

Section-III



Chapter-4

RESULTS

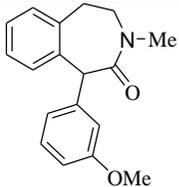
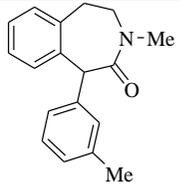
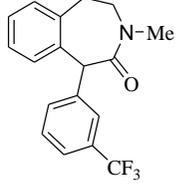
4. RESULTS

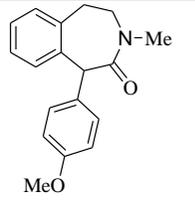
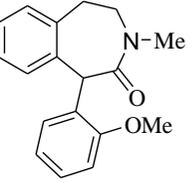
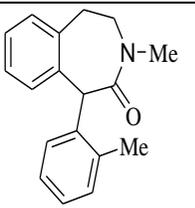
It was found in our earlier studies that benzazepines possessing bulky groups at C-1 and/or N-3 positions exhibited 5-HT activity. So, benzazepines which did not have bulky groups at position 1 and 3 were evaluated for their sensibility towards dopamine receptors.

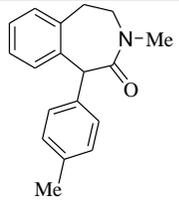
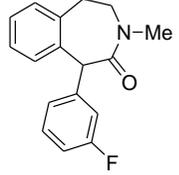
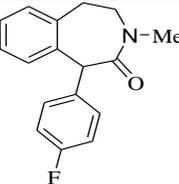
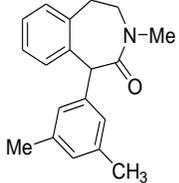
4.1. Effect of compounds (3a-5) on rat superior mesenteric artery

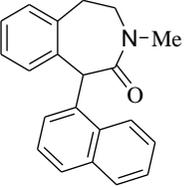
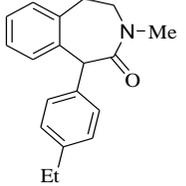
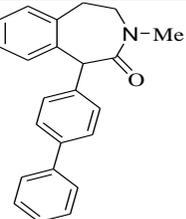
As shown in **Table 1**, the compounds (**3c**, **3d**, **3e**, **3i** and **3k**) were found to be dopamine agonists. Rest of the compounds (**3a**, **3b**, **3f**, **3g**, **3h**, **3j**, **3l**, **3m** and **5**) were found to be inactive or D₃ antagonists as shown in **Table 1**. Compound (**3d**) showed selectivity towards D₁ receptor as evidenced by its pD_2 value (7.00 ± 0.23). It was considered as a potent D₁ agonist as its pD_2 value was almost equivalent to standard **A-77636** (a selective D₁ agonist, 7.42 ± 0.19). Rest of the compounds (**3a**, **3b**, **3f**, **3g**, **3h**, **3j**, **3l**, **3m** and **5**) were excluded from the study as they have shown antagonistic effect on dopamine receptors. The compound (**3d**) was further evaluated using different *in vitro* and *in vivo* studies.

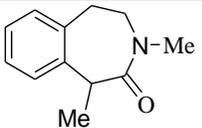
Table 1: Effects of the test compounds on rat superior mesenteric artery preparation. Values are expressed as pD_2 and pA_2 values on D_1 , D_2 and D_3 receptors.

Comp.	Structure	pD_2 values			pA_2 values		
		D_1 receptor	D_2 receptor	D_3 receptor	D_1 receptor	D_2 receptor	D_3 receptor
3a					5.71 ± 0.12	5.68 ± 0.16	5.72 ± 0.05
3b					5.57 ± 0.14	5.6 ± 0.11	5.63 ± 0.13
3c		3.72 ± 0.05	3.55 ± 0.1	3.58 ± 0.14			

Comp.	Structure	pD_2 values			pA_2 values		
		D ₁ receptor	D ₂ receptor	D ₃ receptor	D ₁ receptor	D ₂ receptor	D ₃ receptor
3d		7.00 ± 0.23	4.28 ± 0.12	3.92 ± 0.17			
3e		3.98 ± 0.15	4.06 ± 0.16	3.69 ± 0.07			
3f					5.72 ± 0.14	5.68 ± 0.12	5.74 ± 0.14

Comp.	Structure	pD_2 values			pA_2 values		
		D ₁ receptor	D ₂ receptor	D ₃ receptor	D ₁ receptor	D ₂ receptor	D ₃ receptor
3g					5.73 ± 0.14	5.67 ± 0.07	5.74 ± 0.18
3h					5.64 ± 0.09	5.34 ± 0.10	7.68 ± 0.15
3i		3.71 ± 0.12	3.71 ± 0.11	2.35 ± 0.09			
3j					5.48 ± 0.10	5.51 ± 0.14	5.53 ± 0.13

Comp.	Structure	pD_2 values			pA_2 values		
		D ₁ receptor	D ₂ receptor	D ₃ receptor	D ₁ receptor	D ₂ receptor	D ₃ receptor
3k		4.68 ± 0.07	4.71 ± 0.08	4.73 ± 0.15			
3l					5.74 ± 0.07	5.83 ± 0.10	5.86 ± 0.14
3m					5.71 ± 0.08	5.67 ± 0.16	5.69 ± 0.10

Comp.	Structure	pD_2 values			pA_2 values		
		D ₁ receptor	D ₂ receptor	D ₃ receptor	D ₁ receptor	D ₂ receptor	D ₃ receptor
5					5.58 ± 0.13	5.61 ± 0.11	5.62 ± 0.08
A-77636		7.42 ± 0.19					

4.2 Compound (3d) improved 6-OHDA induced injury in human SH-SY5Y neuroblastoma cell culture

To determine the toxicity of the compound (3d) on human SH-SY5Y neuroblastoma cells, MTT assay was carried out. In a set of experiments human SH-SY5Y neuroblastoma cells were incubated with 100 μM 6-OHDA for 24 h, with or without different concentrations of the compound (3d) and A-77636 (5-20 μM). MTT assay was then performed to assess 6-OHDA induced cytotoxicity, and to assess the neuroprotective role of the compound. As shown in Figure 2, 6-OHDA significantly decreased the cell viability. On the other hand, the cytotoxic effects were reduced by pre-treatment of the cells with the compound (3d) (5-20 μM). The cytotoxic effect of 6-OHDA was significantly blocked by the compound (3d) (Figure 2). This study established that the compound (3d), which was found to be potent D₁ agonist in previous studies, showed protection in 6-OHDA injured human SH-SY5Y neuroblastoma cells dose dependently. The results of MTT assay showed that the compound (5-20 μM) significantly protected human SH-SY5Y neuroblastoma cells from 6-OHDA toxicity.

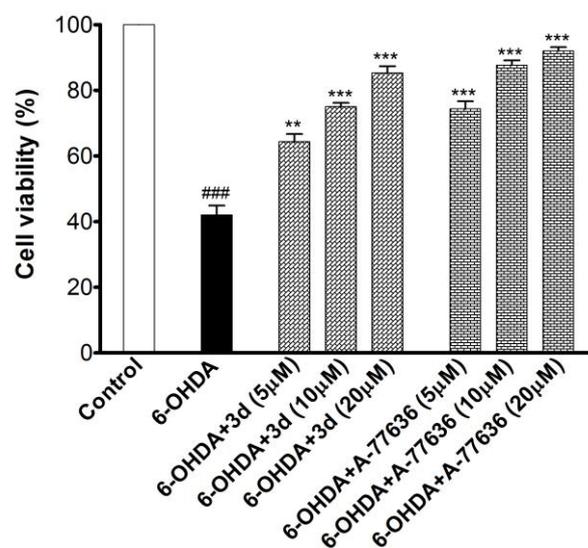


Figure 2: Effect of compound (3d) on the injury induced by 6-OHDA in human SH-SY5Y neuroblastoma culture. In presence of 6-OHDA (100 μM), human SH-SY5Y neuroblastoma cell culture was exposed to compound (3d) *in vitro*. SH-SY5Y neuroblastoma cells were incubated separately with compound (3d) and A-77636 (5-20 μM concentrations) for 2 h, followed by 24 h incubation with 6-OHDA. The percentage cell viability was determined by MTT assay after incubation period.

4.3. Behavioral studies

The rotational (circling) behaviour of rodents with unilateral nigrostriatal damage been utilized broadly to explore nigrostriatal capacity and the activity of dopaminergic compounds [78]. Microinjection of the catecholaminergic neurotoxin, 6-hydroxydopamine (6-OHDA), was given unilaterally in rats. The lesioned rats impulsively display minor ipsilateral (towards the lesioned side) rotation. Obviously because of sensitivity in the lesioned brain, treatment with the dopamine receptor agonist provokes active contralateral rotation [79, 80]. Similar to the above notion, the compound (**3d**) showed contralateral rotations significantly (**Figure 3**). A behavioural tolerance was observed in case of **A-77636** from 5th day of dosing. By the 6th day of dosing, contralateral rotations were almost equivalent to vehicle. This authenticated that the compound (**3d**) was selective D₁ agonist.

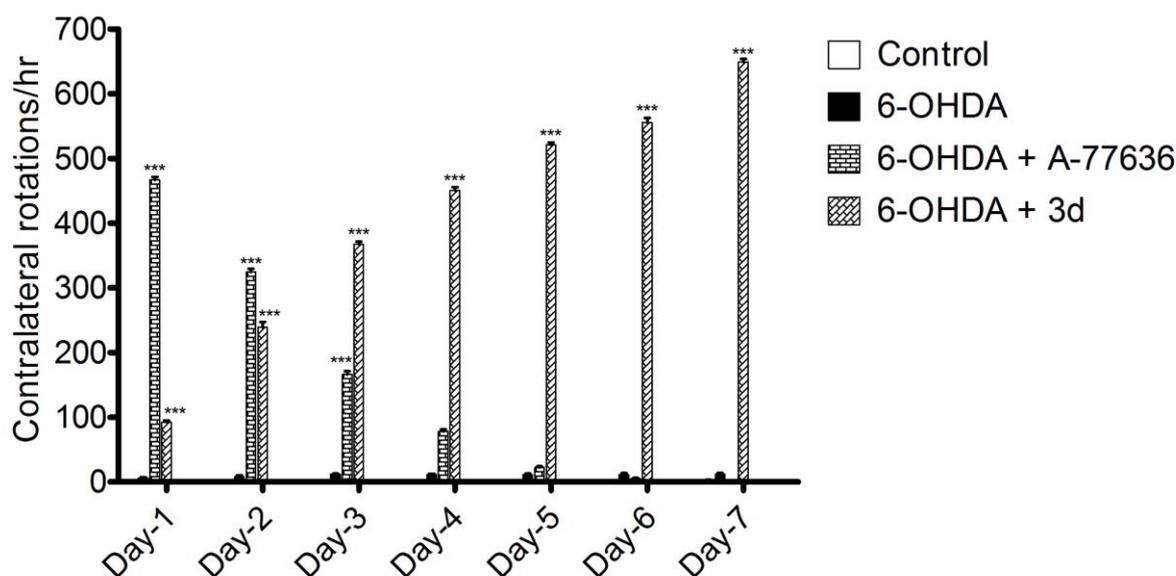


Figure 3: Cumulative rotation (mean \pm S.E.M., $n = 6$ /group) over 1 h produced by daily dosing with **A-77636** and the compound (**3d**) for 7 days. Significant values were compared with *** $p < 0.001$ vs 6-OHDA treated group.

4.4. Antioxidative effects of compound (3d) in 6-OHDA treated rat brain

Oxidative parameters were significantly modified (increased GSH, MDA and catalase levels and decreased MDA level) by the compound (3d) at 5 mg/kg. To evaluate whether compound (3d) exerts antioxidative effects, brain tissues of rat treated with compound (3d) were subjected to colorimetric estimation to establish the antioxidative properties in the brain. The results showed that 6-OHDA treatment significantly ($p < 0.001$) decreased the brain GSH level (1.200 ± 0.1155 nM/mg protein) compared to the control group (3.233 ± 0.1453 nM/mg protein). Treatment with the standard drug (A-77636) and the test compound (3d) significantly increased brain GSH levels (Figure 4a) [A-77636 (3.067 ± 0.18819), and compound (3d) (2.867 ± 0.18519) nM/mg protein] compared to the corresponding 6-OHDA treated group (Figure 4a). Further, 6-OHDA treatment significantly ($p < 0.001$) increased the brain MDA level (3.800 ± 0.15774 nM/mg protein) compared to the control group (2.200 ± 0.1528 nM/mg protein). Treatment with the standard drug (A-77636) and the test compounds (3d) significantly decreased brain MDA levels (Figure 4b) [A-77636 (2.300 ± 0.1528) and compound (3d) (2.400 ± 0.15774) nM/mg protein] compared to the corresponding 6-OHDA treated group (Figure 4b). Further, 6-OHDA treatment significantly ($p < 0.001$) decreased the brain SOD level (29.67 ± 1.202 U/mg protein) compared to the control group (40.67 ± 0.8819 U/mg protein). Treatment with the standard drug (A-77636) and the test compounds (3d) significantly increased brain SOD levels (Figure 4c) [A-77636 (36.67 ± 0.8819) and compound (3d) (37.00 ± 0.25654) U/mg protein] compared to the corresponding 6-OHDA treated group (Figure 4c). Additionally, 6-OHDA treatment significantly ($p < 0.001$) decreased the brain catalase level (2.300 ± 0.15774 U/mg protein) compared to the control group (4.000 ± 0.1155 U/mg protein). Treatment with the standard drug (A-77636) and the test compounds (3d) significantly increased brain catalase levels (Figure 4d) [A-77636 (3.800 ± 0.15444) and compound (3d) (3.567 ± 0.18819) U/mg protein] compared to the corresponding 6-OHDA treated group (Figure 4d). These results revealed the antioxidant property of compound (3d).

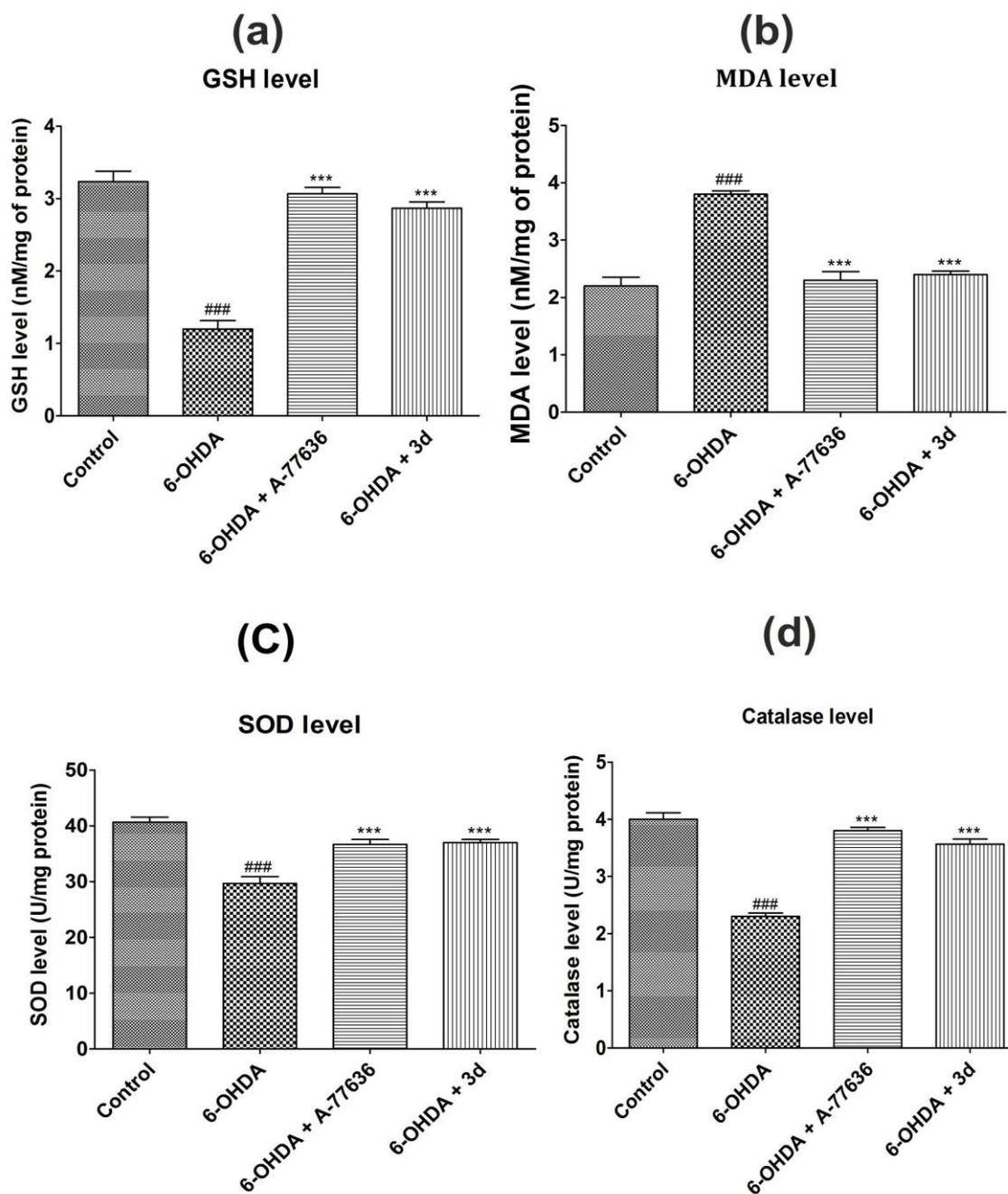


Figure 4: Effect of compound (3d) on antioxidant properties *ex vivo* in 6-OHDA induced Parkinson's rat model. As compared to control group, GSH, SOD and catalase levels significantly decreased in 6-OHDA treated group (a, c, d) and MDA level was significantly increased in 6-OHDA treated group (b). As compared to 6-OHDA treated group, treatment with compound (3d) significantly decreased MDA level (b). Treatment with compound (3d) significantly increased GSH, SOD and catalase levels as compared to 6-OHDA treated group (a, c, d). Values are expressed as mean \pm SEM. (n=6) Significant values were compared with ***p<0.001 vs 6-OHDA treated group & ###p<0.001 vs control group.

4.5. Compound (3d) increased dopamine (DA) level in striatum of 6-OHDA lesioned rats

The unilateral 6-hydroxydopamine (6-OHDA) model has the benefit of giving side-biased motor impairments. The 6-OHDA lesion model now can be accessible in both rats and mice. Unilateral 6-OHDA model is the traditional model for assessing Parkinson's treatments, particularly those proposed to enhance dopamine levels in the striatum. The main playing point of this model is that it is exceptionally sensitive to dopamine agonists. The toxin 6-OHDA is injected unilaterally, while the inverse half serves as an intra-animal control. This injection produces loss of DA neuron on the 6-OHDA-injected side while saving the contralateral DA neurons. Similar to the above note, first, a standard chromatogram was developed to estimate the DA level in the brains of the animals (**Figure 5**). 6-OHDA treatment significantly ($p < 0.001$) decreased the striatum DA level ($2.043 \pm 0.15044 \mu\text{g/gm tissue}$) compared to the control group ($5.940 \pm 0.15033 \mu\text{g/gm tissue}$). Treatment with the standard drug (**A-77636**) and the test compound (**3d**) significantly increased striatum DA levels (**Figure 6**) [**A-77636** (5.457 ± 0.15608) and compound (**3d**) (5.297 ± 0.14631) $\mu\text{g/gm tissue}$] compared to the corresponding 6-OHDA treated group (**Figure 6**). These results revealed the anti-Parkinsonian effect of compound (**3d**).

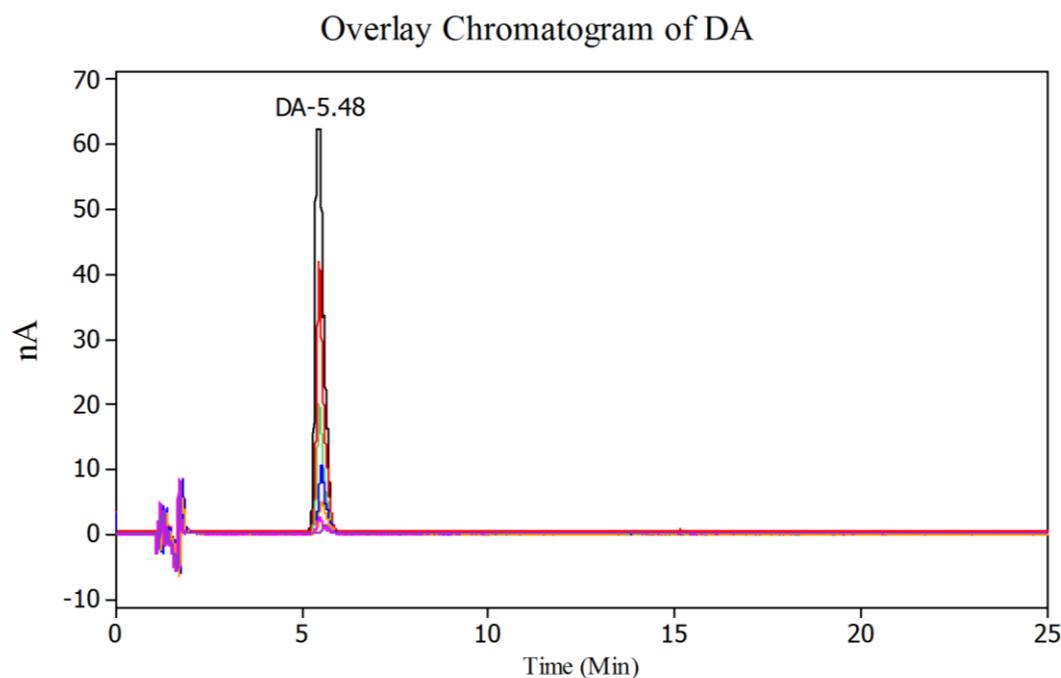


Figure 5: Standard chromatogram of DA (Dopamine)

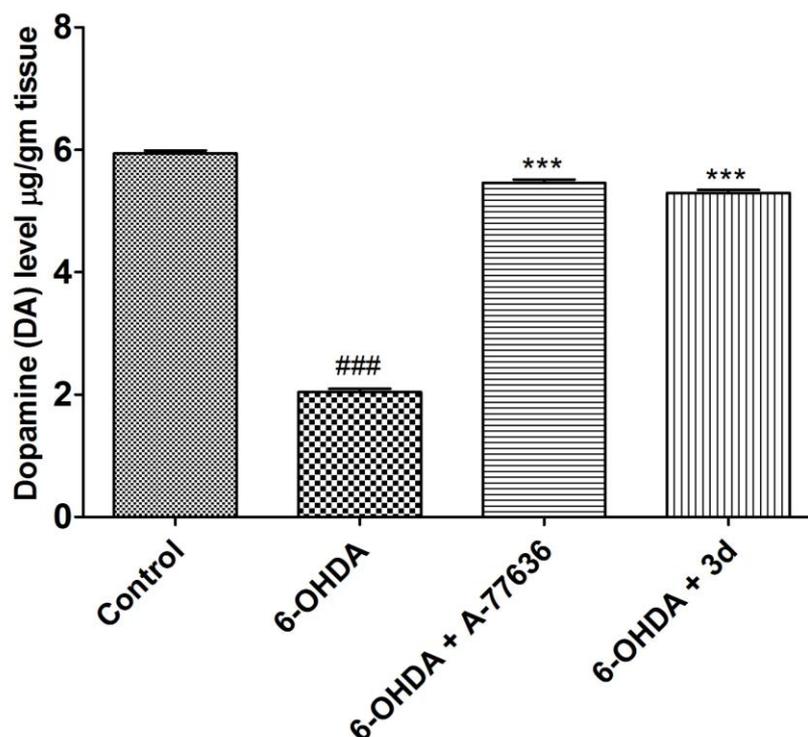


Figure 6: Effect of compound (3d) on dopamine (DA) level *ex vivo* in 6-OHDA induced Parkinson's rat model. As compared to control group, DA level significantly decreased in 6-OHDA treated group. Treatment with compound (3d) significantly increased DA level as compared to 6-OHDA treated group. Values are expressed as mean \pm SEM (n=6). Significant values were compared with ***p<0.001 vs 6-OHDA treated group & ###p<0.001 vs control group.

4.6. Compound (3d) diminishes cleaved caspase-3 level in 6-OHDA induced Parkinson's rat brain

It was explored whether administration of compound (3d) could reduce cleaved caspase-3 in 6-OHDA induced Parkinson's rat brain. Brain sections of rat treated with compound (3d) (5 mg/kg) for 7 consecutive days were subjected to immunohistochemical analysis. Quantitative analysis showed that the number of the cleaved caspase-3 positive cells were significantly reduced in substantia nigra after oral administration of compound (3d) [Figure 7 (A, B)]. A-77636 treatment also reduced the number of the cleaved caspase-3 positive cells. These results pointed towards neuroprotective effect of the compound (3d) in 6-OHDA induced Parkinson's rat brain. This data suggested that treatment with compound (3d) showed neuroprotective role in 6-OHDA induced Parkinson's rat brain at a dose of 5 mg/kg by decreasing the level of cleaved caspase-3.

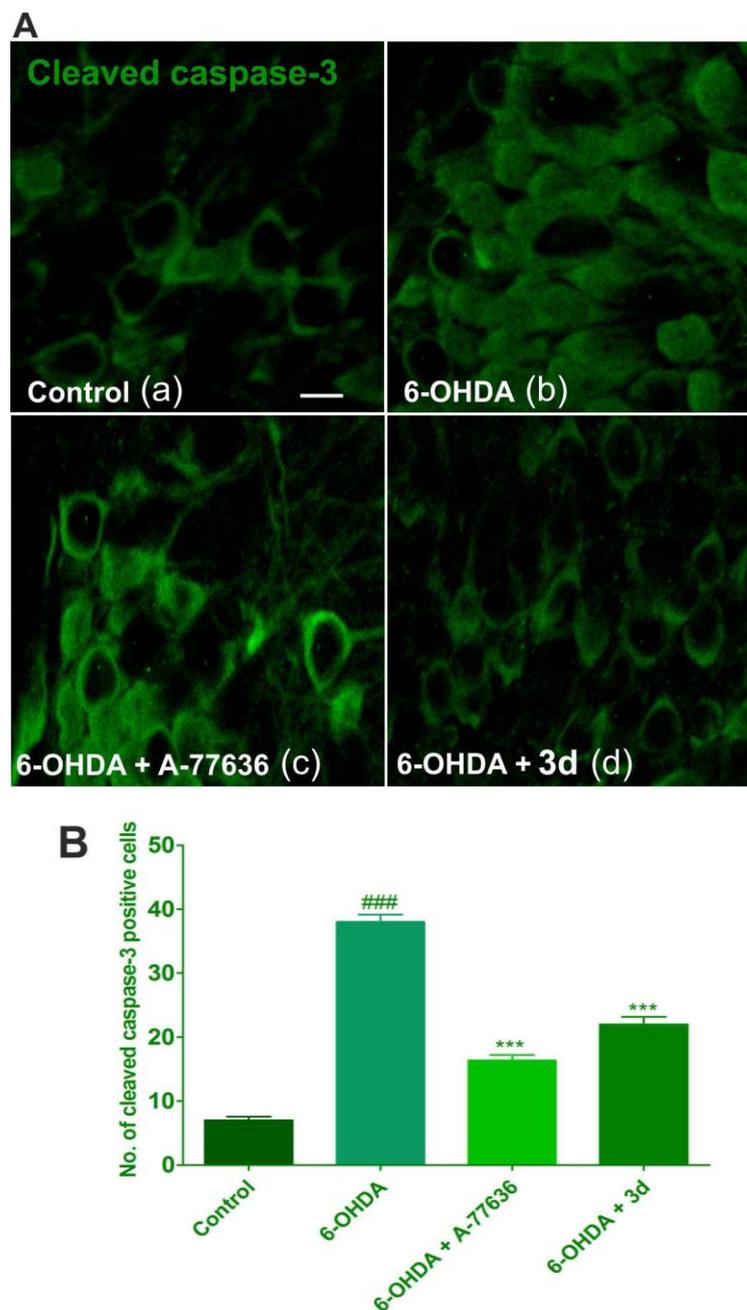


Figure 7: Effect of compound (3d) on the level of cleaved caspase-3 in 6-OHDA induced Parkinson's rat brain. (A) Confocal microscopic images show the distribution of cleaved caspase-3 in 6-OHDA induced Parkinson's rat brain (a) Control, (b) 6-OHDA treated, (c) 6-OHDA + A-77636 and 6-OHDA + compound (3d) (d) treated rats. **(B)** Quantitative analysis suggested that with treatment of compound (3d), there was significant decrease in number of cleaved caspase-3 + neurons in the substantia nigra as compared to the 6-OHDA treated group. Values are expressed as mean \pm SEM (n=6). Significant values were compared with ***p<0.001 vs 6-OHDA treated group and ###p<0.001 vs control group. Scale bar = 50 μ m.