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ORIGINAL PAPER

Gartanin Protects Neurons against Glutamate-Induced Cell Death in HT22 Cells: Independence of Nrf-2 but Involvement of HO-1 and AMPK

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Abstract Oxidative stress mediates the pathogenesis of neurodegenerative disorders. Gartanin, a natural xanthone of mangosteen, possesses multipharmacological activities. Herein, the neuroprotection capacity of gartanin against glutamate-induced damage in HT22 cells and its possible mechanism(s) were investigated for the first time. Glutamate resulted in cell death in a dose-dependent manner and supplementation of 1–10 μM gartanin prevented the detrimental effects of glutamate on cell survival. Additional investigations on the underlying mechanisms suggested that gartanin could effectively reduce glutamate-induced intracellular ROS generation and mitochondrial depolarization. We further found that gartanin induced HO-1 expression independent of nuclear factor erythroid-derived 2-like 2 (Nrf2). Subsequent studies revealed that the inhibitory effects of gartanin on glutamate-induced apoptosis were partially blocked by small interfering RNA-mediated knockdown of HO-1. Finally, the protein expression of phosphorylation of AMP-activated protein kinase (AMPK) and its downstream signal molecules, Sirtuin activator (SIRT1) and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), increased after gartanin treatment. Taken together, these findings suggest gartanin is a potential neuroprotective agent against glutamate-induced oxidative injury partially through increasing Nrf-2-independed HO-1 and AMPK/SIRT1/PGC-1α signaling pathways.

Keywords Gartanin · Oxidative stress · Neuroprotective · Nuclear factor erythroid-derived 2-like 2 · Heme oxygenase 1 · AMP-activated protein kinase

Abbreviations

PD	Parkinson's disease
AD	Alzheimer's disease
ROS	Reactive oxygen species
ΔΨm	Mitochondrial membrane potential
HO-1	Heme oxygenase 1
Nrf-2	Nuclear factor erythroid-derived 2-like 2
AMPK	AMP-activated protein kinase
H_2DCF-DA	H_2DCF-DA dichlorodihydrofluorescein diacetate
DHE	Dihydroethidium
R123	Rhodamine 123
siRNA	Small interfering RNA

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LC3	Microtubule-associated protein light chain 3
PPARα	Peroxisome proliferator-activated receptor α
SIRT1	Sirtuin activator 1
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator-1α

Introduction

Oxidative stress has been implicated in a variety of chronic neurodegenerative disorders, such as Parkinson's disease (PD), Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS) [1]. Excessive reactive oxygen and nitrogen species (ROS and RNS) production damage redox balance, oxidate DNA, especially mitochondrial DNA, protein as well as lipid, and eventually lead to the impairment of biological function [2]. Sustained oxidative stress results in axonal degeneration and neuronal apoptosis and finally induces damage to brain structures [1, 3]. Glutamate is the principal excitatory amino acid neurotransmitter with complex biological activities [4, 5]. However, high concentration of extracellular glutamate is toxic to nerve cells and is thought to be a key contributor in the pathogenesis of AD and PD [6–8]. The pathway of glutamate-induced cell death in the nervous system can be activated by either excitotoxicity or oxidative toxicity (glutamate-induced oxytosis). HT22 cell line is derived from the hippocampus neuronal precursor cells and lacks functional ionotropic glutamate receptors. Glutamate can block glutamate-cystine antiporters, deplete cellular glutathione (GSH), and finally induce necrosis and apoptosis instead of excitocytoxicity in HT22 cells. In addition, glutamate toxicity induces the intracellular ROS accumulation, commonly accompanied by the collapse of the mitochondrial membrane potential ($\Delta\Psi m$). This makes the HT22 cell line serve as an excellent model of glutamate-induced oxidative neurotoxicity [9–12]. Therefore, the glutamate-induced cell death in HT22 cells has been widely used as an in vitro assay to screen neuroprotective compounds and to elucidate its neuroprotective mechanisms [13–15].

Heme oxygenase (HO) is an enzyme that catalyzes the degradation of heme [16]. HO isoforms catalyze the conversion of heme to carbon monoxide (CO) and bilirubin with a concurrent release of iron, which can drive the synthesis of ferritin for iron sequestration [17]. HO is the sole physiological pathway of heme degradation and, consequently, plays a critical role in the regulation of cellular heme-dependent enzyme levels [18]. HO-1, the inducible isoform, is found ubiquitously in all organs with the exception of the adult brain and is rapidly and transiently expressed after various stressor stimuli [19]. Although initial interest in HO-1 focused on its role in heme metabolism, recent studies hat HO-1 is highly induced by agents that cause oxidative stress have renewed interest in the regulation and function of HO-1 [20]. Being one of the most representative vitagenes, HO-1 plays a crucial role in oxidative stress processes, apoptosis and cell differentiation [21]. Now lots of reports have revealed that small molecules, such as neolignans [22], acerogenin A [23], sanguinarine [24], piceatannol [25] could antagonize glutamate-mediated oxidative injury in HT22 neuronal cells through the induction of HO-1.

Herbaceous plants are rich sources of biologically active compounds. Now, to explore natural antioxidants from herbs has become an alternative strategy for devastating degenerative pathologies [26–28]. Mangosteen (*Garcinia mangostana L*), ''the queen of fruits'', is native to Thailand, Malaysia and other tropical countries [29, 30]. The purple pericarp of mangosteen contains rich healthy nutrients and pharmacologically active compounds including prenylated xanthones, triterpenes, tannins and benzophenones [31, 32]. According to the reported work, more than 68 distinct xanthones have been identified in different parts of the mangosteen plant, and among them, 50 xanthones have a higher abundance in pericarp than in other parts [33, 34]. Those xanthones possess a wide range of pharmacological activities, including anti-inflammatory, neuroprotective, cardioprotective, antioxidative, antimicrobial, antiretroviral, antimalarial, analgesic and anti-complement activities, and so on [30, 31, 35–37].

Gartanin is a xanthone isolated from the mangosteen pericarp and was first reported in 1971 [38]. Increasing studies have reported that gartanin exert several biological actions, such as anti-bacterial, anti-cancer, anti-inflammatory and anti-oxidant activity [39–41], but the underlying mechanisms of neuroprotective effects have not yet been clarified. As gartanin contains xanthone nuclear structure, featured phenolic hydroxy and isoprene moieties, which are the characteristics of an antioxidant, its antioxidative activity triggers extensive research interest. Herein, it was the first time to investigate the neuroprotection of gartanin against glutamate-induced cell death in HT22 cells and its possible mechanism(s). Our data indicated that gartanin protected neurons against glutamate-induced cell death in HT22 cells independence of nuclear factor erythroid-derived 2-like 2 (Nrf-2), but involvement of HO-1 and AMP-activated protein kinase (AMPK).

Materials and Methods

Chemicals and Reagents

Gartanin was purified from the pericarps of mangosteen as previously described [39]. HT22 cells were received as a

generous gift from the Second Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China). 2,7-dichlorofluoroescin diacetate (H_2DCF-DA) and DHE were purchased from Molecular Probes Co. (agency in China). Annexin V-FITC/PI kit was purchased from Keygen Biotech (Nanjing, China), GSH assay kits was purchased from Jiancheng Biochemical (Nanjing, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (NY, USA). The following antibodies were used: Bcl-2, SIRT1, phospho-AMPKα (Thr172), Nrf-2 and AMPK (all from Cell Signaling Technologies), Bax (from Abclonal), HO-1 (from Bioworld Technology), and β-actin, PGC-1α (all from Sigma-Aldrich). Others unless stated elsewhere were purchased from Sigma-Aldrich.

Cell Culture

HT22 cells were maintained in DMEM supplemented with 10 % (v/v) FBS and incubated at 37 °C under 5 % CO_2. Cell morphology was observed by an inverse phase contrast microscopy (Olympus, Japan).

Assessment of Cell Viability by 3-(4.5-Dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) Assay

To study the neuroprotective effects of gartanin against glutamate-induced neuronal death and cytotoxicity, HT22 cells were seeded into 96-well plates with a density of 4×10^3 cells/well and incubated overnight. Cells were treated with gartanin or the vehicle control DMSO for 30 min followed with/without 2 mM glutamate for 24 h. 10 μL of 5 mg/mL MTT were added to each well and cells were incubated for 2 h at 37 °C. To dissolved purple formazan crystal, 100 μL DMSO was added to replace the medium. After vigorously shaking for 15 min at 37 °C, the absorbance was measured at 570-nm wavelength using a microculture plate reader (Bio-Tek, USA). The test was repeated three times. All data were represented as folds over control.

Apoptosis Analysis by Flow Cytometry

The apoptotic ratios of cells were determined by the Annexin V-FITC apoptosis detection kit according to the supplier's protocols. HT22 cells were harvested, centrifuged, and resuspended in 200 μL of binding buffer, incubated with 5 μL of AnnexinV-FITC for 10 min, added with 10 μL Propidium Iodide (PI) for 15 min at room temperature, and analyzed by flow cytometry (Molecular Devices, Sunnyvale, CA, USA) immediately. Values were expressed as a percentage of the fluorescence relative to the non-injured control.

Measurement of Intracellular ROS

HT22 Cells were grown in Corning 48-well plates at a cell density 2×10^4 cells/well. After overnight attachment, cells were pretreated with gartanin or the vehicle control DMSO for 30 min and then incubated with/without 2 mM glutamate for 12 h. Cells were washed twice with phosphate-buffered saline (PBS) and then incubated with 10 μM non-fluorescent dye H_2DCF-DA or DHE in serum-free medium for 30 min at 37 °C in the dark. Cells were subsequently washed twice with PBS and photographed using a (200×) fluorescence microscope or analyzed by flow cytometry. Values were expressed as a percentage of the fluorescence relative to the vehicle control.

Determination of ΔΨm

Rhodamine 123 (R123) was used to determine the ΔΨm. R123 is a fluorescent aromatic monovalent cation that accumulates in the matrix of energized mitochondria. After treatment, cells were incubated with 5 μM R123 in PBS for 15 min at 37 °C in the dark, then washed with PBS and incubated at 37 °C for the indicated time before cellular levels of R123 were measured by flow cytometry. Values were expressed as a percentage of the fluorescence relative to the non-injured control.

Western Blot Analysis

HT22 cells were plated in 6-well plates and grown overnight. After treatment, protein was lysed, quantified, and denatured. First, the 20 μg protein were loaded in each well, separated by 10 % SDS-PAGE, and transferred electrophoretically onto the PVDF membranes. Following 2 h blocking with 5 % skim milk, the membranes were incubated of primary antibodies for overnight at 4 °C. Next, the TBST buffer (PBS with 0.01 % Tween 20, pH 7.4) washed membranes three times and then incubated with appropriate horseradish peroxidase-linked anti-mouse or anti-rabbit IgG (1:1000) for 1 h at room temperature. Finally, protein bands were detected using enhanced chemiluminescence (ECL) system (Thermo Scientific) and imaged on BioRad image Quant LAS 4000mini. The intensities of bands were performed using Quantity One Software (Bio-Rad, Hercules, CA, USA).

Transfection and Luciferase Reporter Assay

HT22 cells planted in 48-well plates (1×10^5 cells/well) were transfected with either pARE-luc or pGL6-luc cis

reporter plasmids along with pRL-TK renilla. Transfections were performed using 200 ng DNA, 0.3 μL Lipofectamine™ 3000 (Invitrogen) and 0.4 μL P3000/well according to the manufacturer's instructions. After transfection 24 h, cells were treated with gartanin or *tert*-butyl hydroquinone (t-BHQ). Luciferase activity was detected at 24 h using the dual luciferase assay system (Promega, Madison, WI, USA). The Renilla luciferase activity was normalized to the firefly luciferase activity.

HO-1 Small Interfering RNA (siRNA) Transfection

siRNA duplexes and nontargeting control siRNA were purchased from Jima Biotechnology (Shanghai, China). The sense and antisense strands of murine HO-1 siRNA were as follows: sequence 1, 5′-CCACACAGCACUAUGUAAATT-3′ (sense) and 5′-UUUACAUAGUGCUGUGUGGTT-3′ (antisense); sequence 2, 5′-CCGAGAAUGC UGAGUUCAUTT-3′ (sense) and 5′-AUGAACUCAGCA UUCUCGGTT-3′ (antisense); sequence 3, 5′-GCUGACA GAGGAACACAAATT-3′ (sense) and 5′-UUUGUGUUC CUCUGUCAGCTT-3′ (antisense). The transfection was mediated by Lipofectamine™ 3000 according to the manufacturer's instructions. The cells were transfected at a density of ∼50 % confluence with siRNA duplex (100 nM). After 6 h, media were changed, and the cells were incubated for another 48 h and processed for MTT assay or western blotting analysis.

Statistical Analysis

All analyses were repeated at least in duplicate. Representative experiments are shown and statistical analyses among group were performed by either one- or two-way analysis of variance (ANOVA) with Tukey's post hoc test. Differences were considered statistically significant at $P < 0.05$.

Results

Gartanin Reduced Glutamate-Induced Oxidative Toxicity in HT22 Cells

To determine the effect of gartanin on oxidative stress, cells were exposed to glutamate and cell survival was measured using MTT assay. Glutamate induced cell death in a concentration-dependent manner (Fig. 1a). Incubation with glutamate (2 mM) for 24 h markedly reduced the HT22 neuronal cell viability, and resulted in chromatin condensation and blebbing of cell membrane, like apoptotic cell death (Fig. 1b). These changes were significantly attenuated when cells were pretreated with gartanin (1–10 μM) for 30 min (Fig. 1b, c). The highest concentration (10 μM) of gartanin was not more effective than the concentrations of 3 μM (3 μM gartanin 98.20 ± 2.07 %, VS 10 μM gartanin, 92.57 ± 1.39 %). The reason might be that 10 μM gartanin slightly inhibited cell MTT reduction (Fig. 1d). Therefore, the concentration of 3 μM gartanin was selected as the optimal concentration for further experiments.

Gartanin Decreased Glutamate-Induced Apoptotic Death and Increased the Ratio of Bcl-2/Bax Expression

We examined the effects of gartanin on glutamate-induced apoptosis by Annexin V-FITC/PI double staining analyzed by flow cytometry. Early apoptotic stage is marked by $FITC^+/PI^-$. As shown in Fig. 2a, after 12 h stimulation with glutamate (2 mM), the proportion of the fraction marked by $FITC^+/PI^-$ increased from 1.03 to 16.03 %,whereas cells pretreated with gartanin showed a lower proportion (1.19 %).

A crucial amplification event in the apoptotic cascades is the nearly complete release of cytochrome C from mitochondria [42]. This is regulated by Bcl-2 family proteins, which include anti- and pro-apoptotic members. HT22 cells were treated with 3 μM gartanin for indicated times, and Bcl-2 and Bax protein levels were determined by western blot analysis. Gartanin increased the expression of Bcl-2 but not Bax (Fig. 2b). Glutamate treatment decreased the expression of Bcl-2, whereas the level of Bax was not markedly altered. Gartanin effectively maintained the Bcl-2 protein level down-regulated by glutamate (Fig. 2c).

Gartanin Reduced Glutamate-Induced ROS Accumulation and Mitochondrial Membrane Depolarization

A number of studies have indicated that glutamate-mediated neuronal cell death is closely associated with oxidative stress including excessive ROS production and mitochondrial damage [8, 43]. To test whether gartanin could protect mitochondria against glutamate, we examined glutamate-induced alterations in ROS level and $\Delta\Psi m$ in the presence of 3 μM gartanin in HT22 cells. On the one hand, H_2DCF-DA and DHE staining were performed to measure intracellular ROS production. DCF and DHE fluorescence were analyzed by visual observation of cell morphology through fluorescence microscopy (Fig. 3a) and flow cytometry analysis (Fig. 3b). On the other hand, $\Delta\Psi m$ was measured using R123 dye (Fig. 3c). Data showed glutamate induced intracellular ROS accumulation and mitochondrial membrane depolarization in HT22 cells, which was significantly

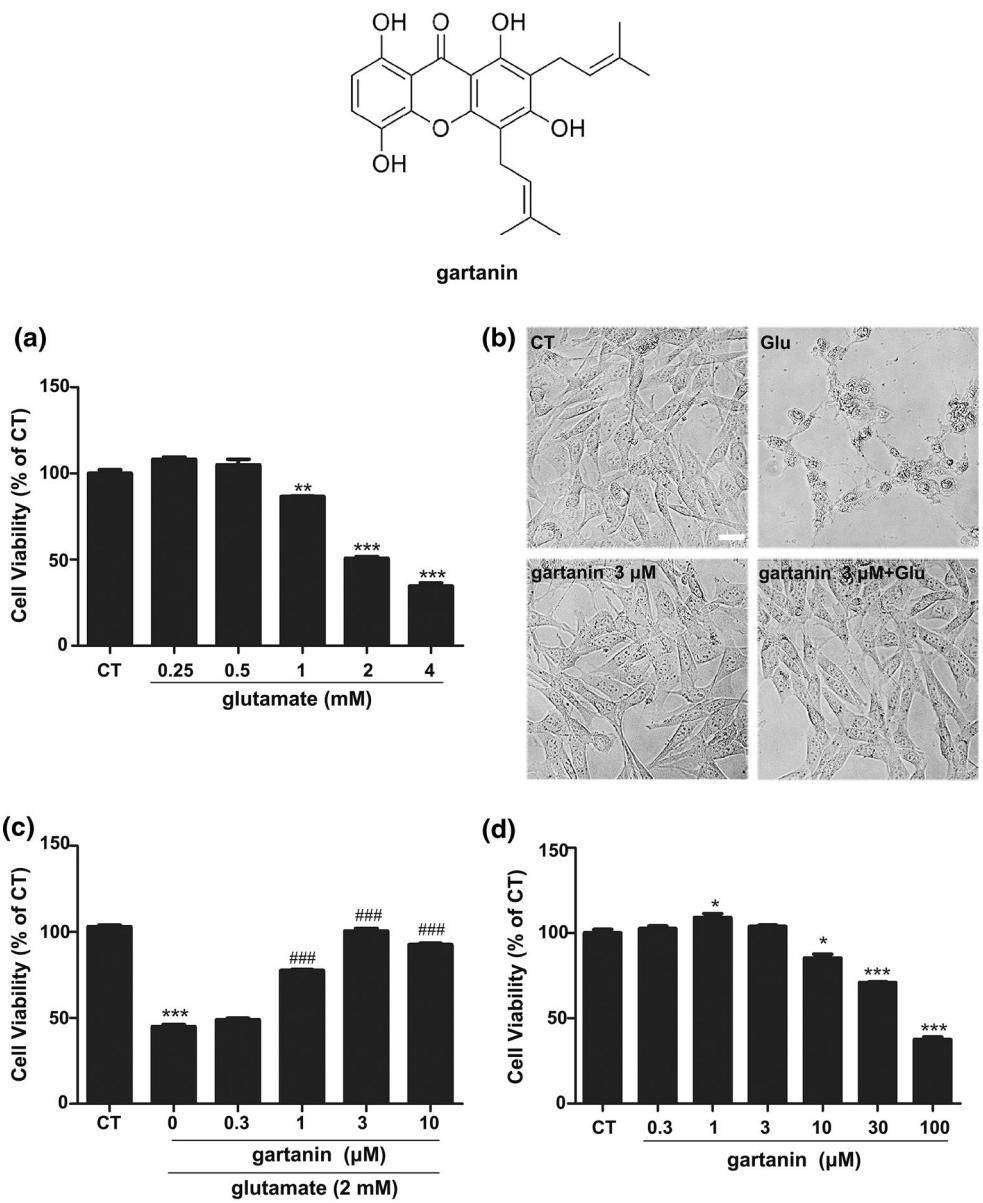

Fig. 1 Gartanin protected HT22 cells against glutamate-induced cell death. **a** Cell viability was measured by MTT assay after exposed to 0–4 mM glutamate for 24 h. **b** HT22 cells were photographed using a photomicroscope (×200) after cells were pretreated with 3 μM gartanin for 30 min and then incubated with 2 mM glutamate for 24 h. **c** HT22 cells were pretreated with 0.3, 1, 3, and 10 μM gartanin for 30 min and then incubated with 2 mM glutamate for 24 h, cells viability was determined by MTT assay. **d** MTT assay was used to measure cell viability after treatment with 0.3–100 μM gartanin for 24 h. Data are presented as mean ± SEM (n = 3). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ versus control group. $^{\#\#\#}P < 0.001$ versus glutamate-treated group. *CT* control, *Glu* glutamate. *Scale bar* 50 μM

reduced by pretreatment with gartanin for 30 min prior to glutamate treatment.

Gartanin Promoted HO-1 Protein Level Independent of Nrf-2 Activation and Mediates it Protection

As we all know, Nrf-2 is an inducible transcription factor and binds to the antioxidant responsive element (ARE) to activate a battery of genes encoding antioxidant proteins and phase II enzymes in response to oxidative stress [44]. We firstly speculated that gartanin could promote Nrf-2-ARE activation, and ultimately protect from oxidative stress-induced cell death. However, gartanin did not show a significant difference on both the protein level and transcriptional activity of Nrf-2 (Fig. 4a–c). Nrf-2/ARE targeting genes didn't change the glutamate-cysteine ligase, modifier subunit (GCLM) and NADPH: quinone oxidoreductase 1 (NQO-1) protein expression (data not shown), while HO-1 protein level increased in a time- and concentration-dependent manners (Fig. 4a, b).

Next, we screened the effective siRNA sequences, HO-1 siRNA sequence 1 and 3 significantly reduced HO-1 protein level (Fig. 4d). MTT assay showed that the protective effect of gartanin was partially blocked by the HO-1 siRNA sequence 1 in HT22, indicating the involvement of HO-1 activation (Fig. 4e).

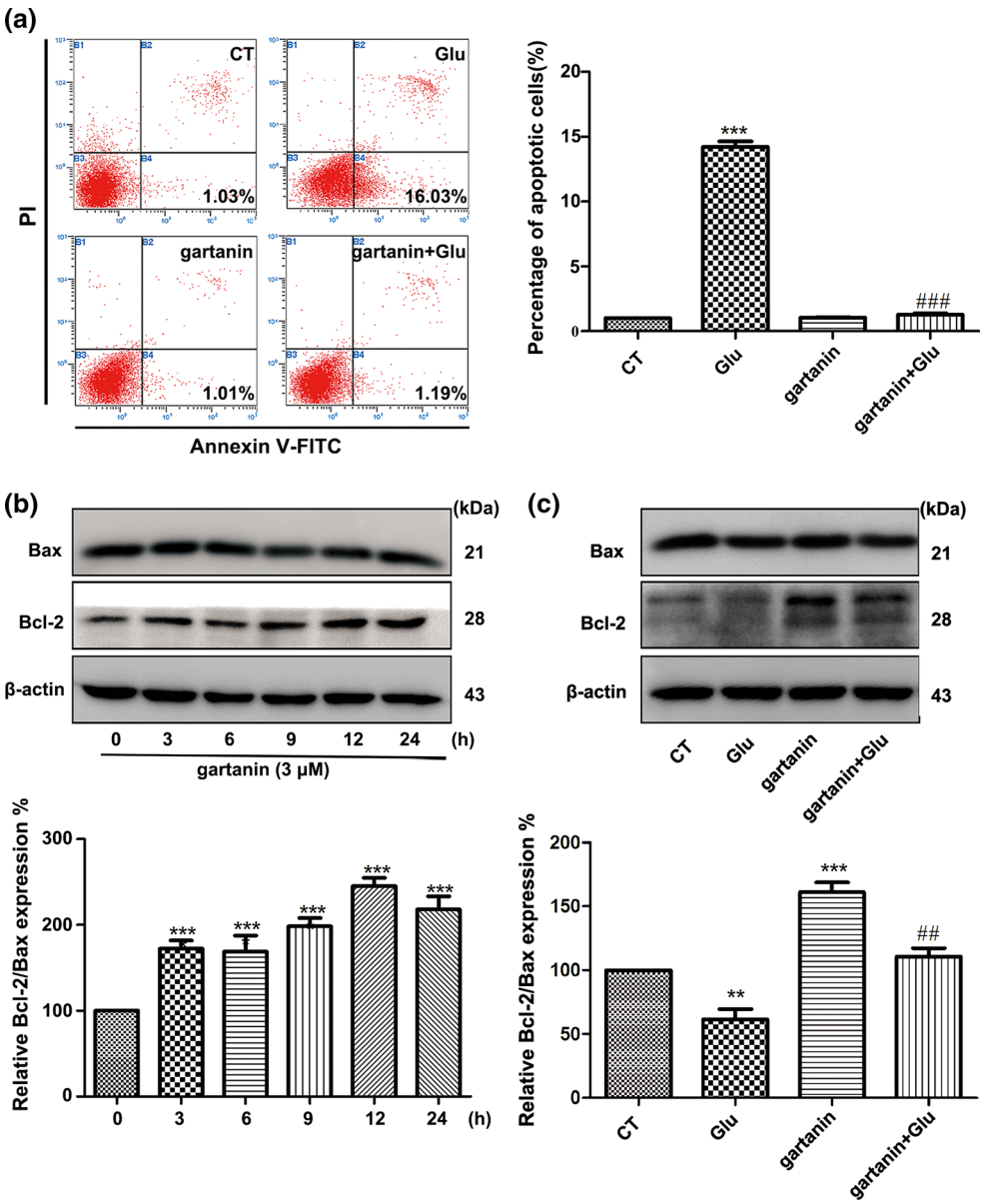

Fig. 2 Gartanin decreased glutamate-induced HT22 cell apoptosis and increased the ratio of Bcl-2/Bax expression. **a** HT22 cells were treated with 3 μM gartanin for 30 min, before exposed to 2 mM glutamate. After 12 h, Annexin V-FITC/PI staining was analyzed by flow cytometry and the percentage of early apoptotic stage demonstrated by $FITC^{+}/PI^{-}$ was analyzed. **b** Bcl-2 and Bax protein level was detected by western blot in HT22 cells treated with 3 μM gartanin for indicated times. The loading control was β-actin. Quantitative analysis was expressed as Bcl-2 relative to Bax following exposure to vehicle control. Data are presented as mean ± SEM (n = 3). *** $P < 0.001$; ** $P < 0.01$, versus control group. ### $P < 0.001$; ## $P < 0.01$ versus glutamate-treated group

AMPK Signaling Pathways May Be Involved in the Protection of Gartanin

To further explore the mechanism responsible for the protective effects of gartanin, we measured a series of signal pathways, which are closely related to neuronal cell survival and death. As shown in Fig. 5a, compared with control, phosphorylated AMPKα at Thr172 was significantly increased at 3–12 h without any change in total AMPK level after gartanin treatment. In addition, SIRT1 Sirtuin activator 1 (SIRT1) and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) protein levels were significantly increased in our culture system, confirming activation of the AMPK pathway.

We investigated whether gartanin acted through AMPK signaling to confer neuroprotection under oxidative toxicity. As shown in Fig. 5b, glutamate induced moderate AMPK activation in HT22 cells, which was dramatically enhanced by gartanin and didn't blocked by Compound C, a well-established AMPK inhibitor. Glutamate-induced HT22 cells damages evidenced by decreased cell viability were further inhibited by gartanin. Compound C robustly strengthened the glutamate tolerance and had no effect on cell viability when applied alone to control cells (10 μM) (Fig. 5c). Furthermore, under glutamate oxidative stress, the compound C didn't inhibit cyto-protection by gartanin, instead increased survival of HT22 cells (Fig. 5c).

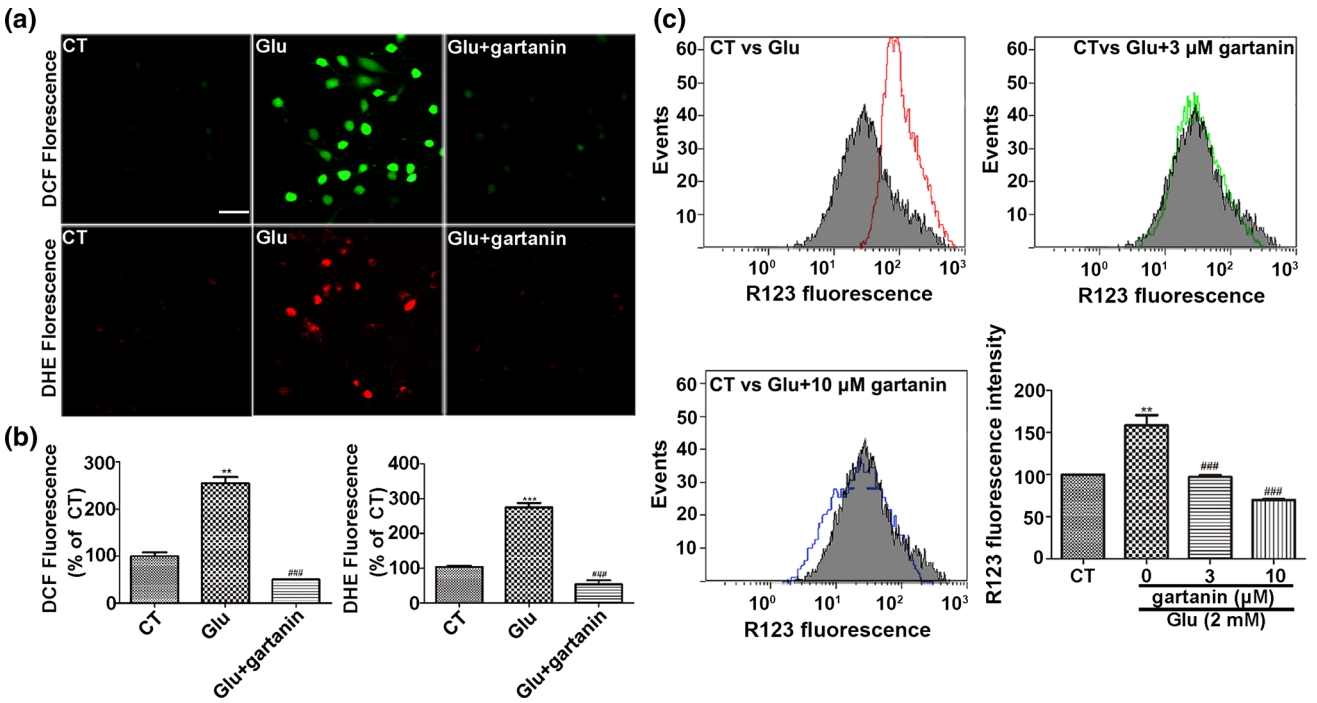

Fig. 3 Gartanin significantly decreased glutamate-induced ROS and stabilized mitochondrial membrane potential. HT22 cells were pretreated with 3 μM gartanin for 30 min and then incubated with 2 mM glutamate for 12 h. **a** DCF and DHE fluorescence were analyzed by visual observation of cell morphology through fluorescence microscopy (×200). **b** The cellular fluorescence intensity was monitored by flow cytometry. **c** R123 fluorescence intensity was detected by flow cytometry. Data are presented as mean ± SEM (n = 3). $^{***}P < 0.001$; $^{**}P < 0.01$, versus control group. $^{\#\#\#}P < 0.001$ versus glutamate-treated group. *Scale bar* 50 μM

Discussion

It is widely known mangosteen peel is one of the most important sources of natural antioxidants [45, 46]. Mangosteen-based functional beverage can enhance plasma antioxidant capacity in healthy adults, which may be due to the enhancement of endogenous antioxidant activity [47]. However, precise mechanisms are still unclear. In neurodegenerative diseases, persistent oxidative stress is a major disastrous element that causes living cells' death. Therefore, antioxidant therapy should be an attractive strategy against neuronal loss. The treatment of HT22 cells with high dose of glutamate resulted in a significant increase in intracellular ROS. In this study, the results of H_2DCF-DA and DHE fluorescence intensity supported that gartanin could decrease the levels of ROS accumulated in cells treated with glutamate.

So far, it is the first time to reveal that gartanin significantly attenuated glutamate-induced oxidative cytotoxicity in vitro. High concentrations of glutamate inhibit glutamate/cystine antiporter and result in reducing intracellular GSH levels. Unfortunately, we found gartanin did not reverse the glutamate-induced intracellular GSH depletion (data not shown). It might quench ROS through a GSH-independent way. It had been previously claimed the Bax expression is not required for oxidative stress-induced HT22 cell death and Bcl-2 can protect cells from oxidative stress as an anti-oxidant protein, so the ratio of the Bax/Bcl-2 determines the cells' resistance to apoptosis triggered by different stimulus [48, 49]. Our findings showed that gartanin could elevated the level of Bcl-2 in time-dependent manner and effectively preserving the Bcl-2 protein level down-regulated by glutamate, whereas it did not affect the expression of Bax, the Bcl-2/Bax protein ratio increased (Fig. 2b, c). Meanwhile, we found gartanin preserved $\Delta\Psi$m in glutamate treated cells. All these indicated that the neuroprotective effects of gartanin against glutamate-induced cell death, to some extent, might exert through increasing mitochondrial anti-apoptotic protein.

Accumulating evidence suggests that Nrf-2 binds to the ARE promoter and gives rise to general antioxidant responses, which represents a promising therapeutic approach to restore the CNS redox balance of neurodegenerative disorders [50]. Among the redox-sensitive inducible enzymes, HO-1 protects neurons against acute insults under stress conditions due to its antioxidant abilities and anti-inflammatory properties [51]. To better understand the neuroprotective mechanism of gartanin, we focused on the stress-sensing transcription factor Nrf-2 and its downstream proteins. We initially speculated that

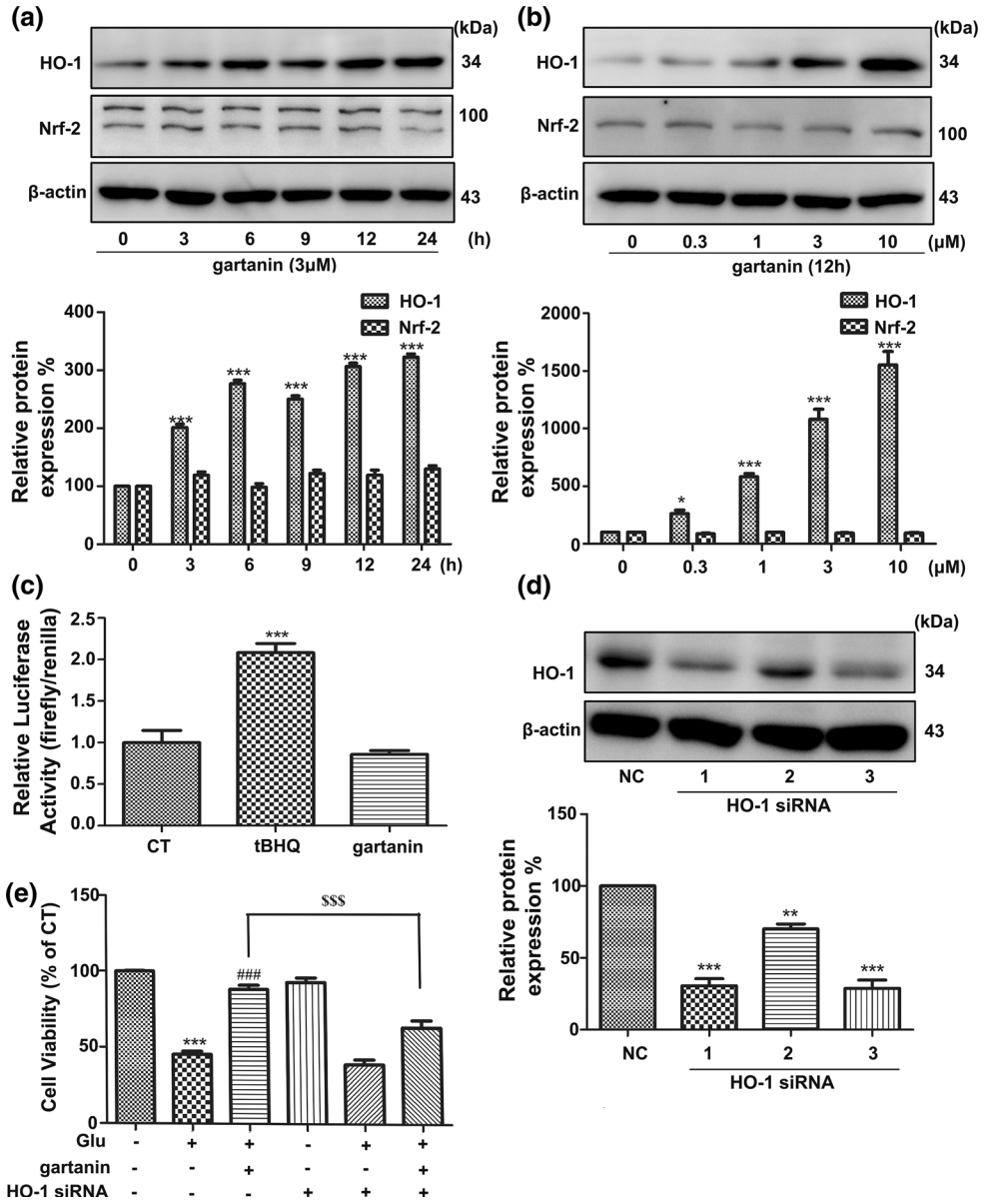

Fig. 4 Gartanin elevated the protein expression of HO-1 and mediated its protection, but had no effects on Nrf-2. **a**, **b** Gartanin elevated HO-1 but not Nrf-2 in a time- and concentration-dependent. Quantitative analysis of the bands was shown. **c** Gartanin had no effect on Nrf-2 transcriptional activity. tBHQ (50 μM) was used as positive control. **d** Western blot analysis of HO-1 protein expression after HO-1 siRNA treated. Lanes 2, 3 and 4 represented three HO-1 siRNA sequences respectively. NC represented nonspecific control siRNA. Quantitative analysis of the bands was shown. **e** Cells were transfected with HO-1 siRNA sequence 1. After 48 h, 3 μM gartanin was added 30 min before the treatment with 2 mM glutamate for 24 h. Cell viability was determined by the MTT assay. Data are presented as mean ± SEM (n = 3). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ versus control group. $^{\#\#\#}P < 0.001$ versus glutamate-treated group. $^{\$\$\$}P < 0.001$ versus gartanin combined with glutamate-treated cells

gartanin could activate Nrf-2/ARE pathway, which ultimately leaded to a powerful antioxidant response. The current result demonstrated gartanin could induce the expression of HO-1 in a time- and concentration-dependent manner (Fig. 2a, b), while the protein level and transcriptional activity of Nrf-2 did not show significant change (Fig. 2a–c). Additionally, we found that siRNA mediated knock-down of HO-1 significantly inhibited gartanin-induced neuroprotective response (Fig. 2e), which suggested that the neuroprotective effects of gartanin partially resulted from the HO-1 in HT22 cells. It has been reported that HO-1 is regulated by various moleculars. Jione Kang's study showed that forkhead box O1 (FoxO1) could directly bind to FRE within the HO-1 gene promoter and stimulate HO-1 gene transcription [52]. Several investigations identified peroxisome proliferator-activated receptor (PPAR) α

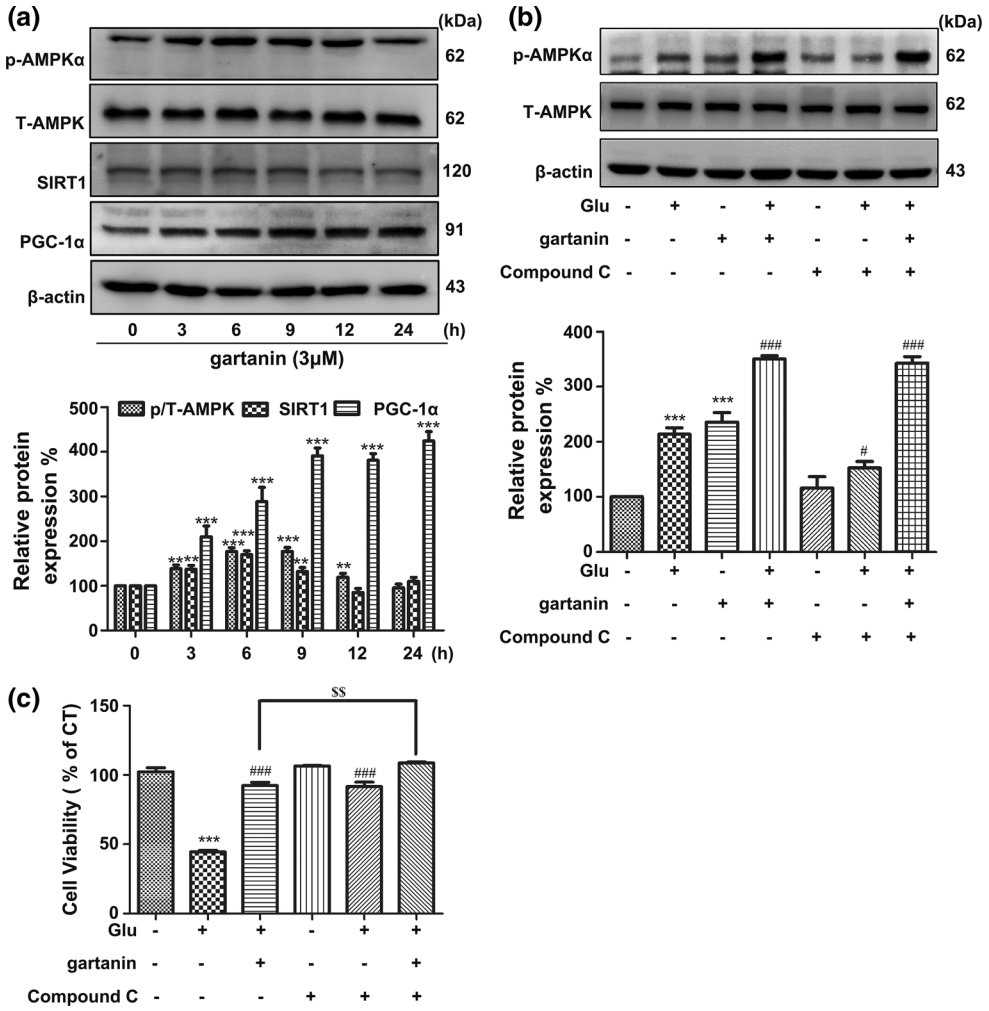

Fig. 5 The neuroprotective effects of gartanin was partially mediated by activating AMPK pathway. **a** Cells were treated with gartanin of 3 μM for different hours, representative western blots showed that gartanin increased pAMPKα (Thr172), SIRT1 and PGC-1α protein level. Quantitative analysis of the bands is shown. **b**, **c** HT22 cells were pretreated with Compound C (10 μM, 1 h) and glutamate (0.5 h), and then were maintained with gartanin for 12 h, western blots analysis of AMPK phosphorylation and MTT assay analysis of cell viability Data are presented as mean ± SEM (n = 3). $^{***}P < 0.001$; $^{**}P < 0.01$ versus control group. $^{\#\#\#}P < 0.001$; $^{\#}P < 0.05$ versus glutamate-treated group. $^{\$\$}P < 0.01$ versus gartanin combined with glutamate-treated cells

and γ are important modulators of HO-1 [53]. Further studies are needed to elucidate how HO-1 is modulated by gartanin.

AMPK was initially characterized as a "fuel gauge" and sensitive to both physiological and pathophysiological stimuli in various cells and tissues [54]. Mitochondrial respiratory chain function and glucose metabolism are closely associated with AMPK-SIRT1-PGC-1α signaling axis [55, 56]. Meanwhile, modest AMPK activation has also been documented to induce neurogenesis and improves cognition [57]. It has also been reported that hydroxytyrosol could activate AMPK with subsequent activation of forkhead transcription factor 3a (FoxO3a) and catalase to reduce intracellular ROS levels in vascular endothelial cells [58]. In this study, the levels of pAMPKα (Thr172) and two other regulators of AMPK signals, SIRT1 and PGC-1α, were significantly increased in the HT22 cells after gartanin treatment. Indeed, it is well-accepted that AMPK activation plays a dual role in the regulation of neuronal survival resulting from difference in downstream targets of AMPK (as a crucial signaling node, AMPK is connected to many downstream effectors), the type of stress, type of cells, and duration of exposure [59–61]. The MTT assay result showed that Compound C remarkably protected the HT22 cells from glutamate toxicity, but the phosphorylation status of AMPKα in cells exposed to gartanin and glutamate was dramatically increased and wasn't inhibited by Compound C. Of note, gartanin also elevated basal AMPK activation levels (Fig. 5b, c). Thus, we suggested that AMPK activation

might play a major role in gartanin protected against glutamate neurotoxicity. The reason that the existing researches failed to consist with result may be that Compound C has off-target effects or gartanin activate AMPK by non-canonical mechanisms that may not involve increases in AMP, ADP or Ca^{2+} levels. Of course, it should also be noted that excessive activation of AMPK would in turn trigger detrimental effects that augment neurodegenerative diseases pathogenesis [62]. Possibly, gartanin specifically induced other activators of AMPK, which are responsible for the neuroprotective activity by unknown mechanisms.

Conclusions

In summary, our research indicated that gartanin exerted strikingly protective effects against glutamate-induced cytotoxicity on HT22 cells, partly through reduced glutamate-induced ROS production, prevented mitochondrial hyperpolarization and activated HO-1 and AMPK. Our results underscored a potential role for gartanin in the prevention of glutamate-induced oxidative stress-associated diseases, such as PD and AD.

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Compliance with Ethical Standards

Conflict of interest All other authors declare no conflict of interest.

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