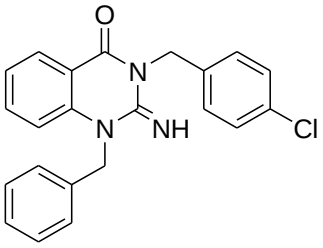
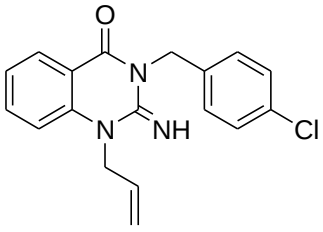
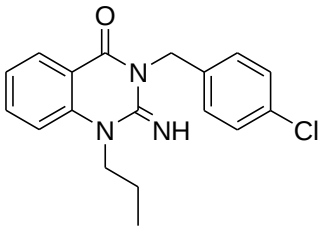
Sr. No	Code	Structure	IC_{50} (µM)*	
			AChE	BuChE
19	2g		>100	>100
20	2h		233.5	>100
21	2i		162	>100
22	2j		142.3	>100
23	2k		>100	>100
24	2l		>100	>100
25	3a		>100	>100

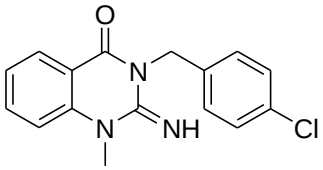
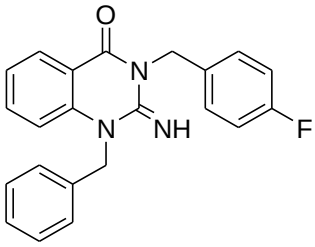
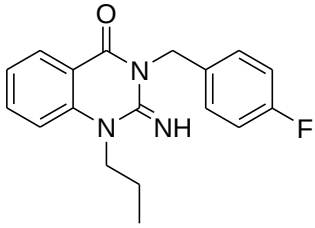
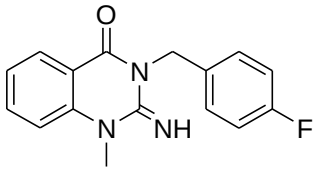
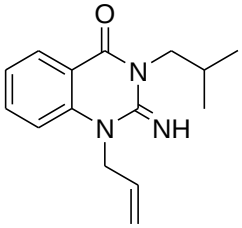
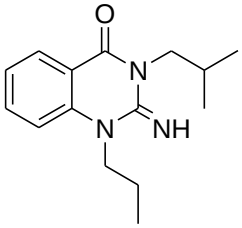
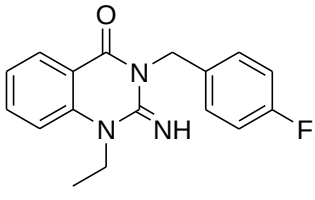
Sr. No	Code	Structure	IC_{50} (µM)*	
			AChE	BuChE
26	**3b**		**6.77**	**22.13**
27	3c		>100	>100
28	3d		7.652	>100
29	3e		>100	>100
30	3f		>100	>100
31	3g		>100	>100
32	3h		>100	>100

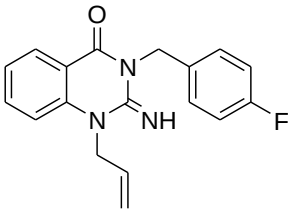
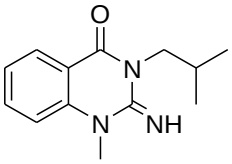
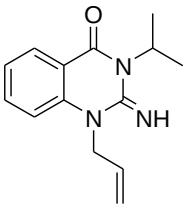
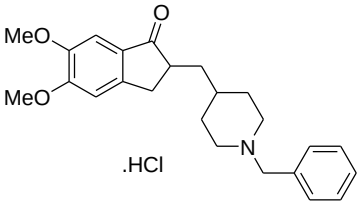
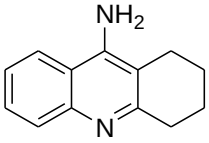
Sr. No	Code	Structure	IC_{50} (µM)*	
			AChE	BuChE
33	3i		>100	>100
34	3j		>100	>100
35	3k		61.62	>100
36	3l		14.13	>100
37	4a		>100	>100
38	4b		>100	>100
39	4c		>100	>100

Sr. No	Code	Structure	IC_{50} (µM)*	
			AChE	BuChE
40	4d		>100	>100
41	4e		>100	>100
42	4f		>100	>100
43	4g		>100	>100
44	4h		>100	>100
45	4i		>100	>100
46	4j		>100	>100

Sr. No	Code	Structure	IC_{50} (µM)*	
			AChE	BuChE
47	4k		>100	>100
48	4l		>100	>100
49	5a		>100	>100
50	5b		>100	>100
51	5c		>100	>100
52	5d		>100	>100
53	5e		>100	>100

Sr. No	Code	Structure	IC_{50} (µM)*	
			AChE	BuChE
54	5f		>100	>100
55	5g		>100	>100
56	5h		>100	>100
57	5i		>100	>100
58	5j		>100	>100
59	5k		>100	>100
60	5l		>100	>100

Sr. No	Code	Structure	IC_{50} (µM)*	
			AChE	BuChE
61	6a		12.95	>100
62	6b		>100	>100
63	6c		>100	>100
64	6d		18.10	>100
65	6e		>100	>100
66	6f		>100	>100
67	6g		>100	>100

Sr. No	Code	Structure	IC_{50} (µM)*	
			AChE	BuChE
68	6h		>100	>100
69	6i		>100	>100
70	6j		>100	>100
71	Donepezil HCl		0.028	3.39
72	Tacrine HCl		0.079	0.0046

*The *in vitro* test compound concentration required to produce 50% inhibition of *h*AChE & equine BuChE.

4.3. Morris water maze test

Morris water maze test was utilized to assess the effect of the compounds (**TRZ-15**, **TRZ-19**, **TRZ-20** and **3b**) using scopolamine induced amnesic mice model. As shown in **Figure 1** and **Figure 2** the escape latency significantly increased in scopolamine treated group as compared to the control group during all trial sessions. This confirmed that scopolamine treatment impaired the memory in mice. On day 5, the escape latency significantly decreased in **TRZ-15**, **TRZ-20 (Figure 5a)** and **3b (Figure 6a)** treated group as compared to the scopolamine treated group in the probe trial session. Assessment of spatial working memory was made by confirming the number of crossings over the platform. The number of crossings was decreased in scopolamine treated group while **TRZ-15, TRZ-20 (Figure 5b)** and **3b** (**Figure 6b**) increased the number of crossings over the platform.

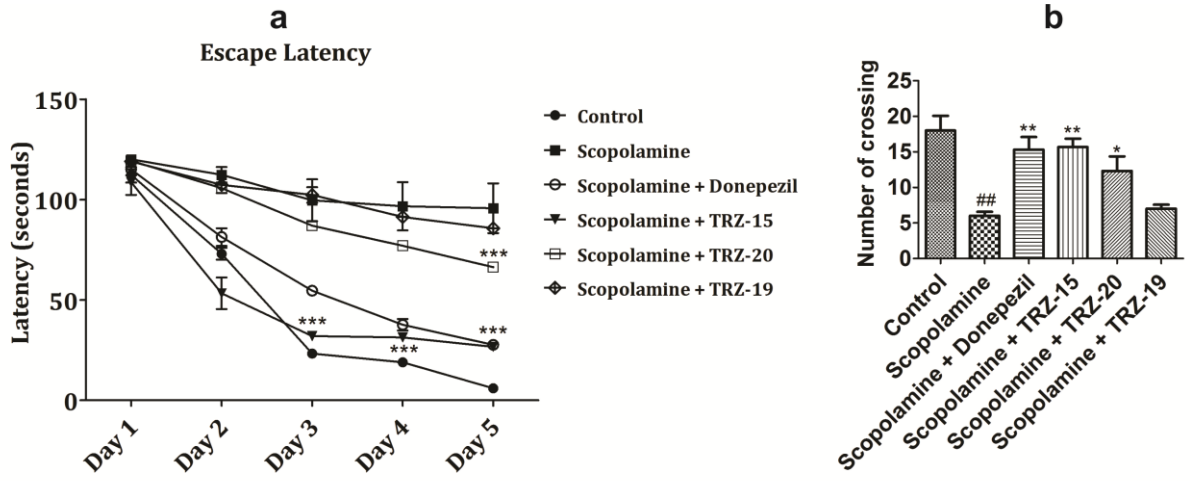


Figure 5: Effect of TRZ-15, TRZ-19 and TRZ-20 on escape latency in training trial and probe trial sessions (a) and the number of crossing platform area (b) for scopolamine induced memory deficit mice. Data are expressed as mean ± SEM. ***$p < 0.001$ versus scopolamine treated group, **$p < 0.01$ versus scopolamine treated group, *$p < 0.05$ versus scopolamine treated group, $^{\#\#}p<0.01$ versus control group.

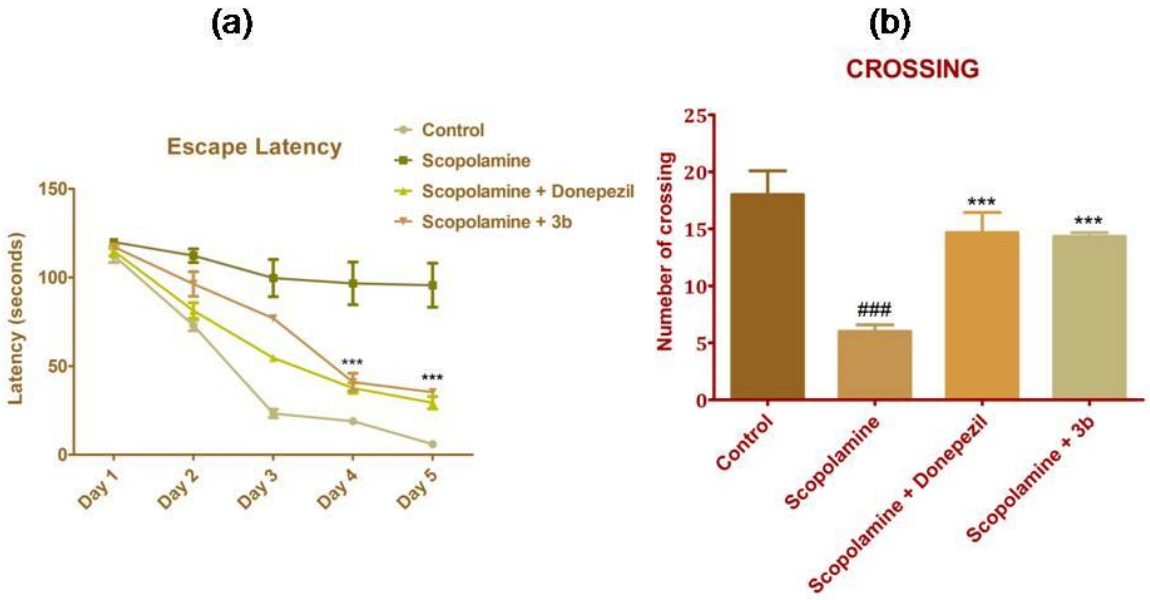


Figure 6: Effect of 3b on escape latency in training trial and probe trial sessions (a) and the number of crossing platform area (b) for scopolamine induced memory deficit mice. Values are expressed as mean ± SEM, (n=6). Significant values were compared with ***$p < 0.001$ versus scopolamine treated group, $^{###}p<0.001$ versus control group.

4.4. Inhibition of AChE and BuChE activities and antioxidative effects of TRZ-15, TRZ-20 and 3b in scopolamine treated mouse brain

AChE & BuChE levels were significantly reduced and oxidative parameters were significantly modified (decreased MDA level and increased catalase level) by the compounds (**TRZ-15**, **TRZ-20** and **3b**) at 20 mg/kg. To evaluate whether **TRZ-15**, **TRZ-20** and **3b** exerts similar inhibitory effects on AChE and BuChE activities as revealed by it in the *in vitro* experiments, brain tissues of mice treated with **TRZ-15**, **TRZ-20** and **3b** were subjected to colorimetric estimation to establish the AChE and BuChE activities in the brain. **TRZ-15**, **TRZ-20** and **3b** at 20 mg/kg reduced AChE level to 0.06681 ± 0.003109 U/ml ($p<0.01$), 0.07601 ± 0.003847 U/ml ($p<0.05$) and 0.06681 ± 0.003109 U/ml ($p<0.001$) respectively, as compared to the scopolamine treated group (0.08760 ± 0.003425 U/ml) [**Figure 7a (TRZ-15** and **TRZ-20), Figure 8a(3b)**], and BuChE level to 0.01562 ± 0.0006051 (20 mg/kg; $p<0.05$), 0.01662 ± 0.0003741 U/ml (20 mg/kg; $p<0.05$) and 0.01562 ± 0.0006051 (20 mg/kg; $p<0.001$ respectively) [**Figure 7b (TRZ-15** and **TRZ-20), Figure 8b (3b)**] as compared to the scopolamine treated group (0.01859 ± 0.0007292 U/ml). This data authenticated the fact that oral doses of **TRZ-15**, **TRZ-20** and **3b** at 20 mg/kg reduced both AChE and BuChE levels, which confirmed their anticholinesterase-like activity. It was then ensued to

demonstrate the effects of **TRZ-15**, **TRZ-20** and **3b** on oxidative parameters. The results showed that scopolamine treatment significantly ($p<0.001$) increased the brain MDA level (3.849 ± 0.07705 nM/mg protein) compared to the control group (2.427 ± 0.1831 nM/mg proteins). Treatment with the standard drug (Donepezil) and the test compounds (**TRZ-15**, **TRZ-20** and **3b**) significantly reduced brain MDA levels [**Figure 7c (TRZ-15** and **TRZ-20), Figure 8c (3b)**] [donepezil (2.059 ± 0.2255), **TRZ-15**, **TRZ-20** (2.265 ± 0.1376, 2.853 ± 0.2051) and **3b** (2.671 ± 0.09037) nM/mg proteins] compared to the corresponding scopolamine treated group (**Figure 7c, Figure 8c**). Further, scopolamine treatment significantly ($p<0.001$) decreased the brain catalase level (1.460 ± 0.05508 unit/mg protein) compared to the control group (2.837 ± 0.1785 unit/mg proteins). Treatment with the standard drug (Donepezil) and the test compounds (**TRZ-15**, **TRZ-20** and **3b**) significantly elevated brain catalase levels [**Figure 7d (TRZ-15** and **TRZ-20), Figure 8d (3b)**] [donepezil (2.393 ± 0.1525), **TRZ-15** (2.277 ± 0.08969), **TRZ-20** (2.070 ± 0.05292) and **3b** (2.310 ± 0.07024) nM/mg proteins] compared to the corresponding scopolamine treated group (**Figure 7d, Figure 8d**).). **TRZ-19** nonsignificantly reduced AChE, BuChE and MDA levels and increased catalase level. **Therefore TRZ-19 was removed from further studies.** These results revealed the antioxidant properties of compounds (**TRZ-15**, **TRZ-20** and **3b)**.

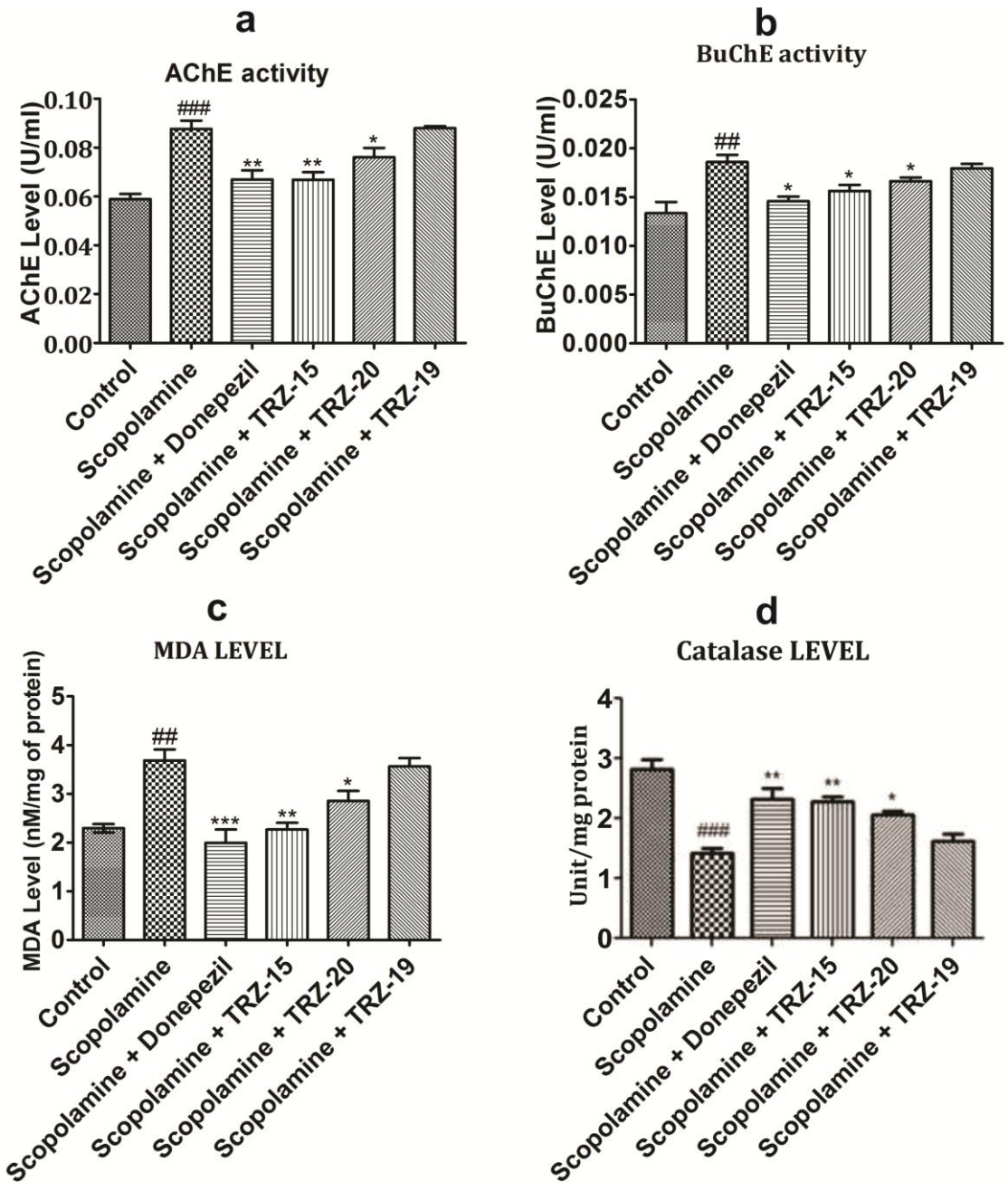


Figure 7: TRZ-15, TRZ-19 and TRZ-20 show their anticholinestrase and antioxidant properties *ex vivo* in scopolamine induced amnesic mice brains. AChE**,** BuChE & MDA levels significantly elevated in scopolamine treated group as compared to the control group (**a, b, c**). Catalase level was significantly decreased in scopolamine treated group (**d**). **TRZ-15** and **TRZ-20** significantly decreased acetylcholinesterase and butyrylcholinesterase levels as compared to scopolamine (**a, b**). **TRZ-15** and **TRZ-20** treatment significantly reduced MDA level as compared to scopolamine treated group (**c**). **TRZ-19** reduced AChE, BuChE and MDA levels non-significantly (**a, b, c**). **TRZ-15** and **TRZ-20** increased catalase level significantly (**d**). **TRZ-19** increased catalase level non-significantly (**d**). ***p<0.001 vs scopolamine treated group, **p<0.01 vs scopolamine treated group, *p<0.05 vs scopolamine treated group, $^{###}$p<0.001 vs control group & $^{##}$p<0.01 vs control group.

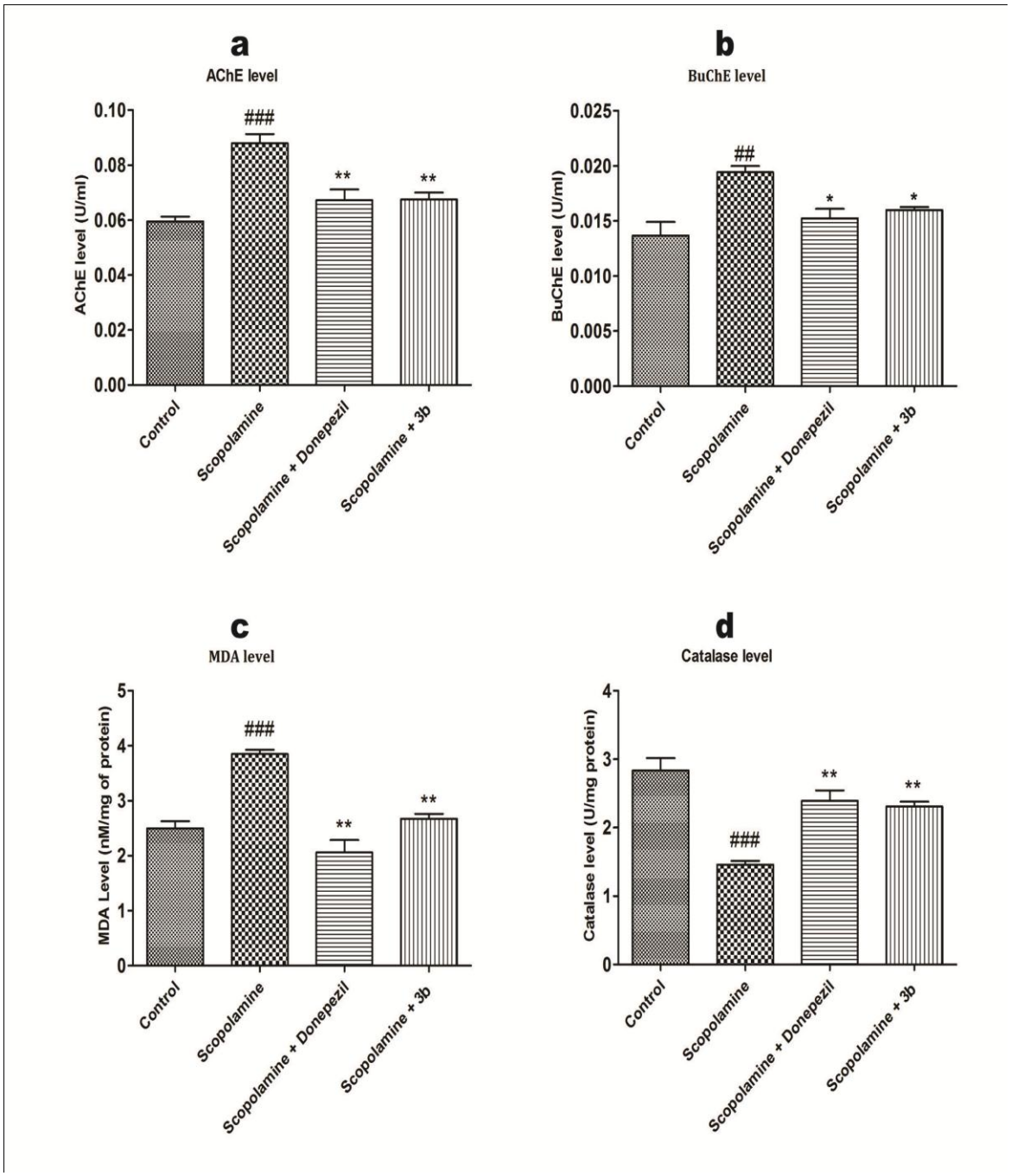


Figure 8: Effect of 3b on anticholinestrase and antioxidant properties *ex vivo* in scopolamine induced memory deficit mice. As compared to control group, AChE, BuChE & MDA levels significantly increased in scopolamine treated group (**a, b, c**) and catalase level was significantly reduced in scopolamine treated group (**d**). As compared to scopolamine treated group, treatment with **3b** significantly decreased acetylcholinesterase and butyrylcholinesterase levels (**a, b**). Treatment with **3b** significantly decreased MDA level as compared to scopolamine treated group (**c**). Treatment with **3b** significantly increased catalase level as compared to scopolamine treated group (**d**). Values are expressed as mean ± SEM. (n=6) Significant values were compared with ***$p<0.001$ vs scopolamine treated group, **$p<0.01$ vs scopolamine treated group, *$p<0.05$ vs scopolamine treated group, $^{###}p<0.001$ vs control group & $^{##}p<0.01$ vs control group.

4.5. Inhibition of hAChE-induced Aβ aggregation

Compound (**3b)** that exhibited good cholinesterase inhibition activity was evaluated to prevent hAChE-induced $A\beta_{1-40}$ aggregation by a thioflavin T (ThT) fluorescence method. The anti-aggregating activity of **3b** along with reference compound tacrine HCl is represented in **Figure 9**. At a concentration of 100 µM, **3b** exhibited 36.52 % inhibition in comparison to the positive control. At a concentration of 100 µM, tacrine exhibited 33.87 % inhibition in comparison to the positive control.

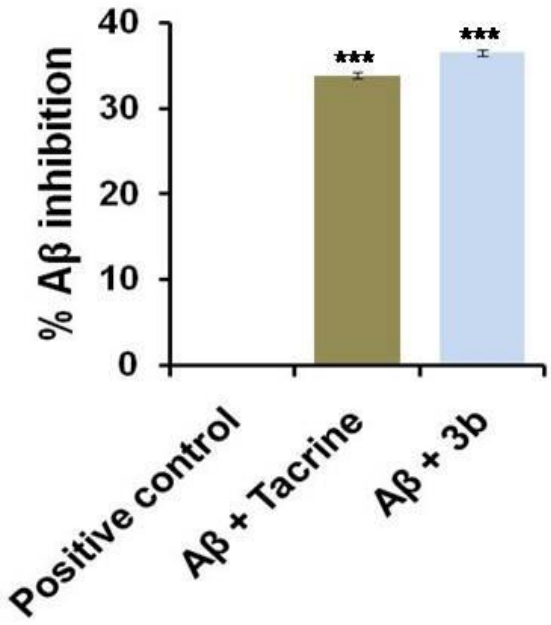


Figure 9: Effect of 3b on $A\beta_{1-40}$ anti-aggregatory property in thioflavin T assay. $A\beta_{1-40}$ anti-aggregatory role of the compound (**3b**) was evaluated by Thioflavin T (ThT) fluorescence method. Almost 36.52% $A\beta_{1-40}$ inhibition was observed by compound (**3b**) as compared with positive control. Values are expressed as mean ± SEM. (n=3). Significant values were compared with ***p<0.001 vs positive control.

4.6. TRZ-15 and TRZ-20 ameliorate $A\beta_{1-42}$ induced injury in rat hippocampal neurons

To determine the toxicity of **TRZ-15** and **TRZ-20** on rat hippocampal neuronal cells, MTT assay was performed. As shown in **Figure 10a**, donepezil, **TRZ-15** and **TRZ-20** at different concentrations (5-40 µM) did not cause significantly noticeable toxicity. In a different set of experiment, rat hippocampal cells were incubated with 16 µM $A\beta_{1-42}$ for 24 h, with or without different concentrations of donepezil, **TRZ-15** and **TRZ-20** (5-40 µM). Again MTT assay was performed to evaluate $A\beta_{1-42}$ induced cytotoxicity, and to assess the neuroprotective role of the compounds. As shown in **Figure 10b**, $A\beta_{1-42}$ significantly decreased the cell viability. On the other hand, the cytotoxic effects were reduced by pre-treatment of the cells with **TRZ-15** and **TRZ-20** (5-40 µM). The cytotoxic effect of $A\beta_{1-42}$ was significantly blocked by **TRZ-15** and **TRZ-20**.

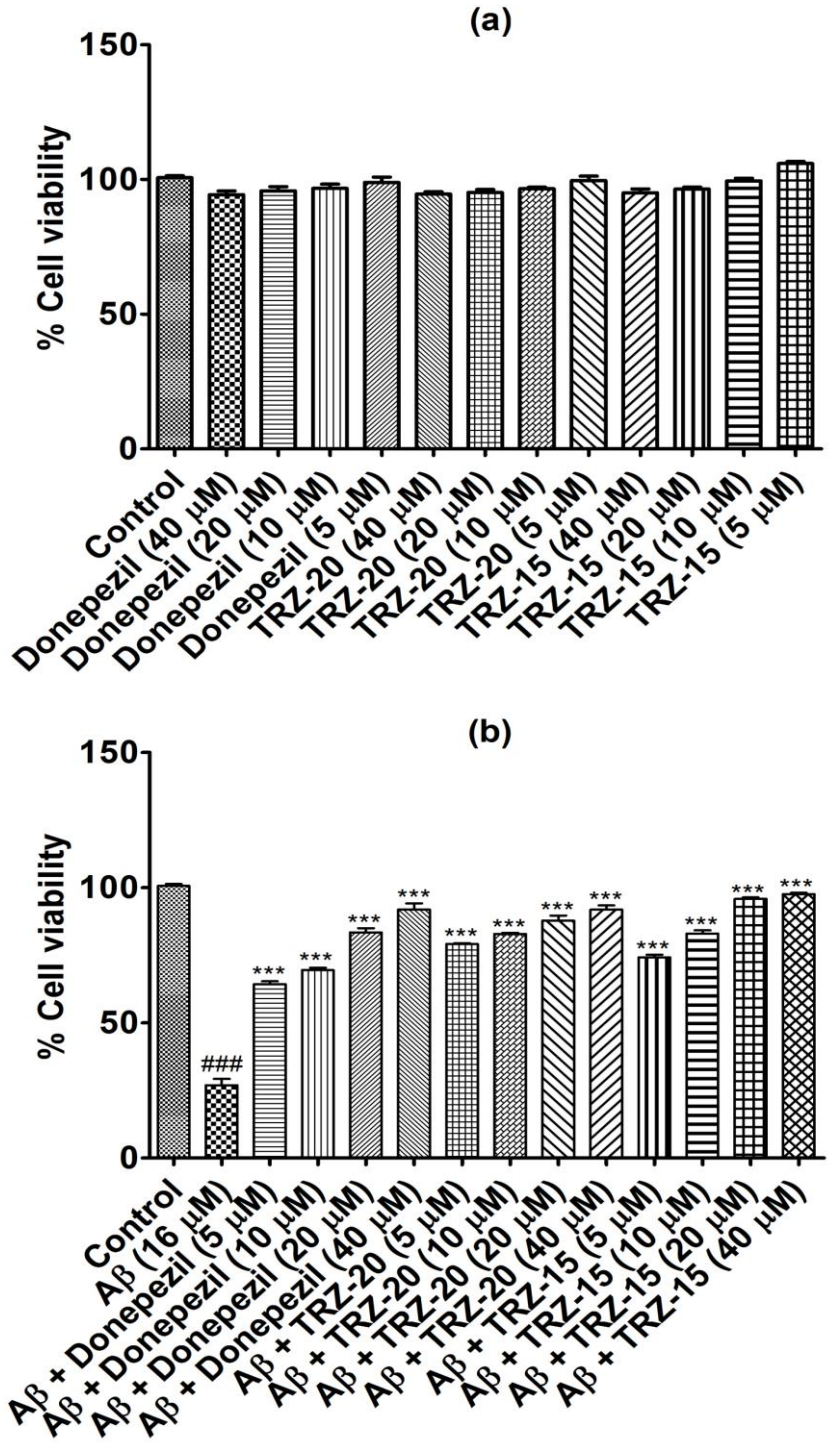


Figure 10: TRZ-15 and TRZ-20 ameliorated $A\beta_{1-42}$ induced injury in rat hippocampal neurons. Rat hippocampal cells were exposed to **TRZ-15** and **TRZ-20** in absence (**a**) and in presence (**b**) of $A\beta_{1-42}$ (16 µM) *in vitro*. Hippocampal cells were incubated with or without **TRZ-15** and **TRZ-20** (5-40 µM concentrations) for 2 h, followed by 24 h incubation with $A\beta_{1-42.}$ After the incubation period, MTT assay was performed to determine percentage cell viability. ***$p<0.001$ vs Aβ treated cells, $^{###}p<0.001$ vs control cells.

4.7. Compound (3b) improves $A\beta_{1-42}$ induced injury in rat hippocampal neuronal cells

To verify the toxicity of **3b** on rat hippocampal neuronal cells, MTT assay was carried out. **3b** significantly blocked the cytotoxic effect of $A\beta_{1-42}$ (**Figure 11**). To evaluate the safety of **3b** on rat hippocampal neuronal cells, MTT assay was performed. As shown in **Figure 11a**, donepezil and **3b** at different concentrations (5-80 µM) did not cause any significant toxicity. Rat hippocampal cells were incubated with 16 µM $A\beta_{1-42}$ for 24 h, with or without different concentrations of donepezil and **3b** (5-80 µM) in different sets of experiments. MTT assay was again performed to assess $A\beta_{1-42}$ induced cytotoxicity, and to evaluate the neuroprotective role of the compounds. As shown in **Figure 11b**, $A\beta_{1-42}$ reduced the cell viability significantly. On the other hand, pre-treatment of the cells with **3b** (5-80 µM) reduced the cytotoxic effects. This shows that the cytotoxic effect of $A\beta_{1-42}$ was significantly blocked by **3b**.

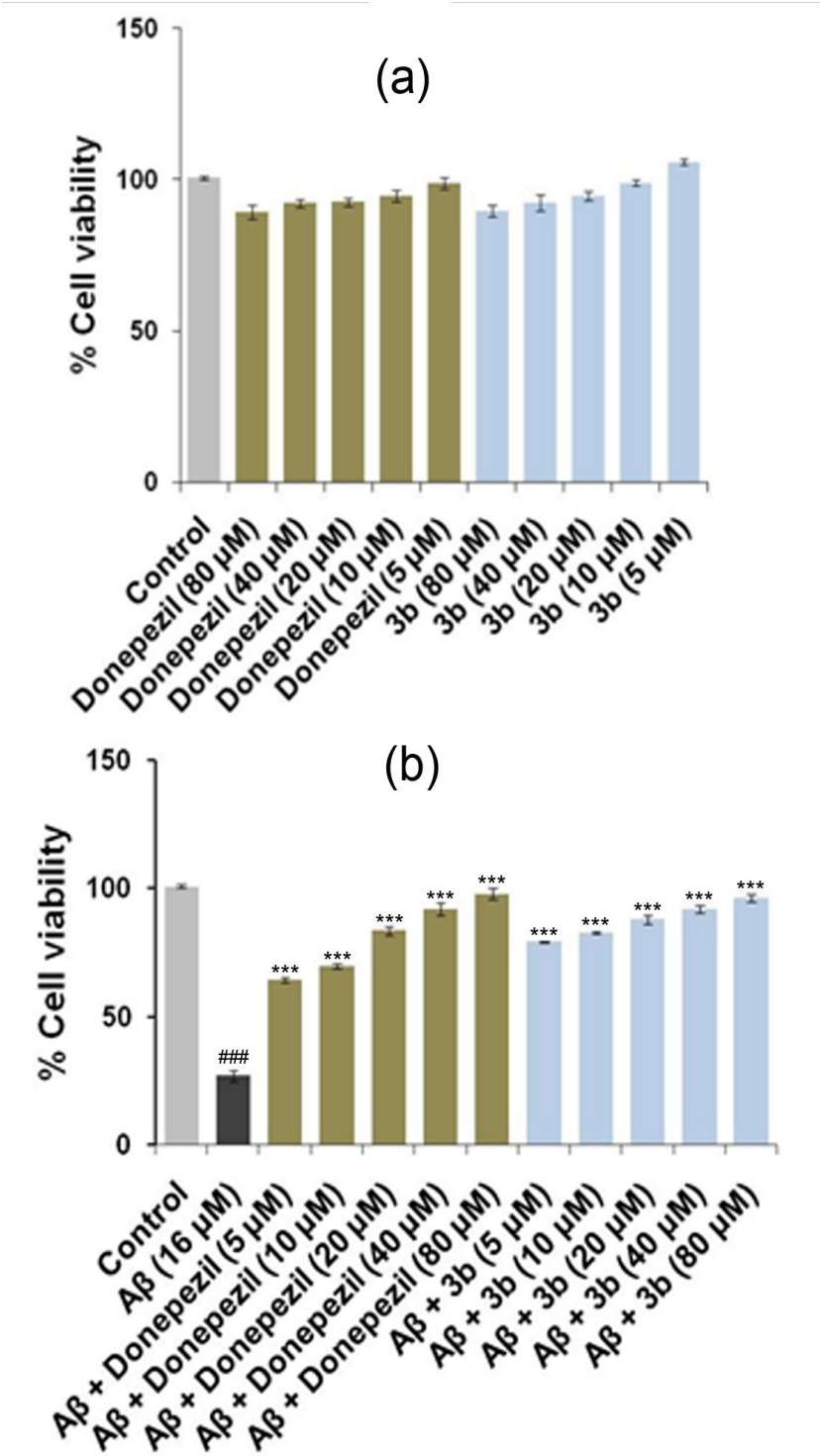


Figure 11: Effect of 3b on the injury induced by $A\beta_{1-42}$ in rat hippocampal neuronal culture. In absence (**a**) and in presence (**b**) of $A\beta_{1-42}$ (16 µM), rat hippocampal neuronal culture was exposed to **3b** *in vitro*. Hippocampal cells were incubated with **3b** (5-80 µM concentrations) for 2 h, followed by 24 h incubation with $A\beta_{1-42}$. The percentage cell viability was determined by MTT assay after incubation period.

4.8. TRZ-15 and TRZ-20 prevent $A\beta_{1-42}$ induced apoptosis

Anti-apoptotic properties of **TRZ-15** and **TRZ-20** were established by quantification of apoptotic nuclei stained with Hoechst in three independent experimental sets (**Figure 12A**). $A\beta_{1-42}$ treatment for 24 h showed increase in number of apoptotic cells, which was not identified in control culture. On pre-treatment with **TRZ-15** and **TRZ-20** at 20 µM concentration, the apoptotic cells were significantly reduced in number compared to $A\beta_{1-42}$ treatment cells alone (**Figure 12B)**.

4.9. Compound (3b) prevents apoptosis induced by $A\beta_{1-42}$

Anti-apoptotic property of **3b** was determined by quantification of Hoechst stained apoptotic nuclei in three independent experimental sets (**Figure 13A**). A 24 h treatment with $A\beta_{1-42}$ increased the number of apoptotic cells, which was not identified in control culture. Pre-treatment with **3b** (20 µM), reduced the number of apoptotic cells as compared to $A\beta_{1-42}$ treatment cells alone (**Figure 13B**).

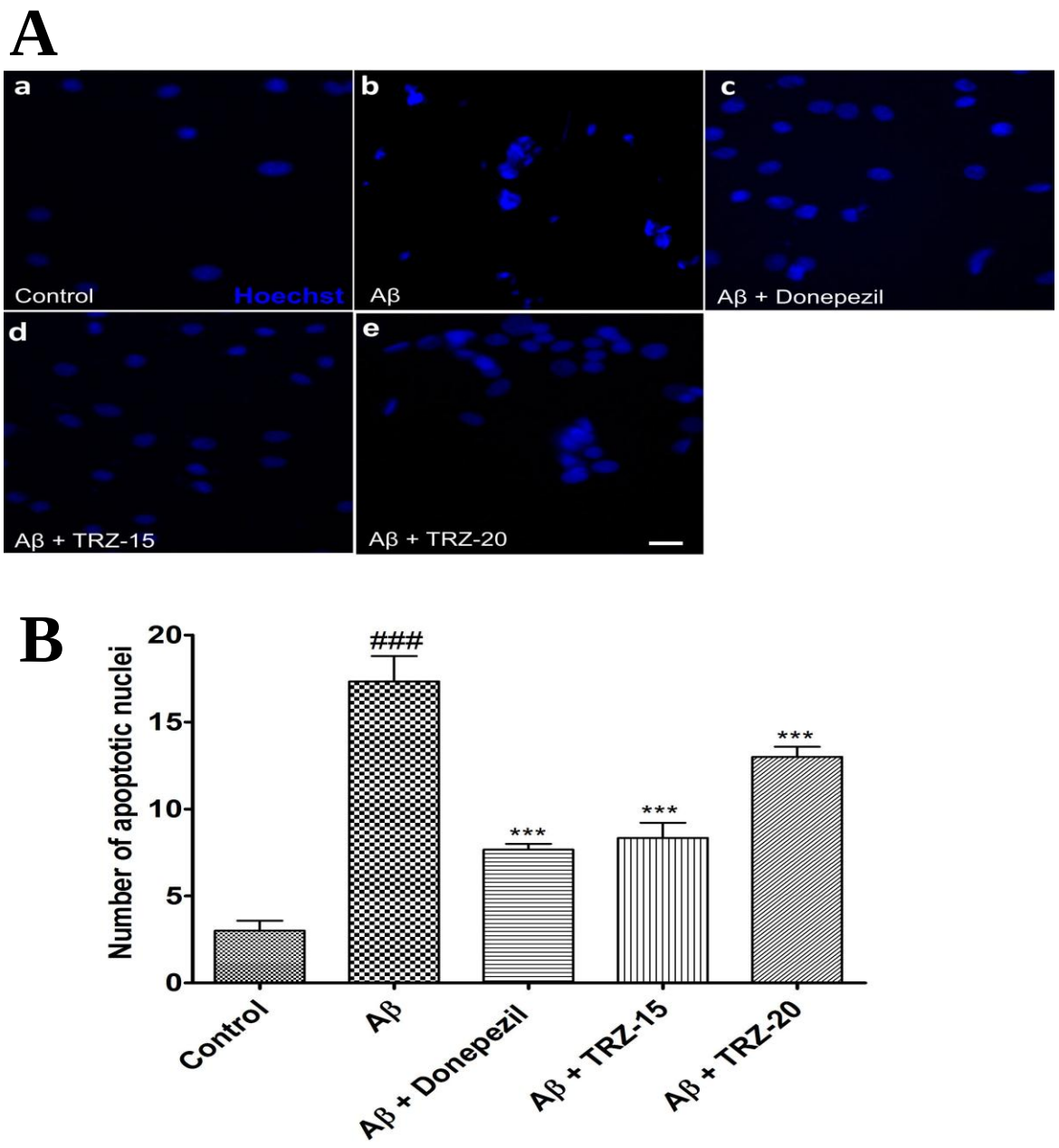


Figure 12: TRZ-15 and TRZ-20 prevent apoptosis and suppresses oxidative injury induced by $A\beta_{1-42}$ in hippocampal neurons. (A) Treatment with **TRZ-15** and **TRZ-20** reduced number of apoptotic cells induced by $A\beta_{1-42}$. **Figure A** showed Hoechst stained hippocampal cells nuclei, (**a**) control, (**b**) $A\beta_{1-42}$ treated cells, (**c**) Aβ + donepezil, (**d**) Aβ + **TRZ-15** and (**e**) Aβ + **TRZ-20** treated cells. The stained nuclei in different groups' clearly demonstrated significant reduction in number of apoptotic nuclei in **TRZ-15** and **TRZ-20** treated cells. (**B**) Quantitative analysis suggested significantly decreased number of apoptotic nuclei in the **TRZ-15** and **TRZ-20** treated rat hippocampal culture. ***$p<0.001$ vs $A\beta_{1-42}$ treated cells, $^{###}p<0.001$ vs control cells. Scale bar=100 µm.

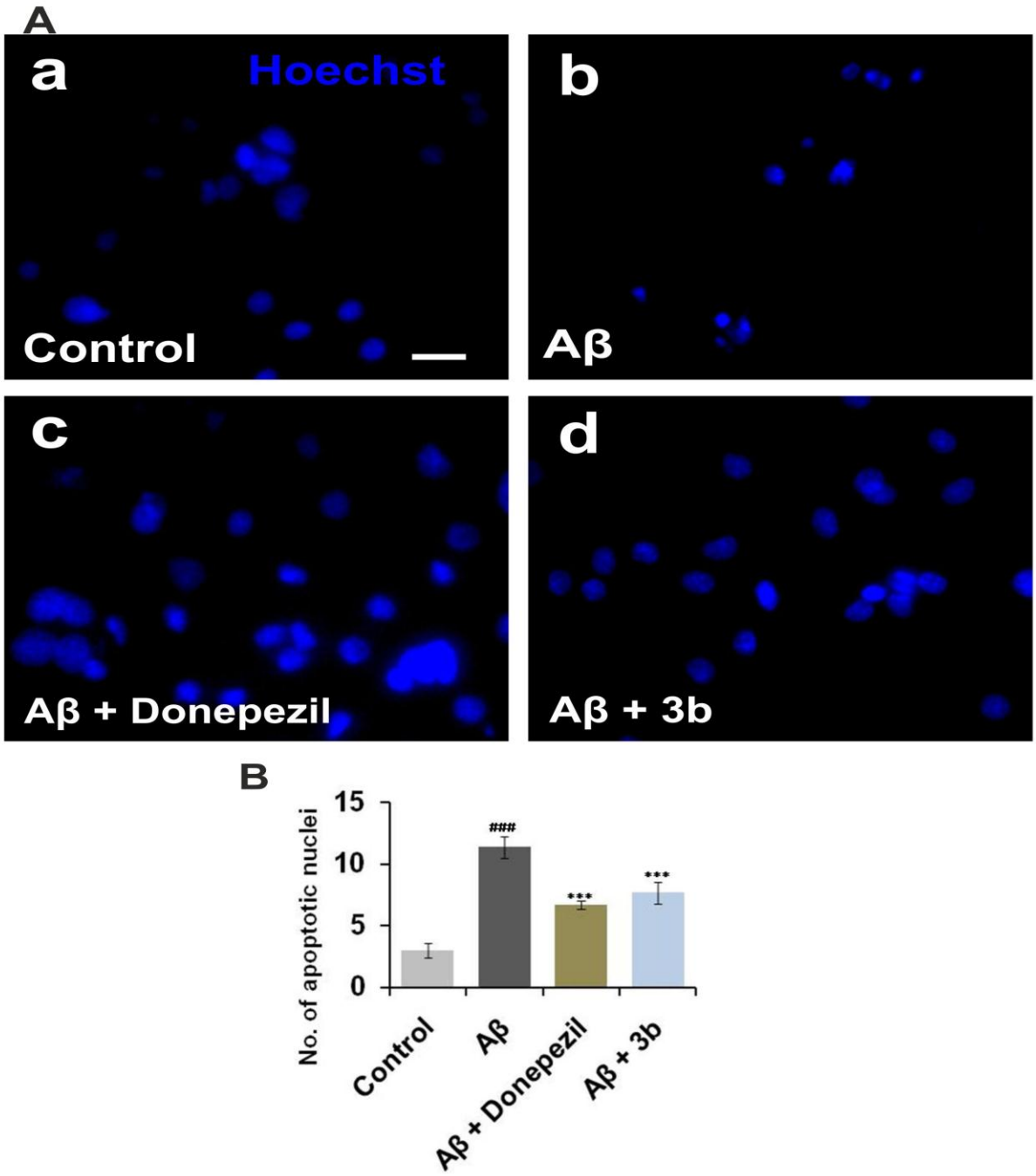


Figure 13: Effect of 3b on apoptosis and oxidative injury in hippocampal neuronal cells induced by $A\beta_{1-42}$. (A) The number of Hoechst stained apoptotic cells induced by $A\beta_{1-42}$ were reduced by treatment with **3b** (**a**) control, (**b**) $A\beta_{1-42}$treated cells, (**c**) Aβ + donepezil and (**d**) Aβ + **3b** treated cells. The stained nuclei in different groups clearly demonstrated significant reduction in number of apoptotic nuclei in **3b** treated cells. (**B**) Quantitative analysis suggested significantly decreased number of apoptotic nuclei in the **TRZ-15** and **TRZ-20** treated rat hippocampal culture. Values are expressed as mean ± SEM. (n=3) Significant values were compared with ***$p<0.001$ vs $A\beta_{1-42}$ treated cells, $^{###}p<0.001$ vs control cells. Scale bar= 100 µm

4.10. TRZ-15 and TRZ-20 suppress $A\beta_{1-42}$ induced oxidative injury

Results indicated that intracellular ROS level was increased 1.9 folds compared to the control cells after exposure of $A\beta_{1-42}$. Pre-treatment with **TRZ-15** and **TRZ-20** at 20 µM concentration suppressed the intracellular ROS elevation. This result demonstrates that **TRZ-15** and **TRZ-20** have the ROS scavenging property (**Figure 14**).

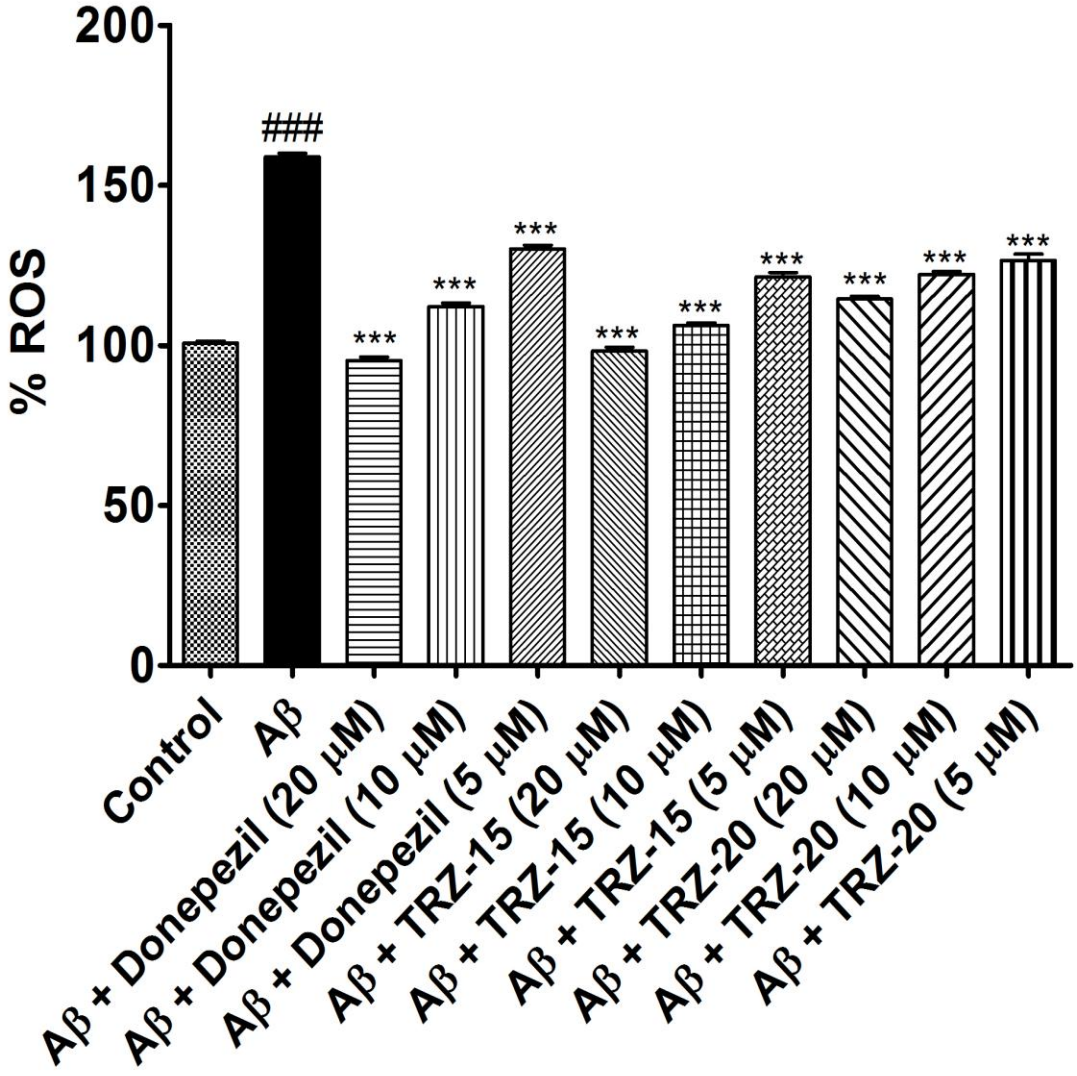


Figure 14: **TRZ-15** and **TRZ-20** showed ROS scavenging property in rat hippocampal cells injured with $A\beta_{1-42}$. **TRZ-15** and **TRZ-20** at (5-20 µM) significantly reduced % ROS in hippocampal neurons injured with $A\beta_{1-42}$ as compared with $A\beta_{1-42}$ treated cells alone. ***$p<0.001$ vs $A\beta_{1-42}$ treated cells, $^{###}p<0.001$ vs control cells.

4.11. Compound (3b) represses oxidative injury induced by $A\beta_{1-42}$

Results signified that intracellular ROS level of $A\beta_{1-42}$ exposed hippocampal cells increased by 1.6 folds as compared to the control cells. Elevated intracellular ROS was suppressed following pre-treatment with **3b** at 20 µM concentration. This result demonstrates the ROS scavenging property of **3b** (**Figure 15**).

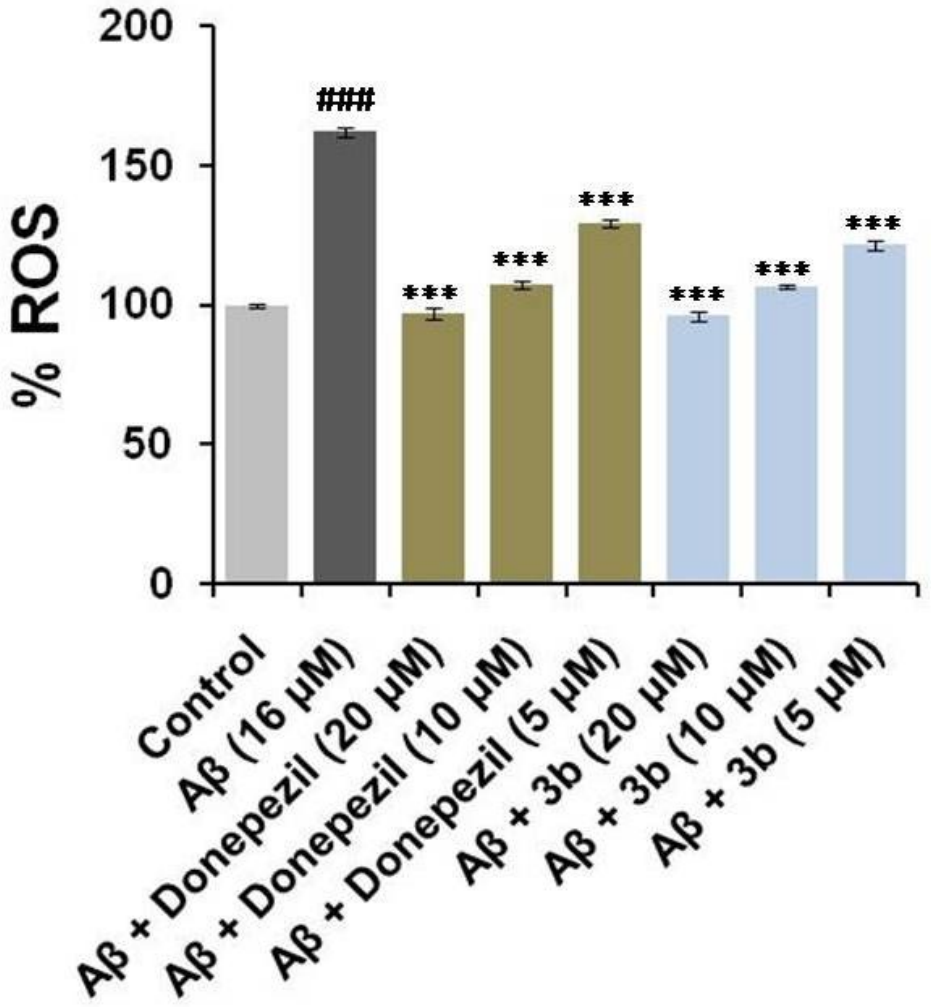


Figure 15: Effect of 3b on ROS in hippocampal neuronal cells induced by $A\beta_{1-42}$. ROS scavenging property in rat hippocampal cells injured with $A\beta_{1-42}$was observed by treatment with **3b.** Treatment with **3b** at different concentrations (5-20 µM) significantly reduced % ROS in hippocampal neurons injured with $A\beta_{1-42}$ as compared with $A\beta_{1-42}$ treated cells alone. Values are expressed as mean ± SEM. (n=3) Significant values were compared with ***$p<0.001$ vs $A\beta_{1-42}$ treated cells, ###$p<0.001$ vs control cells.

4.12. TRZ-15 and TRZ-20 reduced Aβ burden and cleaved caspase-3 expression in rat hippocampal neurons exposed to $A\beta_{1-42}$

Pre-treatment of **TRZ-15** and **TRZ-20** at a concentration of 20 µM prevented axonal and dendritic dystrophy in rat hippocampal neurons and decreased neuritic plaques in neurons exposed to $A\beta_{1-42}$ (16 µM) (**Figure 16A**). Furthermore, neuritic plaques were reduced by almost 90% and 70%, respectively, following **TRZ-15** and **TRZ-20** pre-treatment before $A\beta_{1-42}$ treatment. In a separate experiment, the expression of cleaved caspase-3 is increased in $A\beta_{1-42}$ exposed rat hippocampal cells. Pre-treatment with **TRZ-15** and **TRZ-20** at 20 µM reduced the expression of cleaved caspase-3 in hippocampal cells (**Figure 17A**). Quantification of Aβ plaques & cleaved caspase-3 positive cells was performed and the results showed significant decrease in number of Aβ plaques and cleaved caspase-3 positive cells (**Figure 16B, Figure 17B**) following **TRZ-15** and **TRZ-20** treatment as compared to $A\beta_{1-42}$ treated cells alone. These results indicated the neuroprotective role of **TRZ-15** and **TRZ-20** against $A\beta_{1-42}$ induced apoptotic neurotoxicity.

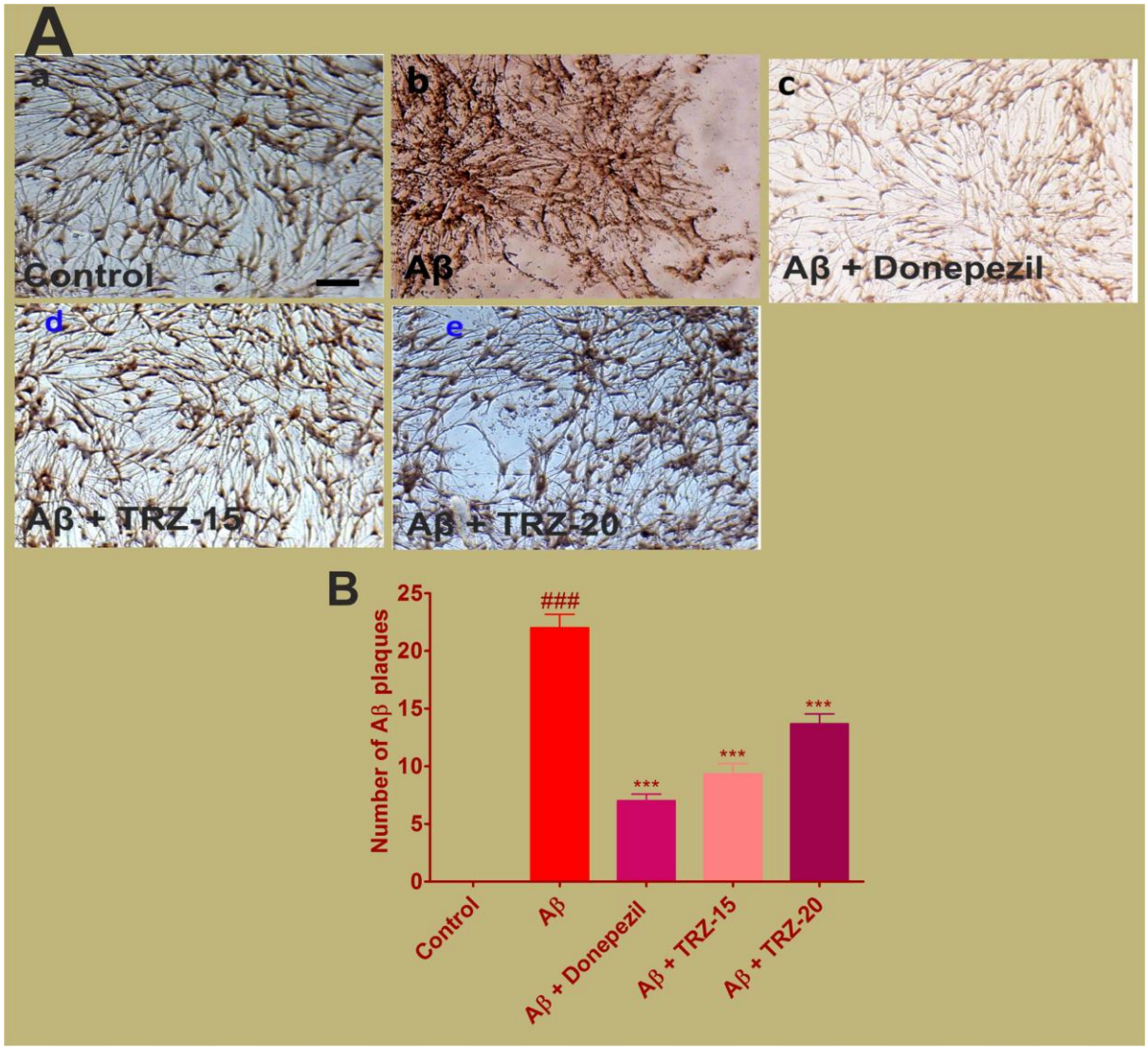
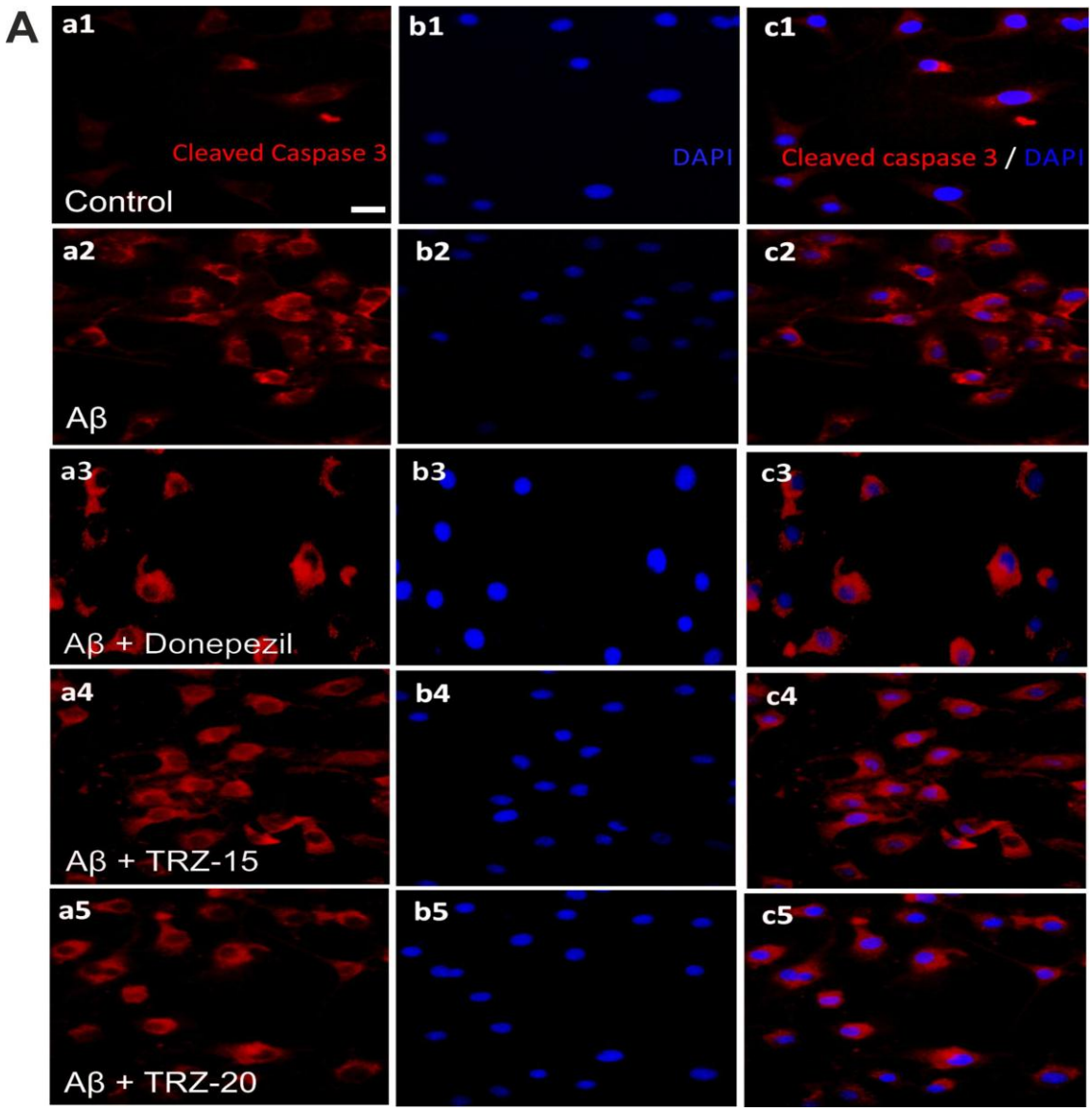


Figure 16: TRZ-15 and TRZ-20 reduce Aβ burden expression in hippocampal neuronal culture using immunocytochemistry. (A) Morphological changes were observed for Abeta antibody using DAB staining protocol in hippocampal neurons. (**a**) Control, (**b**) $A\beta_{1-42}$ treated, (**c**) $A\beta_{1-42}$ + donepezil, (**d**) $A\beta_{1-42}$ + **TRZ-15** and (**e**) $A\beta_{1-42}$ + **TRZ-20**. Cells were incubated with 16 µM concentrations of $A\beta_{1-42}$ for 24 h, (**c-e**) cells were pre-treated with 20 µM concentration of donepezil, **TRZ-15** and **TRZ-20** respectively followed by DAB staining. Reduced number of abeta plaques was observed in **TRZ-15** and **TRZ-20** treatment groups. (**B**) Quantitative analysis showed significant decrease in number of Aβ plaques in the **TRZ-15** and **TRZ-20** treated rat hippocampal culture. Data expressed as mean ± SEM. ***$p < 0.001$ versus $A\beta_{1-42}$ treated group, **$p < 0.01$ versus $A\beta_{1-42}$ treated group, *$p < 0.05$ versus $A\beta_{1-42}$ treated group, $^{###}p < 0.001$ vs control group. Scale bar =100 µm.



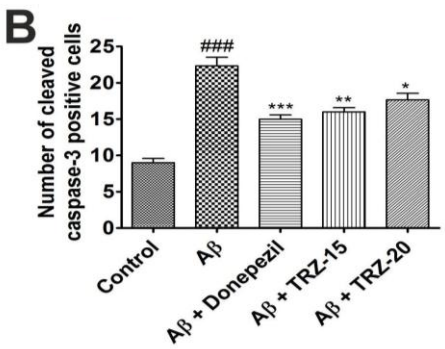


Figure 17: TRZ-15 and TRZ-20 reduce cleaved caspase-3 expression in hippocampal neuronal culture using immunocytochemistry. (A) Reduced number of fluorescence stained cells for cleaved caspase-3 antibody were observed using microscope. (**a1-a5**) control, $A\beta_{1-42}$ treated, $A\beta_{1-42}$ + donepezil, $A\beta_{1-42}$ + **TRZ-15** and $A\beta_{1-42}$ + **TRZ-20** treated hippo-campal cells stained for cleaved caspase-3 antibody, counterstaining of DAPI was used to show the nucleus (**b1-b5**). In the merged images from the two channels, it was evident that the cleaved caspase-3 immunoproducts were mainly located in the neuronal cell bodies (**c1-c5**). **(B)** Quantification of number of cleaved caspase-3 positive cells in six different sets showed that the numbers of cleaved caspase-3 + neurons were decreased following treatment with **TRZ-15** and **TRZ-20**. Data expressed as mean ± SEM. ***$p < 0.001$ versus $A\beta_{1-42}$ treated group, **$p < 0.01$ versus $A\beta_{1-42}$ treated group, *$p < 0.05$ versus $A\beta_{1-42}$ treated group, $^{###}p < 0.001$ vs control group. Scale bar =100 µm.

4.13. Compound (3b) reduced Aβ burden and cleaved caspase-3 positive expression in rat hippocampal neurons exposed with $A\beta_{1-42}$

Pre-treatment with **3b** (20 µM) of rat hippocampal neurons exposed to $A\beta_{1-42}$ (16 µM) prevented axonal and dendritic dystrophy as well as decreased neuritic plaque (**Figure 18A**). Additionally, neuritic plaques were reduced by almost 75% following pre-treatment with **3b** (**Figure 18B**). In a separate experiment, in $A\beta_{1-42}$ exposed rat hippocampal cells, the expression levels of caspase-3 increased. Pre-treated hippocampal cells with **3b** at 20 µM showed reduction in the expression of caspase-3 (**Figure 19A**). Quantification of caspase-3 positive cells showed significant decrease in number of caspase-3 positive cells (**Figure 19B**) following pre-treatment with **3b** as compared to $A\beta_{1-42}$ treated cells alone. These results pointed to the neuroprotective role of **3b** in $A\beta_{1-42}$ induced apoptotic neurotoxicity.

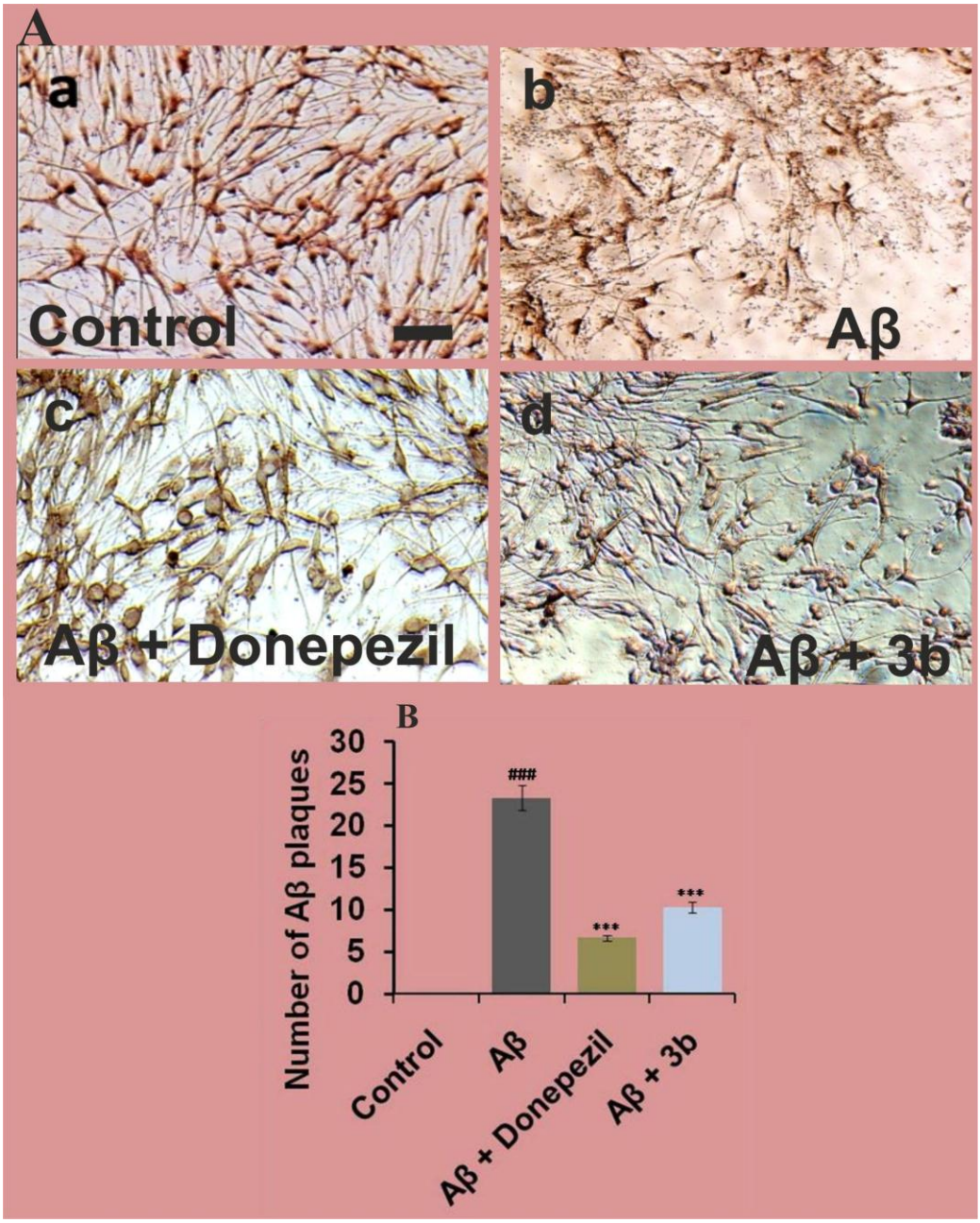


Figure 18: Effect of 3b on Aβ burden in hippocampal neuronal culture using immunocytochemistry. (A) Morphological changes were observed for Aβ antibody using DAB staining protocol in hippocampal neurons. (**a**) Control, (**b**) $A\beta_{1-42}$ treated, (**c**) $A\beta_{1-42}$ + donepezil and (**d**) $A\beta_{1-42}$ + **3b**. For 24h, cells were incubated with 16 µM concentrations of $A\beta_{1-42}$, (**c-d**) cells were pre-treated with 20 µM concentration of donepezil and **3b** respectively followed by DAB staining. Reduction in number of Aβ plaques was observed in **3b** treatment group. **(B)** Quantitative analysis suggested significantly decreased number of Aβ plaques in the **3b** treated rat hippocampal culture. Values are expressed as mean ± SEM. (n = 6) Significant values were compared with ***$p < 0.001$ versus $A\beta_{1-42}$ treated cells $^{###}p < 0.001$ vs control cells. Scale bar = 100 µm

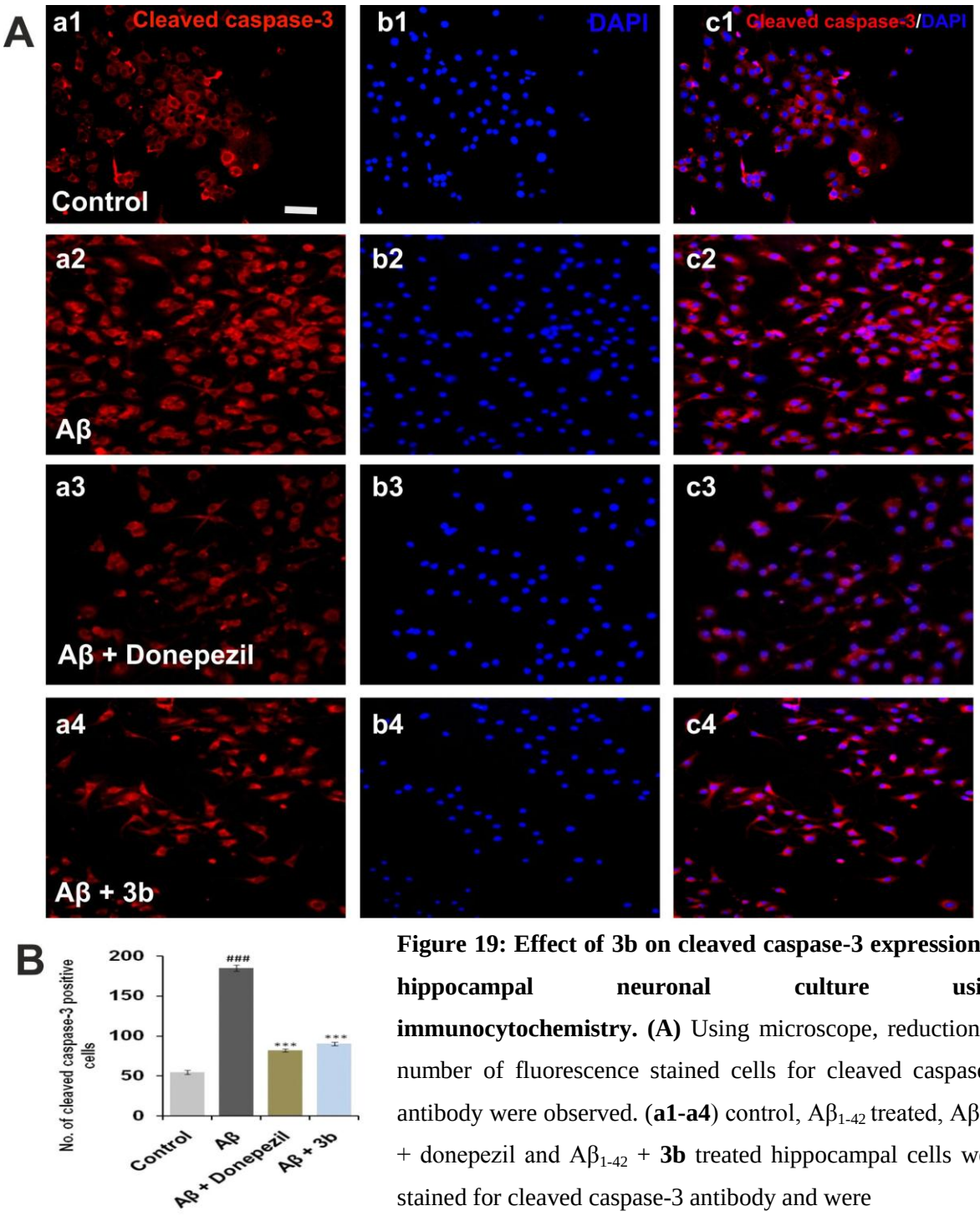


Figure 19: Effect of 3b on cleaved caspase-3 expression in hippocampal neuronal culture using immunocytochemistry. (A) Using microscope, reduction in number of fluorescence stained cells for cleaved caspase-3 antibody were observed. (**a1-a4**) control, $A\beta_{1-42}$ treated, $A\beta_{1-42}$ + donepezil and $A\beta_{1-42}$ + **3b** treated hippocampal cells were stained for cleaved caspase-3 antibody and were counterstained with DAPI to show the nucleus (**b1-b4**).The cleaved caspase-3 immunoproducts were mainly located in the neuronal cell bodies (**c1-c4**) which was evident in the merged images from the two channels. **(B)** With treatment of **3b**, quantification of number of cleaved caspase-3 positive cells in six different sets showed that the number of caspase-3^{+} neurons was decreased. Values are expressed as mean ± SEM. (n=6) Significant values were compared with ***$p < 0.001$ versus $A\beta_{1-42}$ treated cells $^{###}p < 0.001$ vs control cells. Scale bar = 100 µm

4.14. TRZ-15 and TRZ-20 increase hippocampus-dependent learning and memory processes

A significant increase in learning and memory was observed in idioms of increased CAR by 56% and 40% (learning) and 61% and 52% (memory) respectively, in the compound (**TRZ-15** and **TRZ-20)** treated rats as compared to $A\beta_{1-42}$ treated rats ($p < 0.001$ and $p<0.01$; **Figure 20**). These results advocate that **TRZ-15** and **TRZ-20** positively involve hippocampus-dependent learning and memory processes in Aβ induced rats.

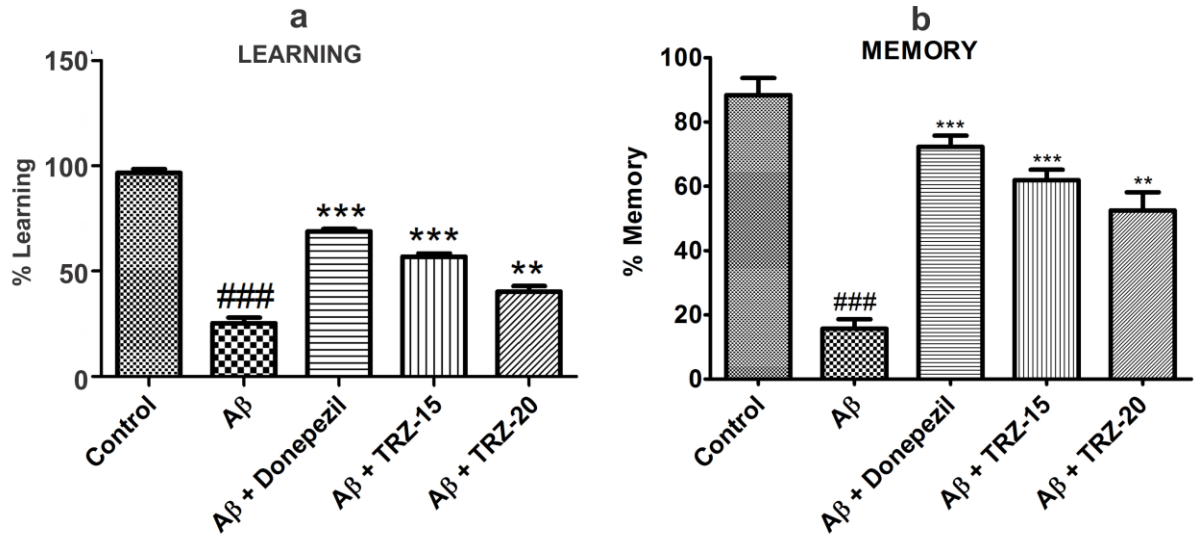


Figure 20: Effect of TRZ-15 and TRZ-20 on hippocampus-dependent learning and memory processes. The cognitive ability of the control, $A\beta_{1-42}$ treated, donepezil, and **TRZ-15** and **TRZ-20** treated animals was measured following assessment of two-way conditioned avoidance behaviour using a shuttle box apparatus. **TRZ-15** and **TRZ-20** caused significant increase in learning and memory as compared to the $A\beta_{1-42}$ treated rats. Values are expressed as mean ± SEM (n=6 rats per group). ***$p < 0.001$ versus $A\beta_{1-42}$ treated group, **$p < 0.01$ versus $A\beta_{1-42}$ treated group and $^{###}p<0.001$ versus control group.

4.15. Compound (3b) augments hippocampus-dependent learning and memory

A significant increase in learning and memory was observed in idioms of increased CAR by 58% and 62% (learning and memory) in the compound (**3b)** treated rats as compared to $A\beta_{1-42}$ treated rats ($p < 0.001$, **Figure 21**). These results advocate that **3b** positively involves hippocampus-dependent learning and memory processes in Aβ induced rats.

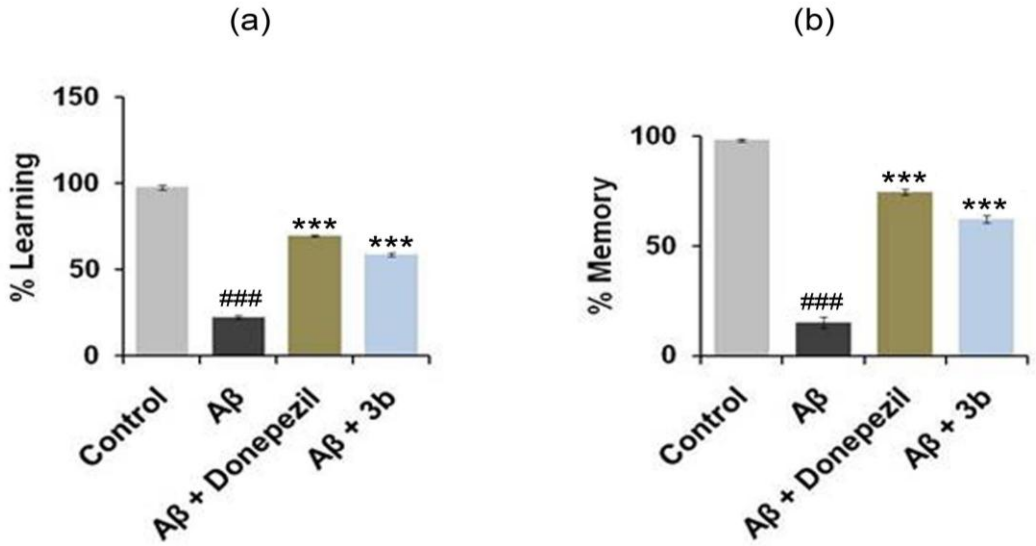


Figure 21: Effect of 3b on hippocampus-dependent learning and memory processes in $A\beta_{1-42}$ induced Alzheimer's rat brain. Using a shuttle box apparatus, the cognitive ability of the control, $A\beta_{1-42}$ treated, donepezil and **3b** treated animals was measured following assessment of two-way conditioned avoidance behaviour. The significant increment in learning and memory was shown by treatment of **3b** as compared to the $A\beta_{1-42}$ treated rats. Values are expressed as mean ± SEM (n=6). Significant values were compared with ***$p < 0.001$ versus $A\beta_{1-42}$ treated group and $^{###}p<0.001$ versus control group.

4.16. TRZ-15 and TRZ-20 diminish Aβ burden in $A\beta_{1-42}$ induced Alzheimer's rat brain

It was explored whether administration of **TRZ-15** and **TRZ-20** could reduce Aβ deposition in $A\beta_{1-42}$ induced Alzheimer's rat brain. Brain sections of rat treated with **TRZ-15** and **TRZ-20** (20 mg/kg) for 14 consecutive days were subjected to immunohistochemical analysis. Qualitative analysis showed that the number and size of the $A\beta_{1-42}$ immunoreactive neuritic plaques were reduced qualitatively in hippocampus after oral administration of **TRZ-15** and **TRZ-20** (**Figure 22** and **Figure 23**). Donepezil treatment also reduced the number and size of $A\beta_{1-42}$ neuritic plaques.

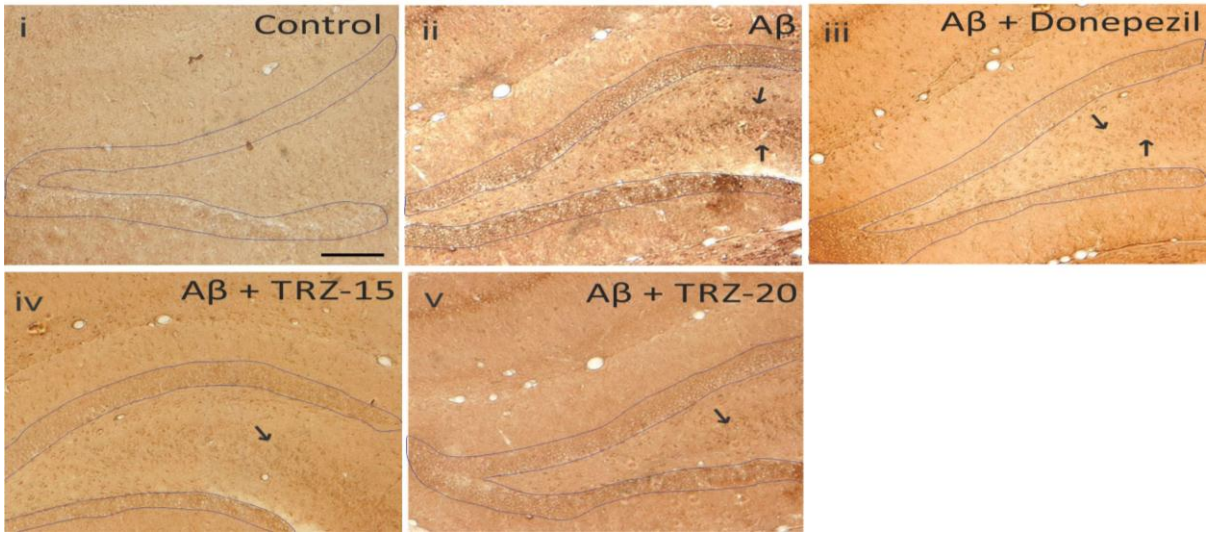


Figure 22: TRZ-15 and TRZ-20 reduce Aβ burden in hippocampal region of Aβ induced Alzheimer's rat brain: Changes in Aβ burden were observed for Aβ antibody using DAB staining protocol in Aβ induced Alzheimer's rat brain. (**i**) Control, (**ii**) $A\beta_{1-42}$ treated, (**iii**) $A\beta_{1-42}$ + donepezil, (**iv**) $A\beta_{1-42}$ + **TRZ-15** and (**v**) $A\beta_{1-42}$ + **TRZ-20.** Arrows identify Aβ plaques. Scale bar =100 µm.

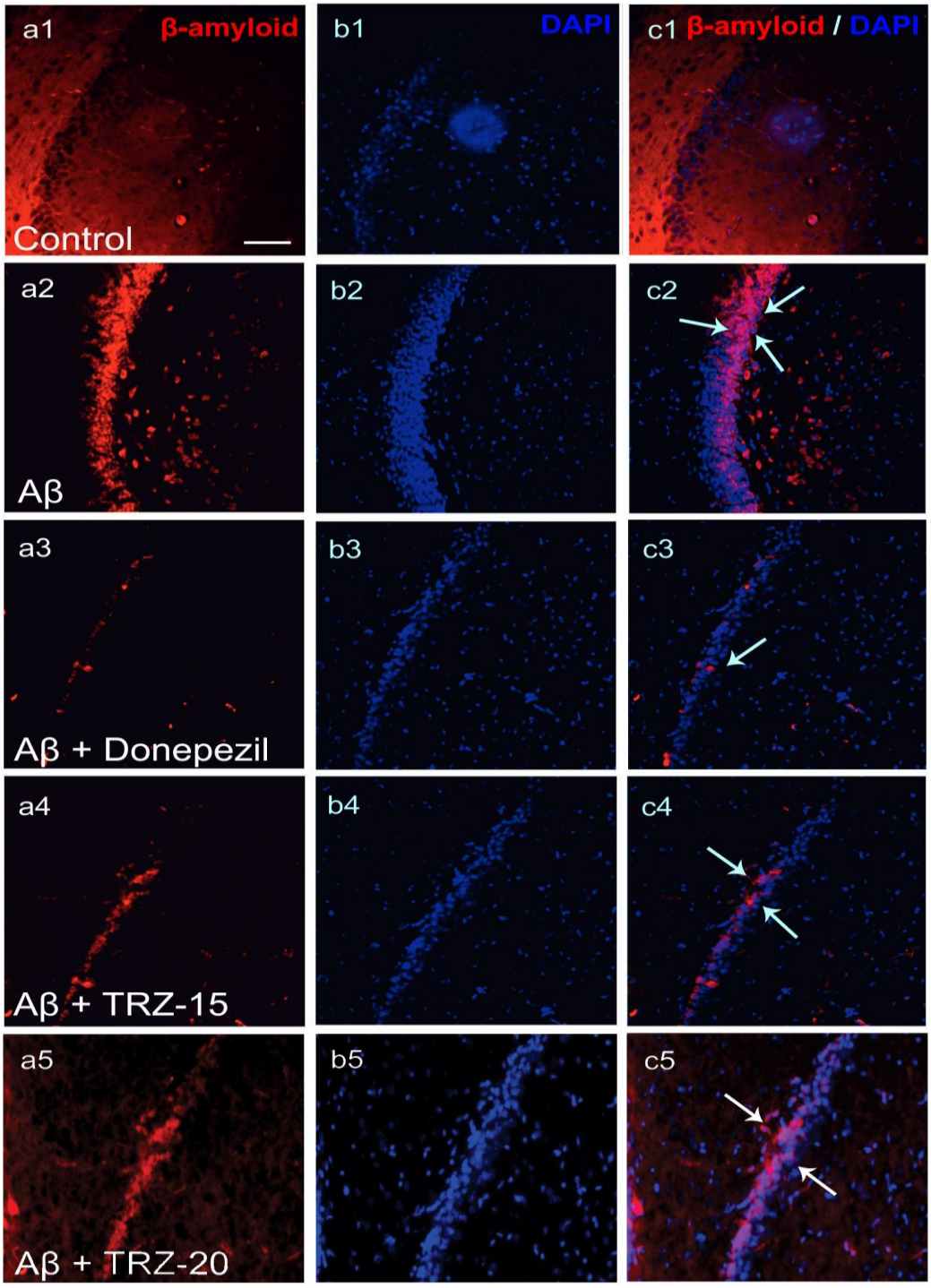


Figure 23: TRZ-15 and TRZ-20 reduce Aβ burden in hippocampal region of Aβ induced Alzheimer's rat brain: Changes in Aβ burden were observed for Aβ antibody using fluorescent staining protocol in Aβ induced Alzheimer's rat brain. (**a1**) Control, (**a2**) $A\beta_{1-42}$ treated, (**a3**) $A\beta_{1-42}$ + donepezil, (**a4**) $A\beta_{1-42}$ + **TRZ-15** and (**a5**) $A\beta_{1-42}$ + **TRZ-20**. Hippocampal region was counter stained with DAPI in each group (**b1-b5**), and finally merged images were obtained (**c1-c5**). After 14 days treatment with the compounds, reduced number of Aβ plaques was observed in **TRZ-15** and **TRZ-20** treatment groups. Arrows identify Aβ plaques. Scale bar =100 µm.

4.17. Compound (3b) reduces Aβ burden in $A\beta_{1-42}$ induced Alzheimer's rat brain

It was investigated whether administration of **3b** could reduce Aβ deposition in $A\beta_{1-42}$ induced Alzheimer's rat brain. Brain sections of rat treated with **3b** (20 mg/kg) for 14 consecutive days were subjected to immunohistochemical analysis. Qualitatively there was reduction in the number and size of the $A\beta_{1-42}$ immunoreactive neuritic plaques in the hippocampal region after oral administration of **3b** (**Figure 24** and **Figure 25**).

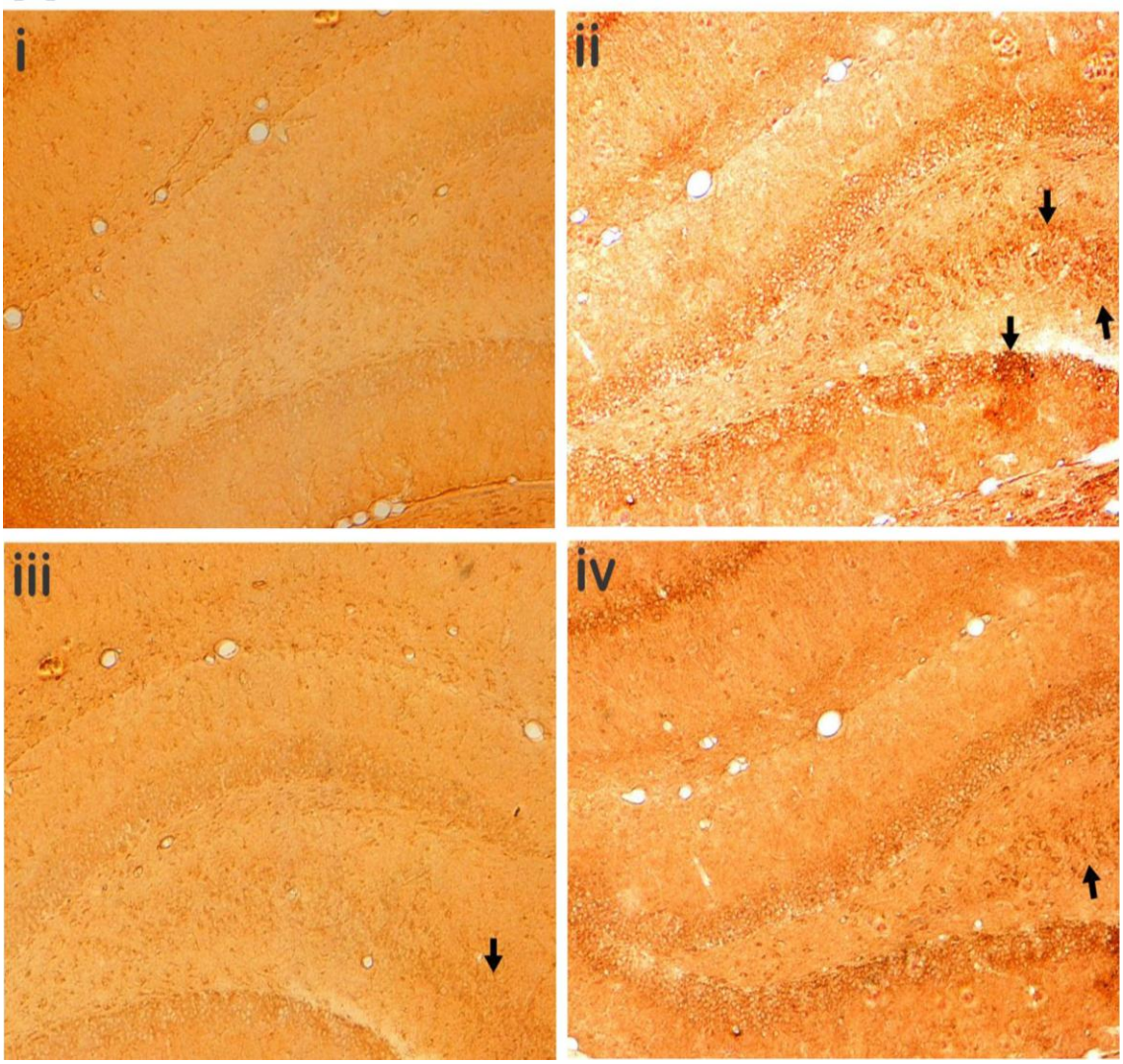


Figure 24: 3b reduces Aβ burden in hippocampal region of Aβ induced Alzheimer's rat brain: Changes in Aβ burden were observed for Aβ antibody using DAB staining protocol in Aβ induced Alzheimer's rat brain. (**i**) Control, (**ii**) $A\beta_{1-42}$ treated, (**iii**) $A\beta_{1-42}$ + donepezil and (**iv**) $A\beta_{1-42}$ + **3b.** Arrows identify Aβ plaques. Scale bar =100 µm.

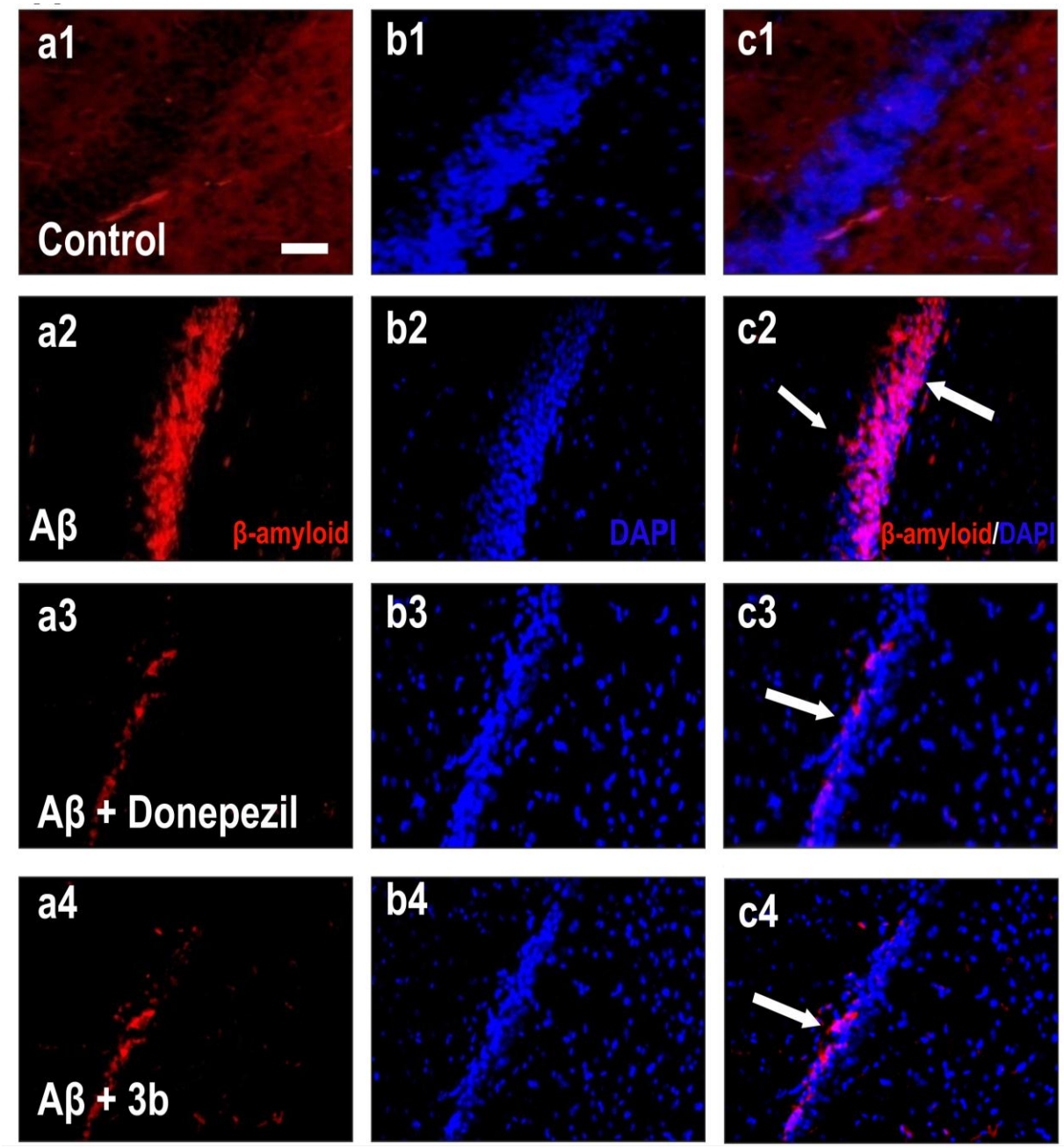


Figure 25: Effect of 3b on Aβ burden in hippocampal region of $Aβ_{1-42}$ induced Alzheimer's rat brain: Changes in Aβ burden were observed for Aβ antibody using fluorescent staining protocol in Aβ induced Alzheimer's rat brain. (**a1**) Control, (**a2**) $Aβ_{1-42}$treated, (**a3**) $Aβ_{1-42}$ + donepezil, (**a4**) $Aβ_{1-42}$ + **3b.** Counterstaining of hippocampal was done with DAPI in each group (**b1-b4**), and finally merged images were obtained (**c1-c4**). After 14 days treatment with the compounds, reduction in number of Aβ plaques was observed in **3b** treatment groups. Scale bar =100 µm.

4.18. TRZ-15 and TRZ-20 decrease neurodegeneration in hippocampal region of $A\beta_{1-42}$ induced Alzheimer's rat brain

TRZ-15 and **TRZ-20** treatment significantly decreased neural degeneration in hippocampal region compared to $A\beta_{1-42}$ treated group (**Figure 26A** (**d, e**)). The number of fluoro-jade^{+} cells was decreased in **TRZ-15** and **TRZ-20** treated groups (*$p < 0.001$ vs Ab_{1-42} treated group) (**Figure 26B**). These results suggest that **TRZ-15** and **TRZ-20** treatment suppresses neural degeneration and apoptosis in proliferating progenitor cells in the hippocampus. Fluoro-jade C staining for degenerating neurons in hippocampal region of brain authenticated the previous finding as the number of fluoro-jade C^{+} cells got significantly decreased after treatment with compounds (**TRZ-15** and **TRZ-20**). These findings suggested the neuroprotective role of **TRZ-15** and **TRZ-20** against $A\beta_{1-42}$ induced neurotoxicity.

4.19. Compound (3b) diminishes neurodegeneration in hippocampus of $A\beta_{1-42}$ induced Alzheimer's rat brain

Significant decrease in neural degeneration was observed following treatment with **3b** in hippocampal region compared to $A\beta_{1-42}$ treated group [**Figure 27A** (**d**)]. There was decrease in the number of fluoro-jade^{+} cells in **3b** treated groups (***$p < 0.001$ vs Ab_{1-42} treated group) (**Figure 27B**). These results offer that **3b** treatment suppresses apoptosis and neural degeneration in proliferating progenitor cells in the hippocampal region.

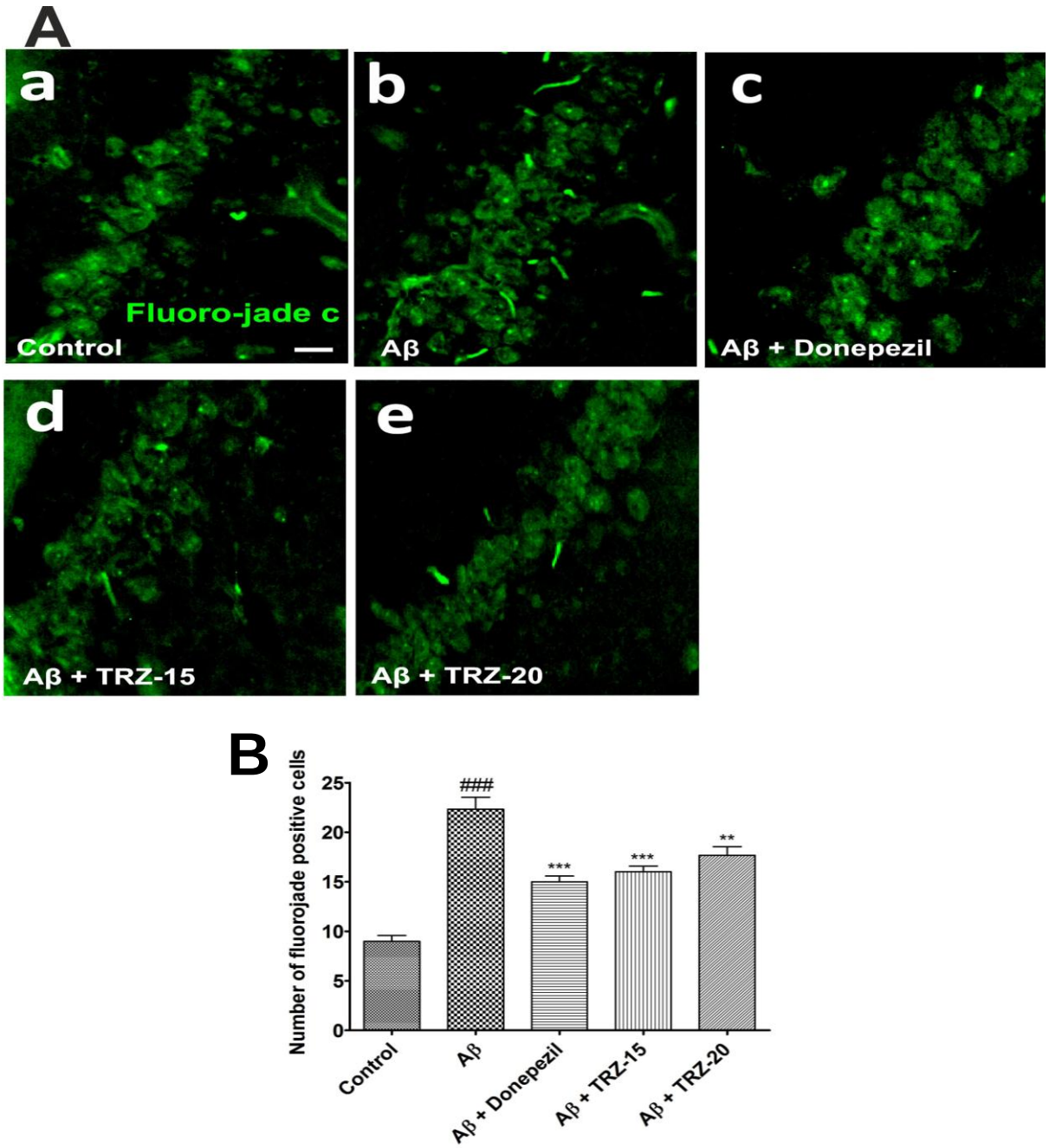


Figure 26: TRZ-15 and TRZ-20 decrease number of degenerating neurons in hippocampal region of $A\beta_{1-42}$ induced Alzheimer's rat brain. (A) Representative photomicrographs of fluoro-jade C stained degenerating neurons in the hippocampus of control (**a**), $A\beta_{1-42}$ treated (**b**), $A\beta_{1-42}$ + donepezil (**c**), $A\beta_{1-42}$ + **TRZ-15** (**d**) and $A\beta_{1-42}$ + **TRZ-20** (**e**) treated rats. **(B)** Quantitative analysis suggested significantly decreased number of fluoro-jade C + degenerating neurons in the hippocampus of **TRZ-15** and **TRZ-20** treated rats. Values are expressed as mean ± SEM (n=6 rats per group). ***$p < 0.001$ versus $A\beta_{1-42}$ treated group. **$p < 0.01$ versus $A\beta_{1-42}$ treated group and $^{###}p<0.001$ vs control group. Scale bar =100 µm.

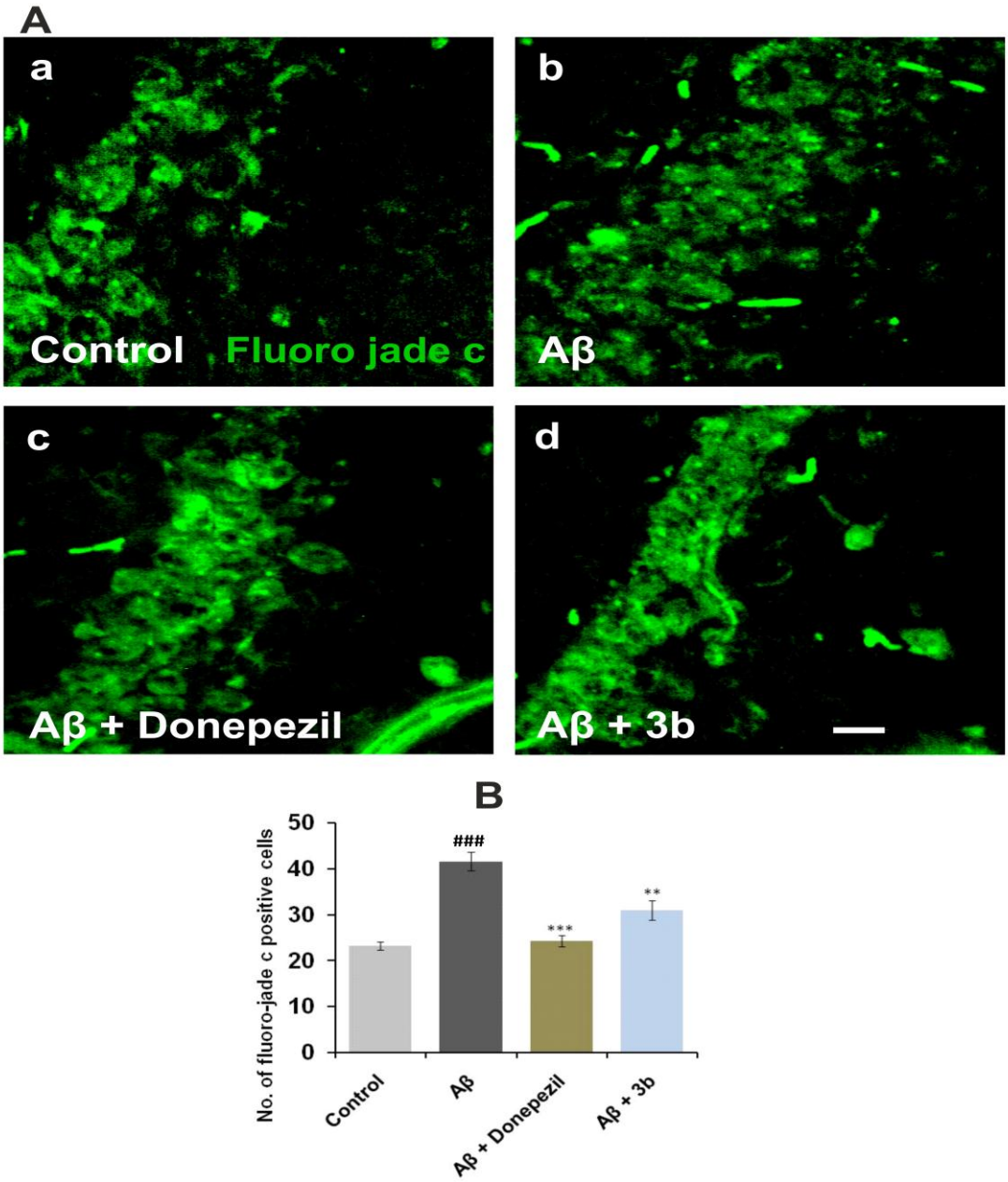


Figure 27: Effect of 3b on number of degenerating neurons in hippocampal region of $A\beta_{1-42}$ induced Alzheimer's rat brain. (A) Photomicrographs representing fluoro-jade C stained degenerating neurons in the hippocampus of control (**a**), $A\beta_{1-42}$ treated (**b**), $A\beta_{1-42}$ + donepezil (**c**) and $A\beta_{1-42}$ + **3b** (**d**) treated rats. **(B)** Quantitative analysis suggested that with treatment of **3b,** there was significant decrease in number of fluoro-jade C + degenerating neurons in the hippocampus as compared to the Aβ treated group. Values are expressed as mean ± SEM (n=6). Significant values were compared with ***$p < 0.001$ versus $A\beta_{1-42}$ treated group. **$p < 0.01$ versus $A\beta_{1-42}$ treated group and $^{###}p<0.001$ vs control group. Scale bar =100 µm.

4.20. TRZ-15 and TRZ-20 stabilize the level of β-catenin in $A\beta_{1-42}$ induced Alzheimer's rat brain

Some recent *in vitro* studies state that cholinergic neuronal loss and destruction of Wnt pathway is due to AChE–Aβ toxicity [78-80]. The finding that the compounds under investigation showed good cholinesterase inhibitory activity and reduced Aβ burden led us to investigate whether the compounds i.e. **TRZ-15** and **TRZ-20** could interfere in Wnt signaling by examining the expression levels of both GSK3b and β-catenin, the two main components in the Wnt signaling pathway.

Subsequently, the distribution of β-catenin was analyzed by immunofluorescence labelling of β-catenin. Confocal microscopic observation showed that more number of β-catenin labelled cells was observed in the hippocampal region of diseased rat brain treated with **TRZ-15** and **TRZ-20** as compared to $A\beta_{1-42}$ induced Alzheimer's rat brain [**Figure 28A (d, e)**]. High-magnification images showed prominently that most of the β-catenin-positive products were located in the cell bodies with a large-sized DAPI-stained nucleus [**Figure 28A (a-e)**], signifying a neuronal cell localization of β-catenin in the rat brain. **TRZ-15** and **TRZ-20** treatment noticeably increased the density of β-catenin immunofluorescence at 20 mg/kg dose [**Figure 28A (d, e)**]. The quantification showed significant increase in number of β-catenin positive cells in **TRZ-15** and **TRZ-20** treated rat brains (**Figure 28B**). These findings point out that **TRZ-15** and **TRZ-20** may play a protective role against $A\beta_{1-42}$ induced neuronal death through activation of β-catenin.

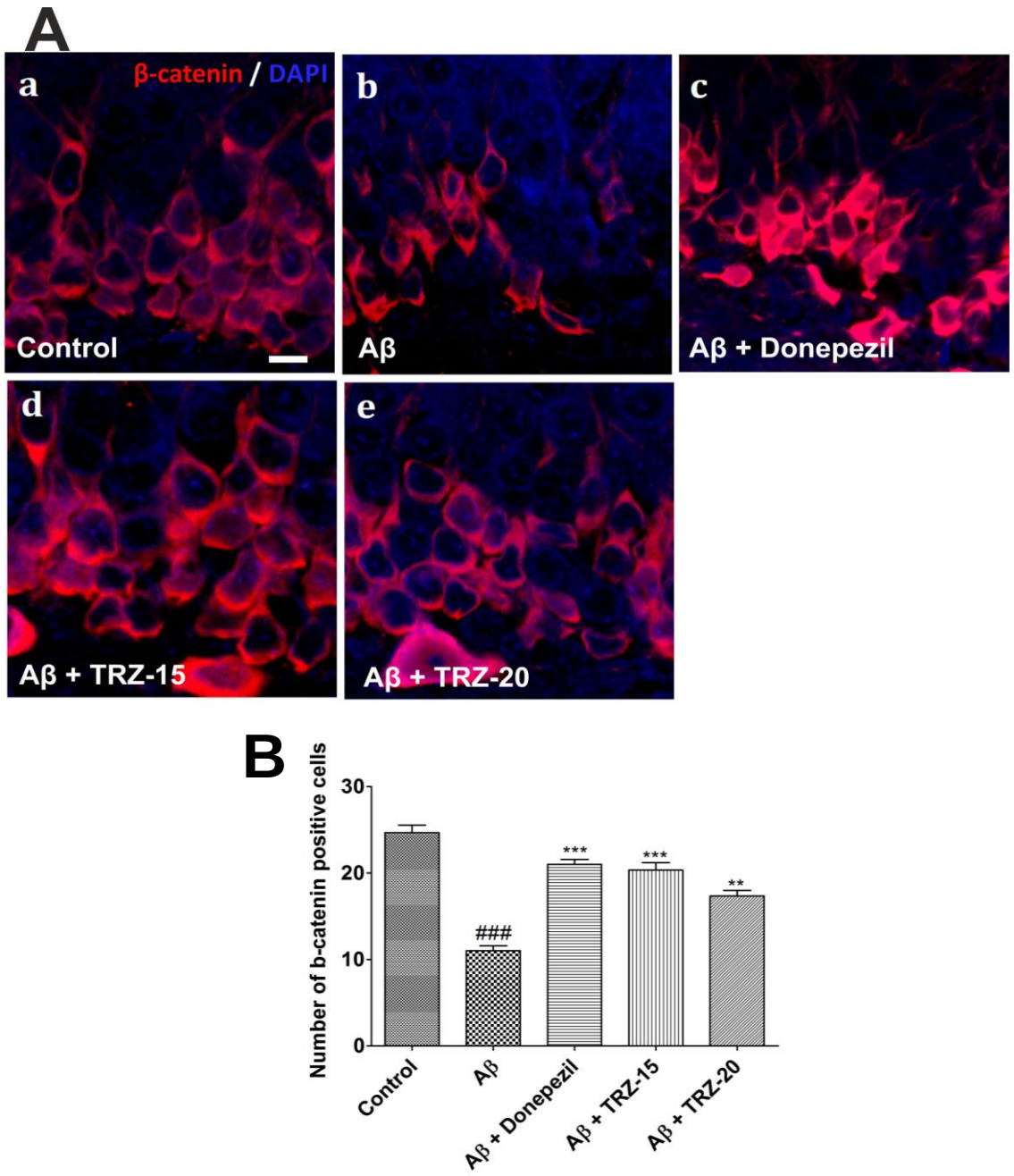


Figure 28: TRZ-15 and TRZ-20 enhance the level of β-catenin and inhibit GSK3a/b by up regulation of GSK3a/b in $A\beta_{1-42}$ induced Alzheimer's rat brain. (A) Confocal microscopic images showing the distribution of β-catenin in $A\beta_{1-42}$ induced Alzheimer's rat brain counterstained with DAPI (**a**), $A\beta_{1-42}$ treated (**b**), $A\beta_{1-42}$ + donepezil (**c**), $A\beta_{1-42}$ + **TRZ-15** (**d**) and $A\beta_{1-42}$ + **TRZ-20** (**e**) treated rats. (**B**) Quantitative analysis suggested significantly increased number of β-catenin + neurons in the hippocampus of **TRZ-15** and **TRZ-20** treated rats as compared to the abeta treated group. In the Aβ treated rat brain, the density of β-catenin immunofluorescence was very weak (**b**), whereas it was very intense in the **TRZ-15** and **TRZ-20** treated Alzheimer's rat brain (**d** and **e**). Scale bar = 20 µm. All values are expressed as mean ± SEM (n=6). ***p <0.001 vs Aβ treated group, **p <0.01 vs abeta treated group and $^{###}p<0.001$ vs control group.

4.21. Compound (3b) inhibits GSK-3 and stabilizes the level of β-catenin in $A\beta_{1-42}$ induced Alzheimer's rat brain

According to some recent *in vitro* studies, AChE–Aβ causes destruction of Wnt pathway and cholinergic neuronal loss [78-80]. The finding that the compound under exploration revealed good cholinesterase inhibitory activity and also reduced Aβ burden led us to investigate whether the compound i.e. **3b** could interfere in Wnt signaling by examining the expression levels of the two main components in the Wnt signaling pathway viz. pGSK3 and β-catenin,.

Further, confocal microscopic observation and immunoblot analysis illustrated that treatment with **3b** increased number of β-catenin labelled cells as observed in the hippocampal region of the diseased rat brain as compared to $A\beta_{1-42}$ induced Alzheimer's rat brain [**Figure 29A** (**d**)]. Consequently, the distribution of β-catenin was analyzed by immunofluorescence technique in **3b** treated Alzheimer's rat brain sections. Confocal microscopic observation demonstrated that more number of β-catenin labelled cells were observed in the hippocampal region of diseased rat brain treated with **3b** as compared to $A\beta_{1-42}$ induced Alzheimer's rat brain (**Figure 29B**). High-magnification images prominently showed that most of the β-catenin-positive products were located in the cell bodies with a large-sized DAPI-stained nucleus [**Figure 29A** (**a-d**)], implying a neuronal cell localization of β-catenin in the rat brain. **3b** treatment markedly increased the density of β-catenin immunofluorescence at 20 mg/kg dose [**Figure 29A**, (**d**)]. These findings indicate that **3b** may play a protective role in $A\beta_{1-42}$ induced neuronal death by activating β-catenin.

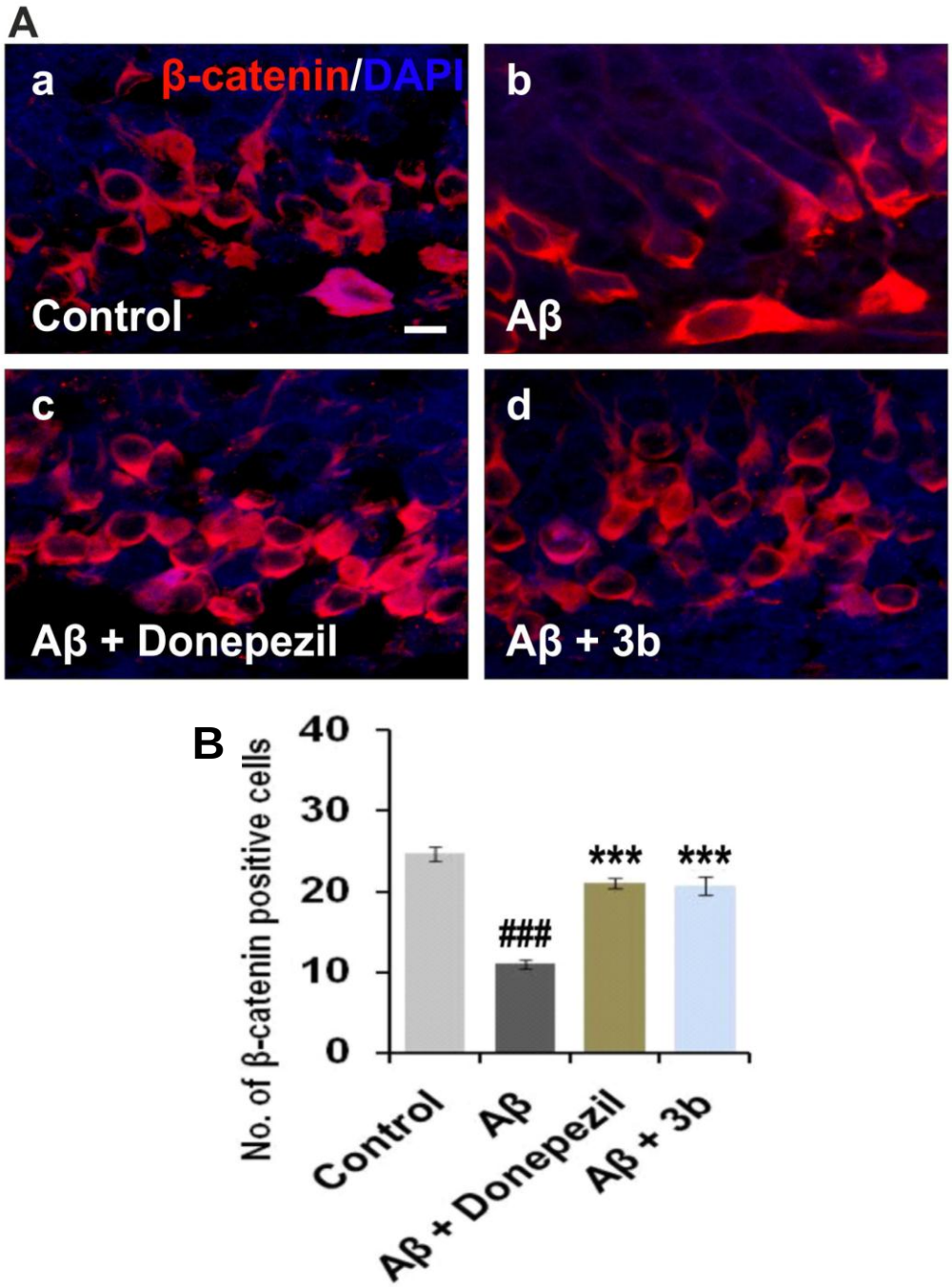


Figure 29: Effect of 3b on the level of β-catenin in $Aβ_{1-42}$ induced Alzheimer's rat brain. (A) Confocal microscopic images show the distribution of β-catenin in $Aβ_{1-42}$ induced Alzheimer's rat brain counterstained with DAPI (**a**), $Aβ_{1-42}$ treated (**b**), $Aβ_{1-42}$ + donepezil (**c**) and $Aβ_{1-42}$ + **3b** (**d**) treated rats. (**B**) Quantitative analysis suggested that with treatment of **3b,** there was significant increase in number of β-catenin + neurons in the hippocampus as compared to the Aβ treated group. Values are expressed as mean ± SEM (n=6). Significant values were compared with ***$p<0.001$ vs abeta treated group and $^{###}p<0.001$ vs control group. Scale bar = 50 μm.

4.22. TRZ-15 and TRZ-20 reduce Aβ, cytochrome c and cleaved caspase levels in $A\beta_{1-42}$ induced Alzheimer's rat brain

Western blot results indicated that there was significant decrease in levels of Aβ, cytochrome c and cleaved caspase-3 in **3b** treated $A\beta_{1-42}$ induced Alzheimer's rat brain (**Figure 30**). Previous results were further substantiated by Western blot analysis for $A\beta_{1-42}$, cytochrome c and cleaved caspase-3 in **TRZ-15** and **TRZ-20** treated $A\beta_{1-42}$ induced Alzheimer's rat brains. Levels of $A\beta_{1-42}$, cytochrome c and cleaved caspase-3 were reduced in donepezil, **TRZ-15** and **TRZ-20** treatment groups as compared to the $A\beta_{1-42}$ treated group. Statistical analysis showed that **TRZ-15** and **TRZ-20** treatment significantly reduced the levels of $A\beta_{1-42}$, cytochrome c and cleaved caspase-3 in $A\beta_{1-42}$ induced Alzheimer's rat brains. These results pointed towards neuroprotective effect of **TRZ-15** and **TRZ-20** in $A\beta_{1-42}$ induced Alzheimer's rat brain. Western blot analysis of Aβ, cytochrome c and cleaved caspase-3 were carried out on hippocampal tissue of animals treated with **TRZ-15** and **TRZ-20** (**Figure 30**). The protein levels of Aβ in **TRZ-15** and **TRZ-20** treated $A\beta_{1-42}$ induced Alzheimer's rat brains with Western blot analyses (**Figure 30B**) were detected first. Levels of Aβ got decreased to 746.3 ± 23.58 % ($p<0.001$) and 922.9 ± 55.16 % ($p<0.001$) in **TRZ-15** and **TRZ-20** treated groups respectively as compared to the Aβ treated group (1903 ± 4.418 %) at a dose of 20 mg/kg (**Figure 30B**). Levels of cytochrome c were significantly decreased to 81.36 ± 0.8670 % ($p<0.001$) and to 104.6 ± 1.313 % ($p<0.001$) as compared to the Aβ treated group (212.2 ± 1.313 %) at a dose of 20 mg/kg in **TRZ-15** and **TRZ-20** treated groups respectively (**Figure 30C)**. The levels of cleaved caspase-3 were also decreased to 34.99 ± 1.947 % ($p<0.001$) and to 139.3 ± 5.064 % ($p<0.001$) as compared to the Aβ treated group (271.9 ± 3.877 %) at a dose of 20 mg/kg in **TRZ-15** and **TRZ-20** treated groups respectively (**Figure 30D**). This data suggested that treatment with **TRZ-15** and **TRZ-20** showed neuroprotective role in $A\beta_{1-42}$ induced Alzheimer's rat brain at a dose of 20 mg/kg by decreasing the levels of Aβ, cytochrome c and cleaved caspase-3.

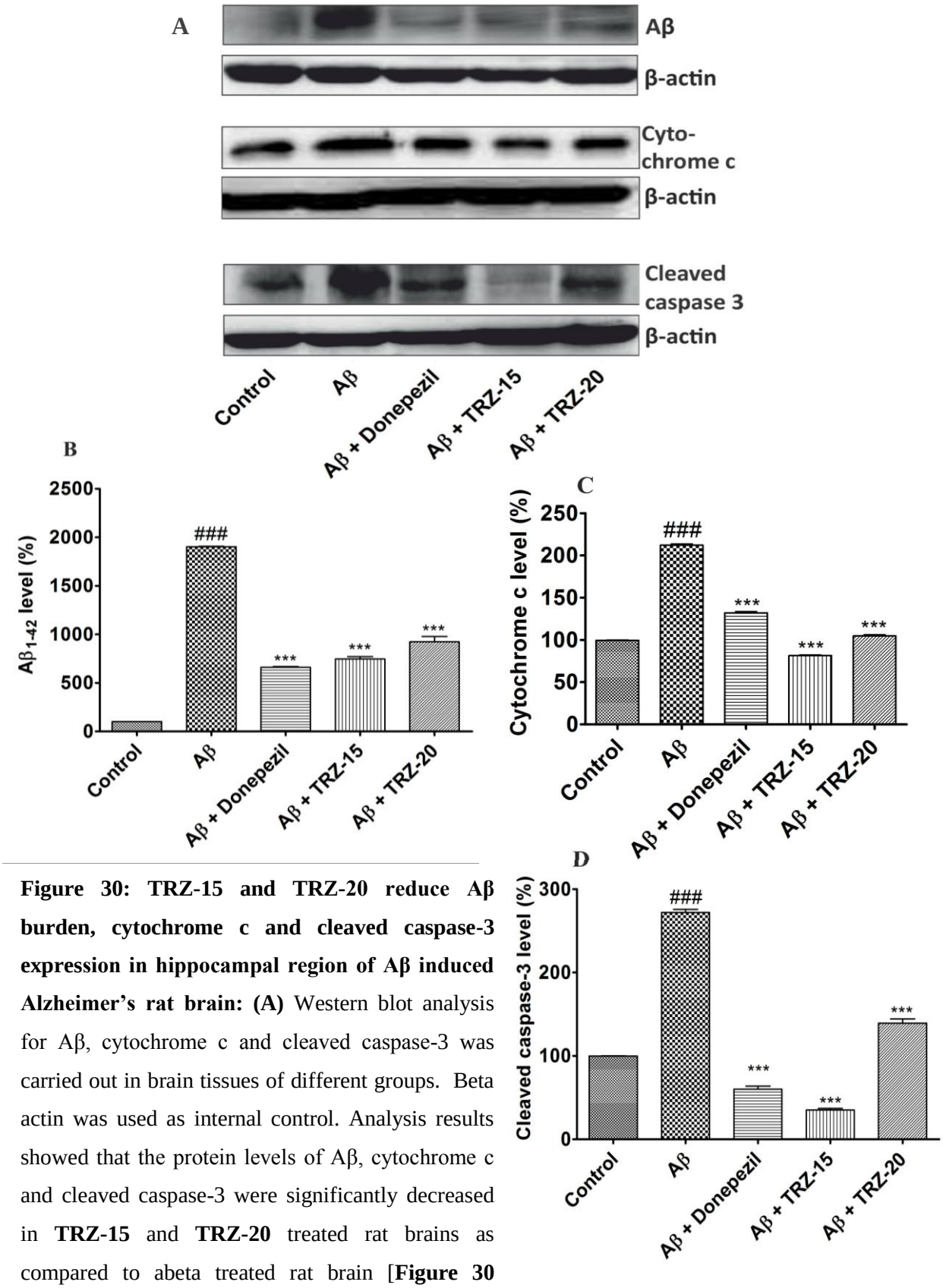


Figure 30: TRZ-15 and TRZ-20 reduce Aβ burden, cytochrome c and cleaved caspase-3 expression in hippocampal region of Aβ induced Alzheimer's rat brain: (A) Western blot analysis for Aβ, cytochrome c and cleaved caspase-3 was carried out in brain tissues of different groups. Beta actin was used as internal control. Analysis results showed that the protein levels of Aβ, cytochrome c and cleaved caspase-3 were significantly decreased in **TRZ-15** and **TRZ-20** treated rat brains as compared to abeta treated rat brain [**Figure 30 (B-D)**]. All values are expressed as mean ± SEM (n=6). ***p <0.001 vs abeta treated group, ###p<0.001 vs control group. Scale bar =100 µm.

4.23. Compound (3b) reduced Aβ, cytochrome c and cleaved caspase-3 levels in $A\beta_{1-42}$ induced Alzheimer's rat brain

Western blot analysis substantiated the previous results as levels of $A\beta_{1-42}$, cytochrome c and cleaved caspase-3 were reduced in donepezil and **3b** treatment groups as compared to the $A\beta_{1-42}$ treated group (**Figure 31A**). It was investigated whether administration of **3b** could decrease Aβ deposition in $A\beta_{1-42}$ induced Alzheimer's rat brain. After 14 days treatment with **3b** (20 mg/kg), brain sections of rat were subjected to Western blot analysis. Western blot analysis for $A\beta_{1-42}$, cytochrome c and cleaved caspase-3 in **3b** treated $A\beta_{1-42}$ induced Alzheimer's rat brain further authenticated the previous results (**Figure 31A**). $A\beta_{1-42}$ and caspase-3 levels were significantly reduced in donepezil and **3b** treatment groups as compared to the $A\beta_{1-42}$ treated group. These results pointed towards neuroprotective effect of **3b** in $A\beta_{1-42}$ induced Alzheimer's rat brain.

The protein levels of Aβ in **3b** treated $A\beta_{1-42}$ induced Alzheimer's rat brain was detected first using Western blot analyses (**Figure 31B**). Levels of Aβ got reduced to 757.3 ± 18.58 % ($p<0.001$) in **3b** treated group as compared to the Aβ treated group (1803 ± 4.418 %) at a dose of 20 mg/kg (**Figure 31B**). The levels of cytochrome-c were reduced to 158.02 ± 1.210 % ($p<0.001$) in **3b** treated group as compared to the Aβ treated group (219.18 ± 0.865 %) at a dose of 20 mg/kg (**Figure 31C**). The levels of cleaved caspase-3 were also reduced to 36.65 ± 1.694 % ($p<0.001$) in **3b** treated group as compared to the Aβ treated group (273.9 ± 3.272 %) at a dose of 20 mg/kg (**Figure 31D**). This data indicated that treatment with **3b** showed neuroprotective role in $A\beta_{1-42}$ induced Alzheimer's rat brain at a dose of 20 mg/kg by reducing the levels of both Aβ and cleaved caspase-3.

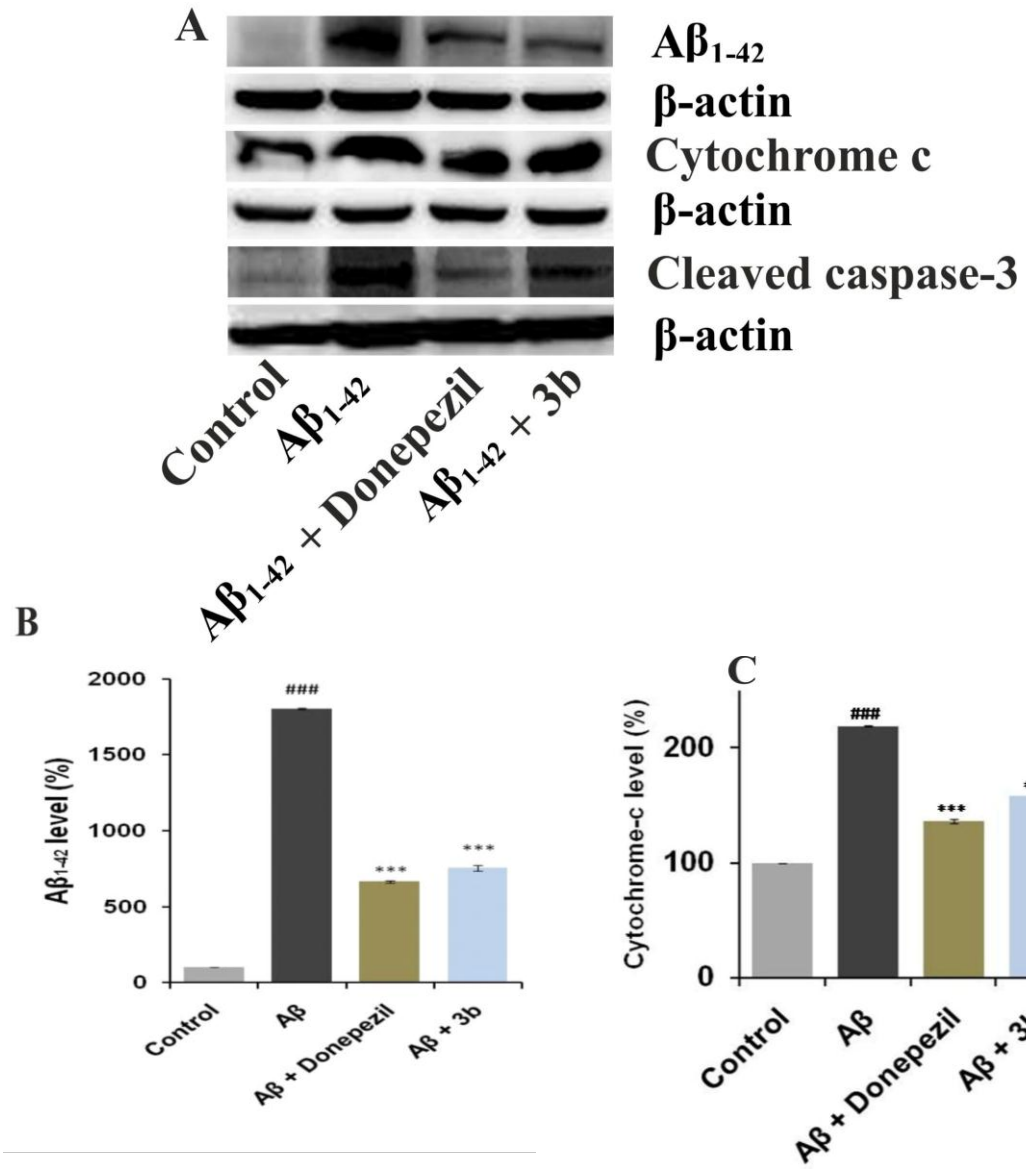


Figure 31: Effect of 3b on Aβ burden, cytochrome c and cleaved caspase-3 levels in hippocampal region of $Aβ_{1-42}$ induced Alzheimer's rat brain: (A) $Aβ_{1-42}$, cytochrome c and cleaved caspase-3 levels in brain tissues of different groups was analysed using Western blot analyses. Internal standard used was Beta actin. The significant depletion of protein levels of $Aβ_{1-42}$, cytochrome c and cleaved caspase-3 were observed in **3b** treated rat brains as compared to Aβ treated rat brain [**Figure 31 (B-D)**]. Values are expressed as mean ± SEM (n=6). Significant values were compared with ***$p<0.001$ vs Aβ treated group, $^{###}p<0.001$ vs control group.

4.24. TRZ-15 and TRZ-20 diminish GSK3 and increase the level of β-catenin in $A\beta_{1-42}$ induced Alzheimer's rat brain

The protein levels of phosphorylated GSK-3b (p-GSK-3b)/GSK-3b ratio were determined first in **TRZ-15** and **TRZ-20** treated $A\beta_{1-42}$ induced Alzheimer's rat brains with Western blot analyses (**Figure 32**). Levels of p-GSK3b/GSK3b ratio were increased to 74.26 ± 0.7784 % ($p<0.001$) and 58.59 ± 0.3543 % ($p<0.001$) by **TRZ-15** and **TRZ-20** at a dose of 20 mg/kg, respectively (**Figure 32C**). The levels of p-GSK3a/GSK3a ratio were also increased to 147.2 ± 1.877 % ($p<0.001$) and 78.37 ± 1.066 % ($p<0.001$) at a dose of 20 mg/kg, respectively (**Figure 32B**). The data suggests that treatment with **TRZ-15** and **TRZ-20** inactivates both GSK3b and GSK3a in Alzheimer's rat brain at a dose of 20 mg/kg. Levels of β-catenin were measured after treatment of **TRZ-15** and **TRZ-20** in $A\beta_{1-42}$ induced Alzheimer's rat brain. Immunoblot results showed that treatment of **TRZ-15** and **TRZ-20** increased the levels of β-catenin (**Figure 32D**), to 186.1 ± 6.747 % (20 mg/kg; $p<0.001$) and 166.4 ± 5.636 % (20 mg/kg; $p<0.001$) respectively compared to the Aβ treated group [44.82 ± 4.652 % (**Figure 32D**)].

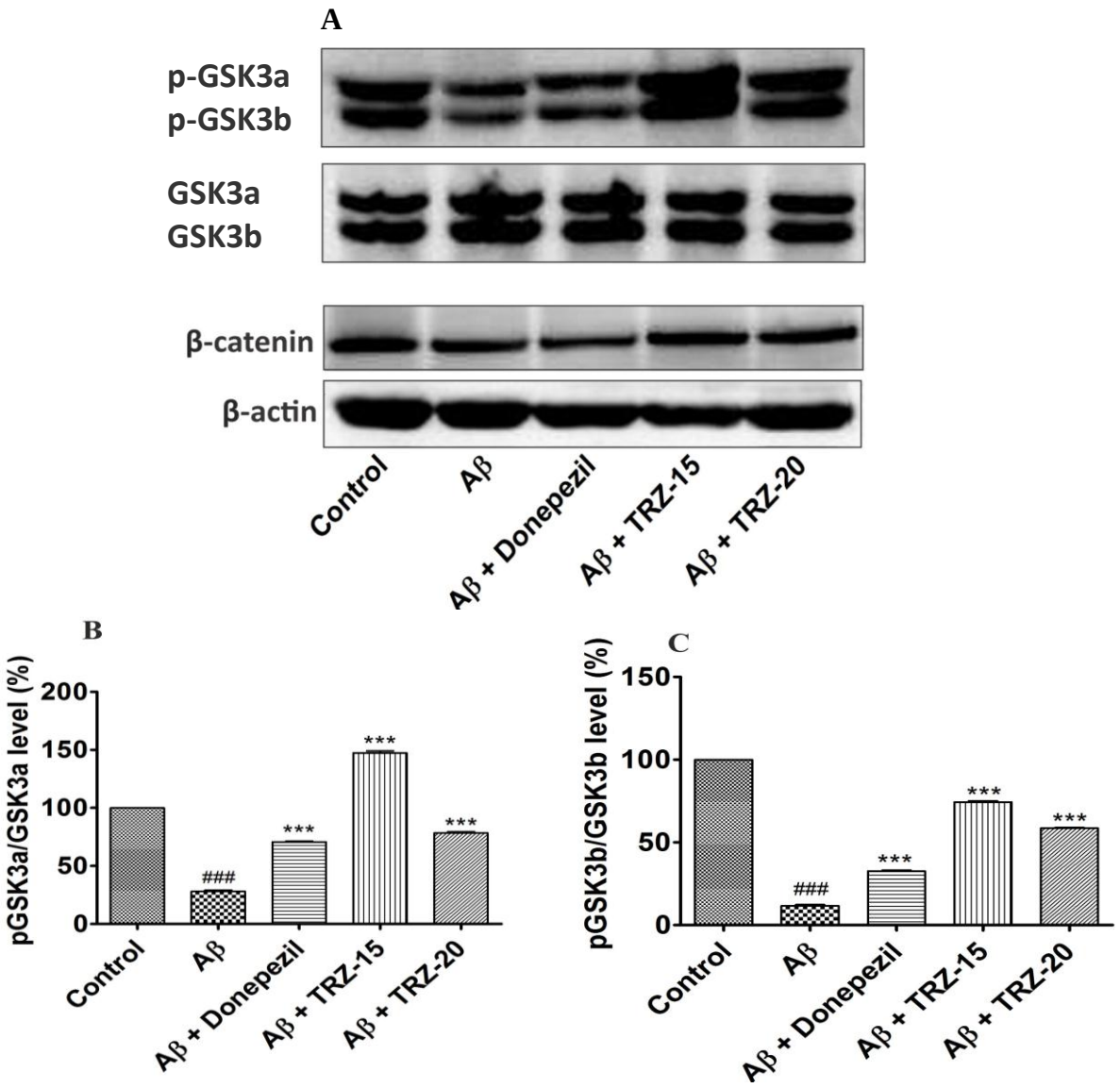


Figure 32: TRZ-15 and TRZ-20 enhance the level of β-catenin and inhibit GSK3a/b by up regulation of GSK3a/b in $A\beta_{1-42}$ induced Alzheimer's rat brain. (A) Protein levels of p-GSka/GSK3a ratio, p-GSKb/GSK3b ratio and β-catenin were examined by Western blot in Alzheimer's rat brain of control and treatment groups. Beta actin was used as an internal control. Analysis showed that the protein levels of p-GSK3/GSK3 ratio and β-catenin were significantly increased in **TRZ-15** and **TRZ-20** treated rat brains as compared to the abeta treated rat brain [**Figure 32 (B-D)**]. All values are expressed as mean ± SEM (n=6). ***p <0.001 vs abeta treated group, **p <0.01 vs abeta treated group and $^{###}$p<0.001 vs control group.

4.25. Compound (3b) inhibits GSK-3 and stabilizes the level of β-catenin in $A\beta_{1-42}$ induced Alzheimer's rat brain

Initially, Western blot analysis was carried out to analyze the protein levels of phosphorylated GSK-3b (p-GSK-3b) in **3b** treated $A\beta_{1-42}$ induced Alzheimer's rat brain (**Figure 33**). Level of p-GSK3b/GSK-3b ratio was increased to 102.5 ± 1.495 % ($p<0.001$) by **3b** at a dose of 20 mg/kg (**Figure 33C**). The level of p-GSK3a/GSK3a was also increased to 102.4 ± 1.489 % ($p<0.001$) at a dose of 20 mg/kg (**Figure 33B**). The data proposed that treatment with **3b** inactivated both GSK3b and GSK3a in Alzheimer's rat brain at a dose of 20 mg/kg.

After 14 days treatment with **3b** (20 mg/kg), brain sections of rat were subjected to Western blot analysis for β-catenin. Immunoblot results illustrated that treatment with **3b** increased the level of β-catenin (**Figure 33D**), to 103.6 ± 2.545 % (20 mg/kg; $p<0.001$) compared to the Aβ treated group [18.8 ± 1.675 % (**Figure 33D**)].

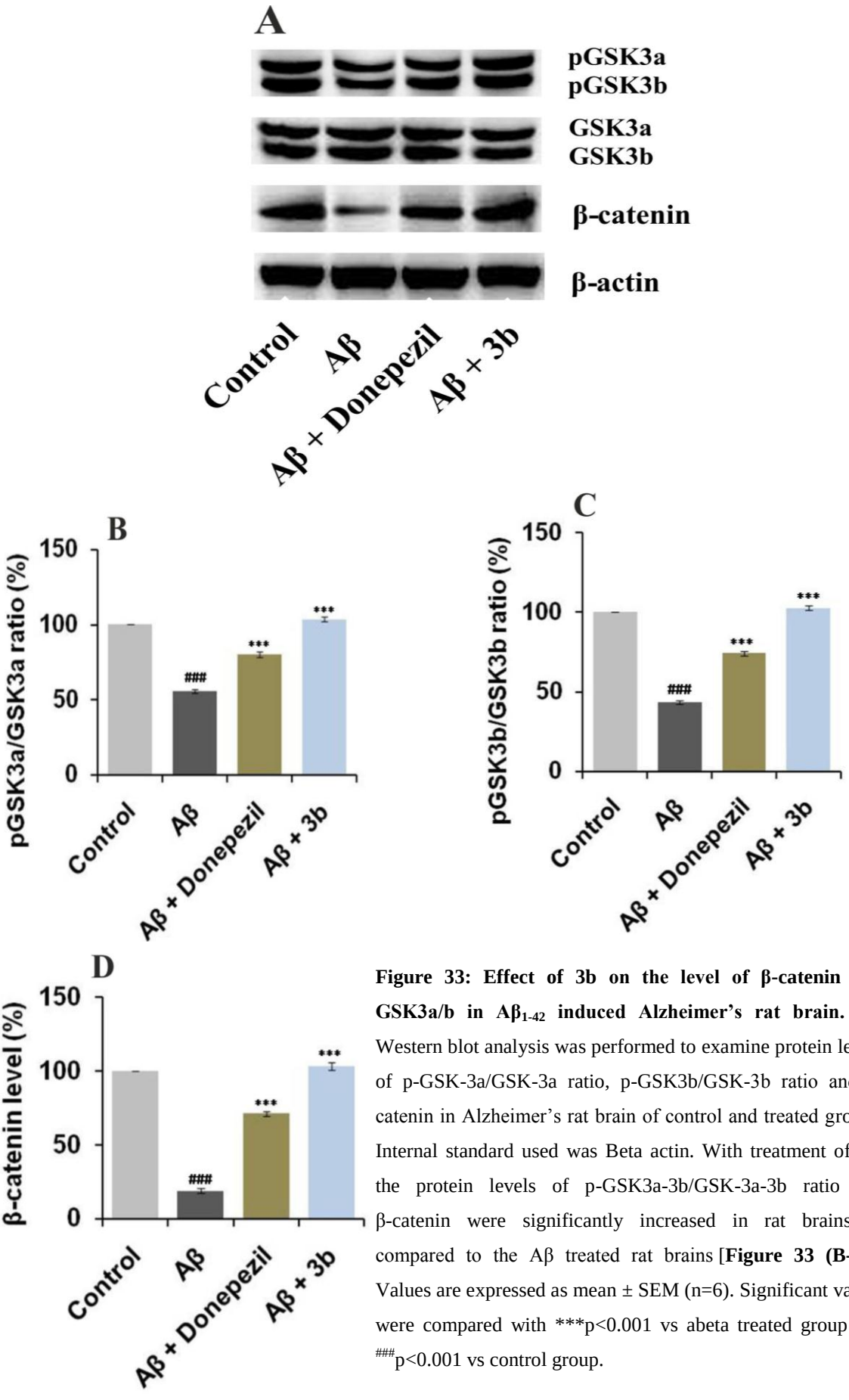


Figure 33: Effect of 3b on the level of β-catenin and GSK3a/b in $A\beta_{1-42}$ induced Alzheimer's rat brain. (A) Western blot analysis was performed to examine protein levels of p-GSK-3a/GSK-3a ratio, p-GSK3b/GSK-3b ratio and β-catenin in Alzheimer's rat brain of control and treated groups. Internal standard used was Beta actin. With treatment of **3b**, the protein levels of p-GSK3a-3b/GSK-3a-3b ratio and β-catenin were significantly increased in rat brains as compared to the Aβ treated rat brains [**Figure 33 (B-D)**]. Values are expressed as mean ± SEM (n=6). Significant values were compared with ***$p<0.001$ vs abeta treated group and $^{###}p<0.001$ vs control group.