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The Neuroprotective Effect of the Extract of Traditional Mongolian Medicine Eerdun Wurile in PC12 Cells

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Abstract: *Background*: Traditional Mongolian medicine Eerdun Wurile is one of the ideal drugs for cerebral stroke and is effective on treating vascular and nervous system diseases. Numerous studies have proved that Eerdun Wurile has neuroprotective effect. However, the biological mechanism of which is still unclear. *Objective*: To investigatethe molecular mechanism underlying the neuroprotective effect of Eerdun Wurile. *Methods*: The extract from Eerdun Wurile was prepared by simulated gastric fluid first and its effect on PC12 cells differentiaton and MAPK phosphorylation was tested by neurite outgrowth assay and western blotting. Also, the target of active compounds was predicted by moleculr modeling system and the content of active compounds was examined by LC-MS/MS. *Result*: Result showed that the extract stimulated the differentiation of PC12 cells, exhibited nerve growth factor (NGF)-like effect. Meanwhile, the extract induced the MAPK phosphorylation in PC12 cells. In-depth analysis by molecular modeling technology predicted that genipin interact with MAPK as a target. Consistent with the prediction, genipin induced the phosphorylation of MAPK in PC12 cells. Besides, genipin was detected both in the extract and Eerdun Wurile pills. *Conclusion*: These results proved that the extract of Eerdun Wurile has neuroprotective effect in PC12 cells, and genipin in the extract and Eerdun Wurile might be one of the active compounds that show neuroprotective effect.

Keywords: Traditional Mongolian Medicine Eerdun Wurile, Molecular Modeling Technology, Genipin, MAPK, PC12 Cells

1. Introduction

Eerdun Wurile is a traditional Mongolian drug effective on the treatment for learning and memory ability decline. It is one of the most ideal drugs for the nervous system diseases and is the main therapeutic drug for cerebral stroke [1].

Eerdun Wurile could increase the content of SOD, scavenge free radicals, inhibit the expression of MDA，NO，ROS and Nogo-A (Neurite outgrowth inhibitor-A), MBP (Myelin basic protein), so that effectively suppress the oxidative damage of tissue and reduce neurons damage [1, 2].

Eerdun Wurile has significant curative effect on memory loss, dizziness, drowsiness, headache and insomnia when used with another Mongolian medicine Zhachong-13 pills. It is also effective on the sequelae of cerebral infarction, neurological dysfunction caused by cerebral hemorrhage, disturbance of consciousness when supplied with other Mongolian medicines. Eerdun Wurile enhances the effect of lipoic acid and mecobalamin on treating diabetic neuropathyneurotoxicity caused by vincristine chemotherapy. Besides, recent studies have proved that the curative effect of Eerdun Wurile on brain infarction and the recovery of cerebral stroke again [3, 4].

Although the clinical effect of Eerdun Wurile has been proved, the biological mechanism is still unclear. The results from recent studies suggest that Eerdun Wurile can promote the secretion of neurotrophic factors on the site of nerve injury, such as the expression of BDNF (brain-derived neurotrophic factor), NGF, bFGF (basic fibroblast growth factor), TGF-β (Transforming growth factor beta) at both protein and gene levels, and decrease IL-1β (Interleukin-1β) and TNF-α level in brain tissue, so as to repair the damaged neurons. Igf2 (Insulin-like growth factor 2) is a regulator of cell proliferation, plays an important role in promoting cell proliferation and differentiation. The expression of Igf2 in cerebral cortex of ischemic stroke model rats was significantly up regulated by Eerdun Wurile [1]. Erdun Wurile could also down regulate the level of Microglia marker Ibal, increase the M2 type microglia marker Arg-1 in MCAO/R damage model rat brain [5]. Eerdun Wurile induces the activation, proliferation and polarization of microglia in the injured area and improves angiogenesis and nerve repair through regulating microglia markers, growth factors and protease [6]. It was also reported that Eerdun Wurile inhibits the retinal ischemia-reperfusion injury causedincrease of Bax, Fas, Fasl and p53 proteins in rat [7]. In rat myocardial ischemia-reperfusion injury model, Eerdun Wurile prohibited the apoptosis of myocardial cells. It is probably contributed to its effect on up regulating Bcl-2, down regulating Bax and caspase3 proteins and the increasing of Bcl-2/Bax proportion [8]. In myocardial tissue injury in rats with myocardial ischemia-reperfusion injury, Eerdun Wurile can down the expression of LC3-IIand Beclin 1 which are autophagy related proteins LC3-II and Beclin1, activate PI3K-Akt-mTOR signaling pathway [9].

In this study, the simulated gastric juice extract of Eerdun Wurile was found to differentiate PC12 cells for the first time. Further analysis by molecular modeling technique predicted that genipin in the extract can bind to MAPK. It was validated that genipin phosphorylate MAPK in PC12 cells. It might be one of the mechanism by which Eerdun Wurile stimulate the differentiation of neurons.

2. Materials and Methods

Eerdun Wurile (Lot No: 20190828) was supplied by national Mongolian Medicne center, Inner Mongolia International Mongolian Hospital (Hohhot, China).

F-12K medium (ZQ-504, Zhong Qiao Xin Zhou Biotechnology, Shanghai, China), MTS (Promega, Germany), methanol (LC-MS 67-56-1 Thermo), formic acid (LC-MS 64-18-6 Thermo), ammonium acetate (LC-MS 631-61-8 Thermo).

2.1. Preparation of the Extract from Eerdun Wurile

The extract was prepared by refereeing the method in literature [10, 11]. The Eerdun Wurile pills were ground very fine powder. Then, 10g of powder was applied with 250 ml ultra pure water in a 500 ml- flask. The ultra pure water used should be adjusted pH to 2.0 using trifluoroacetic acid in advance. The mixture was extracted for 1 h at 40°C in water bath with slight shaking. The supernatants of raw extracts was concentrated by evaporation at ≤ 40°C, then freeze-dried for 24 h, and stored.

2.2. Rat Pheochromocytoma PC12 Cells

Cells were regularly transferred once in two or three days. PBS supplementated with 1 mM EDTA (PBS/EDTA) was used to detach and collect cells before transfer.

PC12 cells were inoculated on 24-well culture plates coated with collagen type1 (rattail) at a density of 1×10^5 cells/well in 0.5ml/well F-12K medium (10% FCS), and incubated for 24h or longer until>80% confluent. Culture medium was removed by aspiration before cells were stimulated by different treatments as descriped in the text. Then, cells were washed twice with F-12K medium without serum carefully, added F-12K medium without serum 0.5ml/well for 24-wellplates, and incubated for 1.5h (serum-starvation). Cells were stimulated by various treatments by applying vehicle control or various treatments following 1.5h serum-starvation. Vehicle control for various stimuli was prepared by mixing the same amount of F-12K medium without serum and methanol.

2.3. Analysis of Cell Viability for the Extract

The CellTiter 96® AQueous One Solution Cell Proliferation Assay MTS (Promega, Mannheim, Germany) was used to determine cell viability. The experiment was done in 96-well microplates in above described medium for PC12 cells. Extracts in medium were filtered through a 0.2 μm filter before treatment. PC12 cells (10.000 cells/100 μL) were seeded and treated after 24 h culture at 80% confluence. Cells were treated with the extract with different concentrations (0.0064, 0.032, 0.16, 0.8, 4 mg/mL) as well as with the vehicle control (medium without extract) for 24 h. Cells stimulated with 100μL medium were used as control. Then, MTS solution was added 10 μL/well and incubated for 4 h at 37°C, 5% CO_2 humidified incubator. The absorbance was measured at 490 nm.

2.4. Analysis of Cell Viability for Genipin

Genipin in medium were filtered through a 0.2 μm filter before treatment. PC12 cells (50.000 cells/100 μL) were inoculated and treated after 24 h culture at 80% confluence. Cells were treated with the genipin at five different concentrations (0.078125, 0.015625, 0.3125, 0.625, 1.25, 2.5, 5 mg/mL) for 24 h. Cells stimulated with 100μL medium were used as control. After incubation, 10 μL of the MTS solution were added to each well and incubated for 4 h at 37°C, 5% CO_2 provided and humidified incubator. The absorbance was measured at 490 nm.

2.5. Neurite Outgrowth Assay in PC12 Cells

PC12 cells were cultured at the density of 5×10^3 cells/well in 0.5 ml/well F-12K medium (10%FCS) in 24-well plates coated with collagen for 24h. Then, cells were then washed

with serum-free F-12K medium (0.5 ml/well) twice and incubated in serum-free F-12K medium (0.5 ml/well) containing vehicle control or NGF (50 ng/ml) and/or extract (0.16 mg/mL) for 48 h. Medium was removed and cells were fixed with 4% paraformaldehyde by adding 0.35 ml/well and keep for 30 min at room temperature. Cells were stained with CBB (250 μl/well) for 15 sec after washed once with PBS (0.5 ml/well). After that, cells were washed with PBS twice, 5ml each well and added PBS, 0.5ml in each well. At last, cells were observed by phase-contrast microscopy and pictures were taken from different region, and cell shaving neuritis was considered as positive and counted. At least 5 of pictures were taken randomly in each well, and total of cells were counted. The percentages of differentiating cells (positive) to total cells were analyzed.

2.6. Western Blotting Analysis

The antibodies used are: phospho-p44/42 (Thr202/Tyr204) MAP kinase/Erk (4370s, CST, 1:1000), P44/42 MAPK (Erk1/2) Antibody (9102, CST, 1:1000), anti-mouse-IgG (H+L) (DyLight™ 800 4xPEG Conjugate, 5257s, CST), (TTBS 1:15000).

After serum-starvation and treatments by different stimuli, medium was removed by aspiration, and PC12 cells were collected in 1×SDS sample buffer 50 μl/well (24-well plates) and heated to boil for 3 min. In each experiment, equal volume (usually 20 μl) of cell lysate was subjected to electrophoresis on 10% acrylamide gel (140 v, 60 min). Proteins were transferred onto polyvinylidene fluoride (PVDF) microporous membrane (Millipore) for 80 min at 60 mA using a semi-dry blotter. Immunoreactive bands were visualized using the SuperSignal®WestPicoLumino/Enhancer (Pierce #1856136) and SuperSignal®WestPico Stable Peroxide (#1856135) solution (mixture of 1ml of each). Imaging was then carried out using LI-COR Odyssey® CLx Imager (Gene Company Limited).

2.7. Virtual Screening for Neuroprotective Candidate Compounds in Eerdun Wurile

The reported neuroprotective compounds contained in Eerdun Wurile were collected from literatures and were subjected to reverse screening for searching the protein target from pharmacophore database by ligand profiler progress Discovery studio (2019 Edition), which contains pharmacophore database with 117,432 pharmacopores based on 7,028 proteins' crystal structure from PDB.

2.8. Molecular Docking Studies

The active compound genipin was docked against the predicted target MAPK (PDB ID, 1tvo) by LibDock. The 3D crystal structure of MAPK was obtained from PDB database. The chemical structure of genipin was obtained from the ChemSpider database, (ID390864).

2.9. Analysis of the Relative Content of Genipin in Eerdun Wurile Pills and the Extract by LC-MS/MS

The powder of Eerdun Wurile pills (100 mg) and the extract (100 mg) were individually grounded with liquid nitrogen and the homogenate was re-suspended with prechilled 80% methanol and 0.1% formic acid by well vortexing. The samples were incubated on ice for 5 min and then were centrifuged at 15000 rpm, 4°C for 5 min.

Some of supernatant was diluted to final concentration containing 53% methanol by LC-MS/MS grade water. The samples were subsequently transferred to a fresh Eppendorf tube and then were centrifuged at 15000 g, 4°C for 10 min. Finally, the supernatant was injected into the LC-MS/MS system analysis. PS: Liquid sample (100 μL) and prechilled methanol (400 μL) were mixed well by vortexing, and then sonicated for 6 min. Repeat this step once again and then operate the same steps as above.

LC-MS/MS analyses were performed using a Vanquish UHPLC system (Thermo Fisher) coupled with an Orbitrap Q Exactive series mass spectrometer (Thermo Fisher). Samples were injected onto a Hyperil Gold column (100×2.1 mm, 1.9 μm) using a 16- min linear gradient at a flow rate of 0.2 mL/min. The eluents for the positive polarity mode were eluent A (0.1% FA in Water) and eluent B (Methanol). The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (Methanol).

The solvent gradient was set as follows: 2% B, 1.5 min; 100% B, 12.0 min; 100% B, 14.0 min；2% B, 14.1 min；2% B, 17 min.

Screening range was: m/z 70-1050. The ESI source were set as follows. Q Exactive series mass spectrometer was operated in positive/negative polarity mode with spray voltage of 3.2 kV, capillary temperature of 320°C, sheath gas flow rate of 35 arb and aux gas flow rate of 10 arb.

2.10. Statistical Analysis

The results shown are from at least three independent experiments. Data were analyzed for statistical significance using Student's *t*-test. Differences were considered significant at $p<0.05$ as indicated.

3. Results

3.1. Effect of the Extract of Eerdun Wurile on Neurite Outgrowth and MAPK Phosphorylation in PC12 Cells

Approximately, 1 g of extract can be obtained from each 10 g Eerdun Wurile pills powder. Optimal concentration of the extract for the treatment of PC12 cells was determined through examining cell viability by exposure of cells to various concentrations of extract. The result showed that the extract at 0.16 mg/ml showed the highest effect on survival of PC12 cells. The survival rate was significantly decreased with the increase of the extract concentration higher than 0.16 mg/ml, up to 0.8 mg/ml. Cell viability was only 53.5%, when the concentration of extract was 4 mg/ml (Figure 1A), (*t*-test, $p<0.0001$, $n=5$).

In order to indicate the effect of extract on the differentiation of PC12 cells, PC12 cells cultured in the serum-free medium were stimulated by the extract (0.16

mg/ml) for 48 h. Results in Figure 1B showed that neurite outgrowth of PC12 cells was significantly induced by the extract compared to the control. Also, number of cells with enlarged cell body increased which shows the tendency of differentiation (bearing short neurites) was observed when the cells were stimulated with the extract compared to the control. Thus, the data strongly suggest that cell differentiation signaling pathway was activated by the extract.

The activation of Ras-MAPK signaling cascade vital is essential for neuronal differentiation. Therefore, we tested possible mechanism for the effect of extract on the phosphory late on MAPK in PC12 cells. The result in Figure 1C showed, the extract significantly induced the phosphorylation of MAPK at 0.16 mg/ml in PC12 cells, similar to NGF. This result suggests that the extract probably induce neurite outgrowth of PC12 cells through activating MAPK signaling pathway. However, which compounds in the extract functions on MAPK phosphorylation was quite complicated since it contains hundreds of small molecules.

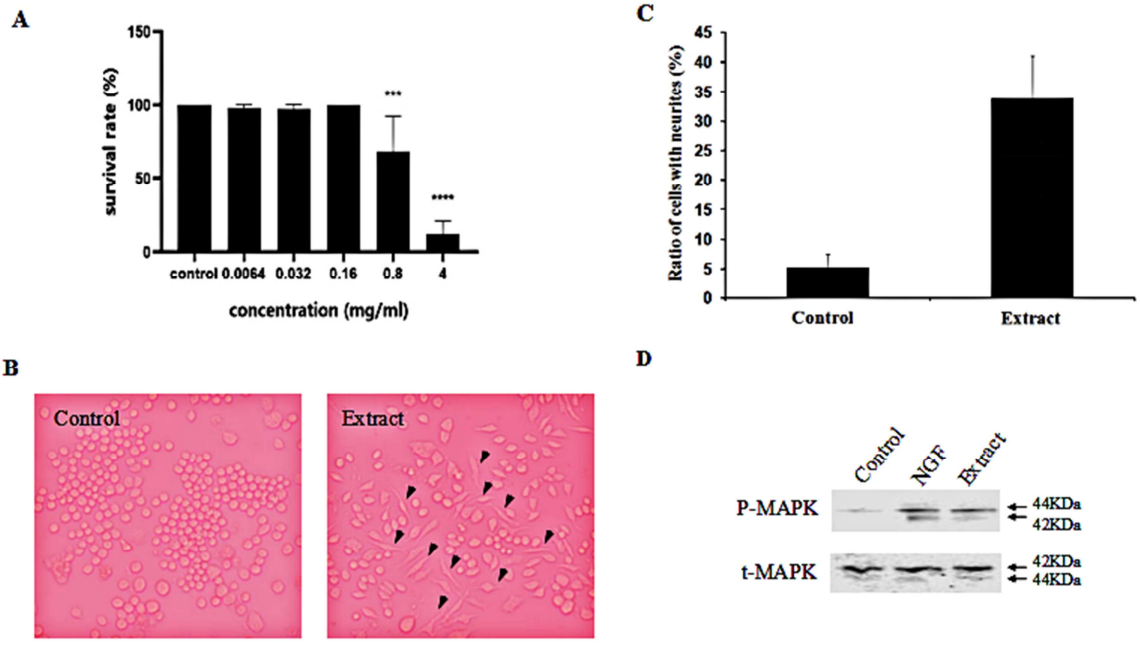

Figure 1. *Effect of the extract of Eerdun wurile on neurite outgrowth and MAPK phosphorylation in PC12 cells.*

A: MTS assay for the extract in PC12 cells; B: Neurite outgrowth assay for the extract in PC12 cells; C: Quantification of cells with neurites outgrowth and tendency; D: Effect of the extract on MAPK phosphorylation. NGF was used as a positive control.

3.2. Screening of Active Compounds Target to MAPK and Molecular Docking Analysis

In order to retrieve the potential active compounds which functions on MAPK in the extract of Eerdun Wurile, reverse screening was carried out using molecular modeling method (Discovery Studio, 2019 version). We selected 35 small molecules (data not shown) which were reported in literatures to be neuroprotective and screened their target proteins individually using the pharmacophore data base in Discovery Studio. We found that 35 molecules showed interaction with 12588 pharmacophore (data not shown). Among them, we focused on the compound genipin, since it showed the interaction with MAPK with the highest fit value. Furthermore, the key residues interaction and molecular binding structure of genipin (Molecular Formula $C_{11}H_{14}O_5$, 226.226 Da, ChemSpider ID390864) (Figure 2A) and MAPK (Figure 2B) were examined by LibDock in Discovery Studio. The LibDock score for genipin and MAPK was 76.79, and relative energy was 0.18. The 3D structure of the interaction of genipin and MAPK was shown in Figure 2C and their 2 D diagram was shown in Figure 2D. The vital amino acids of MAPK targets to genipin were VAL (39), ASP (111) and SER (153).

3.3. Effect of Genipin on the Phosphorylation of MAPK in PC12 Cells

In order to further analyze whether or not genipin functions on MAPK as predicted, the effect of genipin on the viability of PC12 cells was examined first by MTS assay. The significant activity observed neither on proliferation nor on viability when cells were stimulated by genipin at different concentrations from 0.07812 to 5 mg/ml compared to the cells in control group. It indicates that genipin has no cytotoxicity at concentration up to 5 mg/ml (Figure 3A). Therefore, genipin 0.3125 mg/ml was used for the next experiment to analyze the effect of genipin on MAPK phosphorylation.

The result showed that the MAPK was phosphorylated when PC12 cells were stimulated by genipin at 0.3125mg/ml for 120 min, similar to NGF in PC12 cells (Figure 3B), suggesting that genipin directly or indirectly acts on the

phosphorylation of MAPK.

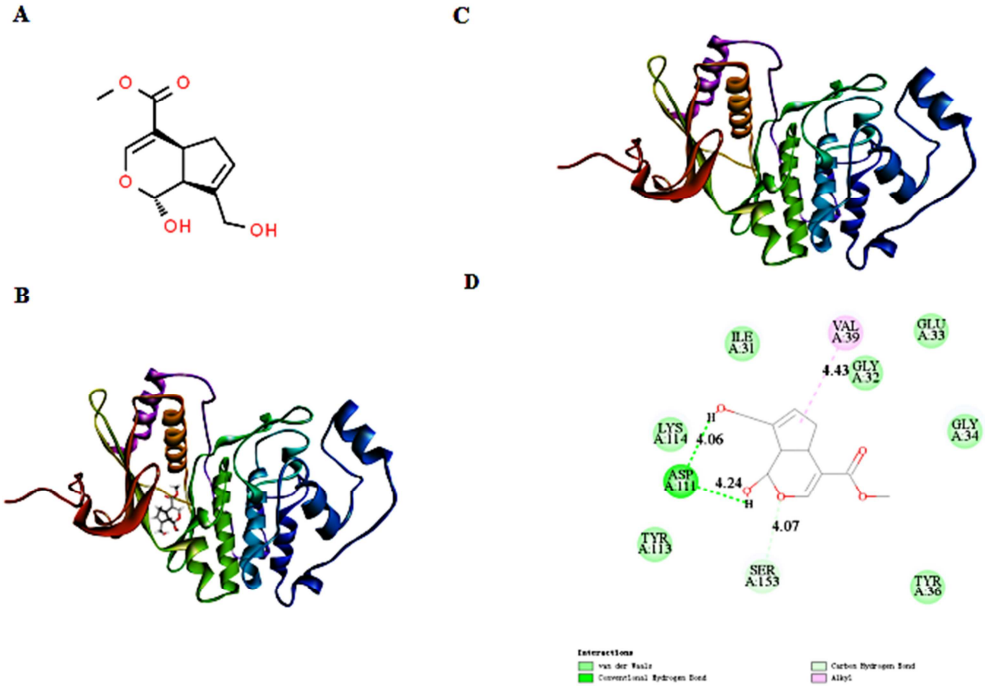

Figure 2. *The analysis of interaction between genipin and MAPK.*

A: The chemical structure of genipin from Chemspider; B: The x-ray 3D structure of MAPK (ERK2) from PDB (1TVO); C: The 3D structure of genipin and MAPK interaction; D: 2D structure of genipin and MAPK interaction.

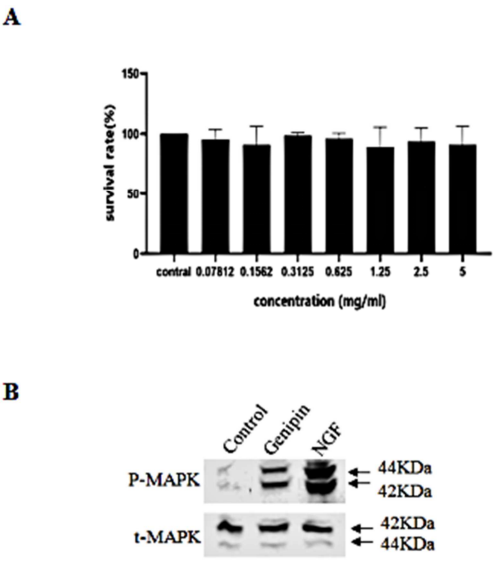

Figure 3. *Effect of genipin on the phosphorylation of MAPK in PC12 cells.*

A: MTS assay for genipin in PC12 cells; B: Effect of genipin on MAPK phosphorylation. NGF was used as a positive control.

3.4. Content of Genipin in Eerdun Wurile Pills and in the Extract

Finally, to investigate the content of genipin in original Eerdun Wurile pills and also to clarify if there is still any amount of genipin in the extract of Eerdun Wurile pills, the relative content of genipin was determined by LC-MS/MS.

Genipin was detected in both original Eerdun Wurile pills and its extract in the negative mode. Result (Figure 4) showed that the relative content of genipin in the extract was significantly higher than that in original Eerdun Wurile pills ($p<0.01$, $n=6$). This suggests that the relative content of genipin has obviously increased after extraction.

Collectively, these results indicate that genipin might contribute to the neuroprotective effect of the extract on the MAPK phosphorylation and neurite outgrowth of PC12 cells. Genipin-MAPK might be one of the molecular-target mechanisms of Eerdun Wurile by which it shows neuroprotective effect.

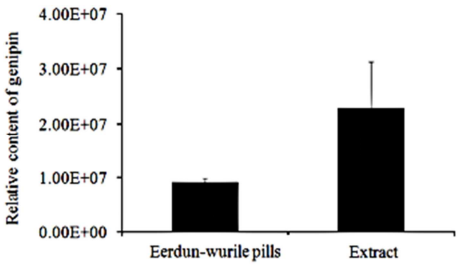

Figure 4. *Determination of the content of genipin in Eerdun Wurile pills and in the extract by LC-MS/MS.*

4. Conclusion

In current study, we have demonstrated that the simulated gastric fluid extract of Eerdun Wurile showed neurotrophin like activity by stimulating the differentiation of PC12 cells,

similar to nerve growth factor. According to reported neuroprotective compounds Eerdun Wurile contains, we screened their targets by molecular modeling technique and found that active compound genipin was predicted to work on MAPK as a target. Although, whether or not genipin bind directly with MAPK, genipin can stimulate the phosphorylation of MAPK. Furthemore, the content of genipin was also detected and in both extract and original drug powder. Therefore, it can be predicted that one of the active compound-target interaction of Eerdun Wurile is genipin-MAPK, by which it showed neuroprotective effect.

5. Discussion

Our result highly suggest that genipin in Eerdun Wurile is one of the active components which shows neuroprotective effect through MAPK signaling pathway. There might be more active compounds functions on multiple targets which make complicated interaction network. It needs further research to clarify. Our result provides a new research idea for investigating such a complicated mechanism for traditional medicines.

Genipin is an aglycone derived from geniposide, contained in *G. jasminoides* fruit extract (GFE). *Gardenia jasminoides* Ellis is also a major and frequently used component for traditional Mongolian and Chinese medicines because of that it has anti-inflammation, antioxidant, neuro protective and liver protective effects etc [12]. It is also used for various traditional medicines to inhibit in flammation in different diseases, such as cancer [13, 14]. Numerous researches have reported the potential of genipin as an anti-cancer agent for bladder cancer, gastric cancer, colon cancer, lung cancer and colorectal cancer by enhancing the anti-cancer effect of related drugs or inhibiting the growth of cancer cells [15-19]. Genipin is also antioxidant and can prohibit mitochondrial uncoupling protein 2, which is a tumor promoter in cancers [1, 18, 20-22]. Besides, genipin was found to be a very good cross link in gagent, since it can provide sustained release of drugs from nanoparticle formulations [23-26]. In addition, it was proved to have nuero protective activity. Hence, it is a potential therapy for neurodegenerative diseases [27].

Our finding is consistent with it. In summary, genipin has showed multiple pharmacological features, including anti-inflammatory, anti-cancer, neuroprotective, neurogenic, antidiabetic, and antidepressant effects. It is very important to further analyze the activity of genipin in Eerdun Wurile to investigate the mechanism by which Eerdun Wurile works in nervous system disease, and improve the impairment of learning and memory ability.

Declaration of Competing Interests

The authors declare no conflict of interest.

Highlights

1. The extract from traditional Mongolian medicine Eerdun Wurile induced differentiation of PC12 cells.
2. The extract from traditional Mongolian medicine Eerdun Wurile induced MAPK phosphorylation in PC12 cells.
3. The screen and prediction by Molecular modeling technology found that genipin act on MAPK as a target.
4. Genipin significantly induced the phosphorylation of MAPK in PC12 cells.
5. The relative content of genipin in the extract and Eerdun Wurile pills was determined by LC-MS/MS.

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