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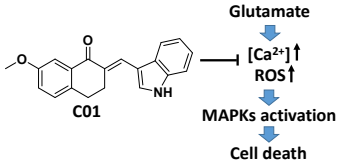

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Synthesis and Biological Evaluation of Chalcone Derivatives as Neuroprotective Agents against Glutamate-Induced HT22 Mouse Hippocampal Neuronal Cell Death

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ABSTRACT

Seventeen chalcone analogues were synthesized from 7-methoxy-3,4-dihydronaphthalen1(2H)-one and various aromatic aldehydes under basic conditions and their therapeutic properties were studied in mouse hippocampal cell line HT-22 against neuronal cell death induced by glutamate. From this study, we selected an analogue C01 as a active compound which showed significantly high neuroprotection. This compound inhibited Ca^{2+} influx and reactive oxygen species (ROS) accumulation inside cells. The glutamate-induced cell death was analyzed by flow cytometry and it showed that C01 significantly reduced apoptotic or dead cell induced by 5 mM glutamate. Western blot analysis indicates that glutamate-mediated activation of MAPKs were inhibited by compound C01 treatment. In addition, the C01enhanced Bcl-2 and decreased Bax, the anti and pro apoptotic proteins respectively. Further analysis showed that, C01 prevented the nuclear translocation of AIF (apoptosis inducing factor) and inhibited neuronal cell death. Taken together, compound C01 treatment resulted in decreased neurotoxicity induced by 5 mM of glutamate. Our finding confirmed that compound C01 has neuro-therapeutic potential against glutamate-mediated neurotoxicity.

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cytotoxicity in the central nervous system (CNS). The neuronal cells are very sensitive to oxidative stress due to their demand for relatively high oxidative energy metabolism.[1] Glutamate is known as an excitatory neurotransmitter in the CNS. However, if the concentration of glutamate is elevated, it can lead to neuronal cell death due to excessive oxidative stress.[2,3,4] Oxidative stress is implicated in various neuropathologies including Alzheimer's disease[5], Parkinson's disease[6], brain ischemic injury[7], and amyotrophic lateral sclerosis[8]. Glutamate-induced oxidative stress is caused by blocking cysteine uptake, which results in diminished the level of glutathione (GST) and accumulation of the cellular reactive oxygen species (ROS).[9,10,11] The homeostatic maintenance of the intracellular calcium ion (Ca^{2+}) levels is crucial in the physiology and pathology of neuronal cell functions. Under glutamate-induced oxidative stress, calcium ion (Ca^{2+}) levels are dramatically increased. Disrupting calcium homeostasis results in neuronal cell death.[10,12,13] Oxidative stress and Ca^{2+} influx induced by glutamate activate various cellular signaling pathways in neuronal cells. The oxidative stress induced by glutamate activate the mitogen-activated protein kinase (MAPK) pathway proteins such as ERK and p38 and JNK.[14] The MAPK signaling pathway has critical roles in many cell types in controlling cell death and survival. In previous reports, ERK activation induced by glutamate-induced oxidative stress has been shown to be related to neuronal cell toxicity.[15,16] Oxidative stress seems to induce and reduce the pro and anti-apoptotic proteins respectively.[17] Furthermore, apoptosis inducing factor (AIF) activated and translocate to the nucleus and causes apoptotic cell death.[18,19]

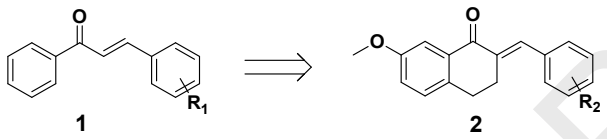
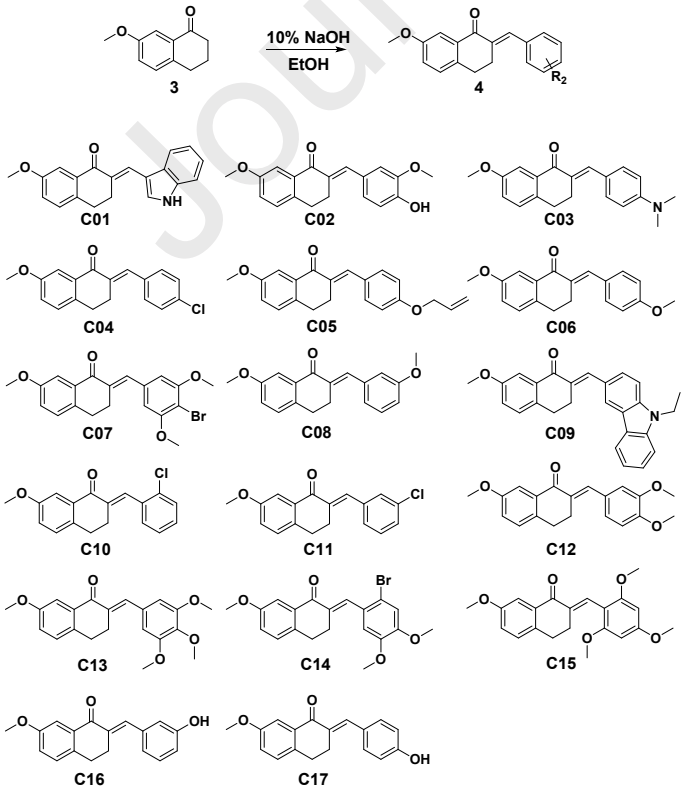


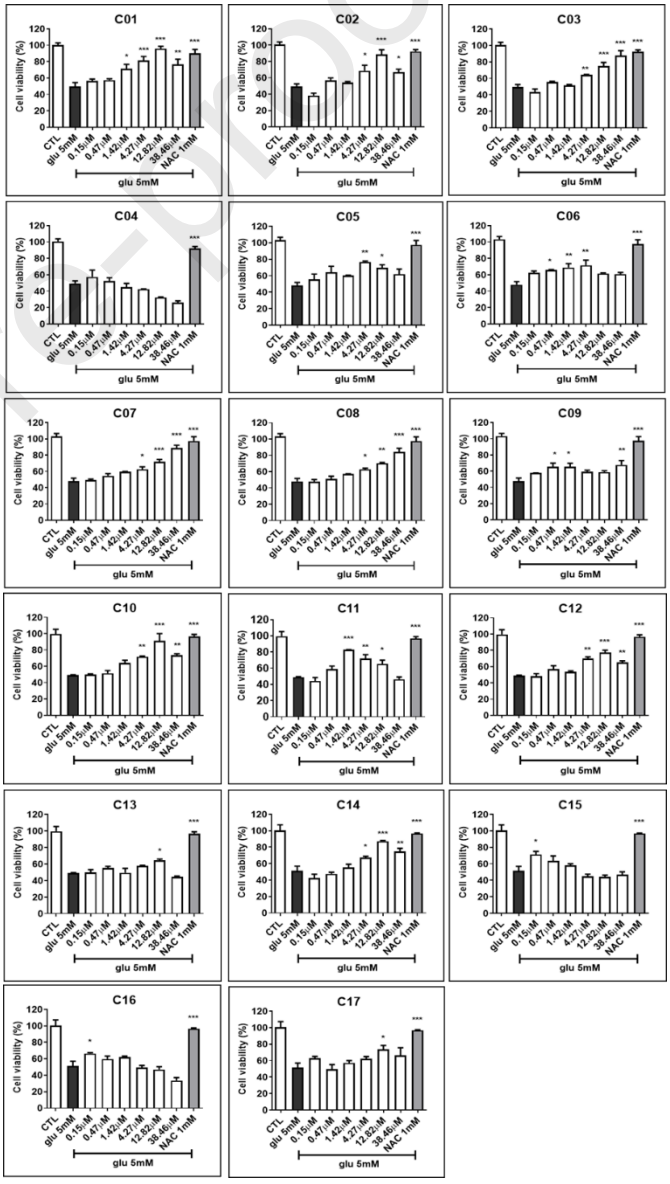
Figure 1. Design of chalcone derivatives.

Chalcones are natural products that possess diverse therapeutic capabilities such as anti-bacterial, anti-inflammatory and anti-cancer, among other.[20] In previous reports, hydroxyl chalcone and its derivatives were shown to rescue the neuronal cells from cell death caused by high concentration of glutamate treatment.[21]



ives may have significant neuroprotective potential. Therefore, we designed and synthesized chalcone derivatives, (*E*)-2-benzylidene-7-methoxy-3,4-dihydronaphthalen-1(2H)-one (2), as novel neuroprotective agents. For the synthesis of (*E*)-2-benzylidene-7-methoxy-3,4-dihydronaphthalen-1(2H)-one derivatives, 7-methoxy-3,4-dihydronaphthalen-1(2H)-one and various aryl aldehydes were used under basic conditions. Compared with a conventional chalcone structure (1), (*E*)-2-benzylidene-7-methoxy-3,4-dihydronaphthalen-1(2H)-one (2) has additional fused cyclohexanone ring, which decreases the flexibility of α,β-unsaturated bond of the chalcone scaffold (Fig. 1).

Figure 2. Synthesis of chalcone derivatives and their structures.



Figu [illegible] that effects of 17 chalcone derivatives were evaluated on HT22 cells treated with 5 mM glutamate. ***P ≤ 0.001 , **P ≤ 0.01 , *P ≤ 0.05

The synthesis of chalcone derivatives was performed under basic conditions using 10% aq. NaOH in EtOH (Fig. 2). These 17 derivatives were initially characterized by liquid chromatography-mass spectrometry (LC-MS) and further by ^{1}H-NMR. Their neuroprotection capacity was studied in HT22 using glutamate. Briefly, the cells were treated with derivatives and 5mM glutamate for 12hrs time and cell viability was measured by counting the live cell stained by calcein AM dye and propedium iodide (PI) which selectively stains dead cells. The 12hr glutamate treatment alone reduced the cell viability significantly to 50%. However, co-treatment of the cells with chalcone derivaitves at the concentration ranging from 4.27μM to 38.46 μM rescued cell viability to more than 80% (Fig. 3)

Among the 17 synthesized chalcone derivatives, compound C01 at 12.8 μM exhibited the most potent protective effects against glutamate-induced cell death (Fig 3A, 3B). In the neuroprotective assay, compounds C03, C07, C08, C12, and C14, which have a methoxy group in the phenyl ring showed potent neuroprotective effects. However, chalcone derivatives C13 and C15, which have a tri-methoxy group on phenyl ring show weak neuroprotective effects. Chalcone derivatives C16 and C17 with hydroxyl groups on the phenyl ring showed no potent neuroprotective effects against glutamate-induced cytotoxicity.

C01 shows neuroprotective effect. Calcein AM and propidium iodide staining of live and dead cells (Scale bars, 200 μm). (B) The co-treatment of HT22 cells with 12.8 μM C01 and 5 mM glutamate (glu) showed more than 80% cell survival. **P≤0.01 than the glutamate. (C,D,E,F) FACS analysis of HT22 cells after staining PI/annexin V. (C) Control. (D) 5 mM glutamate (glu) treatment. The ratio of apoptotic or dead cells is 84.97% in 5 mM glutamate (glu) treated cells, (E) but it was decreased to 8.63% by co-treatment of C01 (12.8 μM). (F) C01 treatment only. The treatment of C01 shows no toxicity in HT22 cells.

The glutamate seems to induce cell death via apoptosis and also necrosis in a different stages of cell death. The flow cytometry analysis using the Annexin-V/PI stains showed that 24 h of glutamate treatment induced apoptotic cell death extensively and reduced the percentage of live cell to 15.03%. In the presence of C01, the live cell population was restored to 91.37%. C01 treatment itself showed no cytotoxic effect on HT22 cells (Fig. 4C, 4D, 4E, 4F). This analysis indicates that C01 rescued the HT22 cells from apoptotic or death cells induced by glutamate neurotoxicity.

Glutamate initially blocks cysteine uptake, which results in depletion of endogenous antioxidant, glutathione, in HT22 cells. Therefore, antioxidants may show neuroprotective effects by reducing cellular concentrations of ROS. We therefore tested the antioxidant effect of C01 using an in-vitro 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay. It revealed that C01 has no free radical scavenging effect (Fig. 1S).

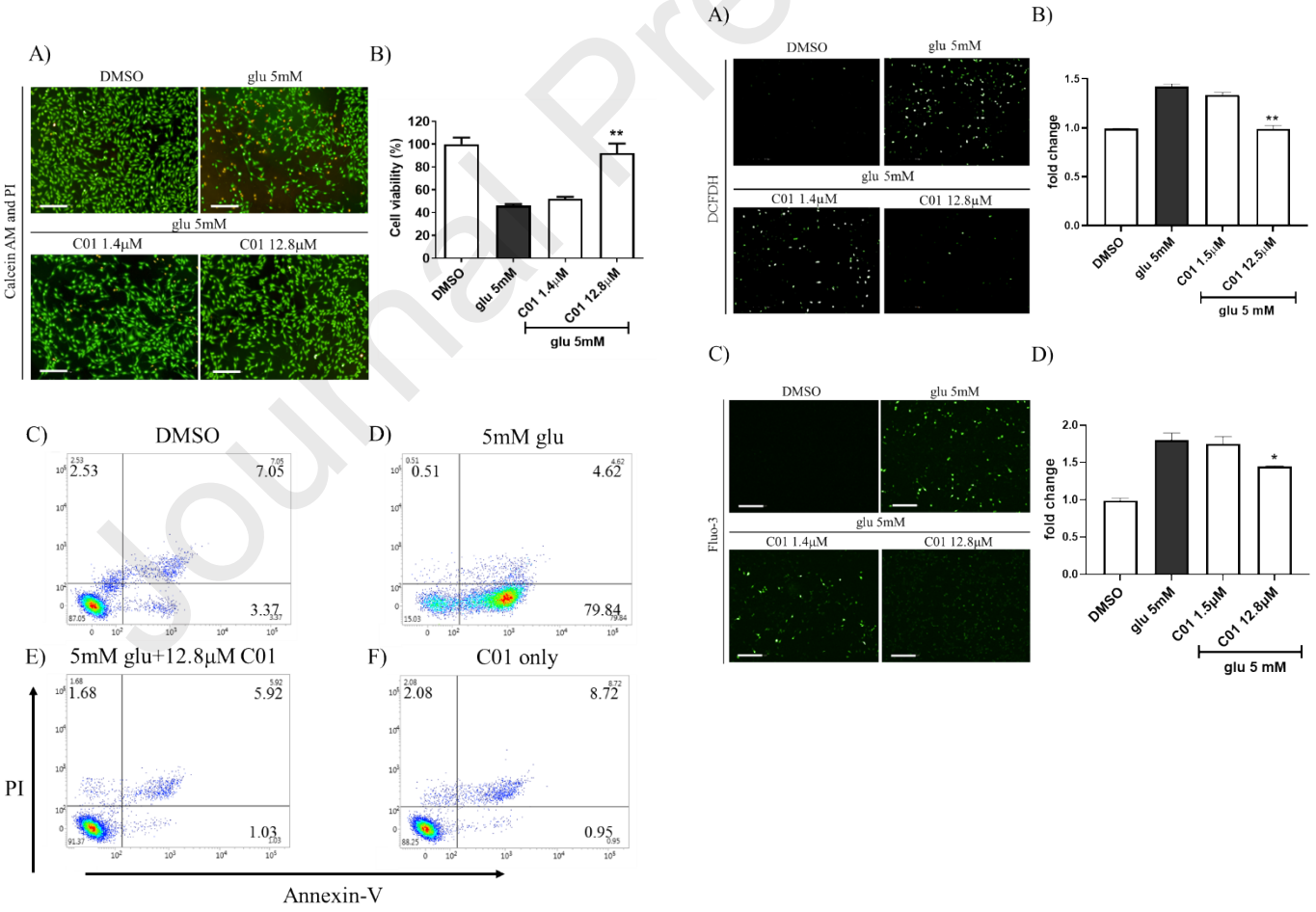


Figure 5. Effect C01 on glutamate-induced elevation of intracellular Ca^{2+} level and reactive oxygen species. (A) Fluorescent image of ROS sensing using DCFDA. (B) Fluorescent image of ROS sensor presented as bar graph. (C)

image of calcium sensor presented as bar graph. $^{**}P \leq 0.006$, $^{*}P \leq 0.03$ compared with the glutamate-treated value, Scale bars, 200 μm.

The excessive glutamate level elevates the intracellular ROS in the neuronal cells. To study the effect of C01 on intracellular ROS generation in HT22 cells we used ROS specific fluorescent dye 2' ,7'-dichlorodihydrofluorescein diacetate (DCF-DA), and we observed that 12.8 μM C01 has significantly attenuated the ROS generation induced by glutamate (Fig. 5A, 5B and 2S).

Ca^{2+} homeostasis is affected by the oxidative stress induced by glutamate. Many literatures indicate that glutamate-induced oxidative stress increases cellular Ca^{2+} uptake, which results in the activation of apoptosis. Ca^{2+} fluorescent sensor Fluo-3 AM was used to measure cellular Ca^{2+} levels. In this experiment, glutamate increased Fluo-3 AM fluorescent intensity in HT22 cells. However, co-treatment of C01 reduced the fluorescent intensity, indicating that C01 inhibited glutamate-induced cellular Ca^{2+} uptake in the HT22 cells (Fig. 5C and 5D).

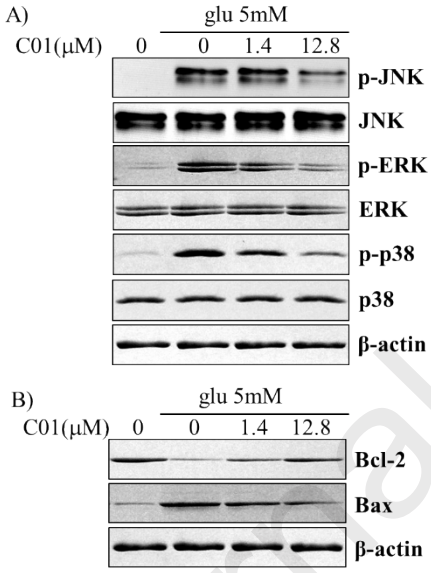


Figure 6. (A-B) Western blot analysis of p-JNK, JNK, p-ERK, ERK (p44/42 MAP kinase), p-p38, p38, Bcl-2, Bax, and β-actin (control) in HT22 cells treated with indicated concentration of C01 and 5 mM glutamate.

The MAPKs pathway associated with neuronal cell death in the downstream of oxidative stress elevated by glutamate. The increased intracellular Ca^{2+} and ROS levels have been suggested to activate MAPK signaling pathway, including p38, ERK1/2, and JNK kinases. Recent studies have indicated that inhibition of MAPK phosphorylation in HT22 cells attenuate glutamate neurotoxicity. Therefore, we investigated the effect of C01 on MAPK activation by western blot analysis. It clearly shows that the glutamate treatment increased activation of MAP kinases whereas C01 co-treatment has reduced the amount of phosphorylation of each MAPKs in concentration dependent manner. (Fig. 6A). Further, the glutamate treatment seems to affect the expression of pro and anti-apoptotic protein levels such as Bcl-2 and Bax. To study the anti-apoptotic effect of C01, we also evaluated Bax and Bcl-2 protein levels by immunoblot. As, 2 protein levels in the HT22 cells (Fig. 6B).

The previous literatures data shows that glutamate induce the cell death via caspase independent AIF apoptotic pathway. So, we studied the C01 effect on AIF translocation to the nucleus by immunoblot and immunostaining (Fig. 7). Our both results have showed AIF translocation to the nucleus by the glutamate treatment. But, the C01 has inhibited the AIF translocation to the nucleus which thereby prevented the apoptotic cell death in the HT22 cells (Fig. 7A, B).

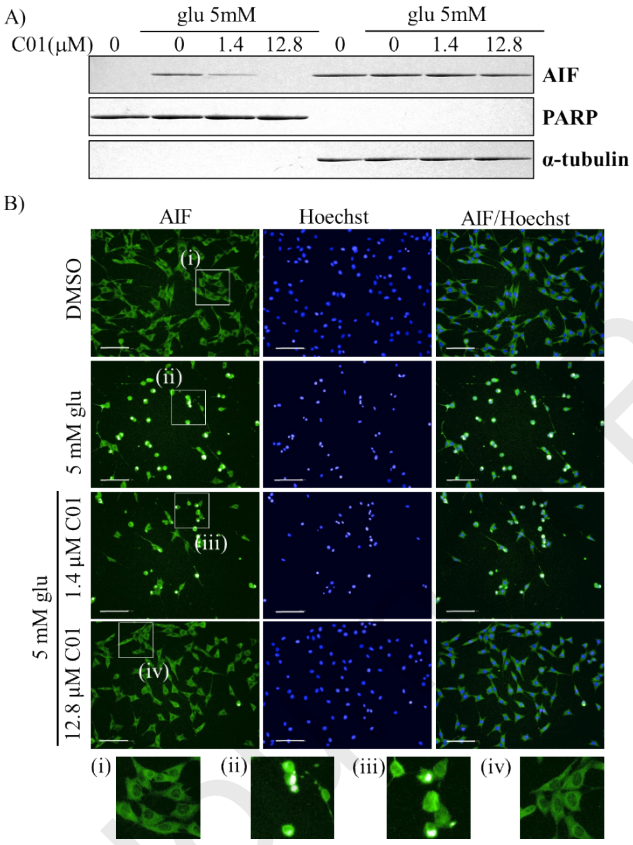
To evaluate the in vivo efficacy of C01, we tested C01 using 6-hydroxyl dopamine (6-OHDA)-treated *C. elegans* (*Caenorhabditis elegans*) model, which mimics oxidative stress-induced dopaminergic neurodegeneration.[22] It is known that 6-OHDA can disrupt dopamine signaling and affect the locomotory behavior of *C. elegans*, such as body bending activity.[23]

When *C. elegans* were treated with only 6-OHDA, the number of body movement of the worms was significantly reduced than the vehicle control group ($P < 0.001$). Cotreatment of the *C. elegans* with C01 (100 μM) significantly increased the number of body movement compared with that of worms treatment of 6-OHDA ($P < 0.001$). These data demonstrated that C01 significantly reduced the neuronal dysfunction mediated by 6-OHDA in the *C. elegans* model (Fig. 8).

Here, we have analyzed the neuroprotective effect of the synthesized chalcone derivatives and found that C01 effectively protected the HT22 neuronal cell death induced by glutamate. Also, C01 treatment successfully reduced ROS accumulation and cellular Ca^{2+} influx of glutamate. Additionally, it regulates the expression levels of pro and anti-apoptotic proteins Bcl-2 and Bax. In the downstream, C01 control the activation of MAP kinase pathway proteins ERK, JNK and p38. Also, the C01 prevented AIF-mediated apoptotic cell death by preventing the AIF nuclear translocation. Further we studied the effect of C01 on NRF2 antioxidant signaling pathway using ARE-luciferase HepG2 stable cell line. The C01 treatment has highly increased the luciferase signal and it indicate that C01 seems to strongly activate NRF2 signaling pathway (Fig. 3S). We also studied the C01 effect on ferroptotic cell death using erastin. Lately some studies showed that inhibition of ferroptosis also prevent glutamate neurotoxicity in rat hippocampal slice cultures. But there was no significant protective effect of C01 against ferroptosis observed. (Fig. 4S). [24]

This study clearly demonstrates the neuroprotective effect of C01 against glutamate neurotoxicity which mainly induced by the elevation of glutamate-induced oxidative stress. This compound seems to strongly influence the anti-oxidant signaling pathways in the downstream and attenuate the oxidative stress in the neuronal cells. As, the oxidative stress is the main cause behind many neurodegenerative diseases, the C01 might hold novel therapeutic capabilities and it has to be evaluated further.

blot analysis shows that 5 mM of glutamate treatment induced translocation of AIF into nucleus. The treatment of 12.8 μM of C01 inhibited translocation of AIF into nucleus. (B) Immunostaining of AIF in HT22 cells showed that 12.8 μM C01 treatment inhibited nuclear translocation of AIF. (i-iv) enlarged image of the boxed region.



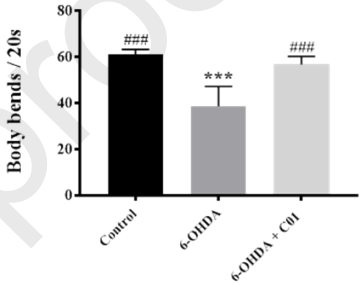


Figure 8. Effect of C01 on 6-OHDA-induced body bending depression in *C. elegans*. Age-synchronized N2 adult worms were treated with 40 mM of L-ascorbic acid, 50 mM of 6-OHDA, and 100 μM of C01 for 1 h. Then, the number of body bending were counted for 20 s. The values shown are the means ± SD (n = 14). The graph is a representative from three independent experiments. $^{***}P < 0.001$, compared to the control. $^{###}P < 0.001$, compared to the 6-OHDA single-treatment group.

Acknowledgments

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Supplementary Material

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