Original Article

Protective Effect of Trillium tschonoskii Maxim Components Against Glutamate-Induced SH-SY5Y Cells Damage Through Regulating Apoptosis

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Abstract

Context: Among the Tujia people, the root or rhizome of *Trillium tschonoskii* Maxim.in Bull.Acad (TTM) is considered a miraculous herb for headaches. Previous studies have shown ethyl acetate extract (TTM1) can protect SH-SY5Y cells against glutamate injury.

Objective: This study clarified TTM1's mechanism against glutamate-induced cell damage, focusing on the regulation of apoptosis. The compounds were separated, identified, and performed molecular docking with pro-apoptotic proteins.

Materials and Methods: SH-SY5Y cells were treated with glutamate (2 mM) for 12 hour, and the effect of TTM1 (2.5, 5, 10, and 20 μg/mL) was evaluated with MTT and LDH release assays, taking EGb761(40 μg/mL) as a control. Cell apoptosis was detected with Hoechst 33258 and Annexin V-FITC and measurements of intracellular calcium and caspase-3. The major components were separated and identified by LCMS-IT-TOF and NMR, then the proapoptotic activity of TTM1 was confirmed by molecular docking method.

Results: TTM1 protected SH-SY5Y cells by resisting apoptosis, TTM1 (10 and 20 μg/mL) decreased apoptotic bodies and nuclear fragments, increased the proportion of normal cells to 68.38 ± 5.63% and 92.80 ± .88%, decreased VA cells to 4.30 ± .76% and 3.58 ± .45% and caspase-3 to .365 ± .034 and .344 ± .047 ng/mL.TTM1 (10 μg/mL) decreased intracellular free calcium to 2.77 ± .40. Polyphyllin VI and pennogenin 3-O-β-chacotrioside were identified in TTM1 at 15.04% and 2.84%, and had potential anti-apoptosis activities.

Discussion and Conclusions: Folk records of TTM for headache may be related to its anti-apoptosis of nerve cells. Identification and content determination of index components based on effective extract provides research paradigms for rare and endangered ethnic plants.

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Keywords
Trillium tschonoskii maxim, ethyl acetate extract, ethnic medicine, headache, apoptosis, polyphyllin VI, pennogenin 3-O-beta-chacotrioside

Introduction

Medicinal plants have historically proven their value as a source of molecules with therapeutic potential, and nowadays many of the drugs in common therapeutic use are directly or indirectly from plants.[1] The historic trial and ancient records of botanicals in a traditional medical system can provide important information regarding the efficacy of traditional medicines,[2] supplying a valuable pool for recognizing and exploring the medicinal value of plants.[2] The genus Trillium consists of 31 species, widely distributed from the western Himalayas to Japan, China, Kamchatka (Russia), and North America.[3] *Trillium tschonoskii* Maxim. (TTM), is an herbaceous plant in midwestern China, known as "Yan Ling Cao" locally, which has been used in China for thousands of years, and it has been widely used in the treatment of hypertension, headache, neurasthenia, giddiness, trauma bleeding, removing carbuncles, and ameliorating pains.[4] *T tschonoskii*, using the root and rhizome as medicine (Figure 1), has been confirmed as a phytotherapeutic remedy for both inflammatory conditions and pain.[5]

Glutamate is the most abundant free amino acid in the brain and in the nervous system, it is the primary excitatory neurotransmitter participating in a wide range of neural functions such as learning and memory, long-term potentiation, and synaptic plasticity.[6-8] Glutamate exerts its actions in the central nervous system (CNS) through 2 principal types of receptors (ionotropic and metabotropic glutamate receptors), including N-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionicacid (AMPA) and mGluR[9] which may modulate glutamate release, thereby modifying postsynaptic excitability. The early observation showed that increasing amounts of glutamate can cause excitotoxicity and neural cell death, that states excessive glutamate causes neuronal dysfunction, degeneration, and chronic neurodegenerative disorders.[10,11] Therefore, neural cell damage caused by glutamate excitotoxicity is used as the main model for evaluating the neuroprotective effect of drugs.[12]

In our previous study, we found that the ethyl acetate extract (TTM1) of *T tschonoskii* protected SH-SY5Y cells against glutamate-induced nerve cell damage.[13] These results suggest that *T tschonoskii* may have a neuroprotective effect. In the present study, we clarified the mechanism of TTM1 against glutamate-induced cell damage, focusing on the regulation of apoptosis. Then, the major components of TTM1 were separated and identified by an established content determination method. This study will provide experimental evidence to clarify the effects on the nervous system of *T tschonoskii* and its related components contributors.

Materials and Methods

Plant Materials

T. tschonoskii was purchased from Anguo Medicinal Material Market in Hebei Province at September 2009 and was identified by Prof. Fengqing Zhou from Shandong University of Traditional Chinese Medicine. The specimens were made into ultrafine powder according to Tujia people's usage habits and stored under vacuum seal and in a cool dry environment. The results from pharmacognostic studies of *T tschonoskii* conducted in our laboratory were reported previously.[14,15]

Preparation of TTM1

The TTM1 from *T tschonoskii* was obtained with the following protocols: the powder of *T tschonoskii* (2 kg), refluxed in about 6 L ethanol (75% v/v), and heated twice by the reflux extraction method for 45 minutes each time. The extracts were filtered, and the 2 filtrates were combined, evaporated to the final weight (47.5 g, 2.375%) under reduced pressure.

The extracts were completely dissolved in deionized water and then consecutively extracted with petroleum ether, ethyl acetate, and then n-butanol separately. The extraction rates of the petroleum ether extract, TTM1 and n-butanol extract were .245%, 2.375%, and 13.48%, respectively. TTM1 was frozen as a lyophilized powder with a Christ Alpha1-2 lyophilizer (CHRIST, Germany) and later prepared to 1 mg/mL with DMEM/F12 (lot: 929215, Gibco, USA) and stored at 4°C for cell experiments.

Cell Culture

SH-SY5Y human neuroblastoma cells were obtained from the Institute of Basic Medicine of the Chinese Academy of Medical Sciences (National Infrastructure of Cell Line Resource, Beijing, China). The SH-SY5Y cells were cultured to approximately 80% confluence in T-25 flasks (Corning, USA) in DMEM/F12 supplemented with L-glutamine and 10% fetal bovine serum (FBS, lot: AUB34148, HyClone, USA) in a 5% CO_2 humidified incubator at 37°C (Binder CB150, Germany). The cell medium was refreshed every day for 3 days, and the cells were observed under a microscope every day. The cells were used at passages 3∼10, and cells at 70 to 80% confluence were sub-cultured under the conditions described above at 1×10^4 cells/well in standard 96-well plates with 200 μL of 10% FBS and DMEM/F12. After 12 hours in the medium with FBS, the cells were cultured in only DMEM/F12 for another 12 hours before drugs were added to the culture.

Figure 1. *Trillium tschonoskii* Maxim and its firstly recorded document. A Habitat photos of *T tschonoskii* Maxim in Taibai Mountain, Shannxi province, China. B *Western Hubei Ethnic Medicine Records*, written by Western Hubei Autonomous Prefecture Institute for drug control in 1985. C Root and rhizome of *T tschonoskii* Maxim.

Effect of TTM1 on Cell Survival Rate

The cells were randomly allocated to 5 different treatment groups as follows: control and TTM1 groups treated with different doses of TTM1 (5, 10, 20, and 40 μg/mL), with 6 wells in each group. The cells incubated with only DMEM/F12 were considered the negative control. The incubation was continued for 24 hours; subsequently, 10 μL of CCK-8 (lot: FQ659, Dojindo, Japan) was added to the culture plates and incubated for another 3 hours. The absorbance (A) value was measured at 450 nm (Flex Station 3, Molecular Devices, USA). Each experiment was repeated 3 times. The following formula was used to calculate the cell survival rate: the cell survival rate (%) = $A_{TTM1}/A_{control} \times 100\%$.

Protective Effect of TTM1 on SH-SY5Y Cells Injured by Glutamate

Treatment of SH-SY5Y Cells with Glutamate, TTM1, and EGb761. SH-SY5Y cells were seeded at 1×10^4 cells/well in 96-well plates and cultured as described above. For the treatment with glutamate, TTM1, and EGb761, the SH-SY5Y cells were divided into 3 groups: (i) control group: the cells were treated with Locke's buffer; (ii) glutamate group: The cells were treated with 2 m*M* glutamate (BioDee, China)[16]; (iii) glutamate + TTM1 group: the cells were treated with 2 m*M* glutamate and 2.5, 5, 10, and 20 μg/mL TTM1; (iv) glutamate + EGb761 group: the cells were treated with 2 m*M* glutamate and 40 μg/mL EGb761 (lot: 1280211, Dr Willmar Schwabe, Germany), 6 wells/group. Each of these agents was added to the medium 12 hours after the cells were seeded. The following analyses were performed 12 hours after treatment.

MTT Assay. Cell viability was measured using 3 - (4, 5-dimethylthiazol - 2 - yl) - 2,5 -diphenyltetrazolium bromide (MTT, AMRESCO, USA) assay. Then, 20 μL of MTT solution (5 g/L) was added into each well and incubated at 37°C for 4 hours. After the removal of culture medium, 100 μL of dimethyl sulfoxide (DMSO, lot: BCBB9519, Sigma, USA) was added into each well to dissolve formazan. The optical density was measured at 510 nm using a FlexStation 3 (Molecular Devices, USA). The absorbance of the control group was considered to show 100% cell viability. Each experiment was repeated 3 times.

LDH Release Assay. Culture solution (A) was carefully removed and stored at −20°C. Then, 150 μL of Locke's buffer was added to cell culture. The cell culture plate was frozen at −80°C for 5 h to disrupt the cell wall. After defrosting, the cell supernatant (B) was removed, and a lactate dehydrogenase assay kit (lot: 20110610, Nanjing Jiancheng Bioengineering Institute, China) was used to detect the LDH in the cells and the LDH released from the cells. The following formula was used to calculate the LDH release rate

$$\text{LDH release rate}\,(\%) = \text{LDH}_\text{A}/(\text{LDH}_\text{A} + \text{LDH}_\text{B}) \times 100\%$$

Hoechst 33258 Staining. SH - SY5Y cells were seeded at 3×10^5 cells/well into 6 - well plates, cultured 12 hours with DMEM/F12 supplemented with 10% FBS, and for another 12 hours with DMEM/F12. The cell grouping and treatment were the same as before, 4 well/group and 2.5 mL each well. Cells were washed twice with phosphate-buffered saline (PBS) and fixed in methanol/acetic acid (3:1, v/v) for 15 minutes at 4°C. Cells were washed twice with PBS after fixative was removed. Cells were stained with 0.5 mL Hoechst 33258 (lot: 110607, Beyotime) for 5 minutes and then washed twice with PBS, added the anti-fluorescence quenching sealer. Examination was conducted under a fluorescent microscope (Olympus, Japan).

Flow Cytometry Analysis of Cell Apoptosis. After treatment, the cell culture medium was removed and centrifuged at 1000 rpm (Eppendorf, Germany) at 4°C for 10 minutes, and the

suspended cells were collected. The cells attached to the culture plate were detached with .125% trypsin (AMRESCO, USA) and centrifuged. The cells were resuspended in 200 µL of binding buffer. Then, all the collected cells were prepared with 10 µL of Annexin V - FITC (lot: A110723, Biosea Biotechnology, China) and incubated in the dark for 10 minutes at room temperature. After 300 µL of binding buffer and 5 µL of PI (lot: A110723, Biosea Biotechnology, China) were added to each tube, apoptotic cells were evaluated with flow cytometry (Beckman Coulter FC500, USA).

Measurements of Intracellular Calcium. After treatment, the cells attached to the culture plate were detached with .125% trypsin and collected and centrifuged at 1000 rpm at 4°C for 10 minutes. Then, the cells were washed 3 times with PBS. All the collected cells were prepared with 5 µmol/L Fluo-4 AM (lot: S1060, Beyotime, China) and incubated in the dark for 40 minutes at 37°C. The cells were then washed 3 times with PBS to remove the Fluo-4 AM, and then, the cells were cultured with PBS at 37°C for 20 minutes. Excitation/emission wavelengths were adjusted to 494/516 nm for detecting the Fluo-4 AM, and intracellular calcium was examined with flow cytometry.

Measurements of Intracellular Caspase-3. After treatment, the cells were detached with .125% trypsin (AMRESCO, USA) and collected into 15 mL tubes after centrifugation. Then, the cells were resuspended in 1 mL of double distilled water and lysed by an ultrasonic probe (BANDELIN SNOOPILY, Mini 20, Germany) for 20 minutes and centrifuged at 10000 rpm at 4°C for 10 minutes. The supernatant was collected and used to examine caspase-3 with an ELISA.

Separation and Identification of the Active Components in TTM1

TTM1 was analyzed using high performance liquid chromatography (HPLC) system (LC 20A, Shimadzu, Japan) and chromatographic separation was performed on an Agilent Eclipse Plus C18 (250 mm × 4.6 mm, particle size 5 µm, Agilent Technologies, California, USA). TTM1 (2 mg) was weighed and dissolved in 5 mL of methanol (Fisher) and subjected to ultrasonication. The sample was filtered through a .22 µm membrane filter. The mobile phase consisted of acetonitrile (A, Fisher, USA) and water (B) in gradient elution (Table 1). The flow rate was 1.0 mL/min, the column temperature was maintained at 30°C, and the sample injection volume was 10 µL.

Table 1. Mobile Phase Condition of HPLC.

Time (min)	A (%)	B (%)
0	35	65
25	45	55
30	90	10
50	95	5

TTM1 was separated and analyzed by silica column chromatography and eluted repeatedly using a column packed with TTM1 (50 g), mixed silica gel, and chromatography silica gel (W 1: 3: 30). Ethyl acetate-methanol and dichloromethane-methanol-distilled water were used as the mobile phase. The flow rate was 5 mL/min. Thin-layer chromatography (TLC) and HPLC were used to track and monitor the flow separations. The same flow separations were combined and purified with TLC, octadecyl-silica gel (ODS) and semipreparative liquid phase chromatography. The purity of the compounds was detected by HPLC according to the peak area. Structural characterization of the compounds extracted from the TTM1 was performed by Agilent Technologies 6540 UHD Accurate-Mass Q - TOF LC/MS (Agilent, USA) and NMR (AVANCE 600 MH, Bruker, Switzerland).

Components of TTM1 as Determined by HPLC Analysis

Component references were obtained from the experiments described above. After freeze-drying, the reference material and TTM1 were weighed precisely (XP205, Mettler Toledo, Switzerland) and configured to a certain concentration with 5 mL of methanol. The HPLC system and separation conditions were the same as before, and the mobile phase consisted of acetonitrile (A, Fisher) and water (B) in gradient elution: 25 minutes, 35%–45% A → 5 minutes, 45%–90% A → 20 minutes, 90%–95% A → 15 minutes, 35% A. The flow rate was 1.0 mL/min, and the column temperature was maintained at 30°C. Different concentration levels of the component references were prepared by diluting the stock solution. The peak area of each solution was plotted against the concentration to obtain the calibration curves. The methodology was examined with a prepared standard curve and precision, stability, repeatability, and sample adding recovery assays; the results of these tests are expressed as the relative standard deviation (RSD) values of the retention time (RT) and peak area (Pa) for each sample.

Molecular Docking

Two chemical constituents of polyphyllin VI and pennogenin 3-O-β-chacotrioside were determined through the separation and purification of TTM1 of *T tschonoskii*, and identified by Q - TOF LC/MS and NMR in the previous experiment. The required target proteins (receptors) were obtained from the RCSB protein database (PDB, http://www.rcsb.org/) and saved in "pdb" formats. The 3D structures of the active compounds were generated by Chem 3D 2014 (PerkinElmer Informatics, Waltham, MA, USA) and saved in "mol2" format. The accurate docking with the components of *T tschonoskii* and the target proteins were performed using AutoDock

vina1.1.2. The tool drugs, Ginkgolide B, Ginsenoside Rg3, and MSN-50 came from literature, and were used to evaluate the effect of molecular docking in this study, which had been proved to have exact docking activities with the target proteins. The PyMol 2.5 software was used to visualize the docking results and establish the docking interaction mode diagram.

Statistical Analysis

The results related to cell experiments are expressed as the means ± SD, and the data of each subject are collected and analyzed by SPSS17.0 (SPSS, Chicago, USA). If multiple sets of variables conform to normal distribution and homogeneity of variance, the significance is evaluated by one-way analysis of variance (ANOVA). Otherwise, statistical evaluation is performed by means of the non-parametric. $P < .05$ is considered to have a significant difference; $P < .01$ indicates an extremely significant difference.

Results and Discussion

TTM1 Prevented a Decrease in SY-SH5Y Cell Viability Induced by Glutamate

TTM1 with the doses of 5, 10, and 20 μg/mL had no influence on the viability of the SH-SY5Y cells, but 40 μg/mL TTM1 inhibited cell proliferation to 86.43 ± 6.25% ($P < .05$). In this experiment, glutamate (2 mM) was used to induce cell damage, the cell survival rate was 61.3 ± 6.4% exposed to glutamate. Compared with the glutamate group, TTM1 (5, 10, and 20 μg/mL) and EGb761 (40 μg/mL) increased cell survival rates to 73.0 ± 5.9%, 89.9 ± 6.9%, 94.5 ± 7.4%, and 91.7 ± 4.1%, respectively ($P < .05$ and $P < .001$). Compared with glutamate group (17.19 ± 3.46%), TTM1 (2.5, 5, 10 and 20 μg/mL) and EGb761 (40 μg/mL) reduced the LDH release induced by glutamate in the SH-SY5Y cells to 8.34 ± 3.71%, 4.91 ± 2.25%, 3.49 ± 2.16%, and 4.83 ± 2.32%, respectively, ($P < .05$ and $P < .001$). TTM1 from *T tschonoskii* protected the SH-SY5Y cells from damage induced by glutamate. These results are shown in Figure 2.

Glutamate induced a decrease of cell viability and LDH release from SH-SY5Y cells, both of which could be prevented by TTM1. A: Safe dose range of TTM1 on normal SY-SH5Y cells was restricted to less than 40 μg/mL for 24 hours according cell viability, $^{*}P < .05$ vs control group, 6 well/group, n = 3. B: TTM1 (5, 10, and 20 μg/mL) and EGb761 (40 μg/mL) could improve cell survival rate of SH-SY5Y damaged by glutamate, $^{\#}P < .05$ vs control group; $^{*}P < .05$ vs glutamate group; $^{***}P < .001$ vs glutamate group, 6 well/group, n = 3. C: TTM1 (2.5, 5, 10 and 20 μg/mL) and EGb761 (40 μg/mL) could reduce the LDH release assay induced by glutamate in SH-SY5Y cells, $^{\#\#\#}P < .001$ vs control group; $^{*}P < .05$ vs glutamate group; $^{***}P < .001$ vs glutamate group, 8 well/group.

TTM1 Inhibited the Apoptosis of SY-SH5Y Cells Induced by Glutamate

Hoechst33258 staining is a classical, rapid, and simple method for detecting apoptosis. The nuclei of apoptotic cells and typical apoptotic bodies stained by Hoechst33258 can be observed under fluorescence microscopy. Compared with the control group, cells treated with glutamate presented apoptotic features of cells, such as cell shrinkage, nuclear chromatin concentration, and broken cell membranes with nuclear lysis, which were relieved in the TTM1 (10,20 μg/mL) treated group. To identify whether TTM1 inhibits apoptosis, the treated cells were stained with Annexin V-FITC/PI, and the population of apoptotic cells was determined based on flow cytometry. The results showed: (i) in glutamate group, the percentage of normal cells decreased to 15.50 ± 3.66%, the percentage of viable cells undergoing apoptosis (VA cells) increased to 36.4 ± 6.72%, and that of non-viable apoptotic (NVA) cells increased to 46.93 ± 3.61%, while the NVA percentage in the control group was 6.00 ± 1.44% ($P < .001$). (ii) TTM1 (2.5, 5, 10, and 20 μg/mL) significantly increased the proportion of normal cells (27.83 ± 3.45%, 28.25 ± 4.74%, 68.38 ± 5.63%, and 92.80 ± .88%) and decreased the proportion of VA cells (4.00 ± 1.28%, 10.28 ± .43%, 4.30 ± .76%, and 3.58 ± .45%) ($P < .05$ or $P < .001$). TTM1 of 10 and 20 μg/mL decreased the proportion of NVA cells to 20.18 ± 2.13% and 2.25 ± .34% ($P < .001$). (iii) TTM1 (20 μg/mL) induced a greater antiapoptotic effect than did TTM1 (10 μg/mL). The results are shown in Figure 3.

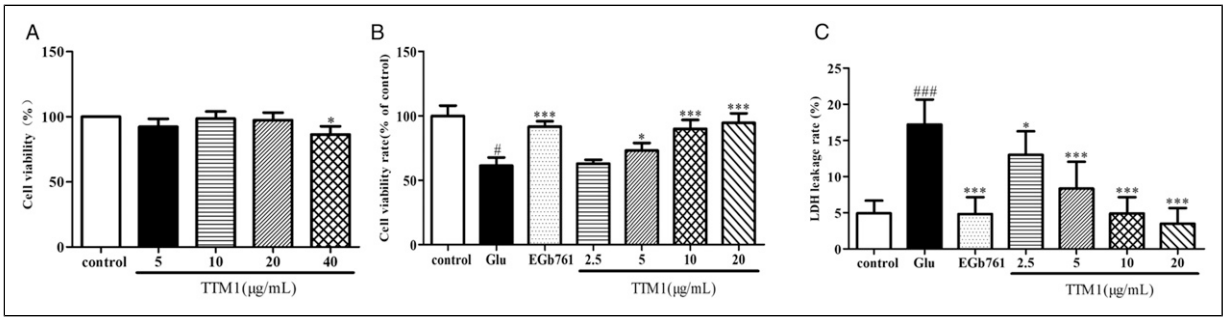

Figure 2. Effect of TTM1 on cell viability and LDH release assay of SH-SY5Y(±SD).

TTM1 Decreased Intracellular Calcium Level in the SY-SH5Y Cells Induced by Glutamate

Calcium is a second messenger that plays a fundamental role in a plethora of cellular processes, including cell proliferation and cell apoptosis. Therefore, to determine the effect of TTM1 on calcium levels in the SH-SY5Y cells, intracellular-free calcium was measured in control group, cells treated with glutamate and cells in the TTM1 group. All the cells were loaded with Fluo-4AM, and the relative fluorescence was measured by flow cytometry. As shown in Figure 4, glutamate (2 m*M*) significantly increased the relative fluorescence of the SH-SY5Y cells, which increased to 32.27 ± 9.13 ($P < .01$), compared with that of the control (13.97 ± 4.63). TTM1 decreased the concentration of intracellular free calcium, and compared with that of the glutamate group, the influence of TTM1 (10 μg/mL) on intracellular free calcium was significant (12.77 ± .40, $P < .01$).

TTM1 Decreased Caspase-3 in the SY-SH5Ycells Induced by Glutamate

Caspase-3 is known as the executor of apoptosis. The content of intracellular caspase-3 in the cells damaged by glutamate was significantly higher than it was in the control group cells ($P < .001$), the contents were separately .856 ± .108 and .378 ± .042 ng/mL. TTM1 (2.5, 5, 10, and 20 μg/mL) decreased the content of caspase-3 (.442 ± .061 ng/mL, .399 ± .028 ng/mL, .365 ± .034 ng/mL, and .344 ± .047 ng/mL) compared with that of the glutamate group, which indicated that TTM1 might inhibit caspase-3-mediated apoptosis (Figure 5).

Isolation and Identification of TTM1

The HPLC results showed 2 single and strong absorption peaks (peak times of 19 and 22 minutes, respectively) representing TTM1, as shown in Figure 6. After purifying the samples, we obtained 2 compounds. Compound A was obtained as a white powder. Its molecular formula was deduced as $C_{39}H_{62}O_{13}$, and the molecular weight was presumed to be 738 based on LCMS - Q - TOF data (*m/z* 761.3708 $[M + Na]^+$). The ^{1}H-NMR and ^{13}C-NMR data showed that ^{1}H - NMR (400 MHz, CD_3OD) δ: 5.42 (1H, brd, $J = 5.1$ Hz, H - 6), 5.23 (1H, brs, H-1′′′′), 4.54 (1H, d, $J = 7.8$ Hz, H-1′), 1.28 (3H, d, $J = 6.2$ Hz, 2′′-O-Rha - CH_3), 1.09 (3H, s, H-19), .93 (3H, d, $J = 7.2$ Hz, H-21), .87 (3H, s, H-18), .84 (3H, d, $J = 6.3$ Hz, H-27); ^{13}C-NMR (100 MHz, CD_3OD) δ: 141.9 (C - 5), 122.6(C-6), 110.9 (C-22), 102.2(C-1′′), 100.5 (C-1′), 91.3 (C - 17), 90.6 (C-16), 79.4(C-2′), 79.2 (C-3), 79.0 (C-4′), 77.7 (C-3′), 74.0 (C-4′′), 72.4 (C-3′′), 72.2 (C-2′′), 71.9 (C - 5′), 69.8 (C-5′′), 67.7 (C-26), 62.8 (C-6′), 53.9 (C-14), 51.5 (C-9), 45.8 (C-13), 45.5 (C - 20), 39.5 (C-4), 38.6 (C-1), 38.0 (C-10), 33.3 (C-8), 33.2 (C-23), 32.9 (C-12), 32.5 (C-7), 32.1 (C-15), 31.3 (C-25), 30.8 (C-2), 29.4 (C-24), 21.7 (C-11), 19.8 (C-19), 17.9 (C-6′′), 17.5 (C-27), 17.4 (C - 18), 9.06 (C-21). The above data were consistent with literature reports,[17] compound A is identified as pinogenin-3β-*O*-α -l-

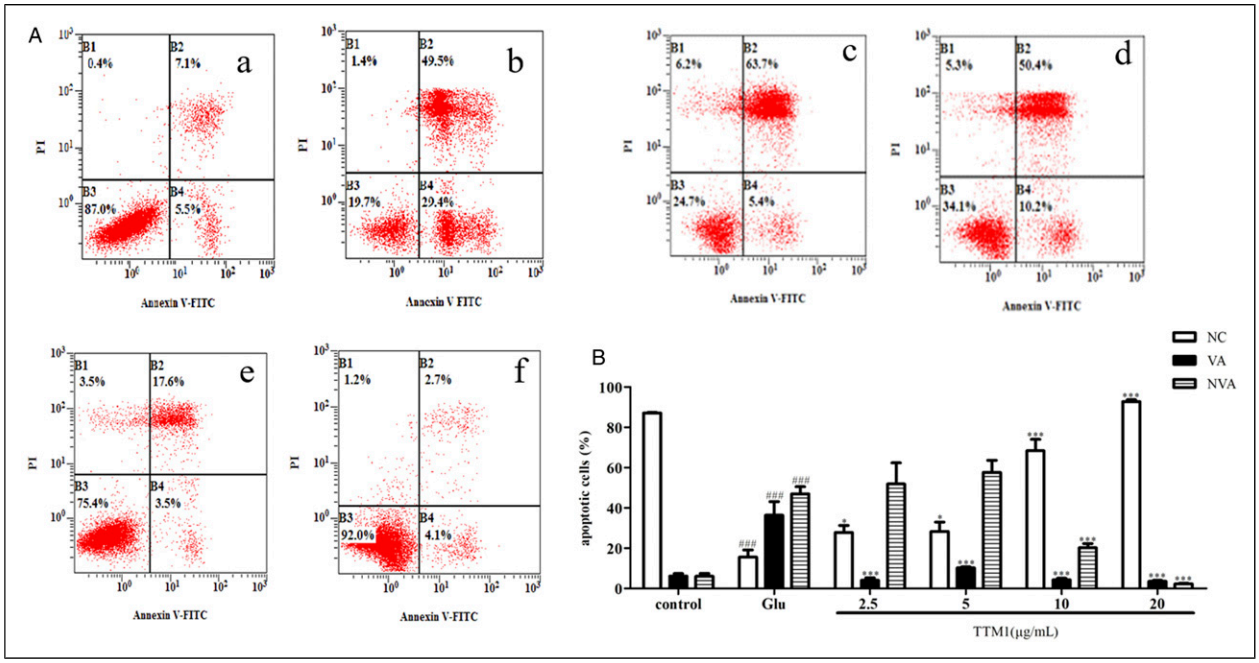

Figure 3. Effect of TTM1 on apoptosis in SH-SY5Y cells exposed to glutamate. Note: A: Apoptosis assay in SH-SY5Y cells treated by glutamate and TTM1 (Annexin V/PI staining assay), control group (a), glutamate group (b), glutamate + TTM1(2.5 μg/mL) group (c), glutamate + TTM1 (5 μg/mL) group (d), glutamate + TTM1 (10 μg/mL) group (e) and glutamate + TTM1 (20 μg/mL) group (f). B: Histogram of apoptosis assay (±SD), $^{###}P < .001$ vs control group; $^{*}P < .05$, $^{***}P < .001$ vs glutamate group, n = 4.

pyranoside-(1→2)- *O*-β -D-glucopyranoside, also called polyphyllin VI, and the LCMS-IT-TOF and NMR spectra shows in Figure 7.

Compound B displayed a molecular of $C_{45}H_{72}O_{17}$ as determined by LCMS-Q-TOF (*m/z* 907.4212 $[M + Na]^+$), and the molecular weight was presumed to be 885. Its ^{1}H-NMR and ^{13}C-NMR data showed that ^{1}H-NMR (400 MHz, CD_3OD)*δ*: 5.42(1H, brd, *J* = 5.1 Hz, H-6), 5.25 (1H, brs, H-1″), 4.88 (1H, brs, H-1‴), 4.54 (1H, d, *J* = 7.8 Hz, H - 1′), 1.30 (3H, d, *J* = 6.3 Hz, 4‴ -O-Rha-CH_3), 1.28 (3H, d, *J* = 6.1 Hz, 2″ -O -Rha-CH_3), 1.09 (3H, s, H-19), .93 (3H, d, *J* = 7.2 Hz, H-21), .87 (3H, s, H-18), .84 (3H, d, *J* = 6.3 Hz, H-27); ^{13}C- NMR (100 MHz, CD_3OD) *δ*: 141.9 (C-5), 122.6 (C-6), 110.9 (C-22), 103.0(C-1‴), 102.3(C-1″), 100.5 (C-1′), 91.3 (C-17), 90.6 (C-16), 80.1(C-4′), 79.3 (C-2′), 79.3(C-3), 78.1(C-3′), 76.6 (C-5′), 74.0 (C-4″), 73.8 (C-4‴), 72.5 (C-3″), 72.4 (C-2″), 72.2 (C-2‴), 72.2 (C-3‴), 70.7 (C-5‴), 69.8 (C-5″), 67.7 (C-26), 62.0 (C-6′), 53.9 (C-14), 51.5 (C-9), 45.8 (C-13), 45.5 (C-20), 39.5 (C-4), 38.6 (C-1), 38.0(C - 10), 33.3 (C-8), 33.2 (C-23), 32.9 (C-12), 32.5 (C-7), 32.1(C-15), 31.3(C-25), 30.8(C-2), 29.4 (C-24), 21.7(C-11), 19.8(C-19), 18.0 (C-6‴), 17.9 (C-6″), 17.5 (C-27), 17.4 (C-18), 9.06 (C-21). The above data were consistent with literature reports,[18] compound B is identified as pennogenin 3-*O*-α -l-rhamnopyranosyl- (1→ 4)- [α-l- rhamnopyranosyl-(1→ 2)]-

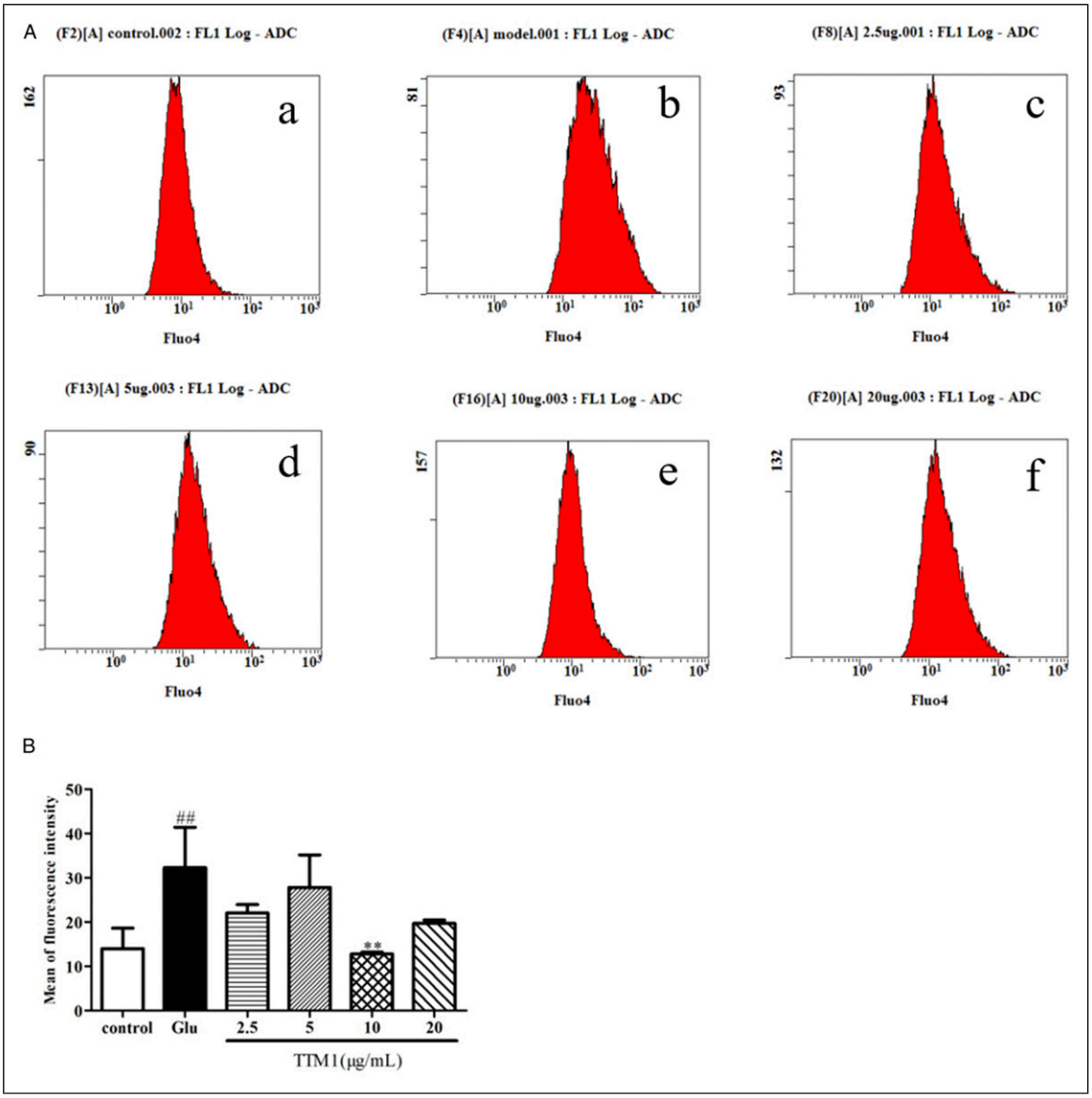

Figure 4. Effect of TTM1 on intracellular calcium in SH-SY5Y cells exposed to glutamate. Note: A: fluorescence intensity of intracellular calcium detected by flow cytometry, control group (a), glutamate group (b), glutamate + TTM1 (2.5 μg/mL) group (c), glutamate + TTM1 (5 μg/mL) group (d), glutamate + TTM1 (10 μg/mL) group (e) and glutamate + TTM1 (20 μg/mL) group (f). B: Histogram of intracellular calcium, $^{\#\#}P < .01$ vs control group. $^{**}P < .01$ vs glutamate treating group.

β-D-glucopyranoside, which may be referred to as pennogenin 3-*O*-β-chacotrioside, and the LCMS-IT-TOF and NMR spectra shows in Figure 8.

Content of 2 compounds in TTM1

Polyphyllin VI and pennogenin 3-*O*-β-chacotrioside were used as references (Figure 9) to generate standard curves and then to calculate the sensitivity, stability, precision, repeatability, and recovery rate; the method was validated in terms of all these parameters. The following the linear regression results were obtained: for polyphyllin VI, $y = 8.3 \times 10^5 \times -3.8 \times 10^5$, $R^2 = .9973$, in the range of .714 ~ 4.76 μg; and for pennogenin 3-*O*-β-chacotrioside, $y = 7.3 \times 10^5 \times -2.9 \times 10^5$, $R^2 = .9994$, in the range of .198 ~ 49.5 μg. The content was accurately determined using the regression equation. For the precision estimation, TTM1 (methanol dissolved, 5.55 mg/mL, 10 μL) was assayed successively 5 times (Table 2). To determine the stability of the extracts, sample extracts were assayed separately at 1, 2, 5, and 24 hours after extraction (Table 3). The values of the relative standard deviation (RSD) for polyphyllin VI and pennogenin 3-*O*-β-chacotrioside were 1.7% and 3.1%, respectively, indicating that the sample extract remained stable for at least 24 hours. To determine the rate of sample recovery, polyphyllin VI (500 μL) and pennogenin 3-*O*-β-chacotrioside (500 μL) were added at known concentrations to the TTM1 sample (5.55 mg/mL, 500 μL) at the beginning of the extraction. The recovery rates for polyphyllin VI and pennogenin 3-*O*-β-chacotrioside were 104.9% (RSD = 1.5%) and 99.3% (RSD = 1.7%), respectively (Table 4). The level of polyphyllin VI and pennogenin 3-*O*-β-chacotrioside in the TTM1 were 15.04% (RSD = 1.07%) and 2.84% (RSD = .95%), respectively, as indicated by 3 successive assays. Results are shows in Table 5.

Polyphyllin VI and Pennogenin 3-O-beta-chacotrioside Target Apoptosis-Related Proteins

Many evidences have reported that glutamate increases cytoplasmic Ca^{2+} concentration, the excessive glutamate concentration causes neuronal cell death, namely excitotoxicity via mitochondria dysfunction,[19] and causing intracellular calcium overload may be one of the key factors to induce apoptosis. These proteins related to apoptosis, including CYTC, AIF1, and BAX were docked with polyphyllin VI and pennogenin 3-O-beta-chacotrioside. Ginkgolide B,[20] ginsenoside Rg,3[21] and MSN-50[22] were reported publicly chemical constituents that can bind to these above proteins, and were used as control drugs in this molecular docking. The results showed in Figures 10 and 11. CYTC, AIF1, and BAX are all pro-apoptotic proteins, compared with the inhibitor drugs, polyphyllin VI, and pennogenin 3-*O*-β-chacotrioside showed good docking abilities with them. There were 6 hydrogen bonds between polyphyllin VI and AIF1, which included ASP (position 44, bond distance = 2.7 Å), GLU (position 45, bond distance = 2.5 Å), GLY (position 70, bond distance = 2.3 Å),

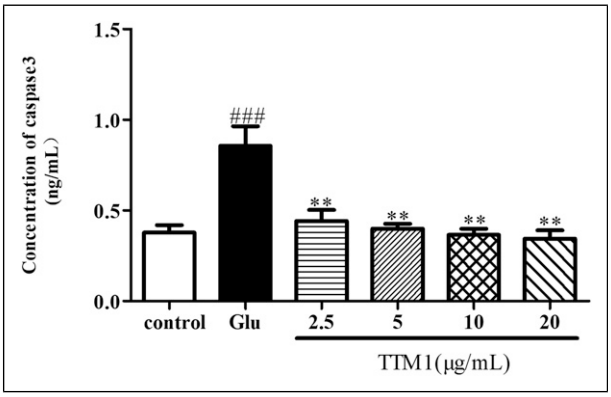

Figure 5. Effect of TTM1 on caspase 3 in SH-SY5Y cells exposed to glutamate (±SD).Note: caspase-3 was calculated according the linear regression equation: y = .1689 x + .1243, $R^2 = .9925$, x represents content of caspase 3 (unit: ng/mL), y represents absorbance under A_{405nm}; ###*P* < .001 vs control group; **P < .01 vs glutamate treating group. n = 4.

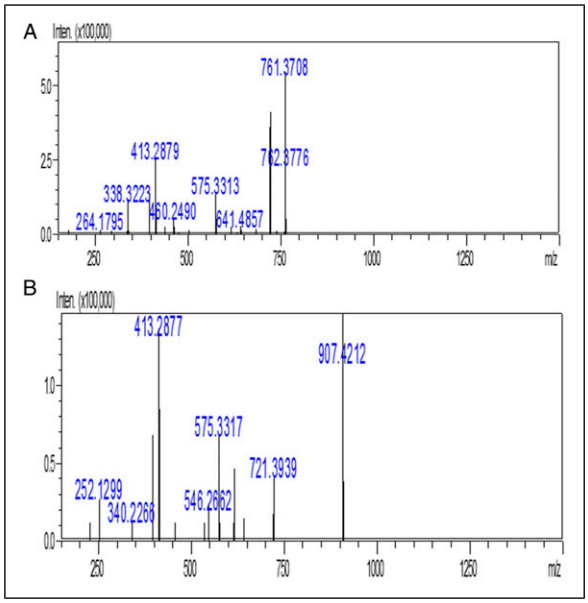

Figure 6. Spectra of compound A and B (+ESI) based on LCMS-IT-TOF. Note: Compound A (a); Compound B (b).

GLY (position 42, bond distance = 2.4 Å), ILE (position 43, bond distance = 2.4 Å), and ARG (position 23, bond distance = 2.1 Å). There were 4 hydrogen bonds between pennogenin 3-*O*-β-chacotrioside and CYTC, which included ARG (position 45, bond distance = 2.5 Å, 2.5 Å), PHE (position 85, bond distance = 2.8 Å), LYS (position 36, bond distance = 2.3 Å). The docking positions of other molecules and proteins are shown in Figure 11.

(A: polyphyllin VI and CYTC; B: pennogenin 3-*O*-β-chacotrioside and CYTC; C: ginkgolide B and CYTC; D: polyphyllin VI and AIF1; E: pennogenin 3-*O*-β-chacotrioside and AIF1; F: ginsenoside Rg3 and AIF1; G: polyphyllin VI and

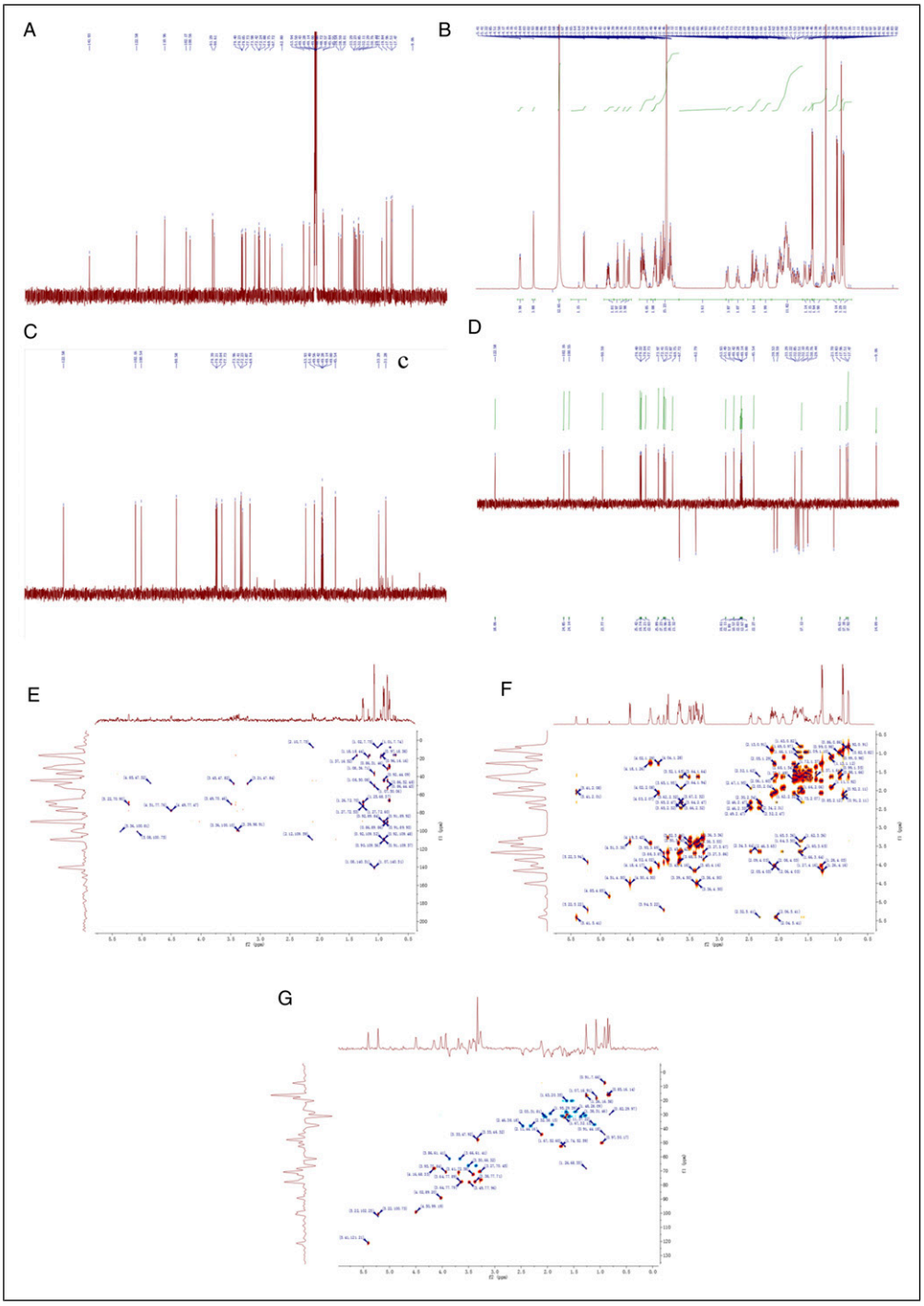

Figure 7. NMR spectrum of compound A. Note: ^{1}H-NMR (CD_3OD, 600 MHz) (a); ^{13}C-NMR (CD_3OD, 150 MHz) (b); DEPT 90 (CD_3OD, 150 MHz) (c); DEPT 135 (CD_3OD, 150 MHz) (d); HMBC (e); H-H COSY(f); HSQC (g).

BAX; H: pennogenin 3-*O*-β-chacotrioside and BAX; I: MSN-50 and BAX; In the overall effect picture of docking, protein is represented by carton model, and small molecules are represented by sticks model; The two-dimensional detailed diagram can be used to observe the hydrophobic interaction between small molecules and protein residues.)

Discussion

In this study, we used glutamate to simulate neuronal excitotoxicity caused by increased glutamate levels in vivo, and administered 2 m*M* glutamate to SH-SY5Y cells, after 12 hours of incubation, the glutamate induced apoptosis in vitro. Glutamate

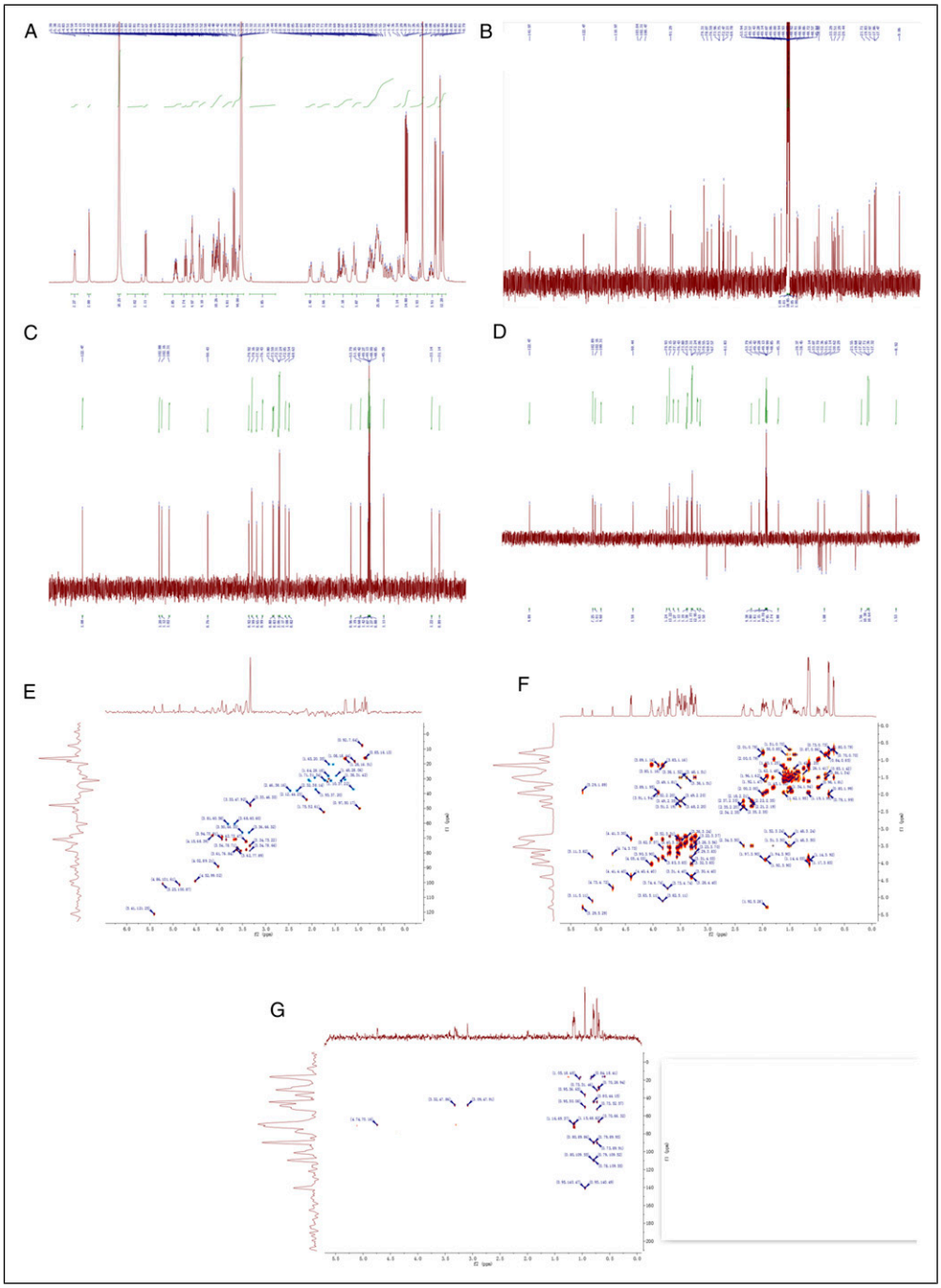

Figure 8. NMR spectrum of compound B. Note: ^{1}H-NMR (CD_3OD, 600 MHz) (a); ^{13}C-NMR (CD_3OD, 150 MHz) (b); DEPT 90 (CD_3OD, 150 MHz) (c); DEPT 135 (CD_3OD, 150 MHz) (d); HMBC(e); H-H COSY(f); HSQC (g).

can mediate excitatory synaptic transmission by activating ionotropic and metabotropic receptors. Ionotropic receptors primarily mediate fast synaptic transmission, and their activation leads to calcium influx, triggered intracellular signaling pathways, and production and release of vasoactive agents such as nitric oxide (NO). TTM1 protected the SH-SY5Y cells from apoptosis induced by glutamate. Our study focused on the effects of TTM1 on the intracellular calcium and caspase-3, which are the executors of apoptosis. Annexin V/PI double staining was used to determine the effect of TTM1 on apoptosis through morphological and quantitative analyses, and TTM1 reduced glutamate-induced apoptosis.

Neuronal cell death or apoptosis may be induced by increased intracellular free calcium. The mechanism by which calcium induces apoptosis or other types of death may include a large amount of calcium accumulation in mitochondria

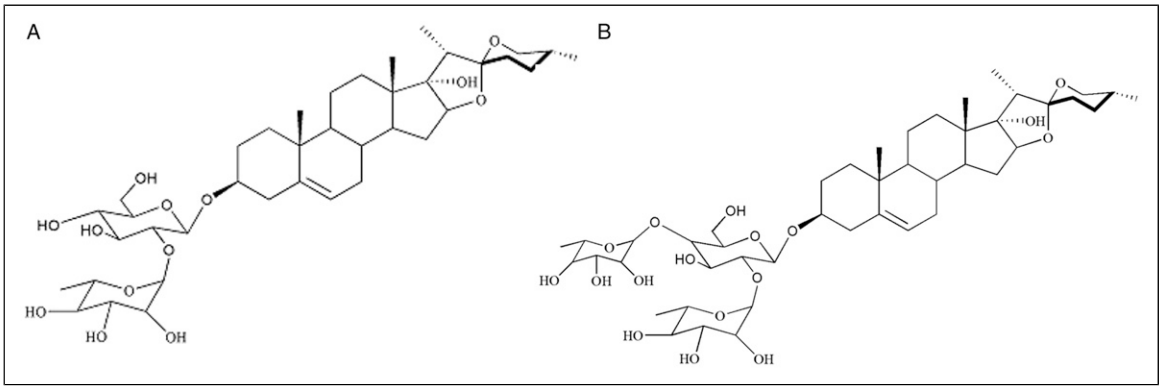

Figure 9. Structure of polyphyllin VI (compound A). Structure of pennogenin 3-O-beta-chacotrioside (compound B).

Table 2. Precision Results of Polyphyllin VI and Pennogenin 3-*O*-β-chacotrioside.

No	Peak Area (A)	Peak Area (B)
1	6965697	844629
2	7029595	869107
3	6843357	868655
4	6830356	849849
5	6756478	823991

Table 3. Stability Results of Polyphyllin VI and Pennogenin 3-*O*-β-chacotrioside.

Time (h)	Peak Area (A)	Peak Area (B)
1	6965697	844629
2	7029595	869107
5	6756478	823991
24	6908573	809233

Table 4. Average Recovery Results of Polyphyllin VI and Pennogenin 3-*O*-β-chacotrioside.

No	Original Quantity (μg)	Additive Quantity (μg)	Detection Quantity (μg)	Recovery Rate	Mean Recovery, %
A1	3.98	1.12	5.378	1.067342132	104.9
A2	3.98	1.12	5.270	1.040178301	
A3	3.98	1.12	5.269	1.039837743	
B1	.794	5.52	6.285	.995	99.3
B2	.794	5.52	6.365	1.009	
B3	.794	5.52	6.178	.976	

interferes with the oxidative phosphorylation process, causing energy metabolism disorders, or it may involve some calcium-induced intracellular proteases that destroy the cell membrane and cytoskeleton.[23] In addition, intracellular calcium overload can activate nonselective ion channels, increasing the permeability of cell membranes to sodium, potassium, and other ions, which causes electrolyte imbalance in cerebral ischemic tissue.[24] TTM1 was confirmed to prevent increases in intracellular calcium in the SH-SY5Y cells, while this inhibition of intracellular calcium was most significant when the concentration of TTM1 was 10 μg/mL. Caspase-3 is required for some typical hallmarks of apoptosis, which is essential for specific processes as sociated with the degradation of the cell and the formation of apoptotic bodies. Pathways of caspase-3 activation have been identified, and they are dependent on or independent of mitochondrial cytochrome c release and caspase-9 function.[25] TTM1 (2.5, 5, 10, and 20 μg/mL) reduced the expression of caspase-3 in injured cells, revealing that TTM1 might play an important anti-apoptotic role.

Table 5. Content Determination of Polyphyllin VI and Pennogenin 3-*O*-β-chacotrioside.

No.	Peak Area	Injection Volume (μg)	Content (%)	Mean (%)
A1	6965697	8.450237349	15.22565288	15.04
A2	6843357	8.302839759	14.96007164	
A3	6830356	8.287175904	14.93184847	
B1	869107	1.587817808	2.860932988	2.84
B2	868655	1.58719863	2.859817352	
B3	849849	1.561436986	2.813399975	

Figure 10. Thermal map of the lowest binding energy of the proteins and components by molecular docking.

Phytochemical examinations have revealed that *T tschonoskii* contains a large number of steroidal saponins, and over 50 saponins, including diosgenyl, pennogenyl, protodiosgenyl, kryptgenin, and trillenogenin saponins, have been isolated from it.[26] Through the separation and purification of TTM1 and analysis based on LCMS-IT-TOF and NMR, 2 components of *T tschonoskii* were obtained and identified: polyphyllin VI and pennogenin 3-*O*-β-chacotrioside. Then, we developed a method of content determination for these 2 components that depended on HPLC, and the contents of polyphyllin VI and pennogenin 3-O-β-chacotrioside in TTM1 were found to be 15.04% and 2.84%, respectively. The anti-apoptosis activities of these 2 components were predicted by molecular docking, the target proteins including CYTC, AIF1, BAX acquired from signal pathways related with calcium and caspase 3, and their specific inhibitors consisted of ginkgolide B, ginenoside Rg3 and MSN-50 were all confirmed by literature.[20-22] The process of apoptosis includes Ca^{2+} increasing, cytochrome C (CYTC) releasing Bcl-2 protein from mitochondria decreasing, and caspase-3, caspase-8, caspase-9 activating.[27] Cytochrome C (Cyt c), located on the outside of mitochondrial inner membrane, is a key factor in mammalian apoptosis signal transduction; Apoptosis-inducing factor (AIF) is fixed in the mitochondrial inner membrane by an amino terminal transmembrane bundle. After the permeability of the mitochondrial membrane changes, the translocation enzyme is activated to expose phosphoserine and transmit apoptosis signals.[28] Bax as a pro-apoptotic protein can be activated by Ca^{2+}, leading to mitochondrial membrane permeability changes, release of cytochrome c and caspase activation.[29-31] The results of molecular docking suggested that polyphyllin VI and pennogenin 3-O-β-chacotrioside showed good docking abilities with these proteins, even better than their inhibitors. Combined with the above cell experiment results of TTM1, it can be concluded that polyphyllin VI and pennogenin 3-O-β-chacotrioside may be one of the material bases of *T tschonoskii* on anti-apoptosis.

Because of damage to the growing environment and the overuse of natural resources obtained in the wild, *T tschonoskii* was included in the List of Rare and Endangered Protected Plants in China (1984) but not in the List of National Key Protected Wild Plants (1999).[32] However, with increasing clinical usage, *T tschonoskii* could possibly become an endangered and rare plant again, new drug research and clinical application of *T tschonoskii* must be balanced with its protection. Thus, we explored and practiced an approach used for endangered and rare plants as follows: (i) a cell model was established to determine the activation factors of disease in vitro, which was used to screen and evaluate the extract; (ii) the easily obtained components of the extracted parts were isolated, purified, and identified, and the content of these components in the active extract were determined. The results from this study will benefit from verification of the efficacy of the extract in vivo and establishment of quality standards for herbal medicine in future research. According to the guidelines of Chinese pharmacopoeia that the detected marker components can reflect the efficacy of TCMs or be related to

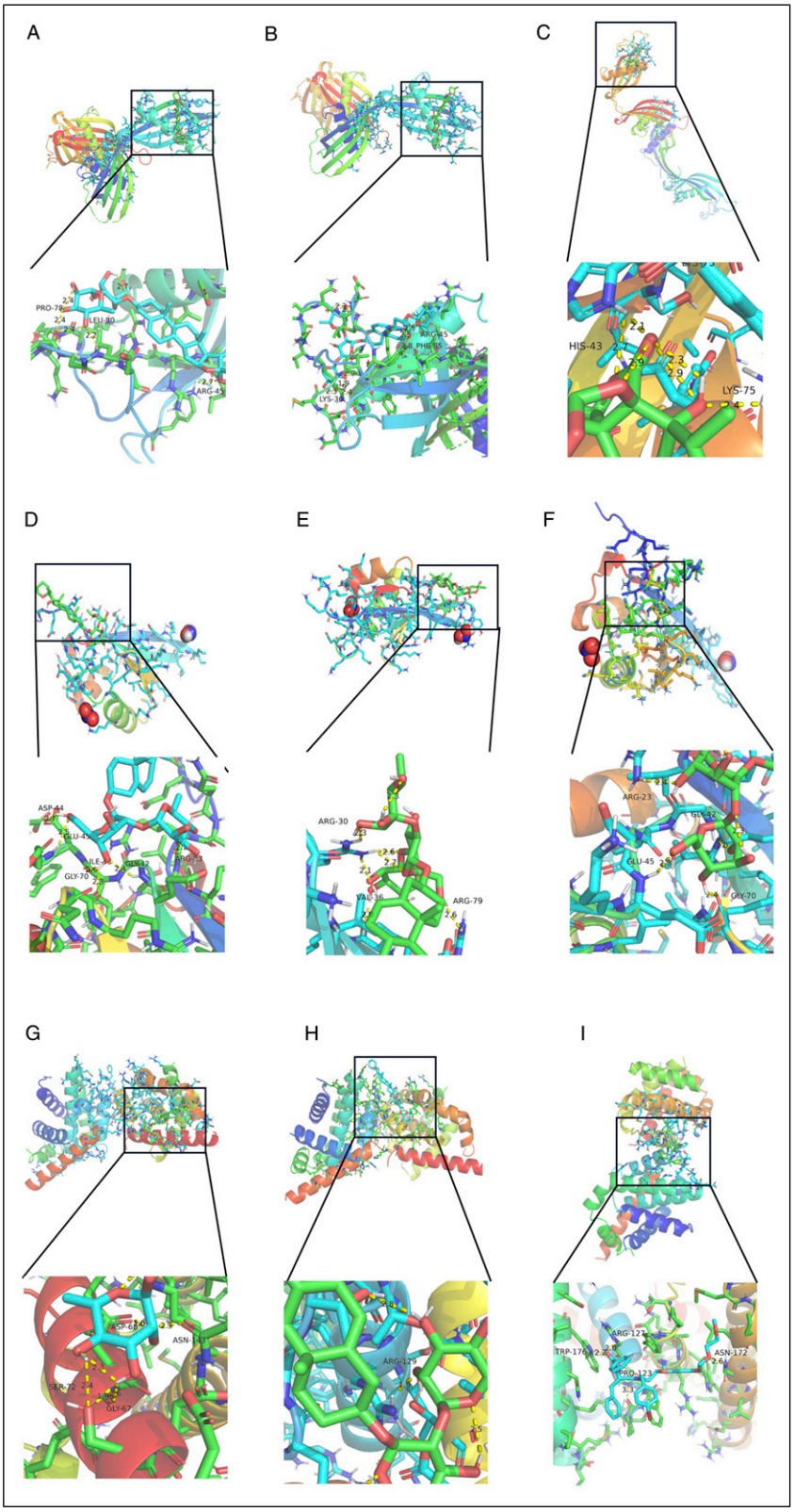

Figure 11. Molecular docking visualization.

certain activities or safety of TCMs,[33] Polyphyllin VI and pennogenin 3-O-β-chacotrioside, which have good resolution and high contents in the active extract of *T tschonoskii*, might be used as reference materials for quality evaluation.

Conclusions

Our results show that TTM1 reduces the apoptosis induced by glutamate either by reducing or decreasing intracellular calcium and decreasing caspase-3 levels in glutamate-injured cells. Polyphyllin VI and pennogenin 3-O-β-chacotrioside might be the index components for contributing to this mechanism of *T tschonoskii.*

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Data Availability

The data used to support the findings of this study are available from the first author upon request.

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