GTS40, an active fraction of Gou Teng-San (GTS), protects PC12 from $\text{H}_2\text{O}_2$-induced cell injury through antioxidative properties

CHEN Lei², WEI Meng-Lin², ZHAO Jiao-Jiao¹, HONG Hao³, QU Wei¹, FENG Feng¹*, LIU Wen-Yuan²*

¹Department of Natural Medicinal Chemistry, China Pharmaceutical University, Nanjing 210009, China;
²Department of Pharmaceutical Analysis, China Pharmaceutical University, Nanjing 210009, China;
³Department of Pharmacology, China Pharmaceutical University, Nanjing 210009, China

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[ABSTRACT] Oxidative stress, a predominant cause of apoptosis cascades triggered in neurodegenerative disorders, has been regarded as a critical inducement in the pathogenesis of Alzheimer’s disease (AD). Gou Teng-San (GTS) is a traditional Chinese herb preparation commonly utilized to alleviate cognitive dysfunction and psychological symptoms of patients with dementia. The present study aimed to investigate the protective effects of GTS40, an active fraction of GTS, on $\text{H}_2\text{O}_2$-induced oxidative damage and identify the potential active ingredients. Our results revealed that GTS40 exhibited radical scavenging activity, elevated cell viability, decreased the levels of intracellular reactive oxygen species (ROS), and stabilized mitochondrial transmembrane potential (MMP) in $\text{H}_2\text{O}_2$-treated PC12 cells. In addition, GTS40 blocked the apoptotic cascade by reversing the imbalance of Bcl-2/Bax and inhibiting the activity of caspase-3. Furthermore, an HPLC-QTOFMS method was developed to characterize major chemical constituents in GTS40. Our results revealed twenty-seven identified or tentatively characterized compounds through comparing their retention time ($t_R$) and MS spectra with reference standards. These results suggested that GTS40 was a promising active fraction that may be beneficial in the prevention and treatment of oxidative stress-mediated neurodegenerative disorders.

[KEY WORDS] GTS40; Oxidative stress; Neuroprotective effects; Apoptosis; HPLC-QTOF-MS/MS


Introduction

Alzheimer’s disease (AD) is the most common mode of irreversible dementia among the elderly, characterized by the progressive impairment of learning, memory, and other cognitive functions [1]. This challenge is creating considerable burdens on patients, caregivers, and society, because more people live long enough to be susceptible. Two critical events existing in the pathogenesis of AD are abundant deposits of senile plaques and neurofibrillary tangles [2]. In addition to that, increasing evidences suggest that oxidative stress is closely implicated in its pathologic changes, and excessive reactive oxygen species (ROS) production results in neuron death [3]. $\text{H}_2\text{O}_2$ is the primary intracellular ROS which can cause protein degeneration, lipid peroxidation, and DNA damage, eventually accelerating oxidative injury and neuronal cell death [4]. Currently, acetylcholinesterase inhibitors and N-methyl-D-aspartate-type (NMDA) glutamate-receptor antagonists are two major class of drugs used for the treatment of AD in the clinic, although these are not always effective. Recently, many researchers have paid attention to naturally occurring phytochemicals [5-6]; supplementation with antioxidants from natural products and herbal preparation may be a reasonable approach to preventive intervention of neurodegenerative diseases such as AD.

Gou Teng-San (GTS, chotosan in Japanese), a traditional Chinese medicine (TCM) formula, is comprised of 11 herbal ingredients, namely Uncariae Uncis cum Ramulus, Aurantii Nobilis pericarpium, Pinelliae tuber, Ophiopogonis tuber, Hoelen, Ginseng radix, Saposhnikoviae radix, Chrysanthemi flos, Glycyrrhizae radix, Zingiberis rhizome, and Gypsum
fibrosum at the proportions of 3:3:3:2:2:2:1:1:5. According to the theory of TCM, this formulation has a potential to benefit “qi”, promote blood circulation by removing blood stasis, and improve stagnation of liver “qi”. GTS is generally prescribed for middle-aged and elderly patients with a weak physical constitution and symptoms associated with hypertension and/or chronic headache. Modern pharmacological studies have also demonstrated the effectiveness of GTS in the amelioration of cognitive deficit and memory dysfunction caused by chronic cerebral hypoperfusion, diabetes, cerebral ischemia, and aging.[7-8]

Although preclinical experimental animal studies have indicated that GTS may be hopeful as an anti-dementia drug,[7], convincing conclusion hasn’t been drawn. Moreover, the components of GTS responsible for the beneficial effect and the clinical application and further pharmaceutical research of GTS remain only partly known, predominantly due to the high complexity of the holistic chemical profile, with the previous study mainly focusing on the gentleman medicine uncaria thorn[9]. All of these have hampered the clinical application and further pharmaceutical research of GTS.

Since Oxidative stress-induced damage plays a key role in the progression of neurodegenerative diseases, the present study aimed at evaluating the protective effects and potential active ingredients of GTS40, an active fraction isolated from GTS, on H2O2-induced Oxidative damage as well as the underlying protective mechanisms. We examined the effectiveness of GTS40 on H2O2-induced cell injury, apoptosis and corresponding changes in apoptotic molecules level such as cleaved caspase-3, Bax and Bcl-2. Furthermore, a HPLC-Q-TOF-MS/MS method was adopted to determine the chemical profile of GTS40 in order to further ascertain the material basis for its neuroprotective activity.

Materials and Methods

Reagents

Methyl thiazolyl tetrazolium (MTT), malondialdehyde (MDA) assay kit, total antioxidant capability (T-AOC) assay kit, Bicinechonic acid (BCA) assay kit, Annexin V-FITC and propidium iodide (PI) apoptosis detection kit, Rhodamine 123, and 2’,7’-dichlorofluorescein diacetate (DCFH-DA) fluorescent dye were purchased from the Beyotime Institute of Biotechnology (Shanghai, China). Rabbit-derived Bcl-2, Bax, and caspase-3 antibodies were supplied by Cell Signaling Technology (Boston, MA, USA), Rabbit-derived β-actin and secondary antibodies were purchased from Bioworld Technology Co., Ltd. (Minneapolis, MN, USA). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grandisland, NY, USA). All other chemicals and solvents used in the present study were of the highest purity grade available commercially.

Preparation and chemical profiling of GTS40

The constituent herbs of GTS were purchased from Nanjing Xian Sheng Zai Kang pharmacy (Nanjing, Jiangsu, China). The herbal materials were identified in our laboratory by Professor FENG Feng. The voucher specimen was deposited at the Department of Natural Medicinal Chemistry, China Pharmaceutical University, Nanjing, China. The preparation method of GTS40 sample were as follows: ten kilograms of the raw medicinal materials were mixed according to the formula in GTS and exhaustively extracted by successive refluxing with water (100 °C, 2 h), 50% ethanol (80 °C, 2 h), and 95% ethanol (80 °C, 2 h). All extracts were combined, and then solvent was evaporated to dryness under reduced pressure at 80 °C to give the crude extract. The yield of extracts was 9.28%. After the crude extract was extracted thrice by equal volume of ethyl acetate, the water fraction was sequentially separated through D-101 macroporous adsorption resin. The different concentrations of ethanol–H2O eluate were collected, finally achieving 6 fractions in total. We had screened the neuroprotective effects of all these fractions on H2O2-induced PC12 injury which leads to the identification of GTS40 with the strongest potency. So we focused on GTS40 for further study.

GTS40 (200 mg) was accurately weighed and dissolved in 10 mL distilled water under ultrasonic water bath for 1 h at 60 °C. The supernatant solution was centrifuged at 12 000 r·min⁻¹ for 1 h, filtered through a 0.22-µm millipore filter and the stored at 4 °C until use. In order to guarantee the quality control of GTS40 used in this study, an HPLC-Q-TOF-MS/MS method was adopted to determine the chemical profile.

Cell culture and treatment

The rat pheochromocytoma line 12 (PC12) cells were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). They were maintained in DMEM supplemented with penicillin (100 U·mL⁻¹), streptomycin (100 µg·mL⁻¹), and 10% (V/V) FBS at 37 °C in a humidified atmosphere containing 5% CO2. When grown to 80% confluence, the PC12 cells was pre-incubated with or without GTS40 (50, 100, 200, 400, and 800 µg·mL⁻¹) for 0.5 h, prior to H2O2 (100 µmol·L⁻¹) exposure for 12 h. The highest concentration of DMSO used in the cell experiment was less than 0.2%.

MTT assay for cell viability evaluation

Cell viability was quantified by the MTT assay[3] with some modifications. After drug treatment, the precipitated formazan crystals were solubilized in DMSO and the absorbance was determined at 570 nm using a 1500 microplate reader (Thermo Fisher Scientific Co.). Absorbance of untreated controls was set to 100% and absorbance of GTS40-treated cells was expressed as a percentage relative to the control. Then, we selected two concentrations (200 and 400 µg·mL⁻¹), which had significant neuroprotective effects, to explore the underlying mechanism in subsequent experiments.

Cell apoptosis assay by annexin V-FITC/PI coupled staining

The PC12 cells were seeded into 6-well plates at a density of 5 × 10⁵ cells/well. When PC12 cell became 80%–85%
confluence and they were treated with or without GTS40 (200 and 400 μg·mL\(^{-1}\)) for 0.5 h, followed by H\(_2\)O\(_2\) (100 μmol·L\(^{-1}\)) exposure for 12 h. After treatment, the cells were harvested and stained with Annexin V-FITC/PI in binding buffer for 20 min\(^{[10]}\). Cell suspensions were then analyzed using a FACS Calibur Flow Cytometer (BD Inc., USA).

**Measurement of Intracellular ROS Production**

The cells were incubated with or without GTS40 (200 and 400 μg·mL\(^{-1}\)) for 0.5 h prior to H\(_2\)O\(_2\) (100 μmol·L\(^{-1}\)) exposure for 4 h. The fluorescent probe DCFH-DA was used to monitor intracellular accumulation of ROS. Briefly, the cells were collected and washed thrice with PBS and then incubated with DCFH-DA solution (10 μmol·L\(^{-1}\)) at 37 °C for 20 min. Finally, the fluorescence intensity of DCF was determined using a FACS Calibur Flow Cytometer (BD Inc.)\(^{[11]}\). ROS generation was quantified by the median fluorescence intensity of 10,000 cells. The control group was set to 100% and the fluorescence intensity of GTS40-treated cells was expressed as a percentage of the control.

**Malondialdehyde (MDA) assay and radical scavenging activity**

The PC12 cells were seeded in 6-well plates at a density of 5 × 10\(^5\) cells/well. After incubation with various concentrations of GTS40 (200 and 400 μg·mL\(^{-1}\)) and H\(_2\)O\(_2\) for 12 h, the cells were washed with PBS twice and then collected and lysed for 30 min at 4 °C. The protein content was measured using a BCA assay kit. The activities of MDA were measured lysed for 30 min at 4 °C. The protein concentration of each sample, and expressed as a percentage of the control.

**Malondialdehyde (MDA) assay and radical scavenging activity**

The PC12 cells were seeded in 6-well plates at a density of 5 × 10\(^5\) cells/well. After incubation with drugs and H\(_2\)O\(_2\), the PC12 cells were harvested and lysed in protein lysis buffer (10 mmol·L\(^{-1}\) Tris-HCl (pH 7.4), 150 mmol·L\(^{-1}\) NaCl, 1 mmol·L\(^{-1}\) EDTA, 1% Triton X-100) including PMSF. The lysate was stood on ice for 30 min and centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant was collected and the concentration was measured by BCA assay kit. The protein samples were separated by SDS-polyacrylamide gel electrophoresis and then transferred onto a PVDF membrane which was subsequently blocked with 5% (W/V) nonfat milk in TBST (Tris-buffer saline containing 0.1% Tween-20) for 2 h at room temperature, and then incubated at 4 °C overnight with respective primary antibodies against caspase-3 (1 : 1,000), Bcl-2 (1 : 1,000), Bax (1 : 1,000) or β-actin (inner control, 1 : 4,000). After washing with TBST thrice, the membranes were probed with a horseradish peroxidase conjugated secondary antibody (1 : 5,000) for 2 h at room temperature. The antibody reactive bands were visualized by using the enhanced chemiluminescence detection reagents on a gel imaging system (Tanon Science & Technology Co., Ltd., Shanghai, China).

**HPLC-Q-TOF-MS/MS analysis**

For characterization of the chemical constituents in GTS40, about 50 mg of GTS40 were accurately weighed and dissolved with 10 mL of 40 % methanol (V/W), and underwent an ultrasonic treatment in 35 °C water bath for 40 min. It was filtered through a 0.45-μm membrane filter before injection. HPLC-Q-TOF-MS was performed on a 6520 Q-TOF spectrometry system with an electrospray ionization (ESI) interface (Agilent Corp., Santa Clara, CA, USA), which was equipped with an on-line degasser, binary solvent delivery system, auto-sampler, column oven, and photodiode-array detection (DAD) system.

The solid-phase was a Megres C\(_{18}\) column (4.6 mm × 250 mm, 5 μm) obtained from Jiangsu Hanbon Sci. & Tech. Corp. (Nanjing, China). The mobile phase was a mixture of solvent A (acetonitrile) and solvent B (0.1% formic acid in water). The gradient program was as follows: 0–28 min, 17%–19% A; 28–55 min, 19%–30% A; 55–85 min, 30%–45% A; 85–88 min, 45%–100% A; and 88–95 min, 100% A. The column temperature was set at 40 °C, and the flow rate was set at 1.0 mL·min\(^{-1}\). The monitoring wavelength of DAD was set at 254 nm, and the sample injection was 10 μL. The QTOFMS operating parameters were set as follows: drying gas (N\(_2\)) flow rate, 8.0 L·min\(^{-1}\); drying gas temperature, 325 °C; nebulizer, 40 psig; sheath gas temperature, 400 °C; sheath gas flow, 10 L·min\(^{-1}\); capillary voltage, 3,500 V; and fragmentor voltage, 100 V. The sample collision energy was set at 35 V. Each sample was analyzed in both positive and negative modes over the range of m/z 100–1,200. All operations, acquisition, and analyses of data were controlled by Agilent Mass Hunter Acquisition Software Ver. A.01.00 (Agilent Technologies, Santa Clara, CA, USA).

**Statistical analysis**

The data were expressed as means ± SEM. Multiple group comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett’s test in order to detect inter-group differences. GraphPad Prism software was used to perform the statistical analysis (Version 5.0; GraphPad Software, Inc., San Diego, CA, USA). A probability (P) value of less than 0.05 was considered statistically significant.
Results

Effects of GTS40 on reducing H$_2$O$_2$-induced cytotoxicity

To assess protective effect of GTS40, the PC12 cells were incubated with various concentrations of GTS40 then exposed to 100 μmol·L$^{-1}$ H$_2$O$_2$ for 12 h. The results indicated that H$_2$O$_2$ significantly decreased cell survival rate compared to the control group. As shown in Fig. 1A, when the cells were pretreated with GTS40 at the concentrations of 50, 100, 200, 400, and 800 μg·mL$^{-1}$, the cell viability was significantly recovered (62%, 69%, 77%, 82%, and 86% of the control value, respectively) suggesting that GTS40 conferred protection against H$_2$O$_2$-mediated cytotoxicity. What is more, the GTS40 alone didn’t exhibit significant cytotoxicity to PC12 during treatment (Fig. 1B).

Effects of GTS40 on H$_2$O$_2$-induced oxidative stress in PC12 cells and ABTS free radical scavenging activity

Oxidative stress induced by H$_2$O$_2$ was evaluated by measuring the amount of intracellular MDA (Fig. 2A) and ROS (Fig. 2B-C). After the PC12 cells were subjected to 100 μmol·L$^{-1}$ H$_2$O$_2$ insult for 12 h, the intracellular ROS and MDA levels were significantly elevated to 243% and 197% of the control, respectively, showing that H$_2$O$_2$ induced marked oxidative stress. The alteration could be reversed effectively by GTS40 pretreatment at the concentrations of 200 and, 400 μg·mL$^{-1}$ for 0.5 h. Intracellular ROS production was significantly reduced to 206% and 147% of the control group, respectively, relative to the H$_2$O$_2$ group. Meanwhile, pretreatment with GTS40 also significantly led to a decrease in MDA level (161% and 134% of the control value, respectively).
The *in vitro* antioxidant capacity of GTS40 was also examined using the ABTS radical scavenging method. Trolox, a water-soluble vitamin E analog, was adopted as a reference standard. The antioxidant activity of GTS40 was expressed as a trolox equivalent, with its relative potency at 50 μg·mL⁻¹ compared with trolox. The results showed that GTS40 could scavenge the ABTS radical with 0.85 trolox equivalent. These experimental findings were indicative that GTS40 treatment could dramatically lower the H₂O₂-induced oxidative injury in PC12 cells, at least by inhibiting the free radical production.

**Effects of GTS40 on MMP in H₂O₂-treated PC12 cells**

It is well known that mitochondria plays a key role in sensing and propagating apoptosis signals and collapse of MMP is generally associated with ROS in the early phase of the oxidant stress-induced apoptosis pathway. As shown in Fig. 3, our results showed H₂O₂ remarkably reduced MMP (55% of the control value) and GTS40 remarkably restored the decrease in MMP level induced by H₂O₂ (69% and 87%, respectively).

**Effects of GTS40 on H₂O₂-induced reduction of MMP.**

Values given are the mean ± SEM (*P < 0.001 vs control, *P < 0.05 and **P < 0.01 vs H₂O₂-treated group). All data are representative of three replications from one experiment.

**Effects of GTS40 on H₂O₂-induced cell apoptosis**

Recent evidence has highlighted that apoptosis initiated by oxidant stress has existed in several neuronal cell types. Accordingly, we additionally investigated the effect of GTS40 on apoptosis induced by H₂O₂ through annexin V-FITC/PI coupled staining. The results of flow cytometry analyses showed H₂O₂ remarkably elevated the numbers of apoptosis. The percentage of early apoptotic cells and late apoptotic/necrotic cells in total cells was increased from 2.71% to 15.7% and 5.9% to 14.7%, respectively. The exposure of PC12 cells to GTS40 remarkably reduced the H₂O₂-mediated cell apoptosis, as shown in Fig. 4.

**Effects of GTS40 on the activity of caspase-3 and the expression of Bcl-2/Bax in H₂O₂-treated PC12 cells**

To further confirm inhibitory effects of GTS40 on H₂O₂-induced cell injury, Western blotting analyses were conducted, including measuring the caspase-3 activity, which is known as a crucial regulator of apoptotic signal pathway, Bax, a proapoptotic protein, and Bcl-2, an antiapoptotic protein. Treating PC12 cells with 100 μmol·L⁻¹ of H₂O₂ for 12 h resulted in a significant augmentation in the activity of caspase-3 (258% of the control value), while it caused a decrease in the ratio of Bcl-2/Bax (39% of the control value), suggesting that H₂O₂ treatment induced cell damage by apoptosis. Pretreatment with GTS40 at the concentrations of 200 and 400 μg·mL⁻¹ significantly decreased the activity of caspase-3 and also accentuated the Bcl-2/Bax expression ratio (62% and 77% of the control value, respectively) as compared with the H₂O₂ group. These experimental results unambiguously indicated the capacity of GTS40 to protect PC12 cells from oxidant stress-mediated cellular injury, as shown in Fig. 5.

**Identification of major components in GTS40 by HPLC-Q-TOF-MS**

HPLC-QTOFMS was developed and optimized to identify chemical ingredients from GTS40. A total of 27 chemical constituents were identified via comparing their retention time (tR) and MS spectra with those of reference standards and data in related studies. The UV chromatograms at 254 nm and MS TIC chromatograms of GTS40 are presented in Fig. 6. The MS data and identification results are placed in Table 1. Peaks 1, 4, 7, 10, 17 and 22 were each unequivocally identified by comparison with the respective standard substances. The rest 21 peaks were identified tentatively by comparing their precise molecular weight, corresponding fragmentation ions with reported literature data [13-19].

**Discussion**

A large number of studies have confirmed that oxidative stress is tightly implicated in the development and progression of many neurodegenerative diseases and numerous drugs exert their beneficial effects mainly through anti-oxidative damage [20-22]. ROS containing H₂O₂, play an important role in oxidative stress-mediated neuron injury [23]. They are mainly developed in the mitochondria, resulting in the free radical attack of cellular lipids and DNA and collapse of MMP, which lead to the intermembrane protein, such as cytochrome c, to be released out of the mitochondria and ultimately triggered caspase-3 activation [24-25]. Caspase-3 activation subsequently initiates DNA fragmentation, nuclear chromatin condensation, and cell apoptosis [26].

Our results from the MTT assay and apoptotic double staining provided the first evidence that GTS40 (an active fraction of GTS) could protect PC12 cells from H₂O₂ (the major component of ROS)-induced oxidative injury. Meanwhile, our results also showed that GTS40 substantially attenuated the increase in MDA production and had a good effect on scavenging ABTS free radicals with 0.85 trolox equivalent. The fluorescent probes used in this study indicated that GTS40 prevented the generation of ROS and the loss of MMP caused by H₂O₂. Based on these findings, we hypothesized that the neuroprotective effect of GTS40 on PC12 in the case of H₂O₂ insult is at least partly attributable to its anti-oxidative properties of inhibiting free radical generations.
Fig. 4  Effect of GTS40 on H₂O₂-induced cell apoptosis by flow cytometry. (A) 100 μmol·L⁻¹ H₂O₂ induced the increase of both the early apoptosis and late apoptosis/necrosis in PC12 cells for 12h incubation. However, the apoptosis of PC12 cells was inhibited by GTS40. (B) Quantitative analysis of the ratio of early apoptosis and late apoptosis/necrosis (*P < 0.001 vs control, ‡P < 0.05, #P < 0.01 vs H₂O₂-treated group). All data are representative of three replications from one experiment.

It is well known that Bcl-2 family plays a pivotal role in the regulation of mitochondria-mediated apoptotic pathway. Anti-apoptotic protein Bcl-2, which locates in the upstream of mitochondria-mediated cell death signal, concentrates much of its efforts to maintain functional integrity of mitochondria by antagonizing with pro-apoptotic protein Bax [27]. In the present study, we found that GTS40 weakened the expression level of Bax and up-regulated the expression of Bcl-2, and thereby improved the expression level of H₂O₂-induced Bcl-2/Bax in PC12 cells, which inhibited the mitochondria depolarization, ROS production and the release of intermembrane proteins, ultimately arrested the down-
stream executive caspase-3 activation and cell death \cite{28}.

Fig. 5  Effects of GTS40 on the expression of caspase-3, Bcl-2 and Bax in PC12 cells exposed to 100 μmol·L\(^{-1}\) \(\text{H}_2\text{O}_2\). (A) PC12 cells were lysed after treating with GTS40 and \(\text{H}_2\text{O}_2\), the protein was detected by western blot. (B–C) Quantitative analysis of the expression of caspase-3, Bcl-2/Bax and the values given are the mean ± SEM (*\(P < 0.05\), **\(P < 0.01\) vs \(\text{H}_2\text{O}_2\)-treated group). All data are representative of three replications from one experiment.

From the HPLC-QTOFMS experiment results, a total of twenty-seven compounds were identified by comparing retention time \((t_R)\) and MS spectra with standards and the published relevant data, including 5 alkaloids, 9 flavones, 12 saponins and 1 phenylpropanoid. The results showed that these identified compounds are primarily derived from Glycyrrhiza and Uncaria thorn according to previous literatures.

The main constituents of GTS40 are triterpene saponins, among which Glycyrrhizin acid has the highest abundance with the relative content up to 15% by the normalization method of chromatographic peak area.

These 27 identified compounds were the representatives of major ingredients in GTS40. The majority of them had been reported as the main bioactive constituents of the individual herbs\cite{1,29-32}. Isorhynchophylline exerts neuroprotective effect against Aβ\(_{25-35}\)-induced neurotoxicity in PC12 cells via inhibiting oxidative stress and suppressing the mitochondrial pathway of cellular apoptosis \cite{1}. Nar-ningin has been found to have antioxidant activities, whose IC\(_{30}\) value was 9 026 μmol·L\(^{-1}\) \cite{29}. Ononin is found to inhibit superoxide-induced damage to PC12 cells through preventing a decrease in the activities of antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) \cite{30}. Some papers have reported that Diammonium glycyrrhizinate, the salt form of Glycyrrhizin acid, which is abundant in GTS40, could attenuate Aβ\(_1–42\)-induced neuronal injury by preventing mitochondrial dysfunction and oxidative stress \cite{31}. Some findings suggest that liquiritin might be a potential agent against cerebral I/R injury in mice by its antioxidant and antiapoptosis properties \cite{32}. In the present study, all of these active monomers were detected in GTS40. Based on previous publications and our experiment results, we infer that these identified ingredients can potentially serve as the main bioactive compounds of GTS40 contributing to the neuroprotective effect against \(\text{H}_2\text{O}_2\)-triggered oxidative injury.

Taken together, the present study was conducted to explore the protective effects and potential active ingredients of GTS40 for the treatment of \(\text{H}_2\text{O}_2\)-triggered oxidative injury. Our results showed that GTS40 significantly elevated the cell viability in \(\text{H}_2\text{O}_2\)-treated PC12 cells. GTS40 exerted its beneficial effects mainly through inhibiting oxidative damage and maintaining the functional integrity of mitochondria, which in turn blocked the apoptosis cascades. In fact, many mechanisms and target proteins are tightly involved in \(\text{H}_2\text{O}_2\)-induced cell toxicity in addition to oxidative damage. For future study, we will focus on effects of GST40 on low \(\text{H}_2\text{O}_2\)-induced signal transduction associated with pathophysiologically relevant pathway. Our results also indicated GTS40 was the major active fraction of GTS for preventing the \(\text{H}_2\text{O}_2\)-induced injury, with 27 characterized chemicals being potential active constituents responsible for its protective effect. We expect the information generated from the present study can provide scientific basis for the application of GTS40 in the management of oxidative stress-associated neurodegenerative disorders such as AD.

Acknowledgments

The authors are grateful to ZHAO Yu-Rong, WEI Meng-Ling, CHEN Fang, and ZHAO Jiao-Jiao for fruitful discussions.
Fig. 6  HPLC-Q-TOF-MS analysis of GTS40. A) HPLC chromatogram at 254 nm; B) Negative ion mode MS spectra; C) Positive ion mode MS spectra

Table 1  Identification of main components in GTS40 by HPLC-Q-TOF-MS

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<tr>
<th>No.</th>
<th>tR/min</th>
<th>Molecular formula</th>
<th>Quasimolecular ion (m/z) Measured ppm</th>
<th>Product ions (m/z)</th>
<th>Identification</th>
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<tr>
<td>1a</td>
<td>5.575</td>
<td>C_{16}H_{18}O_{9}</td>
<td>353.088 [M - H] -2.28</td>
<td>191, 127</td>
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<td>2</td>
<td>15.131</td>
<td>C_{27}H_{32}N_{2}O_{10}</td>
<td>545.213 [M + H] + -1.50</td>
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<td>15.572</td>
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<td>549.161 [M - H] -0.88</td>
<td>429, 417, 255, 135, 119</td>
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<td>4a</td>
<td>16.300</td>
<td>C_{21}H_{22}O_{9}</td>
<td>417.119 [M - H] -0.85</td>
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<td>C_{27}H_{34}N_{2}O_{10}</td>
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<td>Product ions (m/z)</td>
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<td>11</td>
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<td>13</td>
<td>41.992</td>
<td>C$<em>{22}$H$</em>{32}$O$_{4}$</td>
<td>431.133 8 [M + H]$^+$</td>
<td>269</td>
<td>Ononin</td>
</tr>
<tr>
<td>14</td>
<td>42.928</td>
<td>C$<em>{22}$H$</em>{32}$O$_{4}$</td>
<td>417.119 S [M – H]$^+$</td>
<td>255, 135, 119</td>
<td>Isoliquiritin</td>
</tr>
<tr>
<td>15</td>
<td>45.696</td>
<td>C$<em>{15}$H$</em>{28}$N$<em>{2}$O$</em>{4}$</td>
<td>255.066 9 [M – H]$^+$</td>
<td>119</td>
<td>Liquiritigenin</td>
</tr>
<tr>
<td>16</td>
<td>65.619</td>
<td>C$<em>{14}$H$</em>{26}$O$_{2}$</td>
<td>983.449 1 [M – H]$^+$</td>
<td>821, 351</td>
<td>Licorice saponin A3</td>
</tr>
<tr>
<td>17*</td>
<td>68.375</td>
<td>C$<em>{14}$H$</em>{34}$O$_{18}$</td>
<td>879.403 0 [M – H]$^+$</td>
<td>351, 193</td>
<td>22β-acetoxyglycyrrhizic acid or isomer</td>
</tr>
<tr>
<td>18</td>
<td>73.732</td>
<td>C$<em>{16}$H$</em>{32}$O$_{4}$</td>
<td>955.489 4 [M – H]$^+$</td>
<td>351, 193</td>
<td>Yunganoside A1/C1/B1</td>
</tr>
<tr>
<td>19</td>
<td>74.695</td>
<td>C$<em>{16}$H$</em>{32}$O$_{4}$</td>
<td>819.381 2 [M – H]$^+$</td>
<td>351, 193</td>
<td>Licorice saponin E2</td>
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<tr>
<td>20</td>
<td>75.184</td>
<td>C$<em>{15}$H$</em>{28}$N$<em>{2}$O$</em>{4}$</td>
<td>837.391 9 [M – H]$^+$</td>
<td>351, 193</td>
<td>Licorice saponin G2</td>
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<tr>
<td>21</td>
<td>78.934</td>
<td>C$<em>{15}$H$</em>{28}$N$<em>{2}$O$</em>{4}$</td>
<td>837.391 9 [M – H]$^+$</td>
<td>351, 193</td>
<td>Isomer of licorice saponin G2</td>
</tr>
<tr>
<td>22*</td>
<td>79.812</td>
<td>C$<em>{16}$H$</em>{30}$O$_{6}$</td>
<td>823.413 2 [M + H]$^+$</td>
<td>453</td>
<td>Glycyrrhizic acid</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>821.397 7 [M – H]$^+$</td>
<td>469, 351, 193</td>
<td>Isomer of licorice saponin B2</td>
</tr>
<tr>
<td>23</td>
<td>80.896</td>
<td>C$<em>{16}$H$</em>{32}$O$_{18}$</td>
<td>807.417 6 [M – H]$^+$</td>
<td>351, 193</td>
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</tr>
<tr>
<td>24</td>
<td>83.364</td>
<td>C$<em>{16}$H$</em>{32}$O$_{4}$</td>
<td>807.417 4 [M – H]$^+$</td>
<td>351, 193</td>
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<tr>
<td>25</td>
<td>84.085</td>
<td>C$<em>{16}$H$</em>{32}$O$_{4}$</td>
<td>823.409 6 [M + H]$^+$</td>
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<tr>
<td></td>
<td></td>
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<td>821.399 1 [M – H]$^+$</td>
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</tr>
<tr>
<td>26</td>
<td>85.270</td>
<td>C$<em>{16}$H$</em>{30}$O$_{4}$</td>
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<td>27</td>
<td>88.108</td>
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<td>823.4147 [M–H]$^+$</td>
<td>351, 193</td>
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</tr>
</tbody>
</table>

a) Identified by comparing with reference substances.

References


[14] Lin LZ, Harly JM. Identification of the phenolic components

![Image](276x41 to 319x63)


