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Pharmacological Study of Phenolic Components in Parkinson's Disease

Xin Dong[1], Huishan Li[1], Xueliang Shang[2], Daliang Guo[2], Yuxuan Liu[2*]

1. Tianjin University of Traditional Chinese Medicine, College of Traditional Chinese Medicine, Tianjin 301617, China.

2. Tianjin University of Traditional Chinese Medicine, Tianjin 301617, China.

Abstract: In this study, cell experiments were conducted to investigate the effects of extracts on cell viability and apoptosis of Parkinson model in vitro, as well as the expression of cysteine protease-3 (Caspase-3) and B lymphocytoma-2-associated X protein (BAX). The results showed that extract of phenols could improve the loss of cell viability and apoptosis induced by MPP+, and inhibit the enhanced expression of Bax and Caspase-3 by MPP+. The potential targets and signaling pathways of phenols in the treatment of Parkinson's disease were predicted by network pharmacology.

Keywords: Gastrodia elata Bl; Phenols; Caspase-3; BAX; Parkinson's disease; Network pharmacology

Introduction

The pathogenesis of PD in modern medicine is not clear, and it is generally believed to be caused by various factors such as genes, environment and lifestyle.As a traditional Chinese medicine, gastrodia elata has the effect of calm and calm. Therefore, doctors combine gastrodia elata with other Chinese medicine to treat PD, such as [1], gastrodia elata drink [2], qi, blood and [3], have achieved good results in clinical treatment.We found that the phenolic components of Gastrodia elata in Gastrodia elata have good effects on CNS diseases such as PD, [4].Pharmacological experiments proved that gastrodin could improve that in model animals Movement disorders, reduces loss of dopamine neurons [5-7]; vanicol improves cell survival in PD model cells [8]; vanillin inhibits neuroinflammation caused by microglial activation, weakens dopamine neuronal degeneration, and plays a potential role in treating PD [9,10].

In this experiment, we proved that the extract can improve the cell survival rate and inhibit apoptosis of PD cell model, predict the potential target and pathway of PD, and explore the mechanism of action in treating PD.

1. Instruments and materials

1.1 Clone

PC 12 cells.

1.2 Laboratory apparatus

Table 1: Experimental Instrument Information

Instrument name	Manufacturer	Model
Micropipet	ThermoFisher	
Superclean bench	Suzhou Group Antai Air Technology Co., LTD	SW-CJ-1FD
CO_2constant-temperature incubator	SANYO	MCO-15AC
Inverted microscope	OLYMPUS	IX51
Conventional centrifuge	Eppendorf	5702R
ELIASA	Thermo	MULTISKAN MK3
Electrophotometer power supply	Beijing 61 Instrument Factory	DYY-7C
Vertical electrophoretic tank	Beijing 61 Instrument Factory	DYCZ-24DN
Transfer electrophoresis instrument	Beijing 61 Instrument Factory	DYCZ-40
Horizontal shaking bed	Jiangsu Haimen Qlinbel Instrument Manufacturing Co., LTD	TS-1
pH count	Metter-Toledo GmbH, Germany	LP115
Magnetic stirring apparatus	Jiangsu Jintan City Zhongda Instrument Factory	T8-1
Centrifuge	Hunan Xiangyi Laboratory Instrument Development Co., Ltd	HI650
Biological microscope	Olympus	BX53

1.3 Experimental reagents and consumables

Table 2. Experimental reagents and consumables

Name of reagents and consumables		Vender	Art.No
0.25% Trypsin		Gibco	15050065
Cell culture dishes, cell culture flasks		Corning	
FBS		Gibco	10091-148
1640		Gibco	11875-093
PS		Gibco	15070-063
Phosphatase inhibitors		Shanghai Biyuntian Biotechnology Co., Ltd	S1873
PMSF		Aladdin Reagent (Shanghai) Co., Ltd	P105539
RIPA lysate		Shanghai Biyuntian Biotechnology Co., Ltd	P0013B
BCA protein concentration determination Kit		Shanghai Biyuntian Biotechnology Co., Ltd	P0010
TEMED		Sinopharm Group Chemical Reagent Co., LTD	80125336
SDS loading buffer	Trise-Base	Biofroxx	1115GR500
	HCl	Xinyang city chemical reagent factory	GB622-89
	Dithiothreitol	Biofroxx	1111gr005
	SDS	Sinopharm Group Chemical Reagent Co., LTD	30166428

	Bromophenol blue	Sinopharm Group Chemical Reagent Co., LTD	71008060
	Glycerol	Sinopharm Group Chemical Reagent Co., LTD	10010618
30% Acrylamide		Biosharp	BL513b
TG	Trise-Base	Biofroxx	1115GR500
	Glycocoll	Biofroxx	1275GR500
	SDS	Sinopharm Group Chemical Reagent Co., LTD	30166428
Electrophoretic transfer buffer	Tris-base	Biofroxx	1115GR500
	Glycocoll	Biofroxx	1275GR500
	Carbinol	Sinopharm Group Chemical Reagent Co., LTD	10014118
PBS	NaCl	Sinopharm Group Chemical Reagent Co., LTD	10019318
	KCl	Sinopharm Group Chemical Reagent Co., LTD	10016318
	$Na_2HPO_4.12H_2O$	Sinopharm Group Chemical Reagent Co., LTD	10020318
	KH_2PO_4	Sinopharm Group Chemical Reagent Co., LTD	10017618
TBST	Tris-base	Biofroxx	1115GR500
	NaCl	Sinopharm Group Chemical Reagent Co., LTD	10019318
	Glacial acetic acid	Sinopharm Group Chemical Reagent Co., LTD	10000218
	Twain 20	Sinopharm Group Chemical Reagent Co., LTD	30189328
Protein marker (14-120KD)		Beijing Total Gold Biotechnology Co., Ltd	DM111
PVDF membrane(0.45μm)		Millipore	IPVH00010
PVDF membrane(0.22μm)		Millipore	ISEQ15150
Rabbit polyresistance GAPDH	37KD	Hangzhou Xianzhi Biological Co., Ltd	AB-P-R 001
		Detection of antibodies	
RabMab cleaved caspase3 （17/19KD)		Cell signaling	9664
Rabbit polyresistance，Bax （21KD)		Wuhan Sanying Biotechnology Co., LTD	50599-2-AP
HRP-labeled sheep anti-mouse secondary antibody		Wuhan Doctor De Biological Engineering Co., Ltd	BA1051
HRP labeled sheep anti-rabbit secondary antibody		Wuhan Doctor De Biological Engineering Co., Ltd	BA1054
ECL, the substrate solution		Beijing Priilai Gene Technology Co., Ltd	P1050

X-ray film	Ruike (Xiamen) Medical Equipment Co., LTD	6535876
Development-fixing kit	Tianjin Hanzhong Photography Materials Factory	
Slides and cover slips	Jiangsu Shitai Experimental Equipment Co., Ltd	
Paraformaldehyde	Sinopharm Group Chemical Reagent Co., LTD	80096618
Concentrated normal goat serum (closed)	Wuhan Doctor De Biological Engineering Co., Ltd	AR1009
Fluorescence (Cy3) labeled sheep anti-rabbit IgG	Wuhan Doctor De Biological Engineering Co., Ltd	BA1032
Fluorescence (Cy3) labeled sheep anti-mouse IgG	Wuhan Doctor De Biological Engineering Co., Ltd	BA1031
Triton X-100	Shanghai Biyuntian Biotechnology Co., Ltd	ST795
DAPI	Shanghai Biyuntian Biotechnology Co., Ltd	C1002
Anti-fluorescence ququenching agent	southernbiotech	0100-01
Triton X-100	Shanghai Biyuntian Biotechnology Co., Ltd	ST795
TUNEL Apoptosis detection kit	Roche Applied Science	12156792910

2. Method

2.1 Effect of Gastrodia elata phenolic extracts on PD cell models

2.1.1 Cell culture

PC 12 cells were removed from liquid nitrogen, quickly placed into a 37℃ water bath, gently shaking the frozen tube, transferred to a centrifuge tube containing 5 mL medium, collected, centrifuged at 1000 r/min for 5 min, the supernatant was discarded, suspended with complete medium containing 10% FBS (1640 + 10%FBS + 1% penicillin-streptomycin), and mixed with 37℃ 5%CO_2 saturated humidity.The cells reach a density of, 80%, on fine Cell passage: discard medium and wash it with PBS; add 1~2m L 0.25% trypsin digested cells, observe under microscope for 30~60s, see cells separated and rounded, or digestion; add complete medium, blow cells to make single cell suspension, passaged in 1:3 ratio, expand culture under saturated humidity of 37℃ and 5% CO_2.

2.1.2 Cell viability detection

Cell viability was determined by the 3- (4,5-dimethyl-2-thiazole) -2,5-diphenylbromide thiazole blue (MTT) reduction method.

Cells with good growth were connected to 5103,96-well plate with blank group and 37℃ overnight (100 L sterile PBS in the wells around the wells); media with different drugs to each well in 37℃, 5%CO_2 incubator for some time.Subsequently, 10 μL MTT was added to each well and 37℃ for 4h and 150 μL DMSO was added for 10min; the absorption value of each well was measured with a microplate reader and the wavelength was set at 568 nm; Each experiment was repeated three times.

2.1.3 TUNEL Apoptosis detection

Slides of scrambled cells were immersed in 4% paraformaldehyde (pH 7.4) and fixed in the solution for 25 min at room temperature, and subsequently washed three times with PBS for 5 min each.Cell tiles were immersed in 0.1% TritonX-100 solution prepared with PBS for 10 min (operated on ice) and then washed twice with PBS for 5 min each.The TUNEL reaction mixture was prepared, and the treatment group was mixed with 50 μL TdT + 450 L fluorescein-labeled dUTP solution; but was negative Control group add only 50 L of fluorescein-labeled dUTP solution, positive control group first add 100 μL DNaseI at 15 to 25℃ for 10 min; drying of glass slides add 50 μL TUNEL reaction mixture (50 L of fluorescein-labeled dUTP solution) to the specimen, incubated at 37℃ for 60 min, washed three times with PBS, 5 min each.Samples were incubated with DAPI for 5 min and stained by PBST Wash the excess DAPI 4 times in 5 min; drain the liquid with suction paper and seal with sheet liquid containing anti-fluorescent quencher.Images were acquired under a fluorescence microscope.

2.1.4 Immunofluorescence detection

Slides of scrambled cells were washed three times with PBS for 3 min; the slides were fixed with 4% paraformaldehyde for 15 min and PBS for 3 min; 0.5%Triton X-100 (PBS) for 20 min; PBS for three times and PBS for 3 min and closed at room temperature for 30 min Primary antibody (1:100) and placed in a wet box, 4℃ incubation overnight; PBST immersion slide for 3 times, 3 min, suction paper on excess liquid after fluorescence (Cy3) labeled sheep anti-rabbit IgG secondary antibody (1:100), fluorescence (Cy3) labeled sheep anti-mouse IgG (1:100), wet box 37℃ incubation for 1h, PBST immersion section for 3 times, 3 min; Samples were incubated with DAPI for 5 min, and the excess DA was washed away 4 times in PBST 5 min PI; drain the liquid on the climbing sheet with suction paper and seal the sheet with sealing sheet liquid containing anti-fluorescent quencher.The images were collected under a fluorescence microscope.

2.1.5 Western blot detection

Cellular proteins were extracted, separated by SDS-PAGE, electrically transferred to PVDF membrane, blocked with blocking solution containing 5% skim milk powder for 2 h, phosphorylated protein blocked with 1% BSA; primary antibody was added, 4℃ incubated overnight; secondary antibody (1:50000), incubated at room temperature for 2 h; development, scanned, and film gray was analyzed by BandScan.

2.1.6 Data statistics

All data were averaged as ±S.E.M. Representation, cell viability differences between groups were determined by one-way analysis of variance (One-Way ANOVA).All analyses were performed using the SPSS (23.0) software.

2.2 Network pharmacology predicts the mechanism of action of phenolic components in PD

2.2.1 Database

PubChem(https://pubchem.ncbi.nlm.nih.gov/)

PharmMapper(http://lilab-ecust.cn/pharmmapper/index.html)

TTD(Therapeutic Target Database,http://db.idrblab.net/ttd/)

Drugbank(http:/www.drugbank.ca)

Genecards(http://www.genecards.org)

DisGeNET(https://www.disgenet.org/home/)

OMIM(Online Mendelian Inheritance in Man, http://www.omim.org)

STRING(Search Tool for the Retieval of Interacting Genes/Proteins,http://string-db.org/cgi/input.pl)

Matescape（http://metascape.de）

2.2.2 Screening of the active ingredients

By searching and consulting the literature related to the chemical composition of gastrodia elata in "CNKI", "Wanfang" and "Pubmed", the phenolic compounds verified by clinical studies or pharmacological experiments were selected.

2.2.3 Screening of target sites

2.2.3.1 Target prediction of the active components of Gastrodia elata

Drug active components act by acting on relevant targets, so this study queries the targets potentially regulated by active components by PharmMapper to select targets with scores> 0.7.

2.2.3.2 Collection of the PD targets

Using TTD [11], Drugbank[12], Genecards[13], DisGeNET[14], and OMIM [15] databases to search targets related to PD with "Parkinson's disease", select genes with high likelihood by scores, and construct the disease gene database.

2.2.3.3 PD-related drug-active component targets

The action target gene number of "2.2.3.1" and the PD target of "2.2.3.2" were crossed to obtain the potential action target of the treatment of PD.

2.2.4 PPI Network and Network Topology Analysis

A network of PPI (Protein-Protein Interaction Networks) was constructed through the STRING[16] database.

2.2.5 GO and KEGG analysis

In order to reveal the potential action mechanism of phenolic components on PD, the main action pathway analysis and GO functional enrichment analysis of the potential targets of PD were conducted using Matescape[17] database.

2.2.6 The "Component-target-pathway" network construction

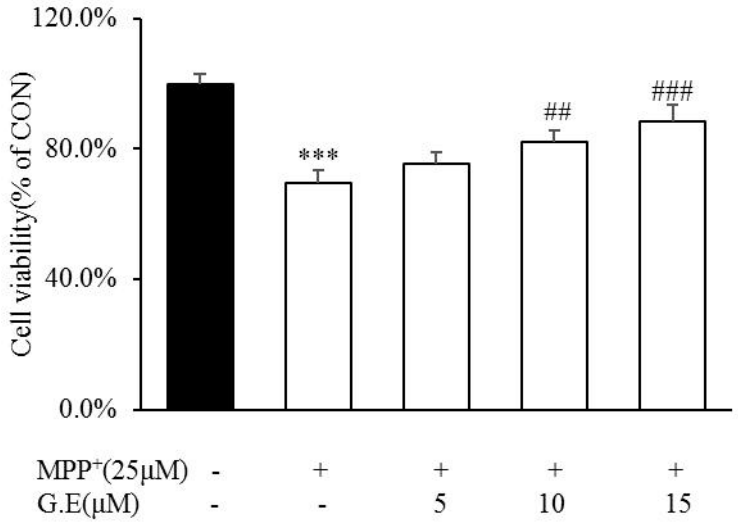
The "component-target-pathway" network was constructed according to "" Cytoscape3.6.1 " software.

3 Results and analysis

3.1 Effect of Gastrodia elata phenolic extracts on PD cell models

3.1.1 Phenolic extracts ameliorated MPP + -induced loss of nerve cell viability

The effect of Gastrodia elata phenolic extracts on MPP + -induced PC12 cells was determined by MTT reduction.When cell survival in control conditions was defined as 100% survival, the activity of PC 12 cells was significantly reduced to 69.4 ± 3.9% after 48 h of 25 μM MPP + treatment.Before addition of MPP +, 4 h pretreatment with Gastrodia phenolic extracts (5,10,15 g / mL) increased MPP + -induced cell survival to 75 .3 ± 3.7%, 82.0 ± 3.8%, and 88.3 ± 5.3%, and were presented in a dose-dependent manner.This result suggests that Gastrodia elata phenolic extract was protective against MPP + -induced nerve cells.



G.E represents Gastrodia elata phenolic extract;**** indicates the p <0.001 of MPP + group compared to control, n=3;# # indicates p <0.01 for MPP + + GE10μM compared to MPP + group, n=3; # # # indicates p <0.001 for MPP + + GE15μM compared to MPP + group, n=3

Figure 1.Cell survival rate of PC12 cells under different conditions

3.1.2 Apoptosis detection

TUNEL detection results are shown in Figure 2.The apoptosis rate was increased in the model group compared to the control group, and decreased in cells treated with Gastrodia elata phenolic extracts.

3.1.3 Effect of Gastrodia elata phenolic extracts on the expression of apoptosis-related proteins

Whether Gastrodia elata phenolic extracts affected the expression of Bax and Caspase-3 in MPP + -treated cells was investigated by Western blot and immunofluorescence assays.As shown in Figure 3, Bax expression was increased in the MPP + -treated group when compared to the control group, which is consistent with previous studies on [18,19]. Bax expression was inhibited by phenolic extract treatment and 25 μM MPP + treatment improved Caspase-3 activity, while addition of different concentrations of phenolic extracts enabled MPP + -induced PC 12 Lower expression of Caspase-3 in the cells.

The results of immunofluorescence are shown in Figure 4, and the intracellular staining intensity was significantly enhanced after MPP + treatment.In the treated group of phenolic extracts, the fluorescence intensity decreased with the increasing concentration of phenolic components.

In conclusion, the results indicate that the inhibition of MPP + by phenolic extracts on the enhanced expression of Bax and Caspase-3.

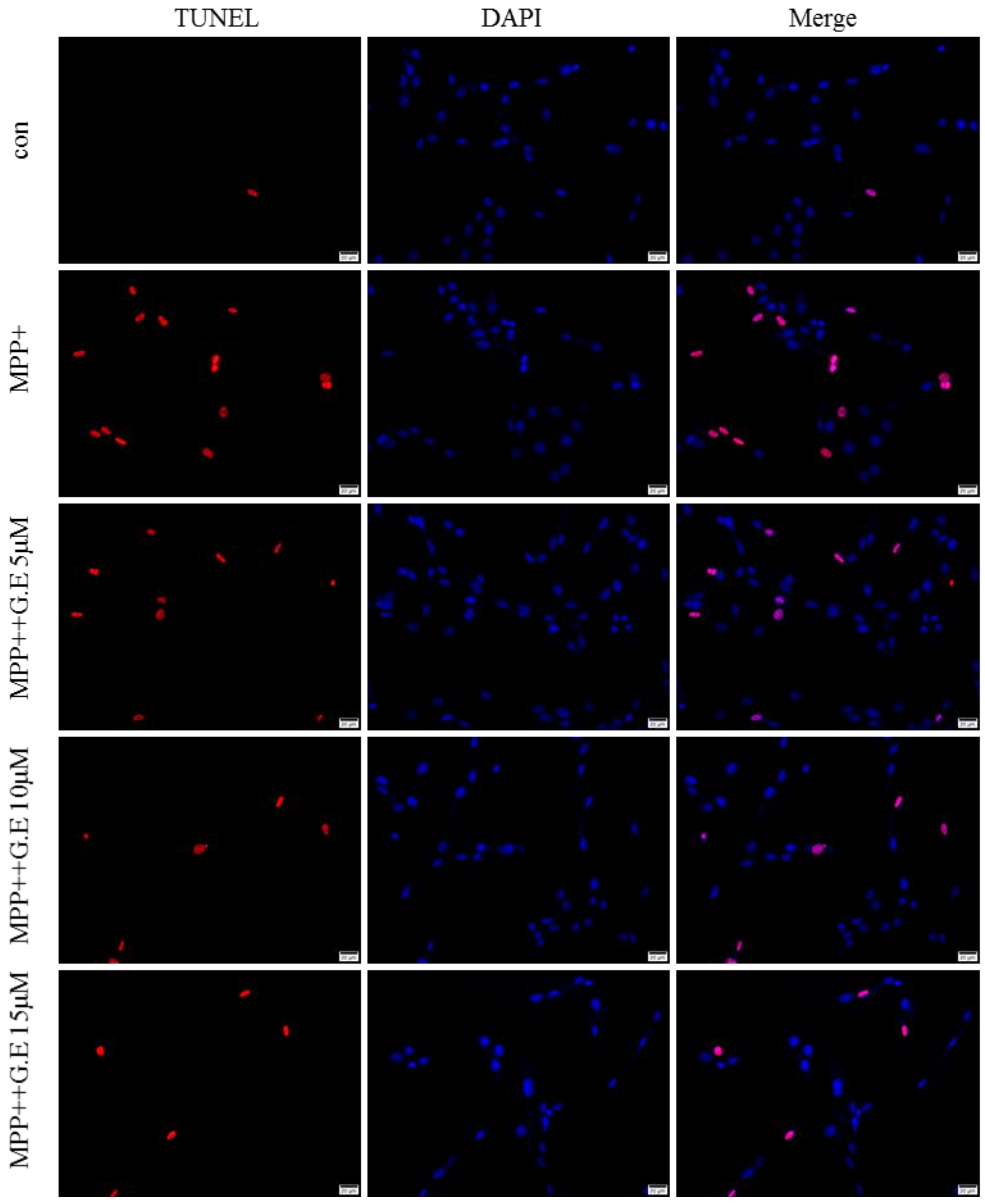


Fig 2 TUNEL Detection (400)

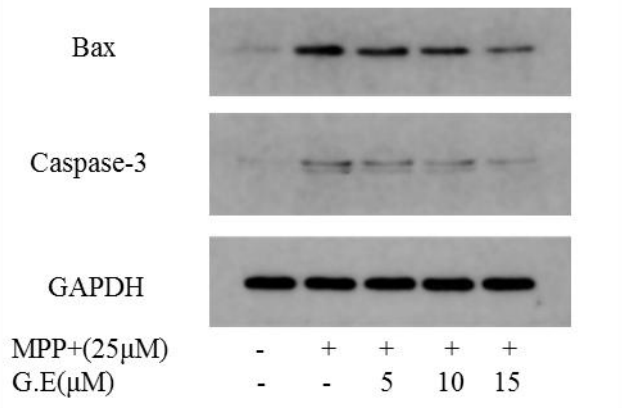


Figure 3 Effect of Gastrodia elata phenolic extracts on the expression activity of Bax and Caspase-3

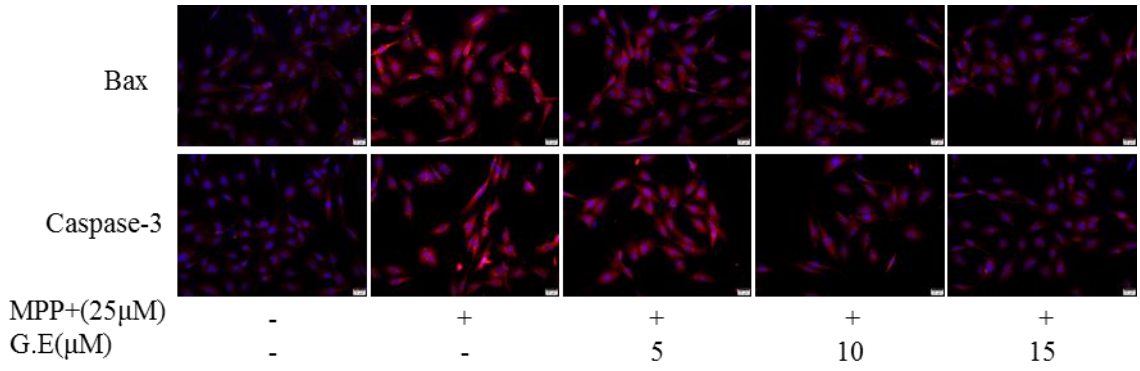


Figure 4 Micrographs of cells in each group visualized by immunofluorescence (400)

3.2 Network pharmacology prediction results

3.2.1 Potential targets of phenolic components of Gastrodia elata for the PD treatment

Previous experiments have proved that the phenolic extracts of Gastrodia elata contain 9 active ingredients in Table 3.A total of 125 targets were obtained by PharmMapper.The intersection of the active components and the PD targets yielded 43 potential targets of the treatment of PD, as shown in Figure 5.

3.2.2 PPI Network and Network Topology Analysis

The PPI network data constructed from the STRING database were imported into Cytoscape3.6.1 to obtain the PPI network maps, as shown in Figure 6.A.It consists of 41 nodes and 163 edges where nodes represent proteins and degree values represent the number of lines connected to 1 node were used to assess the importance of each node in the network.Larger nodes and redder colors indicate larger degree values. Each edge represents the interaction relationship between protein and protein. The thicker the line, the more red the color, the stronger the correlation, the thinner the line The weaker the correlation degree is.

All nodes in the PPI network are analyzed with three topological parameters: Degree, CC, Closeness Centrality, and BC, Betweenness Centrality.Take the median of 3 parameters as the card value, and the nodes with greater than 3 card values are selected as the core target (Figure 6.B).The result filter threshold is set to meet Degree Value 6, Node tightness 0.476, and Node Mediation 0.0144 The core targets are 16, as shown in Table 4 below.

Table 3 List of the active phenol ingredients of Gastrodia elata

ID	name	CAS
MOL1	Gastrodin	62499-27-8
MOL2	4-Hydroxybenzyl alcohol	623-05-2
MOL3	4-Hydroxybenzaldehyde	123-08-0
MOL4	Vanillyl alcohol	498-00-0
MOL5	Vanillin	121-33-5
MOL6	Parishin	62499-28-9
MOL7	Parishin B	174972-79-3
MOL8	Parishin C	174972-80-6
MOL9	Parishin E	952068-57-4

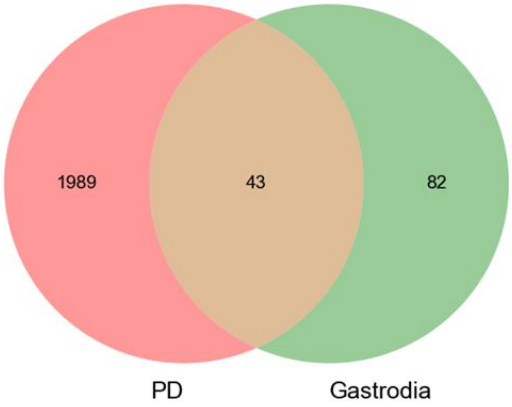


Figure 5 Wayne diagram of the target of Gastrodia elata phenolic components in the treatment of PD

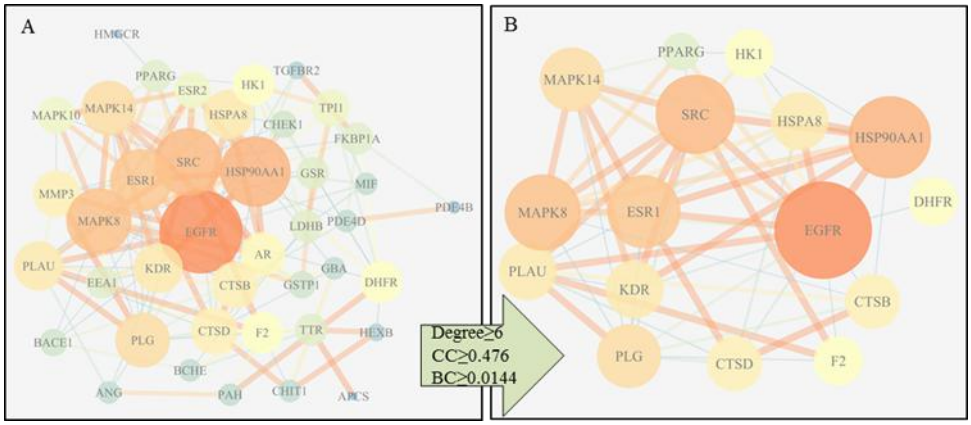


Figure 6 Topological screening process of the PPI network diagram

Table 4 Core target information of phenolic components for PD

Uniport ID	Gene name	Protein name	Betweenness Centrality	Closeness Centrality	Degree
P00533	EGFR	Epidermal growth factor receptor	0.191721	0.689655	24
P07900	HSP90AA1	Heat shock protein HSP 90-alpha	0.114298	0.606061	19
P12931	SRC	Proto-oncogene tyrosine-protein kinase Src	0.113682	0.625	18
P45983	MAPK8	Mitogen-activated protein kinase 8	0.056607	0.588235	17
P03372	ESR1	Estrogen receptor	0.031152	0.571429	16
P00747	PLG	Plasminogen	0.075991	0.571429	13
Q16539	MAPK14	Mitogen-activated protein kinase 14	0.017241	0.540541	13
P35968	KDR	Vascular endothelial growth factor receptor 2	0.019249	0.526316	11
P00749	PLAU	Urokinase-type plasminogen activator	0.018531	0.540541	11
P11142	HSPA8	Heat shock cognate 71 kDa protein	0.026039	0.512821	11
P07858	CTSB	Cathepsin B	0.034104	0.540541	10
P07339	CTSD	Cathepsin D	0.0438	0.526316	10
P00734	F2	Prothrombin	0.06427	0.526316	8
P00374	DHFR	Dihydrofolate reductase	0.068667	0.526316	8
P19367	HK1	Hexokinase-1	0.019337	0.5	8
P37231	PPARG	Peroxisome proliferator-activated receptor gamma	0.05061	0.47619	6

3.2.3 Biological function annotation

In order to reveal the potential mechanism of phenolic components on PD, 43 potential targets of Gastrodia elata for PD were analyzed by Matescape database. A total of 12 pathways for the treatment of PD were selected according to P <0.01, minimum count of 3 and enrichment factor> 1.5, as shown in Figure 7. The first three are "cancer signaling pathway", "estrogen signaling pathway" and "endocrine resistance".

GO functional enrichment analysis of 43 potential targets of Gastrodia elata for PD, including 15 GO Molecular Functions, 20 GO Biological Processes and 8 GO Cellular Components at P <0.01,3 and enrichment factor> 1.5, showing biological functions as shown in Figure 8.

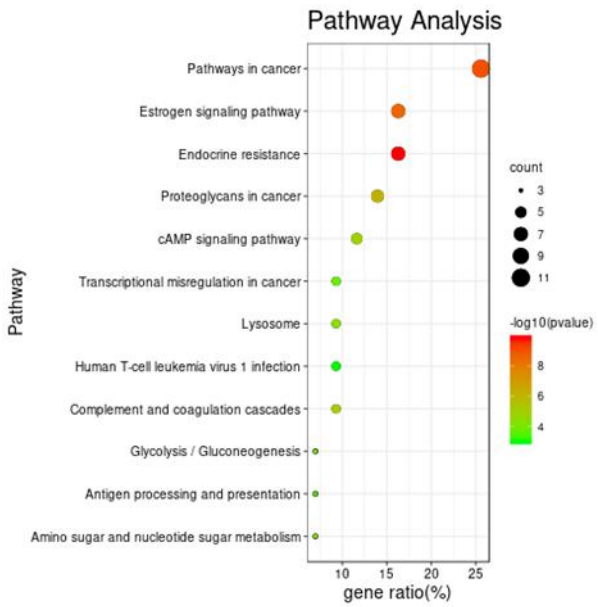


Figure 7 KEGG pathway enrichment analysis of Gastrodia elata phenolic components and PD intersection targets

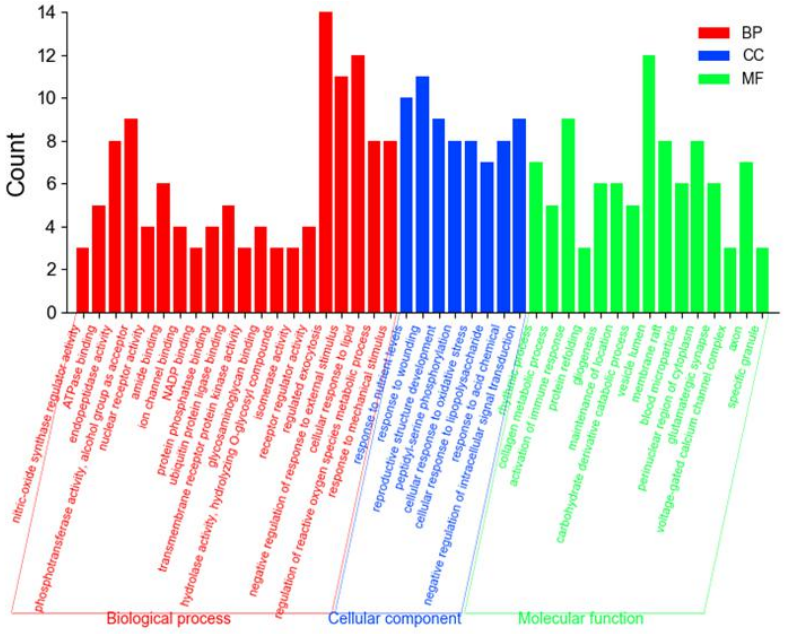


Figure 8 GO enrichment analysis of intersection targets of phenolic components and PD

3.2.4 The "Component-target-pathway" network construction

Cytoscape3.6.1 software was used to correlated 9 active components, 43 targets and 12 KEGG pathways to build a "component-target-pathway" network.The network results showed that 43 targets connected a total of 8 active components, of which 16 core targets connected 5 active components, mainly 4-hydroxybenzaldehyde, balisenside A, balisenside B, balisenside C and balisenside E.The network consists of 65 nodes with 219 edges.

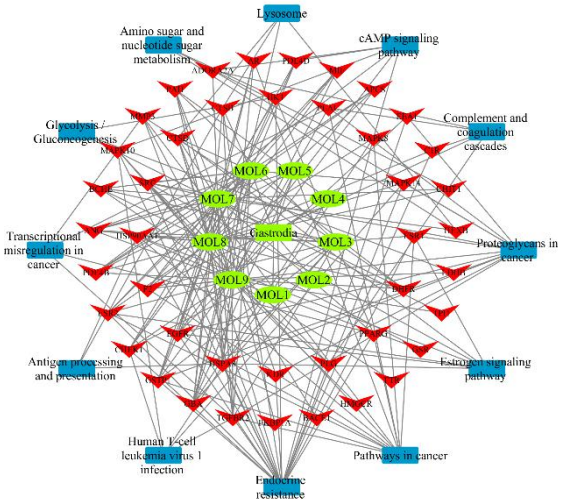


Figure 9 Component-target-pathway Network

Oval nodes represent Gastrodia elata and phenolic components, V-shaped nodes represent the intersection targets of active components and PD, and square nodes represent KEGG enriched pathways.

4. Discussion

The presence of a proapoptotic environment in the nigrostriatal region of PD patients is an important cause of PD, [20].Apoptosis is an important way for the body to maintain cell homeostasis by timely removal of aging and abnormal cells.Caspase-3 and Bax play a key role in the apoptosis process in dopaminergic neurons.Bax has proapoptotic activity, and one of the main mechanisms for inducing the mitochondrial apoptotic pathway is the elevated [21,22] at Bax levels.Caspase-3 is an important biomarker of neuronal apoptosis and [23], the executor of apoptosis.Caspase-3 It can inhibit the activity of poly (ADP-ribose) polymerase and increase the activity of endonuclease, leading to DNA fragmentation and cause apoptotic [24]. Previous studies have found that gastrodin increased Bcl-2 mRNA expression, reduced Bax mRNA expression, inhibited Caspase-3 activation and inhibited PARP shear [25] in MPP + -induced SH-SY5Y cell model; vanillin increased Bcl-2 expression, decreased Bax expression in Wistar rat model Cyt-C expression, downregulation of Caspase-3, Caspase-8 and Caspase-9 expressed [26]; vanicol reduced [27] of Bax / Bcl-2 in MPP + -induced MN9D cell model.In this experiment, the phenolic extracts of Gastrodia elata downregulated the expression of Bax, inhibited the MPP + -induced Caspase-3 activation, and improved cell survival.This suggest that phenolic components of Gastrodia elata have neuroprotective effects in in vitro PD models, possibly by inhibiting apoptosis-induced neuronal death.

When predicting active ingredients through network pharmacology, Integrative Pharmacology-based Research Platform of Traditional Chinese Medicine (TCMIP) or Swiss ADME[29,30] database are often used to screen the known chemical components in TCM using drug-forming rules.However, by using this method, most of the phenolic components of Gastrodia elata have been confirmed to have pharmacological activity The components were screened, such as gastrodin, balisenside, etc.; selected substances lacking known reports, non-major components, such as 4- [(4- (ethoxy methyl) benxy) methyl) phenol, palmitic acid, double (hydrophenyl) methane, affecting the reliability of the prediction results.Therefore, in

this study, the phenolic compounds verified by clinical studies or pharmacological experiments as the active components of Gastrodia elata were used for subsequent network pharmacological prediction.

5. Conclusion

(1) The results of cell experiments showed that the phenolic components of Gastrodia could improve the MPP + -induced loss of nerve cell viability and downregulate the expression of Bax and Caspase-3. It is speculated that the phenolic components of Gastrodia elata may have a neuroprotective effect by inhibiting apoptosis.It provides a basis for further study of the phenolic components of Gastrodia elata in the treatment of PD.

(2) Through literature search, the phenolic compounds verified by clinical studies or pharmacological experiments mainly include gastrodin, 4-hydroxybenzol, 4-hydroxybenzaldehyde, vanillol, vanillin, balisenside A, balisenside B, balisenside C and balisenside E.Gastrodia phenolic extracts contain these phenolic components.Using the prediction of network pharmacology, the potential core targets of these components for PD are mainly EGFR, HSP90AA1, SRC, MAPK8, and ESR1.

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About authors: 1. Xin Dong(2000-), female, Inner Mongolia, research direction: pharmacological research.
2. Xueliang Shang, Research direction: Chinese medicine.
3. Corresponding Author: Liu Yuxuan (1969-), male, professor, research direction: Chinese medicine, Chinese medicine research and development.
4. Guoda Liang (1979-), male, lecturer, research direction: Chinese medicine application.