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Protective effects of *Dioscorea bulbifera* and *Zingiber officinale* mixed extracts against glutamate-induced cell death in HT22 cells

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Abstract

This study aimed to evaluate the neuroprotective effects of mixed extracts from *Dioscorea bulbifera* and *Zingiber officinale* (DBZO) against glutamate-induced excitotoxicity in HT-22 cells and to elucidate the associated molecular mechanisms. Neurotoxicity and protective effects were assessed using MTT and LDH assays, while cellular morphology was analyzed via microscopy. DBZO extract significantly restored axonal integrity disrupted by glutamate exposure. A DCFDA assay confirmed that DBZO reduced reactive oxygen species (ROS) generation in a concentration-dependent manner, underscoring its antioxidant capacity. Western blot analysis demonstrated that DBZO markedly decreased glutamate-induced neuronal death at 0.25 and 0.5 mg/mL. The observed neuroprotection was associated with the inhibition of the MAPK signaling cascade and the downregulation of apoptotic markers, including Caspase-3 and PARP. Moreover, DBZO activated the PI3K/Akt/mTOR survival pathway, enhancing neuronal viability. It also boosted antioxidant defenses by modulating Keap1 and NQO1 expression, thereby reducing oxidative damage. Collectively, these findings suggest that DBZO confers neuroprotection by regulating oxidative stress and apoptosis through NRf2/NQO-1 signaling. Due to its strong antioxidant and antiapoptotic properties.

Keywords *Dioscorea bulbifera*, *Zingiber officinale*, HT-22, Glutamate, Neuroprotection, PI3K/Akt/mTOR

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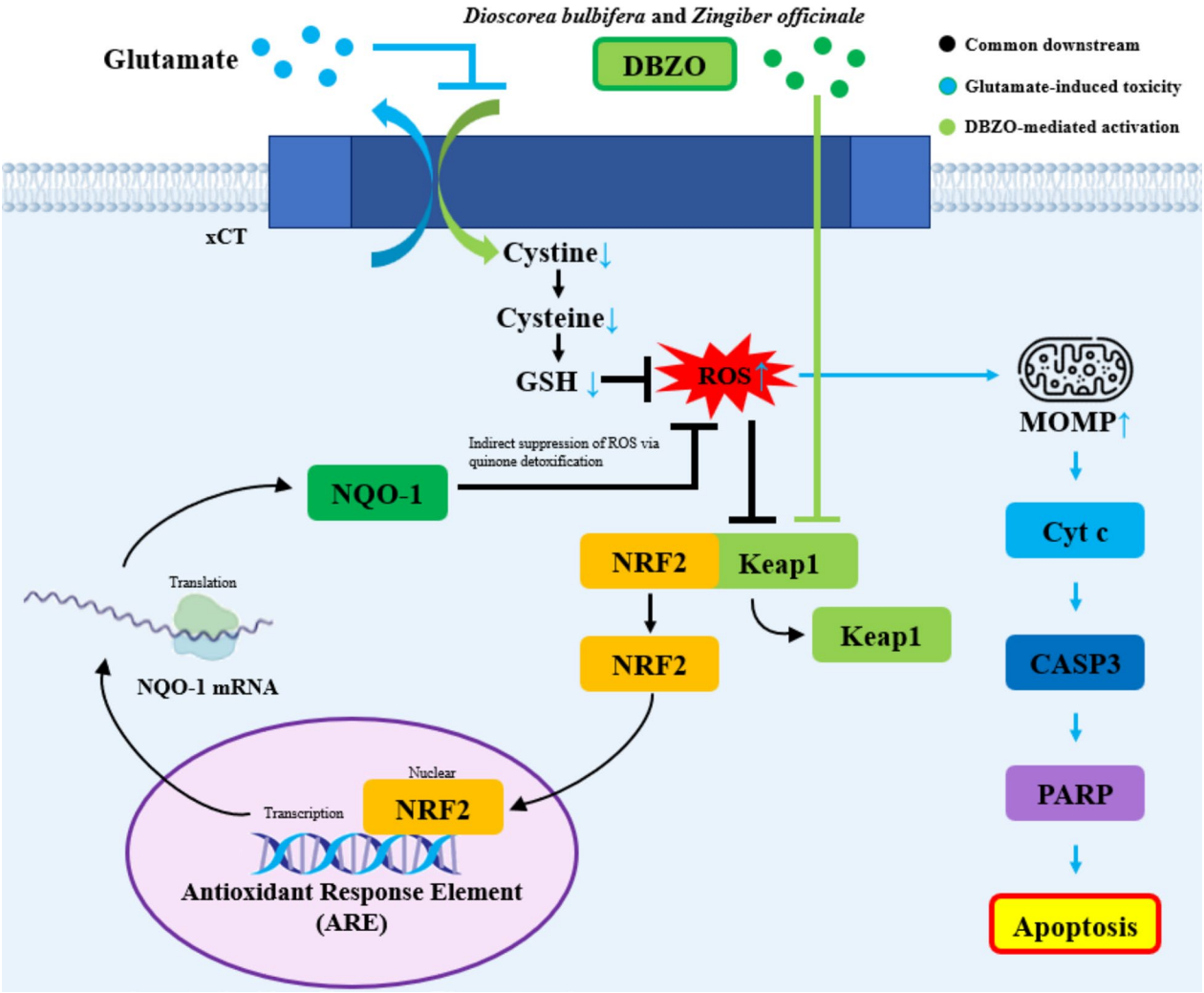
Graphical Abstract



Introduction

South Korea is projected to become a super-aged society by 2025, with individuals aged 65 and older comprising 20.6% of the total population [1]. As a result, cognitive impairment, one of the most prevalent health issues in later life, is emerging as a growing public health concern [2]. Alzheimer's disease and other progressive neurodegenerative disorders are the primary causes of dementia, collectively accounting for approximately 50–75% of all cases. These conditions are typically characterized by progressive cognitive decline, including memory loss, reduced problem-solving abilities, language impairments, and difficulties in performing everyday tasks [3, 4].

Glutamate is one of the most prominent excitatory neurotransmitters in the central nervous system (CNS), where it plays an essential role in synaptic plasticity, learning and memory. However, excessive extracellular accumulation of glutamate can lead to neuronal cell death through mechanisms such as mitochondrial dysfunction, increased production of reactive oxygen species (ROS), and impaired autophagy. These processes contribute to excitotoxicity, a pathological phenomenon closely linked to the development of various neurological disorders, including Alzheimer's disease, ischemic stroke, and Parkinson's disease [5–7].

When excitotoxicity is induced by glutamate treatment in HT-22 cells—a mouse hippocampal neuronal cell line—the central hub gene most actively involved in the interaction network has been identified as one strongly associated with Alzheimer's disease. Due to the

similarities in signaling pathways and gene expression profiles, HT-22 cells have also been proposed as a promising *in vitro* model for anti-Alzheimer's drug screening [8].

The PI3K/Akt signaling pathway plays a pivotal role in regulating essential cellular functions such as proliferation, differentiation, apoptosis, metabolism, and angiogenesis [9]. Inhibition of Keap1 leads to the activation of Nrf2 signaling, which upregulates the expression of downstream antioxidant genes such as NQO1, HO-1, SOD, and GPx, thereby reducing reactive oxygen species (ROS) levels and alleviating oxidative stress [10].

Dioscorea bulbifera L., commonly known as air potato or bitter yam, is widely distributed across Asia and Australia. In traditional Chinese medicine, it has been used to treat colorectal and thyroid cancers [11], while in Thai medicine, it serves as a diuretic, anthelmintic, antiaging agent, and a remedy for wounds and inflammation [12]. Notably, *D. bulbifera* exhibits potent antioxidant [13] and anti-inflammatory properties [14]. *Zingiber officinale* has recently been shown to possess potent antioxidant and anti-inflammatory properties, as demonstrated through network pharmacology-based investigations [15]. Previous studies have demonstrated that the combination of herbs such as *D. bulbifera* and *Z. officinale*, which share antioxidant and anti-inflammatory properties, can more effectively alleviate pathological symptoms than single-herb use due to synergistic interactions among their bioactive compounds [16]. Recent studies suggest that antioxidant intake may offer neuroprotective benefits and help attenuate cognitive decline [17, 18].

Materials and methods

Sample preparation

DBZO extract sample was prepared and provided by the Gyeongnam Anti-Aging Research Institute laboratory. Fresh *D. bulbifera* was obtained from a local market in Jinju, Republic of Korea. The samples were sliced and dried in a dry oven at 45 °C for 48 h. Dried *Z. officinale* was purchased from Donghae Corporation in Seoul, Republic of Korea. The dried *D. bulbifera* and *Z. officinale* were mixed at a weight ratio of 9:1. This mixture underwent reflux extraction using 50% ethanol at 90 °C for 4 h. The extract was then filtered, evaporated, freeze-dried, and stored at − 80 °C for subsequent experiments.

Cell culture

HT-22 cells (a mouse hippocampal neuronal cell line) were generously provided by Professor Kyung-Ho Seok (Kyungpook National University School of Medicine). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; D6546, Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin (Gibco-Invitrogen, Carlsbad, CA, USA). Cultures were maintained in a humidified incubator at 37 °C with 5% CO_2. Cells were seeded at a density of 5×10^3 cells per well in 96-well plates and 3×10^5 cells per dish in 60-mm dishes. After 24 h of seeding, cells were cotreated with DBZO extract and glutamate for an additional 24 h.

Cytotoxicity assays (MTT, LDH)

Following 24 h of treatment, cellular morphology was examined using an optical microscope. Cytotoxicity was evaluated using MTT and EZ-Cytox kits (DoGenBio, Seoul, Korea). For the MTT assay, a 5 mg/mL MTT solution was added to each well and incubated in the dark at 37 °C with 5% CO_2 for 2 h. Absorbance was then measured at 540 nm using a microplate reader (SpectraMax i3x, Molecular Devices, Sunnyvale, CA, USA). LDH release was assessed by adding LDH Reaction Mixture to the culture supernatant, followed by incubation at room temperature in the dark for 30 min. Absorbance was recorded at 450 nm.

Analysis of antioxidant activity

To evaluate intracellular ROS generation, cells were seeded in 96-well plates and 4-well chamber slides. A 5 μM working solution of 2′,7′-dichlorofluorescein diacetate (H_2DCFDA), prepared in PBS from an ethanol stock, was added to the cells and incubated for 20 min at 37 °C in the dark. After incubation, cells were washed with cold PBS, and ROS levels were measured using a microplate reader (excitation: 492 nm, emission: 590 nm). Green fluorescence intensity was also visualized at 530 nm using a fluorescence microscope (ECLIPSE Ti2, Nikon Corporation, Tokyo, Japan).

Western blot analysis

Cells were lysed on ice using RIPA buffer supplemented with protease inhibitor cocktails I and II for 30 min. Protein concentrations were measured using the BCA assay, and equal amounts of proteins were separated by SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and blocked with 5% BSA in TBS-T for 1 h. Membranes were then incubated overnight at 4 °C with primary antibodies, as listed in Table 1. Following washes, membranes were incubated with appropriate secondary antibodies at room temperature for 1 h. Protein bands were detected using an ECL detection system (Pierce, Rockford, IL, USA) and imaged with a digital imaging system (Q9 Alliance, UVITEC Ltd., Cambridge, UK). Band intensities were quantified using ImageJ software (version 1.54v) Table 2.

Table 1 List of Abbreviations

Abbreviation	Full term
DBZO	*Dioscorea bulbifera* and *Zingiber officinale*
ROS	Reactive oxygen species
MAPK	Mitogen-activated protein kinase
PARP	Poly (ADP-ribose) polymerase
PI3K	Phosphoinositide 3-kinase
Akt	Protein kinase B
mTOR	Mammalian target of rapamycin
NQO1	NAD(P)H quinone dehydrogenase 1
Keap1	Kelch-like ECH-associated protein 1
Nrf2	Nuclear factor erythroid 2–related factor 2
DCFDA	2′,7′-Dichlorofluorescein diacetate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
LDH	Lactate dehydrogenase
xCT	Cystine/glutamate antiporter
EAATs	Excitatory amino acid transporters
VGLUTs	Vesicular glutamate transporters
mGluRs	Metabotropic glutamate receptors
MOMP	Mitochondrial outer membrane permeabilization
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
ECL	Enhanced chemiluminescence
SEM	Standard error of the mean

Table 2 Primary antibodies used for Western blot

Antibody	Product no	Dilution rate	Corporation
Caspase-3	#4927	1:1000	Cell signaling
C-Caspase-3	#8592	1:1000	Cell signaling
PARP	#9542	1:1000	Cell signaling
ERK1/2	#4695	1:1000	Cell signaling
p-ERK1/2	#4370	1:1000	Cell signaling
JNK	#9252	1:1000	Cell signaling
p-JNK	#4668	1:1000	Cell signaling
p38	#8690	1:1000	Cell signaling
p-p38	#4511	1:1000	Cell signaling
PI3K	#4263	1:1000	Cell signaling
p-PI3K	#17366	1:1000	cell signaling
Akt	#4691	1:1000	Cell signaling
p-Akt	#4060	1:1000	Cell signaling
Keap1	#8047	1:1000	Cell signaling
Actin	#3700	1:1000	Cell signaling
NQO-1	#11451-1-AP	1:1000	Proteintech

Instrumental parameters for qualitative analysis

The analysis was performed utilizing an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm; Waters, Milford, MA, USA) on a UPLC-Q-TOF/MS system (Waters, U.S.A). The column was maintained at a temperature of 40 °C, and the mobile phase consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B), delivered at a flow rate of 0.35 mL/min. The chromatographic separation involved an initial hold at 1% solvent B for 1 min, followed by a linear gradient increase to 100% solvent B over 7 min (from 1 to 8 min). This high concentration of solvent B was maintained for 1 min (from 8 to 9 min), then decreased to 1% over 0.5 min (from 9 to 9.5 min), and finally held at 1% for an additional 2.5 min (from 9.5 to 12 min). The column effluent was subsequently analyzed using the Q-TOF-MS in positive electrospray ionization (ESI) mode. The mass spectrometry parameters were as follows: capillary voltage, 3 kV; source temperature, 100 °C; desolvation temperature, 400 °C; cone gas flow, 30 L/h; desolvation gas flow, 800 L/h; collision energy, 20–40 eV; and a mass-to-charge ratio range of 50–1500 m/z.

Statistical analysis

All data are presented as the mean ± standard error of the mean. Statistical analysis was performed using GraphPad PRISM version 5.0 (GraphPad Software, San Diego, CA, USA). One-way analysis of variance followed by Tukey's post hoc test was conducted. A p-value of < 0.05 was considered statistically significant.

Results

DBZO inhibits glutamate-induced excitotoxicity in HT-22 cells

To investigate the protective effects of *D. bulbifera* and *Z. officinale* extract (DBZO) against glutamate-induced excitotoxicity, cell viability was assessed following treatment with glutamate, DBZO, and their combination. Glutamate reduced HT-22 cell viability in a dose-dependent manner, with 5 mM glutamate resulting in 63.4% (± 8.14) viability (Fig. 1A). DBZO alone did not exhibit significant cytotoxicity up to 1 mg/mL and even promoted cell growth at certain concentrations, although further investigation is required to determine whether this effect is beneficial or adverse (Fig. 1B). Cotreatment of DBZO with 5 mM glutamate produced dose-dependent neuroprotective effects (Fig. 1C). These findings were further validated by the LDH assay, which produced results consistent with the MTT assay (Fig. 1D). Morphological changes were also observed under an optical microscope (Fig. 1E), collectively supporting the conclusion that DBZO confers neuroprotection in HT-22 cells under excitotoxic conditions.

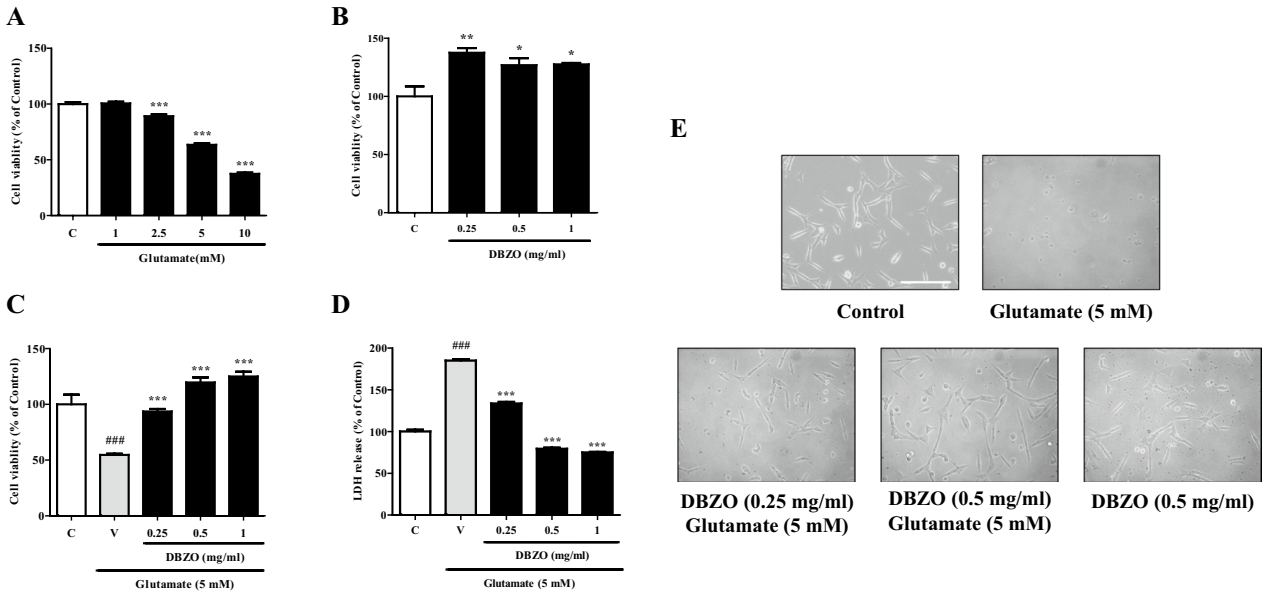


Fig. 1 Cytotoxicity assessment of glutamate and DBZO in HT-22 cells. Cytotoxic effects of glutamate and DBZO were evaluated using the MTT assay, and the neuroprotective potential of DBZO against glutamate-induced excitotoxicity was assessed (**A**–**C**). Additional confirmation of cytotoxicity was obtained via the LDH assay (**D**). Morphological changes in HT-22 cells following treatment were observed under an optical microscope (**E**). Data are presented as mean ± SEM from three independent experiments. $^{###}p < 0.001$ vs. control (**C**); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. glutamate-treated group(V). Scale bar = 100 μm

DBZO reduces glutamate-induced oxidative stress (ROS) in HT-22 cells

To determine whether DBZO mitigates glutamate-induced ROS accumulation, HT-22 cells were treated with glutamate and DBZO for 24 h, and ROS levels were measured using DCFDA staining. Excess extracellular glutamate inhibits the cystine/glutamate antiporter (xCT), which disrupts glutathione (GSH) synthesis and leads to ROS buildup [19]. As expected, glutamate significantly increased ROS levels (Fig. 2A), whereas DBZO cotreatment reduced ROS accumulation in a dose-dependent manner (Fig. 2B), indicating that DBZO possesses antioxidant properties in this context.

Glutamate-induced cell death is mediated by ROS and mitochondrial dysfunction via cytochrome c/Caspase-3/PARP activation

Previous studies indicate that increased ROS levels reduce mitochondrial membrane potential (MOMP), release cytochrome c, and activate mitochondrial-dependent apoptosis pathways such as Caspase-3 [20]. In our study, glutamate markedly increased the expression of cytochrome c, Caspase-3, cleaved Caspase-3, and PARP proteins, indicating activation of apoptosis. DBZO cotreatment attenuated this upregulation, implying its role in inhibiting glutamate-induced apoptotic signaling (Fig. 3A, B).

DBZO inhibits apoptotic MAPK pathways activated by glutamate-induced excitotoxicity

The MAPK pathway plays a critical role in regulating cell survival, differentiation, and immune responses [21]. Specifically, JNK and p38 are known to promote apoptosis via the JNK/Cytochrome c/Caspase-9 axis [22, 23]. In this study, glutamate treatment markedly increased the levels of phosphorylated JNK, ERK1/2, and p38, whereas cotreatment with DBZO reduced their expression (Fig. 4A, B). These results indicate that DBZO may confer neuroprotection by suppressing the JNK-mediated apoptotic signaling pathway.

DBZO protects against glutamate-induced cell death by modulating the PI3K/Akt pathway

The PI3K/Akt signaling cascade is crucial for neuronal survival and is known to be downregulated following glutamate exposure [24]. Consistent with prior reports, glutamate reduced PI3K protein levels in our study (Fig. 5A). However, Akt phosphorylation at Ser473 and Thr308 was increased by glutamate treatment, and this was attenuated by co-treatment with DBZO (Fig. 5A, B).

DBZO enhances antioxidant defense by promoting Nrf2 nuclear translocation via Keap1 suppression

Under oxidative stress, Keap1 inhibition facilitates the nuclear translocation of Nrf2, leading to the expression of antioxidant enzymes such as NQO-1 [25]. Our results

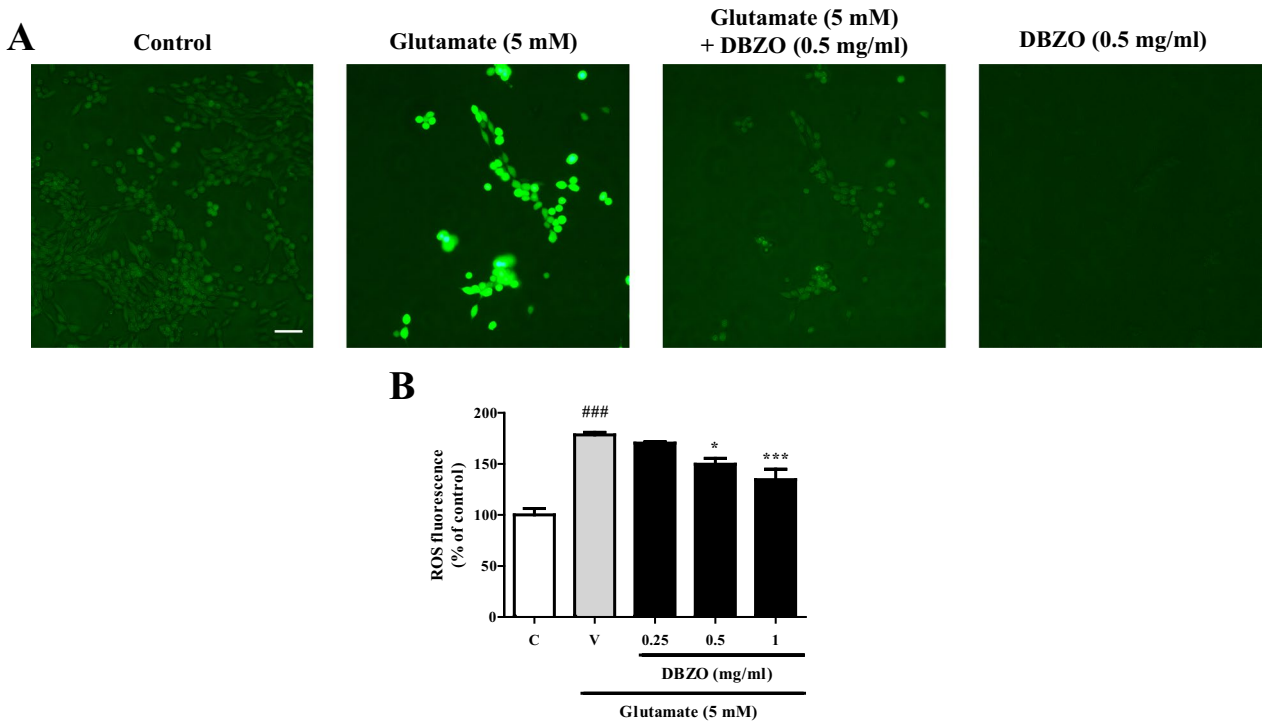


Fig. 2 Measurement of reactive oxygen species (ROS) levels induced by glutamate and DBZO in HT-22 cells. Cells were stained with DCFDA, and fluorescence intensity was visualized by microscopy (**A**) and quantified using a microplate reader (**B**). Data are presented as mean ± SEM from three independent experiments. $^{###}p < 0.001$ vs. control (**C**); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* glutamate-treated group. Scale bar = 100 μm

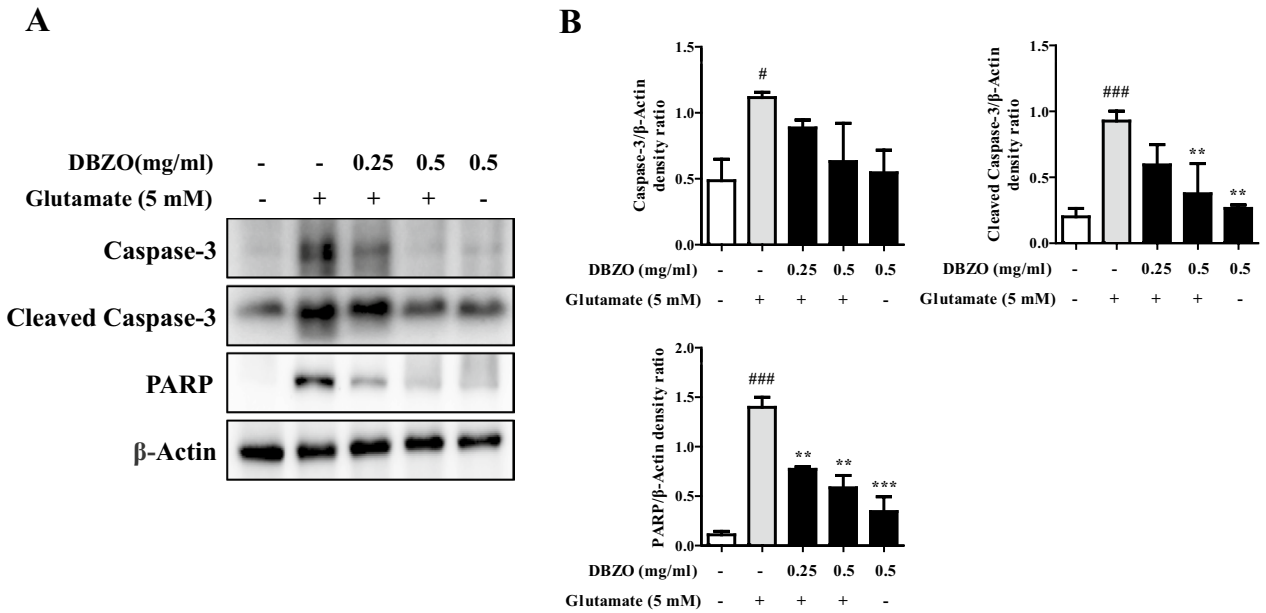


Fig. 3 DBZO suppresses glutamate-induced apoptosis by modulating Caspase-3 and PARP signaling in HT-22 cells. Western blot analysis was performed to detect the expression levels of Caspase-3, cleaved Caspase-3, and PARP following treatment (**A**). Band intensities were quantified using ImageJ software (**B**). Data are presented as mean ± SEM from three independent experiments. $^{#}p < 0.05$, $^{##}p < 0.01$, $^{###}p < 0.001$ *vs.* control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. glutamate-treated group. Scale bar = 100 μm

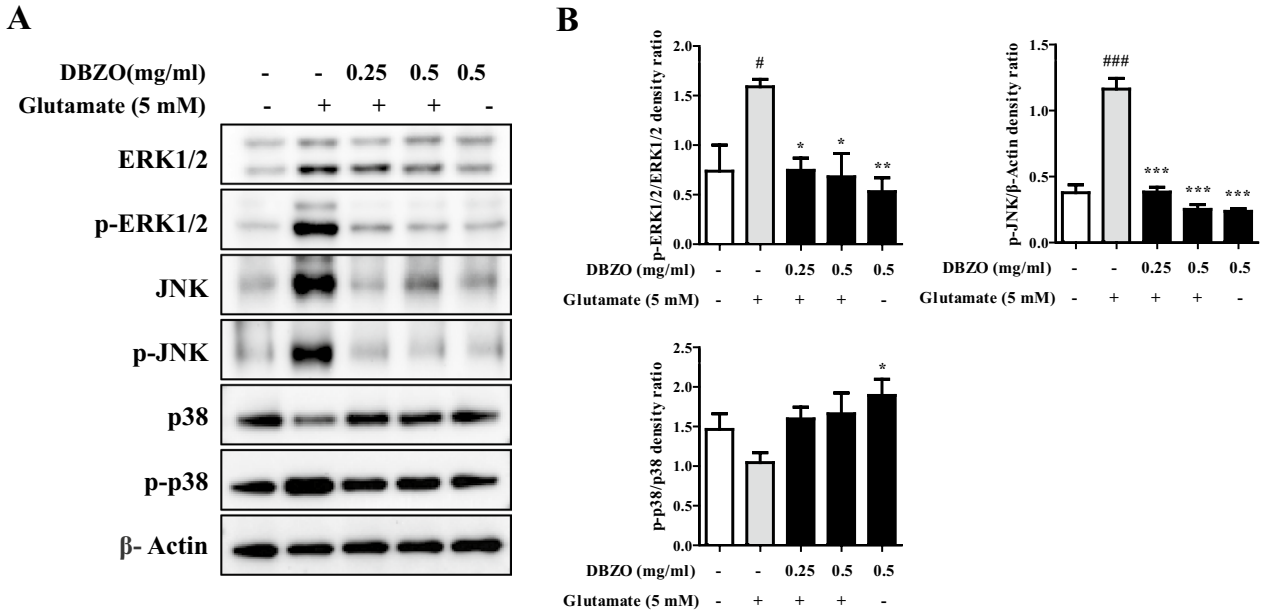


Fig. 4 Glutamate activates apoptosis via the JNK/p38 MAPK pathway, which is inhibited by DBZO. Western blot analysis was conducted to evaluate the phosphorylation of ERK1/2, JNK, and p38 (**A**), and the corresponding protein expression levels were quantified (**B**). Data are shown as mean ± SEM from three independent experiments. $^{###}p < 0.001$, $^{#}p < 0.05$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. glutamate-treated group

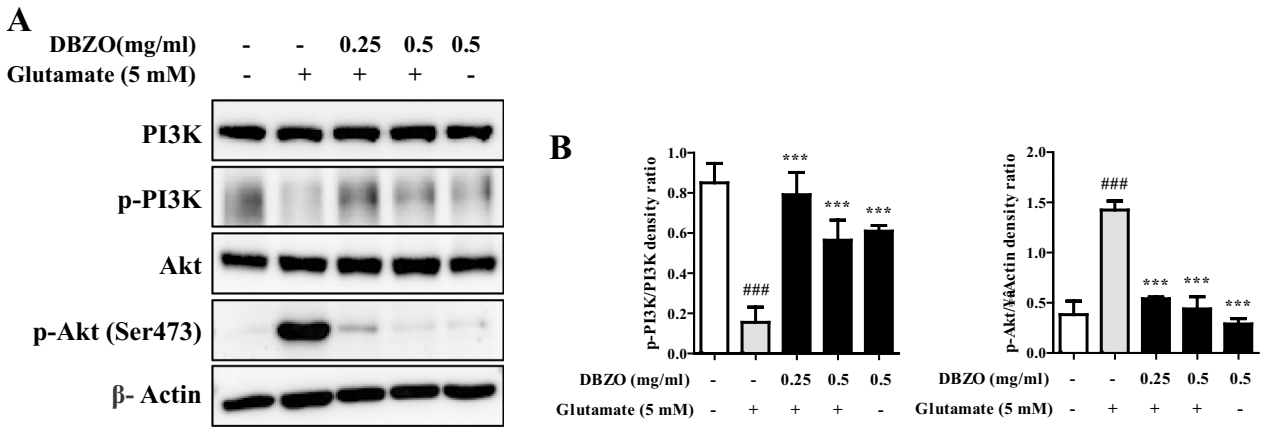


Fig. 5 DBZO protects HT-22 cells from glutamate-induced cytotoxicity via activation of the PI3K/Akt signaling pathway. Protein levels of PI3K and Akt (total and phosphorylated forms) were analyzed by Western blotting (**A**), and the results were quantified (**B**). Data are presented as mean ± SEM from three independent experiments. $^{###}p < 0.001$ vs. control group; $^{***}p < 0.001$ vs. glutamate-treated group

show that DBZO decreased Keap1 expression, promoted Nrf2 nuclear translocation, and subsequently elevated NQO-1 levels, indicating that DBZO activates antioxidant defense mechanisms to exert its cytoprotective effect (Fig. 6A, B).

Qualitative determination of bioactive compounds

The bioactive compounds of DBZO were elucidated through UPLC-QTOF/MS, revealing a distinct set of compounds. Their identification relied on specific retention times (RTs) and mass spectrometric data. Catechin, with an RT of 3.40 min, yielded an adduct ion at 291, producing fragments at 139 and 273. Astragalin (kaempferol-3-glucoside) was detected at 3.99 min (RT), presenting an adduct ion of 449 and fragments at 257, 269, and 287. 6-Gingerol exhibited an RT of 5.88 min, characterized by an adduct ion of 317 and fragments at 115, 117, and 145. 8-Gingerol

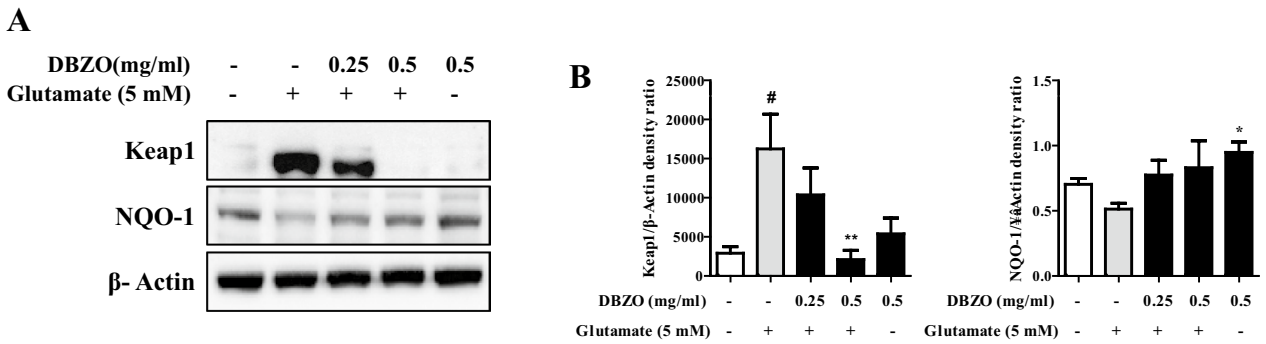


Fig. 6 DBZO enhances antioxidant defense through the Keap1-Nrf2/NQO1 signaling pathway. Western blotting was used to assess the expression of Keap1, Nrf2, and NQO1 in HT-22 cells (**A**), with band intensities quantified using ImageJ (**B**). Data are shown as mean ± SEM from three independent experiments. $^{\#\#}p < 0.01$ vs. control group; $^{*}p < 0.05$ vs. glutamate-treated group

had an RT of 6.66 min, with an adduct ion of 345 and fragments at 115 and 145. Lastly, 6-shogaol was found at 6.82 min (RT), showing an adduct ion of 277, with fragments at 94, 122, 137, and 177. These findings correlate with existing literature [26–28] and are comprehensively detailed in Fig. 7.

Discussion

Previous research has highlighted the neuroprotective effects of *D. bulbifera* (DBZO), attributed to its antioxidant and anti-inflammatory properties [29]. These effects are largely due to its rich content of natural antioxidants, including flavonoids, phenolic acids, tocopherols, vitamin C, and tannins, which help mitigate oxidative stress and protect neural tissues [30]. However, such effects have not yet been explored in HT-22 cells. Therefore, the

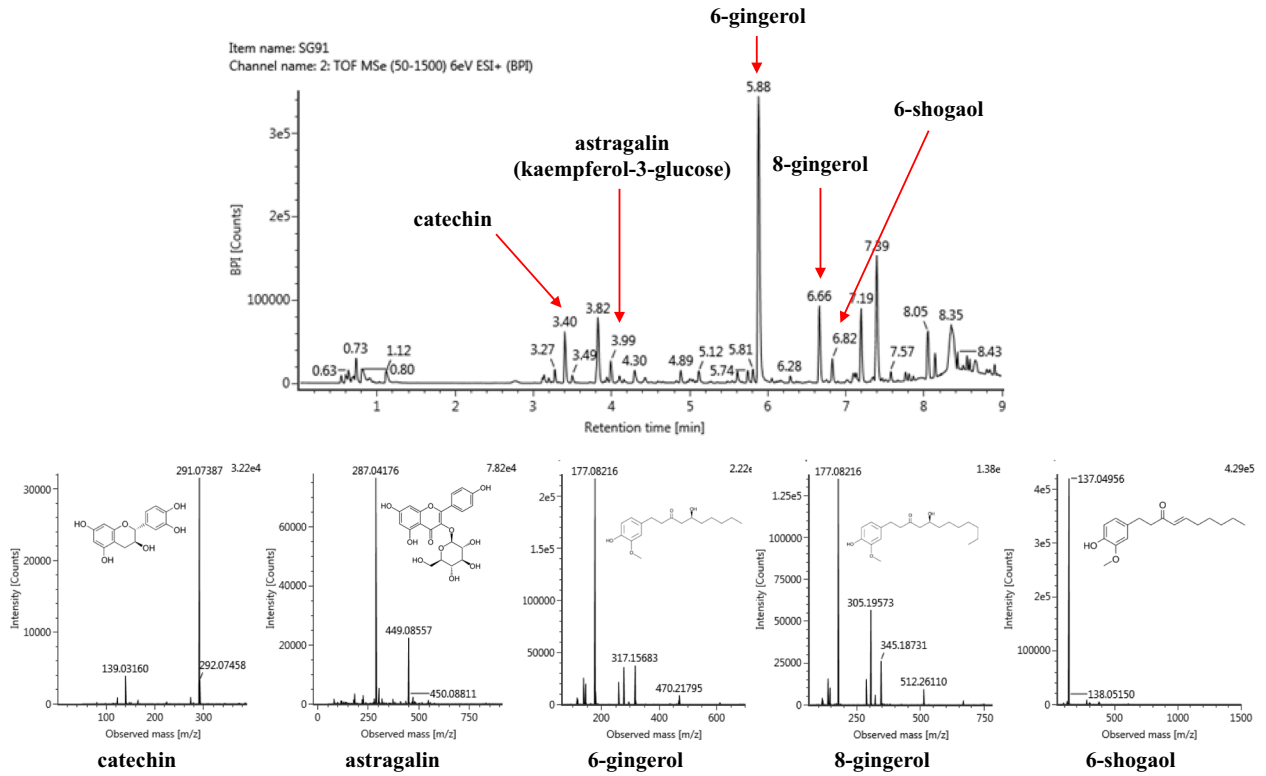


Fig. 7 UPLC-Q-TOF/MS chromatogram and mass Spectra of the DBZO combined extract

present study aimed to evaluate whether DBZO exerts neuroprotective effects in the HT-22 hippocampal neuronal cell model. To determine the optimal concentration of DBZO, we initially conducted dose–response cytotoxicity assays with glutamate and DBZO individually (Fig. 1A, B). Subsequent cotreatment experiments demonstrated that DBZO significantly attenuated glutamate-induced neurotoxicity in a dose-dependent manner (Fig. 1C–E).

Under normal physiological conditions, glutamate plays a vital role in the CNS by regulating neurogenesis, synapse formation, neurite outgrowth, and neuronal survival [31]. These functions involve various glutamate transporters including EAATs, VGLUTs, and the cystine/glutamate antiporter (xCT), as well as glutamate receptors such as NMDA, AMPA, mGluRs, and voltage-dependent ion channels [29]. However, excessive extracellular glutamate inhibits the xCT antiporter, leading to reduced intracellular cysteine levels. This impairs the synthesis of glutathione (GSH), a key cellular antioxidant, thereby elevating oxidative stress [32]. In this study, glutamate treatment significantly increased ROS levels, confirming the induction of oxidative stress in HT-22 cells. Cotreatment with DBZO markedly reduced ROS accumulation (Fig. 2A, B), indicating that DBZO effectively mitigates glutamate-induced oxidative stress.

Oxidative stress can diminish mitochondrial membrane potential, leading to the release of cytochrome c and activation of intrinsic apoptotic pathways involving Caspase-3 and PARP [33, 34]. Consistent with this mechanism, glutamate significantly upregulated Caspase-3 and PARP expression, whereas DBZO cotreatment effectively suppressed their activation (Fig. 3A, B). We further examined the MAPK signaling pathway, as JNK and p38 are known to promote apoptosis through Caspase-3 activation, while ERK1/2 generally supports cell survival. Glutamate markedly increased JNK phosphorylation and showed a trend toward elevated ERK1/2 and p38 phosphorylation. These changes were mitigated by DBZO, suggesting that DBZO suppresses apoptosis by modulating MAPK signaling (Fig. 4A, B).

The PI3K/Akt pathway plays a crucial role in supporting neuronal survival and inhibiting apoptosis [35]. Consistent with previous findings, glutamate reduced PI3K expression, which was restored by DBZO treatment. Interestingly, Akt phosphorylation was increased at both Ser473 and Thr308. This may be attributed to several factors, including PI3K-independent Akt activation [36, 37], ROS accumulation due to impaired antioxidant defense systems, and subsequent inhibition of Akt dephosphorylation caused by PTEN or PP2A inactivation [38]. However, these interpretations remain hypothetical and require further experimental validation.

Notably, co-administration of DBZO restored PI3K expression and normalized excessive Akt phosphorylation, suggesting that the neuroprotective effect of DBZO may be associated with the regulation of this survival pathway (Fig. 5A, B).

Keap1 is a negative regulator of Nrf2, a transcription factor that induces antioxidant enzymes such as HO-1 and NQO1 [39]. During oxidative stress, Keap1 is inhibited, allowing Nrf2 to translocate to the nucleus and activate cellular antioxidant defenses. Our data show that DBZO reduced Keap1 expression and increased NQO1 levels, indicating that DBZO enhances antioxidant capacity through the Keap1-Nrf2 signaling axis (Fig. 6A, B). Taken together, our findings indicate that DBZO exerts neuroprotective effects in HT-22 cells by attenuating glutamate-induced oxidative stress and apoptosis. These protective actions are mediated through the modulation of MAPK and PI3K/Akt signaling pathways, along with activation of the Keap1-Nrf2 antioxidant axis.

Thus, this study identified the signaling pathway through which DBZO attenuates glutamate-induced oxidative stress and demonstrated its potential neuroprotective effects.

In conclusion, this study demonstrates that DBZO confers neuroprotection in HT-22 cells by mitigating glutamate-induced oxidative stress and mitochondrial dysfunction. DBZO effectively suppressed ROS production, thereby preventing key features of mitochondria-dependent apoptosis, including ATP depletion and loss of mitochondrial membrane potential.

Furthermore, DBZO modulated essential intracellular signaling mechanisms—specifically, it inhibited JNK activation and enhanced the Keap1/NQO-1 antioxidant pathways—resulting in decreased ROS levels and suppression of apoptotic cascades. These results are limited to in vitro experiments, and additional in vivo studies are needed to evaluate the efficacy of DBZO at therapeutically relevant concentrations.

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Not applicable.

Author contributions
TWO conceived of the idea presented. TWO, SWI, WMJ and DYL carried out the experiments and verified the analytical methods. In more detail, TWO and SWI studied cell experiments and related mechanisms, and WMJ and DYL studied the analysis of extracts. SWI, TWO wrote the manuscript with input from all authors. DYL, TWO contributed to the interpretation of the results. All authors discussed the results and provide critical feedback to the final manuscript. All authors read and approved the final manuscript.

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on the bioactivity of Dioscorea bulbifera extract and its efficacy in preventing multiple diseases).

Data availability
All data discussed in the paper are available in the main text and Supplementary Information. Materials used in the paper are available upon request from the corresponding authors.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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