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Ameliorative effect of *Gastrodia elata* Blume extracts on depression in zebrafish and cellular models through modulating reticulon 4 receptors and apoptosis

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ABSTRACT

Ethnopharmacological relevance: Gastrodia elata Blume (*G. elata*), a traditional Chinese herb, known as "Tian Ma", is widely used as a common medicine and diet ingredient for treating or preventing neurological disorders for thousands of years in China. However, the anti-depressant effect of *G. elata* and the underlying mechanism have not been fully evaluated.

Aim of the study: The study is aimed to investigate the anti-depressant effect and the molecular mechanism of *G. elata in vitro* and *in vivo* using PC12 cells and zebrafish model, respectively.

Material and methods: Network pharmacology was performed to explore the potential active ingredients and action targets of *G. elata* Blume extracts (GBE) against depression. The cell viability and proliferation were determined by MTT and EdU assay, respectively. TUNEL assay was used to examine the anti-apoptotic effect of GBE. Immunofluorescence and Western blot were used to detect the protein expression level. In addition, novel tank diving test was used to investigate the anti-depressant effect in zebrafish depression model. RT-PCR was used to analyze the mRNA expression levels of genes.

Results: G. elata against depression on the reticulon 4 receptors (RTN4R) and apoptosis-related targets, which were predicted by network pharmacology. Furthermore, GBE enhanced cell viability and inhibited the apoptosis in PC12 cells against CORT treatment. GBE relieved depression-like symptoms in adult zebrafish, included increase of exploratory behavior and regulation of depression related genes. Mechanism studies showed that the GBE inhibited the expression of RTN4R-related and apoptosis-related genes.

Conclusion: Our studies show the ameliorative effect of *G. elata* against depression. The mechanism may be associated with the inhibition of RTN4R-related and apoptosis pathways.

1. Introduction

Depression is one of the most common neuropsychic diseases with serious mental disorder related to mood in global (Cuijpers et al., 2021; Gialluisi et al., 2021; Gronemann et al., 2021). The main clinical manifestations are characterized by depressed mood and interest, cognitive dysfunction, and even suicidal impulse, which seriously affect patients'

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Abbreviations:

GBE	*G. elata* Blume extracts
TCAs	tricyclic antidepressants
MAOIs	monoamine oxidase inhibitors
PPI	protein-protein interaction
BSA	Bovine serum albumin
PBS	phosphate-buffered saline;
FBS	Fetal bovine serum
DMSO	Dimethyl sulfoxide;
PS	penicillin-streptomycin
MAO	monoamine oxidase
COMT	catechol ortho-methyltransferase
vmat2	vesicular monoamine transporter 2
pomc	proopiomelanocortin
mr	Mineralocorticoid receptors
prl	prolactin

daily life and work. According to the prediction by the WHO, more than 300 million people of all ages worldwide suffer from varying degrees depression, and the prevalence of depression is increasing year by year (Moreno-Agostino et al., 2021). The etiology and pathogenesis of depression is multifactorial, including biochemistry, genetics, psychosocial factors, etc. (Paudel et al., 2020). Nevertheless, considerable evidences indicate that the occurrence and development of depression disorder are closely related to changes in the central nervous system, especially the apoptosis of the nerve cells (Mishra et al., 2021; Somelar et al., 2021). The mainstream treatment strategies for depression include tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs) etc. However, despite their efficacy, TCAs are associated with several side effects including sleepiness, weight gain, gastrointestinal issues, and sexual dysfunction (Schechter et al., 2005; Wilson and Mottram, 2004). Hence, there is a pressing need of exploring and developing novel anti-depressants which could overcome the limitation of current mainstream therapy. In this regard, wealthy amount of researches have been conducted on plant-based remedies as alternative treatments for alleviating stress and depression (Maia Oliveira et al., 2021; Ngadni and Akhtar, 2021; Sithisarn et al., 2013). In addition, plant-based remedies has been reported to have decent tolerability, low toxicity, and safety, which increased public attention globally (Mischoulon, 2018).

G. elata, a traditional Chinese herb, known as "Tian Ma", is mainly distributed in China, Southeast Asia, and Oceania (Li et al., 2016). It has been used as a common medicine and diet ingredient for treating or preventing numerous neurological disorders for thousands of years in China (Kong et al., 2019; Luo et al., 2018). The herbal plant has shown a beneficial effect in disorders of the neurological system like Parkinson's disease (Lin et al., 2020), dementia (Heese, 2020), and seizure (Yang et al., 2021a). *G. elata* was listed as a top-grade herbal medicine in Shen-nong Ben-ts'ao Jing. According to Chinese Pharmacopoeia Commission reported, *G. elata*'s dried rhizome has been used by traditional healers in China for treating various central neuron diseases including headache and migraine (Liu et al., 2018). The main active constituents of *G. elata* contain phenolic, alcohols, polysaccharides, and organic acids. Polyphenols, the secondary metabolites of *G. elata* have various pharmacological properties such as anti-oxidant, anti-convulsant, pain relief, and vision adjustment (Kim et al., 2020).

Nowadays, network pharmacology, which is based on database of genes, proteins, and diseases, has become an emerging field which can reveal the complex biological processes and disease from a systematic perspective (Gong et al., 2021). Network pharmacology can predict the potential active ingredients of the herbs and the pharmacological mechanism. Zebrafish (*Danio rerio*) is an ideal and promising vertebrate model in the field of pharmacology, development, and disease studies (Al-Samadi et al., 2019; Haque and Ward, 2018; Hill et al., 2005; Hoo et al., 2016; Jin et al., 2021; McGrath and Li, 2008; Van Sebille et al., 2019) due to the advantages of high reproductive rate, rapid organogenesis, transparent embryos, and easy maintenance (Heideman et al., 2005; Sieber et al., 2019). High degree of genetic conservation is an additional advantage, which shares the similar genes, proteins, and molecular pathways with mammals (Crawford et al., 2008; Jia et al., 2019). In the field of nervous and cognitive studies, zebrafish has become increasing popular because of its elaborate brain structure and neurochemistry (Angelopoulou et al., 2020). There are more and more molecular and behavioral studies of learning and memory changes in the psychopharmacological field associated with the nervous diseases such as anxiety, depression, mood disorder, and Alzheimer's disease (Dang and Paudel, 2021; Jin et al., 2020b; Reddy et al., 2021; Tang et al., 2019).

Despite of arrays of pharmacological effect of *G. elata,* no study till date has reported the anti-depressant effect of *G. elata.* In this study, the potential active components contributing to the anti-depressant effect of *G. elata* were predicted by the network pharmacology. The anti-depressant effect of *G. elata* were assessed *in vitro* using PC12 cells. In addition, the anti-depressant effect and the molecular mechanism were further assessed *in vivo* using zebrafish model. By combining network pharmacology and the experiment *in vitro* and *in vivo*, this study revealed the ameliorative effect of *G. elata* on depression, reflecting its potential as a putative candidate against depression.

2. Materials and methods

2.1. The selection of G. elata components

The *G. elata* components were found from traditional Chinese medicine system pharmacology (TCMSP, http://tcmspw.com/tcmsp.php) and literature mining. The *G. elata* components were screened by the Drug-likeness (DL $\geq$ 30%) and Oral availability (OB $\geq$ 0.18). The targets (Norm Fit $\geq$ 0.7) of the *G. elata* were obtained from PharmMapper (http://lilab-ecust.cn/pharmmapper/index.html).

2.2. Target fishing

The potential targets related to the depression were found and filtrated by 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/) and OMIM (Online Mendelian Inheritance in Man, http://www.omim.org). Depression, depressive disorder or depressive were used as the key words.

The targets selected above were imported into the Cytoscape 3.6.1 software to choose the targets shared by *G. elata* compounds and depression. Then selected target proteins were used to build a protein-protein interaction (PPI) network model on the String (https://string-db.org/) platform. Venn Figure was drawn by online software of FUNRICHNEW.exe.

2.3. Go analysis

The selected target protein information was imported into the database for annotation, visualization, and integrated discovery (DAVID, http://david.abcc.ncifcrf.gov) to get the pathways of the target enrichment. OFFICIAL GENE SYMBOL and *Homo sapiens* were selected as the background. $P < 0.05$ was the screening condition, which excluded a wide range of pathways.

2.4. Experimental method

2.4.1. Materials

The rhizomes of *G. elata* (Sichuandong Tianma) were provided by

Hebeidazhong Pharmaceutical Co., Ltd (No. 20190905). CORT was purchased from MCE, USA (No. 108718) and reserpine was purchased from Aladdin, China (No. J1817170). EdU assay kit was purchased from Ribobio, China. Bovine serum albumin (BSA) (Invitrogen, CA); phosphate-buffered saline (PBS), penicillin-streptomycin (P/S) and 0.25% (w/v) trypsin/1 mM EDTA were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS), Dimethyl sulfoxide (DMSO), MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-bromide], protease and collagen were supplied by Sigma (St Louis, MO). The primary antibodies (anti-GAPDH, anti-PKC, anti-RTN4R, anti-S1PR2, anti-P65, anti-Bax and anti-Caspase3) were purchase from Proteintech, China. Antirabbit IgG, HRP-linked antibody, Anti-rabbit IgG (H + L) (Alexa Fluor® 488 Conjugate) and DY-554 Phalloidin were all purchased from Cell Signaling Technology (Berverly, MA, USA). Alexa Fluor 488 goat anti-rabbit and Cy3 goat anti-mouse was obtain from Wuhan Boster Biological Technology (Wuhan, China). HPD100 resin was purchased from Solarbio (Beijing, China). Trizol reagent, reverse transcriptase kit and the SYBR Green system were purchased from Takara (Dalian, China). The reference substance of Gastrodin was purchase from ChemFaces (Wuhan, China).

2.4.2. The extraction of G. elata Blume

G. elata extracts (GBE) was obtained as the following method. Briefly, GBE was extracted with 10 times the 59% ethanol solution at 54 °C for 89 min. The column was prepared using 100 g HPD100 resin in 3.0 × 25 cm and the height was 20 cm. Then 100 mL extraction at the concentration of 0.15 g/mL was enriched by column chromatography. The GBE was eluted by 300 mL 30% ethanol solution at the flow rate of 3 BV/h. The GBE was freeze-dried using a freeze dryer to prepare a powder. The dried GBE was dissolved in distilled water and stored at −20 °C for subsequent experiments. The content and the ingredients of GBE were determined by HPLC methods according to the reference substance in 2.4.1.

2.4.3. Cell culture and treatment

PC12 cells were cultured in DMEM medium supplemented with 10% FBS, 100 IU/mL penicillin-streptomycin (PS) and 2.5 ng/mL NGF in an incubator containing 5% CO_2 at 37 °C. For the subsequent experiments, cells were divided with the following groups and treated for 24 h: Control group (medium only), cortisol (CORT) group (400 μM), low-dose group (CORT + GBE 15 μg/mL), medium-dose group (CORT + GBE 30 μg/mL) and high-dose group (CORT + GBE 45 μg/mL).

2.4.4. Animal care

Wild-type zebrafish AB line (6 months old) was kept according to standard procedures as previously described (Wang et al., 2020). In brief, the adult fish was maintained under constant temperature (28.5 °C) under a 14 h/10 h light/dark photoperiod in an automatic zebrafish housing system (ESEN, Beijing, China). The fish was fed twice a day with commercial fish food supplemented with living brine shrimp. Entire experiments using adult zebrafish was approved by the Animal Ethical and Welfare Committee (AEWC) in Biology Institute of Shandong Academy of Science.

2.4.5. MTT assay

Cell viability was determined by MTT assay using the earlier reported protocol (Yang et al., 2021b). In brief, after treatment of different concentrations of GBE for 24 h, 10 μL MTT (5 mg/mL) were added to each well of 96-well plate. After treating with MTT for 4 h, the supernatant in each well was removed and 200 μL DMSO was added for 10 min on a shaker. Then the absorbance was detected at 570 nm in a spectrophotometer. The experiments were carried out in 5 repeats. GBE alone at 0, 15, 30, 45, 60, 75, 90 μg/mL were used to study the effect of GBE alone on PC12 cells.

2.4.6. EdU assay

EdU assay was used to study the effect of GBE on PC12 proliferation according reported previously (Huang et al., 2018). Briefly, 5×10^4 cells/well were plate into 96-well. After incubated with different doses of GBE for 24 h, the cells were incubated with 50 μM EdU for 2 h, fixed with 4% PFA for 20 min, penetrated with 0.5% Triton X-100 for 10 min, incubated with Apollo reaction mix for 30 min and then stained with Hoechst33342 for 20 min. Then microscopy was performed with a fluorescence microscope. The proliferation rate was calculated by the ratio of the cells stained with EdU to those stained with Hoechst33342.

2.4.7. Cell apoptosis assay

Anti-apoptosis effect of GBE in PC12 cells was assessed by TUNEL method following the earlier reported study (Wu et al., 2014). Briefly, PC12 cells treated with GBE for 24 h were rinsed by PBS and then fixed in 4% PFA for 20 min. Then the cells were washed with PBS for twice and permeabilized with 0.1% Triton X-100 in PBS for 5min. After washing with PBS for twice, the apoptosis cells were detected by cell apoptosis detection kit according to the manufacturer's instructions (Beyotime, Shanghai, China). Microscopy was taken under a fluorescent microscope at excitation of 488 and 512 nm/emission (ZEISS LSM 510 META, Germany). The apoptosis rate was determined by the ratio of the cell number of apoptotic cells stained with FITC to total cells stained with DAPI.

2.4.8. Immunofluorescence

Immunofluorescence was performed according to previous reports (Liu et al., 2012). Briefly, after treatment with GBE for 24 h, PC12 cells were rinsed with PBS, fixed in 4% PFA, permeabilized in PBS with 0.1% sodium citrate/0.1% Triton X-100 and blocked with 5% BSA. Then the cells were incubated with primary antibodies (mouse anti-Caspase3 at 1:200, rabbit anti-Bax at 1:500 (Proteintech, China) at 4 °C overnight. After incubating with secondary antibodies (1:200) labeling with Alexa Fluor 488 goat anti-rabbit and Cy3 goat anti-mouse, the cells were stained with DAPI for 10 min. Then the microscopy was performed under a Zeiss LSM 510 confocal microscope.

2.4.9. Western blotting

After treatment with the GBE, protein isolation and western blotting was performed as previous report (Mossa et al., 2021). Briefly, the cells were lysed in RIPA lysis buffer containing 0.1% PMSF and the protein content was measured by BCA assay kit (Boster, CA, USA). Then equal amount of protein was subjected to SDS-PAGE gel electrophoresis and then transferred onto PVDF membranes. After blocking with 5% non-fat milk in TBST, the membrane was incubated with the following primary antibodies: anti-GAPDH, anti-PKC, anti-RTN4R, anti-S1PR2, anti-P65, anti-Bax and anti-Caspase3 at the ration of 1: 5000. Then after incubated with the secondary antibody conjugated with HRP, the protein bands were revealed using an ECL advanced Western blotting detection kit and quantification of relative protein levels was assessed by ImageJ (Maryland, USA). GAPDH was served as internal control.

2.4.10. Novel tank diving test

The depressive status in the zebrafish was assessed by the novel tank diving test as described earlier (Tang et al., 2019). The adult zebrafish with either sex was treated with 20 mg/L reserpine for 20 min in 1 L water. Then the fish were exposed to cultured water only or GBE groups (25 mg/L, 50 mg/L and 100 mg/L). The exposure solution was changed every day which helped to maintain the constant concentration. In addition, the adult fish were feed by transferring to the feeding tank with live brine shrimp. All groups were treated for 7 consecutive days. Prior to behavioral test, the zebrafish were habituated to the tank for 2 min. Then video recording was made for 3 min using a video-tracking system (Viewpoint, Lyon, France). The behavioral test was performed in 8 tails of zebrafish of each group. The behavior tests were also performed on groups of GBE alone at the concentration of 100 and 120 mg/L after

treatment for 7 days. Zebralab (Viewpoint, Lyon, France) was used to analyze the data including total duration time (s), numbers, total swimming distance (m) in the top and latency to the top (s).

2.4.11. Real-time quantitative PCR

After the behavioral assays, the fish was firstly anaesthetized in tricaine (0.16%). Then the fish was immediately sacrificed by decapitation on ice. Then the brain tissue of the fish was collected in a tube and quickly stored at −80 °C for the total RNA isolation. The tissue was homogenized in a crusher and extracted using TRIzol reagent. The quality of extracted RNA was evaluated on the basis of OD260/OD280 ratio. Then cDNA synthesis was performed by the PrimeScript™ RT Master Mix (Takara, Tokyo, Japan). Real-time quantitative PCR was carried out by SYBR Green Labeling System (Takara, Dalian, China). *rpl13a* was served as internal control and the runs were performed in triplicate. The sequences of the primers were shown in the supplementary information (Table S1).

2.4.12. Statistical analysis

The results were analyzed with the one-way analysis of variance (ANOVA) by Graph Pad Prism 7.0 (GraphPad Software; CA, USA) and presented as the mean ± SEM. Statistical differences with $P < 0.05$ was considered to be significant.

3. Results

3.1. The main active compounds of G. elata and their targets

As shown in Table 1, 8 polyphenols were found from the *G. elata* as evidenced from the TCMSP database. 125 targets (score > 0.7) of these 8 *G. elata* compounds were found from PharmMapper, SEA, and STITCH databases (Fig. 1A).

3.2. Potential targets shared by of G. elata and depression

A total of 1235 targets related to depression were screened out from the TTD, Drugbank, Genecards, DisGeNET, and OMIM databases (Fig. 1). UniProt was used to identify the targets, remove the duplicates, and take the intersection with the targets regulated by the active ingredients of *G. elata*. As a result, 15 targets were selected out with common intersection between depression and *G. elata* (Fig. 1 and Table 2). Cytoscape 3.6.1 software was used to construct the network of Herbs-ingredients-targets-disease. As shown in Figs. 1B, 23 nodes representing 8 *G. elata* ingredients (square-shaped node) and 15 shared targets (V-shaped node) were found, respectively. The 63 edges indicated the relationship between the targets and the *G. elata*.

3.3. GO pathway analysis

To precisely understand the functions of these targets, we used DAVID online analysis to perform a functional enrichment analysis of the target genes. Herein, GO terms ($P < 0.001$) showed that the targets were mainly associated with the following items: response to steroid hormone, negative regulation of response to external stimulus, ATPase binding, amide binding, oxidoreductase activity, hydrolase activity, acting on ester bonds, organic hydroxyl compound biosynthetic process, organophosphate catabolic process, small molecule catabolic process, and carbohydrate metabolic process (Table 3).

Table 1
List of active ingredients of *G. elata* polyphenols.

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 B	174972-79-3
MOL7	Parishin C	174972-80-6
MOL8	Parishin E	952068-57-4

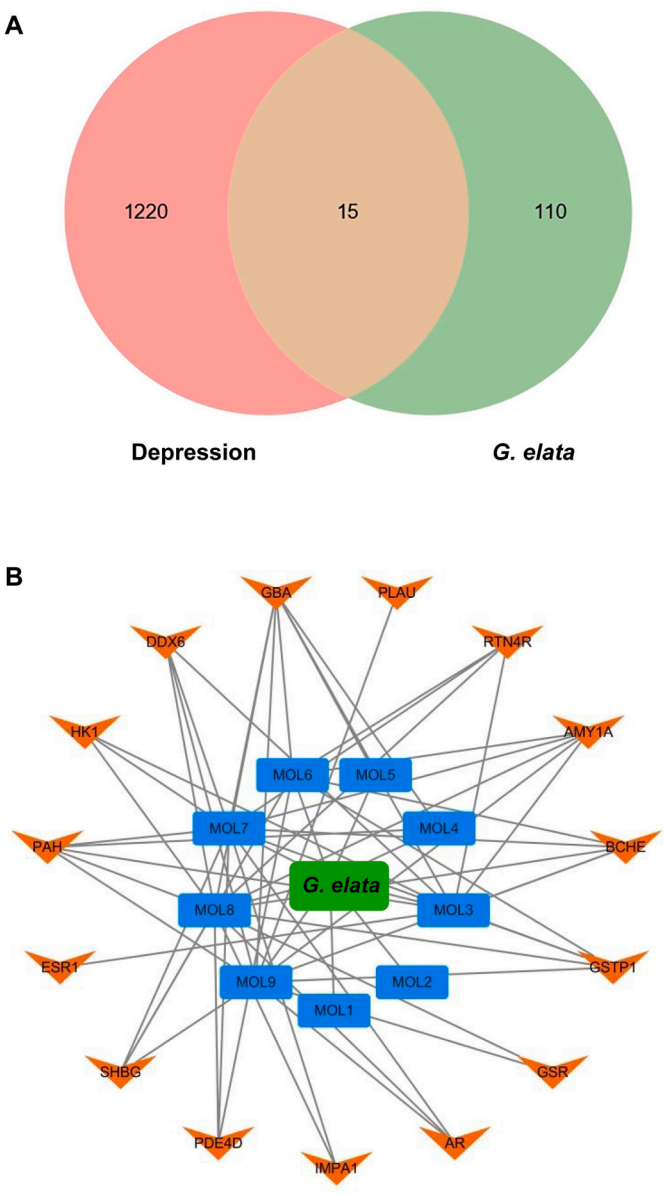

Fig. 1. Venn diagram and the herbs-ingredients-targets network.
A: Venn diagram of targets of *G. elata* and depression. Pink: the number of depression targets. Green: the number of *G. elata* targets. Brown: the number of targets shared by depression and *G. elata*. B: Herbs-ingredients-targets network. Blue: the active ingredients of *G. elata*. Red: target protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. The in vivo and in vitro experiments

3.4.1. Protective effect of GBE in CORT-treated PC12 cells

The bioactivity of the TCM mainly depends on the active ingredients. Previous reports have shown that the compounds of *G. elata* including Gastrodin, 4-Hydroxybenzyl alcohol, Vanillyl alcohol, 4-Hydroxybenzaldehyde, and Vanillin were the important active compounds for treated or prevented numerous neurological disorders (Kong et al., 2019; Luo et al., 2018; Blaikie et al., 2020). Here, the extracts of *G. elata*

Table 2
Infographics of targets for the treatment of depression with *G. elata*.

Name	Betweenness Centrality	Closeness Centrality	Degree
GBA	0.07922	0.55814	7
GSTP1	0.019895	0.510638	5
BCHE	0.019895	0.510638	5
AMY1A	0.019895	0.510638	5
PAH	0.031835	0.510638	5
RTN4R	0.009898	0.470588	4
DDX6	0.012562	0.489796	4
SHBG	0.009665	0.470588	4
HK1	0.004963	0.45283	3
PDE4D	0.00322	0.436364	3
AR	0.004491	0.45283	3
IMPA1	0.000443	0.421053	2
GSR	0.001901	0.436364	2
ESR1	0	0.358209	1
PLAU	0	0.369231	1

Table 3
GO pathway enrichment analysis.

GO ID	Category	Description	Count	Log10 (P)
0048545	GO Biological Processes	response to steroid hormone	5	−6.22
0032102	GO Biological Processes	negative regulation of response to external stimulus	4	−4.4
0051117	GO Molecular Functions	ATPase binding	3	−4.93
0033218	GO Molecular Functions	amide binding	3	−2.97
0016491	GO Molecular Functions	oxidoreductase activity	3	−2.16
0016788	GO Molecular Functions	hydrolase activity, acting on ester bonds	3	−2.15
1901617	GO Biological Processes	organic hydroxyl compound biosynthetic process	3	−3.5
0046434	GO Biological Processes	organophosphate catabolic process	3	−3.44
0044282	GO Biological Processes	small molecule catabolic process	3	−2.77
0005975	GO Biological Processes	carbohydrate metabolic process	3	−2.36

were obtained and used for further studies *in vivo* and *in vitro*. The results of the HPLC showed that Gastrodin, as the reference substance, was 20.49 mg/g (data not shown).

Previous reports have shown that elevated CORT values were found in 73% of depressed patients compared to control subjects, and CORT levels were positively related to severity of depressive symptoms (Stetler and Miller, 2011). According to the previous reports, we used 400 μM CORT to generate the model *in vitro* (He et al., 2021). The effect of GBE on PC12 cells viability was assessed by MTT method. As shown in Fig. 2, compared with the control group, the cell viability of the CORT group was reduced significantly ($P < 0.001$). However, in comparison to the CORT group, there was a significant increase in the GBE treated group at 30 μg/mL ($P < 0.01$) and 45 μg/mL ($P < 0.001$). The effect of GBE alone on PC12 cells was further assessed, the results showed that there was no pro-proliferative effect of GBE on the PC12 cells under the concentration of 90 μg/mL ($P > 0.05$) compared with the control group (Fig. S2). EdU assay results showed that there was no significant difference in the pro-proliferative effect of GBE under the concentration of 45 μg/mL ($P > 0.05$) in the presence of CORT, while the CORT could significantly inhibit the proliferation of PC12 cells ($P < 0.001$) (Fig. S3). These results indicated that GBE protected the cells against the toxicity induced by CORT.

3.4.2. GBE inhibited apoptosis induced by CORT

Previous studies showed that CORT could induce cell apoptosis in PC12 cells, so we evaluated the anti-apoptotic effect of GBE by TUNEL methods. The apoptotic cells with red fluorescence signal in GBE groups were significantly reduced compared with the CORT groups (Fig. 3A). As shown in Fig. 3B, there was a concentration-dependent decrease in the apoptosis rate in the 15 μg/mL GBE ($P < 0.05$), 30 μg/mL GBE ($P < 0.01$), and 45 μg/mL GBE ($P < 0.001$) treated groups as compared to the CORT-treated groups. These results suggested that GBE protected the PC12 cells by inhibiting the apoptosis induced by CORT.

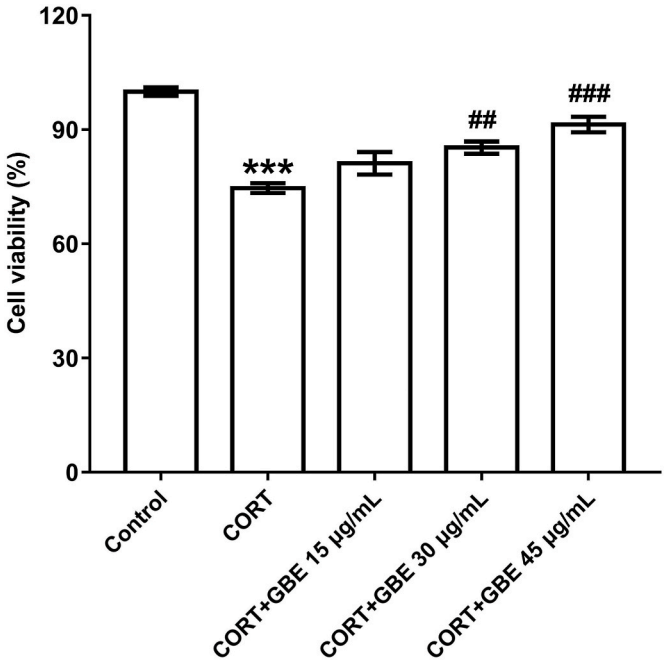

Fig. 2. Protective effect of GBE on the cell viability against CORT. Cell viability was determined by MTT Assay in differentiated PC12 cells treated with GBE (15, 30, 45 μg/mL) with or without 400 μM CORT. The control was treated with medium only. The data are expressed as the mean ± SEM. ***$P < 0.001$ vs Control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs CORT.

3.4.3. GBE inhibited the activation of Bax and Caspase3 by CORT

Bax and Caspase3 are key markers and important pro-apoptotic proteins in the apoptosis. Therefore, the protein expression of Bax and Caspase3 were investigated by immunofluorescence assays. In comparison to the control, there was a significant increase of the fluorescence intensity of Bax ($P < 0.001$) and Caspase3 ($P < 0.001$) in the CORT group (Fig. 4C and D). This increase in the fluorescence intensity of Bax was dose-dependently reduced upon treatment with 15 μg/mL GBE ($P < 0.05$), 30 μg/mL GBE ($P < 0.01$), and 45 μg/mL GBE ($P < 0.001$). Similarly, 15 μg/mL GBE ($P < 0.05$), 30 μg/mL GBE ($P < 0.01$), and 45 μg/mL GBE ($P < 0.01$) significantly decreased the fluorescence intensity of Caspase3 in comparison to the CORT group (Fig. 4C and D).

Western blotting was carried out to further verify the inhibitory effect of GBE on Bax and Caspase3 expression. There was a significant increase in the protein expression of Bax ($P < 0.001$) and Caspase3 ($P < 0.001$) in the CORT group when compared to the control group (Fig. 4F and G). This increase in the protein expression of Bax was reduced upon treatment with 15 μg/mL GBE ($P < 0.01$), 30 μg/mL GBE ($P < 0.01$), and 45 μg/mL GBE ($P < 0.001$). Similarly, 15 μg/mL GBE ($P < 0.05$), 30 μg/mL GBE ($P < 0.001$), and 45 μg/mL GBE ($P < 0.001$) significantly decreased the protein expression of Caspase3 in comparison to the CORT group (Fig. 4F and G). These results further indicated that GBE inhibited the apoptosis induced by CORT to protect the PC12 cells.

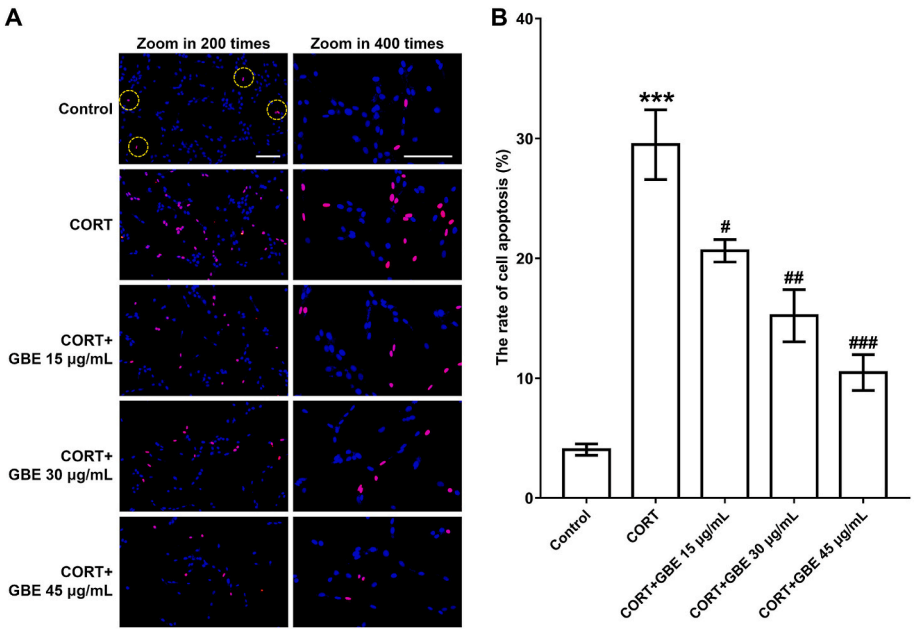

Fig. 3. Inhibitory effect of GBE on the apoptosis induced by CORT.
(A) Representative image of TUNEL staining. (B) Quantitative analysis of TUNEL staining. ***P < 0.001 vs Control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs CORT. Original magnification, ×200 and × 400. Scale bar: 100 μm.

3.4.4. GBE inhibited the expression of RTN4R, S1PR2, PKC, P65 induced by CORT

The protein expression levels of RTN4R, S1PR2, PKC, and P65 were determined to unravel the underlying molecular mechanism behind the anti-depressant effect of GBE. In comparison to the control group, there were significantly increased expressions of RTN4R ($P < 0.001$), S1PR2 ($P < 0.001$), PKC ($P < 0.001$), and P65 ($P < 0.001$) in the CORT group (Fig. 5B–E). On the contrary, 15 μg/mL GBE ($P < 0.001$), 30 μg/mL GBE ($P < 0.001$), and 45 μg/mL GBE ($P < 0.001$) significantly reduced the expressions of RTN4R, PKC, and P65, respectively (Fig. 5B, D-E). In addition, there was a significant decline in the expression level of S1PR2 upon treatment with 15 μg/mL GBE ($P < 0.01$), 30 μg/mL GBE ($P < 0.001$), and 45 μg/mL GBE ($P < 0.001$) (Fig. 5C).

3.4.5. Behavioral tests of adult zebrafish

Previous reports have shown that reserpine was a vesicular monoamine transporter (VMAT) inhibitor that produced a pathological effect by blocking VMAT. Long-term use of such drugs could cause depression (Tang et al., 2019). In order to assess the anti-depressant effect of GBE *in vivo*, the novel tank tests were performed using zebrafish model. As shown in Fig. 6, the exploratory behaviors of swimming trajectories in the top of the tank were significantly reduced in the reserpine group when compared to the control group ($P < 0.001$). However, compared with reserpine group, GBE increased the time spent and distance travelled in upper zone of the tank at 25 mg/L ($P < 0.001$), 50 mg/L ($P < 0.05$), and 100 mg/L ($P < 0.05$) (Fig. 6B and C). The time when the zebrafish first reached the top was significantly decreased upon GBE treatment when compared to the reserpine group at the concentration of 25 mg/L ($P < 0.001$), 50 mg/L ($P < 0.01$), and 100 mg/L ($P < 0.01$) (Fig. 6D and E). In addition, compared with the reserpine group, GBE increased the number of the zebrafish s between the top and the bottom of the tank at 25 mg/L ($P < 0.01$) and 50 mg/L ($P < 0.05$) (Fig. 6F and G). Worth to mention here was that the anti-depressant effect of GBE was not concentration-dependent in zebrafish model. We also found that zebrafish treated with GBE alone (120 mg/L) for 7 consecutive days decreased the time spent, distance travelled, and shuttle numbers in upper zone of the tank ($P < 0.05$), while there was no significant difference at the concentration of 100 mg/L ($P > 0.05$) (Fig. S4). Our results showed that GBE possessed the anti-depressant effect *in vivo*, which is consistent with the *in vitro* findings.

3.4.6. Modulation in the gene expression upon treatment with GBE

Several key genes (*mao*, *vmat2*, *prl*, *pomc*, *hcrt*, and *mr*), which linked to the function of the nervous system, were studied to precisely understand the mechanism behind the anti-depressive effect of GBE in zebrafish. There was a significant downregulation in the expression of *mao* ($P < 0.001$), *vmat2* ($P < 0.001$), and *prl* ($P < 0.05$) in the reserpine group when compared to the control group (Fig. 7A–C). These downregulated expression were in turn increased upon treatment with different concentration of GBE (Fig. 7A–C). There was no statistically significant expression of *mr* in the reserpine group in comparison to the control group and the GBE-treated groups (Fig. 7D). However, there was a significant upregulation in the mRNA levels of *hcrt* ($P < 0.001$) (Fig. 7E), and *pomc* ($P < 0.001$) in the reserpine group when compared to the control group (Fig. 7E and F). Specifically, significant reductions in the expression of *hcrt* and *pomc* were observed upon treatment with 25 mg/L GBE ($P < 0.001$), 50 mg/L GBE ($P < 0.001$), and 100 mg/L GBE ($P < 0.001$) (Fig. 7E and F).

The mRNA levels of genes (*mao*, *vmat2*, *prl*, *mr*, *hcrt* and *pomc*) involved in depression after the treatment of GBE with reserpine. The data were expressed as the mean ± SEM. *P < 0.05, ***P < 0.001 vs Control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs Reserpine.

4. Discussion

Depression, a common psychiatric disorder, is plausibly caused due to the loss of neurons and synapses in the brain and spinal cord, which was characteristic with high mortality and morbidity (Xue et al., 2021; Zhao and Zhang., 2020). There is a lack of effective therapy against depression and the mainstream anti-depressant therapies were associated with side-effect, low efficacy, etc (Tian et al., 2019). *G. elata* is a traditional Chinese medicine with several reported pharmacological effects, including anti-convulsive effect (Zhao et al., 2019), neuroprotective effect (Luo et al., 2018; Zheng et al., 2020), protective effect on cerebral artery occlusion (Lu et al., 2020; Seok et al., 2019), and ameliorative effect on memory (Ye et al., 2018). Polyphenols, such as

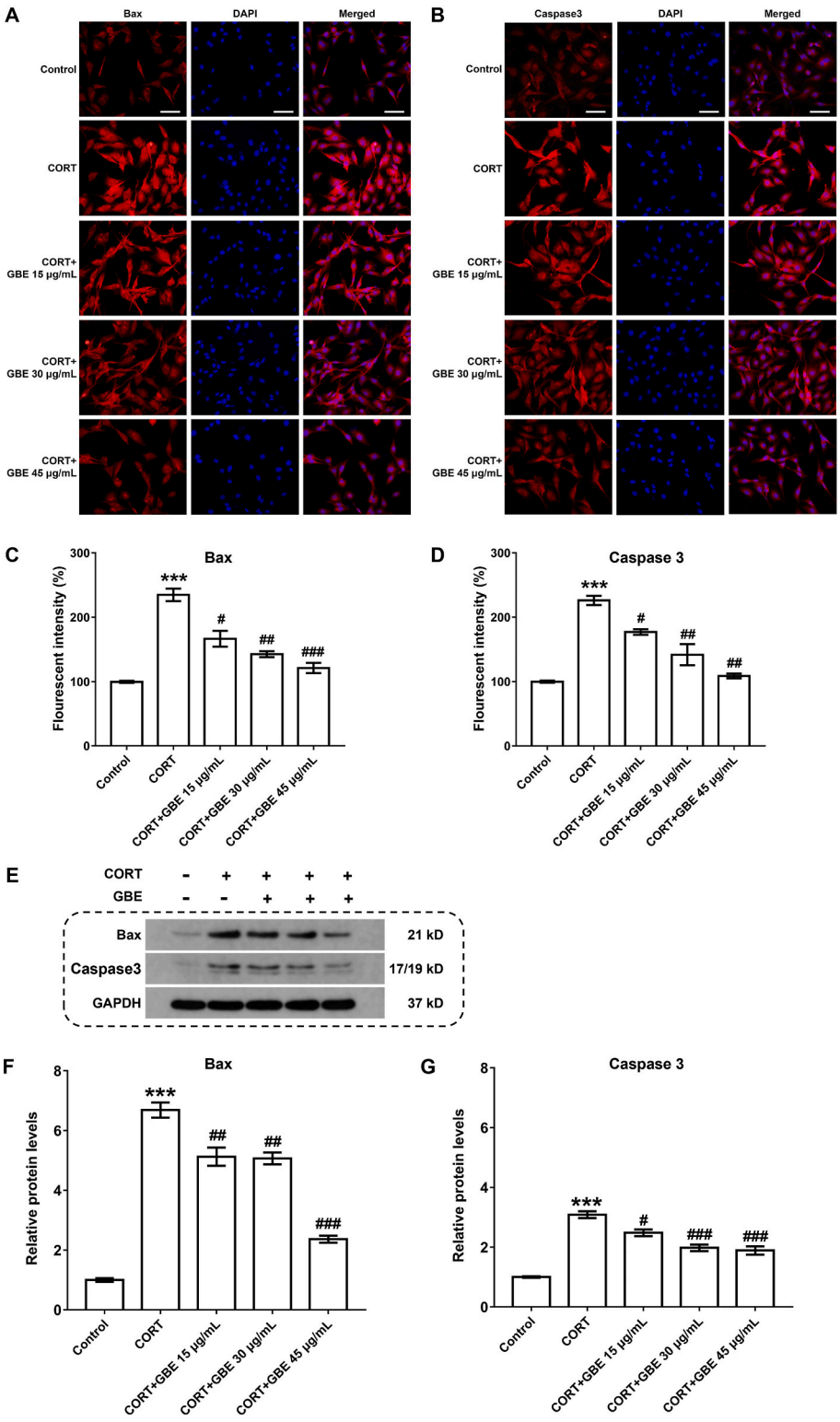

Fig. 4. The inhibitory effect of GBE on the Bax and Caspase3 expression. (A–B) Reprehensive image of immunofluorescence staining of Bax and Caspase3 in PC12 cells. (C–D) Quantitative analysis of the relative expression of Bax and Caspase3 (E) Reprehensive Western blot image of Bax and Caspase3. (F–G) The quantitative analysis of the relative expression of Bax and Caspase3. $^{***}P < 0.001$ vs Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs CORT. Scale bar: 50 μm.

Gastrodin, are the crucial active ingredients in the *G. elata* and possess high safety and low toxicity. Despite the plethora of pharmacological effect of *G. elata*, its protective effect against depression and its associated underlying mechanism have not yet been fully understood. Herein, we have utilized multiple approaches to elucidate the anti-depressant effect of *G. elata.* Precisely, network pharmacology was applied to explore the potential effect and active targets of *G. elata* against depression. In addition, the anti-depressant effect of *G. elata* and its underlying mechanisms were investigated *in vivo* and *in vitro* using PC12 cellular model and zebrafish model, respectively.

CORT is a hormone secreted by the human adrenal glands, which was associated with depression. Patients with depression often produce excess CORT (Bakusic et al., 2020; Scherf-Clavel et al., 2020). In our current investigation, CORT were used to induced depression *in vitro*. The results showed that GBE exerted protective effect against depression induced by CORT. Previous results have shown that neuronal structure

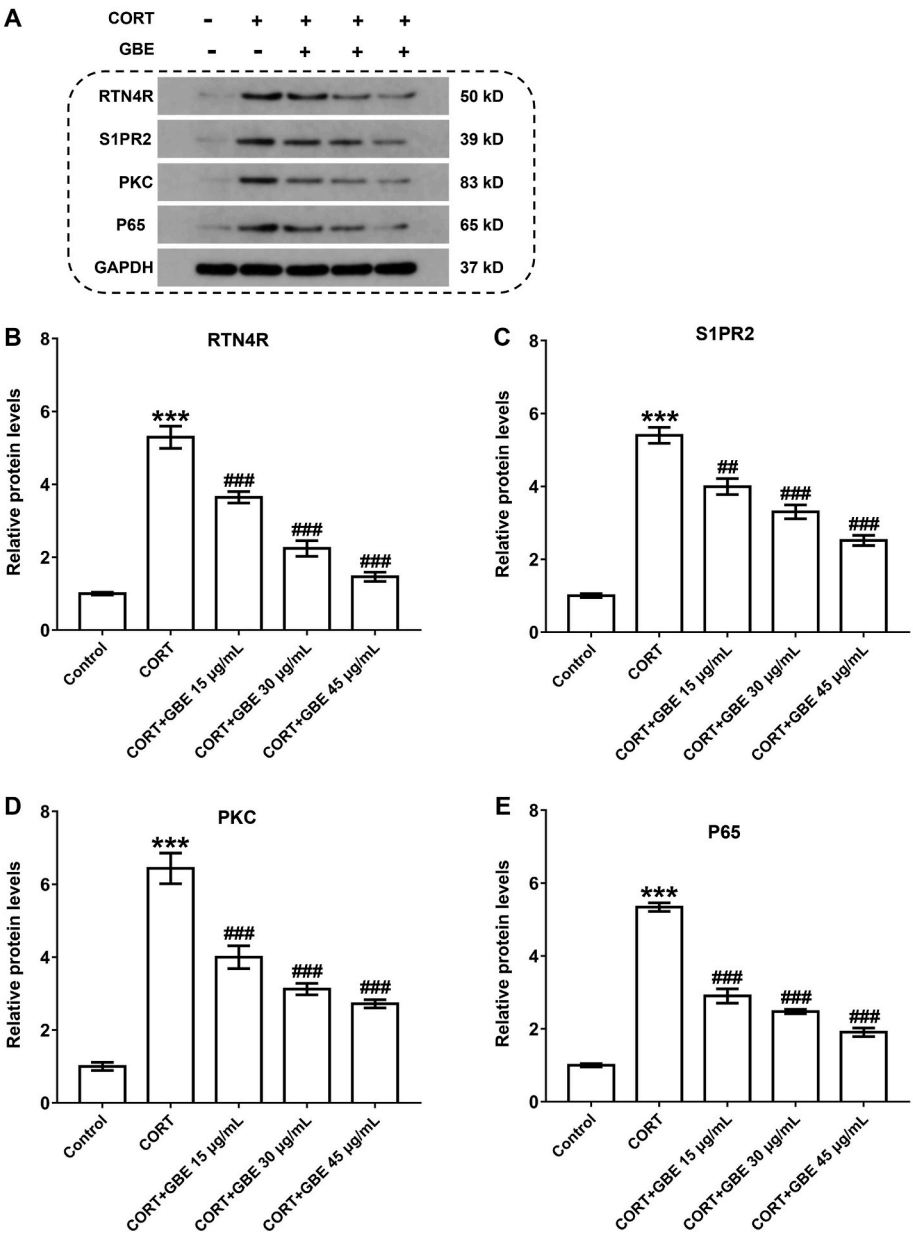

Fig. 5. The effect of GBE on the protein expression. (A) Reprehensive W
estern blot image of RTN4R, S1PR2, PKC and P65. (B–E) Quantitative analysis of RTN4R, S1PR2, PKC and P65. The protein levels were normalized to GAPDH. ***
P <
0.001 vs Control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs CORT.

changes and even neuronal apoptosis occurred due to the neuro-inflammatory responses induced by chronic stress (Fan et al., 2018). In our study, the TUNEL assay results showed that CORT induced the apoptosis and the expression of Caspase 3 and Bax, while GBE inhibited the activated apoptosis. Bax and Caspase3, acting as key pro-apoptotic proteins are important markers of the activation and execution of apoptosis (Angelopoulou et al., 2020; Jin et al., 2020a; Wu et al., 2019; Zhou et al., 2020). Based on these findings, we indicated that the neuroprotective effect of *G. elata* against depression is possibly due to its anti-apoptosis effect.

Inflammatory mediator activated the NF-κB, and then NF-κB p65 subunit translocates into the nuclear and regulates the expression of various genes, including pro-inflammatory cytokines and pro-apoptotic-related genes. Our results showed that the activation of NF-κB p65 induced by CORT was significantly inhibited upon GBE treatment. Based on our findings, it can be speculated that GBE suppressed the inflammatory response, which might be induced by CORT, and then led to the further inhibition of apoptosis process.

Nogo receptor (NgR), coded by RTN4R gene, which plays important roles in the process of neurodevelopment and refinement of neuronal connectivity via regulating neuronal development and the regeneration, sprouting and plasticity of neuron cells in the hippocampus (Kimura et al., 2017; Ullah et al., 2021; Xu et al., 2018). Thus, RTN4R-related signaling may act to stabilize brain wiring both in adulthood and during up-growth and may relate to nerve cell apoptosis (Lo Bianco et al., 2017). The dysregulation of RTN4R is involved with the cognitive and behavioral impairment including impaired working memory and slower learning ability. RTN4R was selected out by network pharmacology. Therefore, we further checked the protein levels of RTN4R. The results showed that increased expression of RTN4R by CORT could be reversed upon GBE treatment. S1PR2 regulated the proliferation, survival and migration of a variety of neuron cells by activated the expression of PKC pathway (Dong et al., 2020; Liu et al., 2020). S1PR2 also inhibited the NF-κB signal pathway to reduce endothelial cell inflammation (Pang and Li, 2020). Our results suggested that the inhibited expression of RTN4R, PKC, S1PR2 and NF-κB p65 were associated with the protective effect of GBE against depression.

The structure and functional damage of the neurons might further

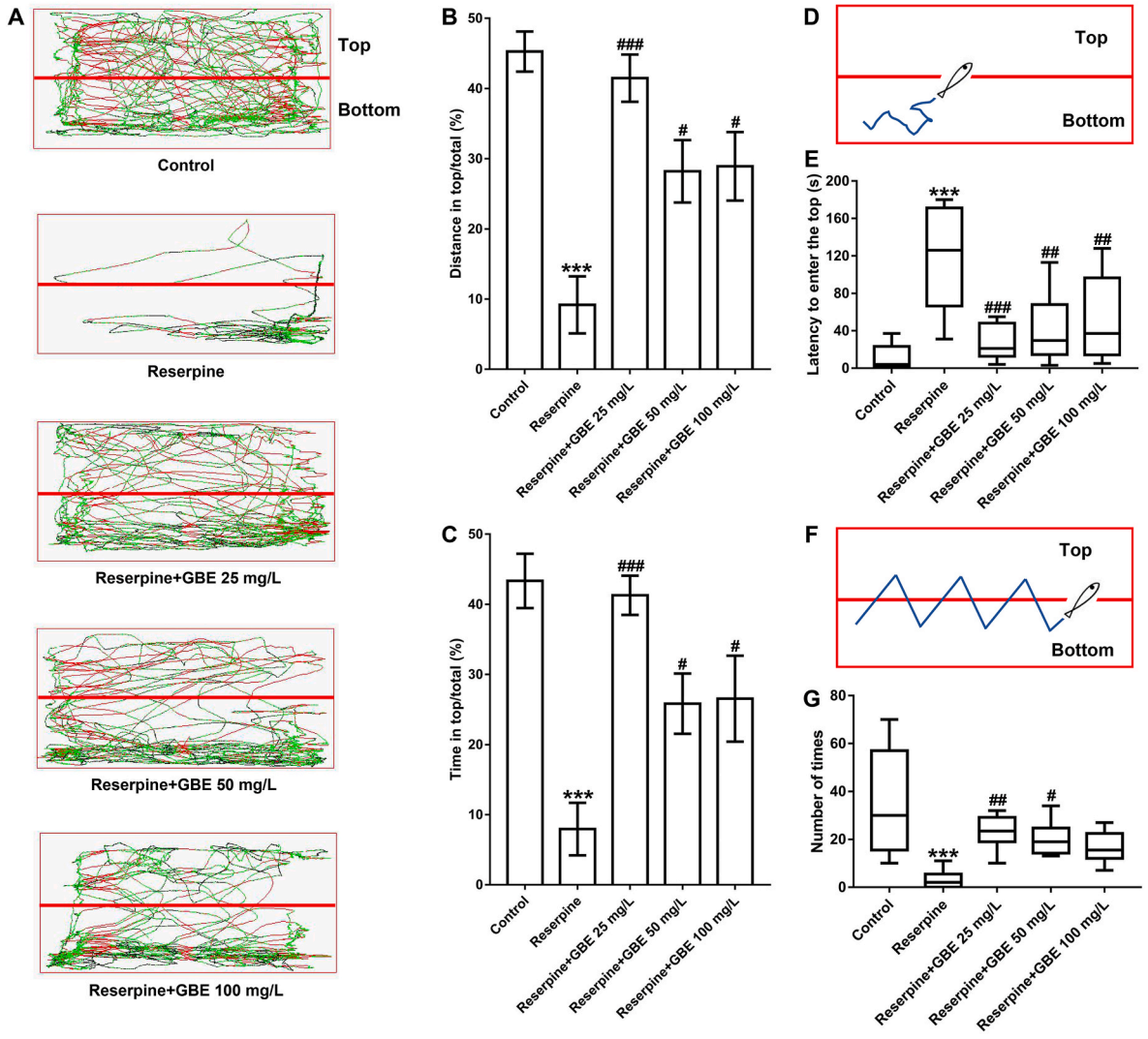

Fig. 6. The relieving effect of GBE on depression-like behavior in adult zebrafish.
(A) Representative swimming trajectories in novel tank diving test. (B–
C) The zebrafish swimming distance and the zebrafish's swimming time in the top. (D–
E) The time when the zebrafish first reached the top. (F–
G) The number of times the zebrafish shuttles between the top and the bottom. ***P < 0.001 vs Control; $^{\#}$P < 0.05, $^{\#\#\#}$P < 0.001 vs Reserpine.

cause the depression-related behaviors (Fan et al., 2018). In the current study, novel tank experiment was used to assess the protective effect of GBE treatment against reserpine-induced depression in the zebrafish (Tang et al., 2019). The indicators of the depression in the zebrafish were assessed according to the exploration and motion behavior. As previously reported, after 7 days treatment, reserpine induced the depression-like behavior of the adult zebrafish (Zhang et al., 2018). The total distances travelled and the exploration behavior including time spent, distance travelled in the top, and the latency to the top, etc. were significantly reduced by reserpine, while the freezing behavior (freezing bouts and duration) notably increased. In contrast to the reserpine group, the GBE-treated groups showed alleviated behavior patterns. These results indicated that GBE inhibited the depressive behavior in zebrafish.

In order to precisely illustrate the underlying molecular mechanism, the genes involved in the synthesis or degradation of 5-HT and other monoamine neuro-transmitters were detected by RT-PCR. *vmat2* (vesicular monoamine transporter 2) can transport monoamine, an important signal molecular, which is produced by dopamine, serotonin, and norepinephrine. *vmat2* also protected monoamine from oxidation by monoamine oxidase (MAO) (Tang et al., 2019) and catechol ortho-methyltransferase (COMT) (Vasireddy et al., 2020). Our results showed that reserpine inhibited the expression of *mao*, *prl*, and *vmat2*, while GBE treatment reversed this decrease. *vmat2* inhibitors reduced dopamine neurotransmission and deplete monoamines such as dopamine, norepinephrine, serotonin, and histamine (Tang et al., 2019; Vasireddy et al., 2020). Previous reports have shown that the dysregulation in the proopiomelanocortin (*pomc*) gene was associated with depressive disorder (Zheng et al., 2020). Mineralocorticoid receptors (*mr*) mediate neuronal changes required for learning and memory, which was densely distributed in the hippocampal region (Keller et al., 2017; Vrijsen et al., 2015). Hormone prolactin (*prl*) regulates neuroendocrine and emotional stress responses and is involved with depression (Zamorano et al., 2014). These results showed that the regulation of the nervous function key genes were involved in the anti-depressant activity of GBE.

5. Conclusion

Summing up, the potential ingredients and targets against depression

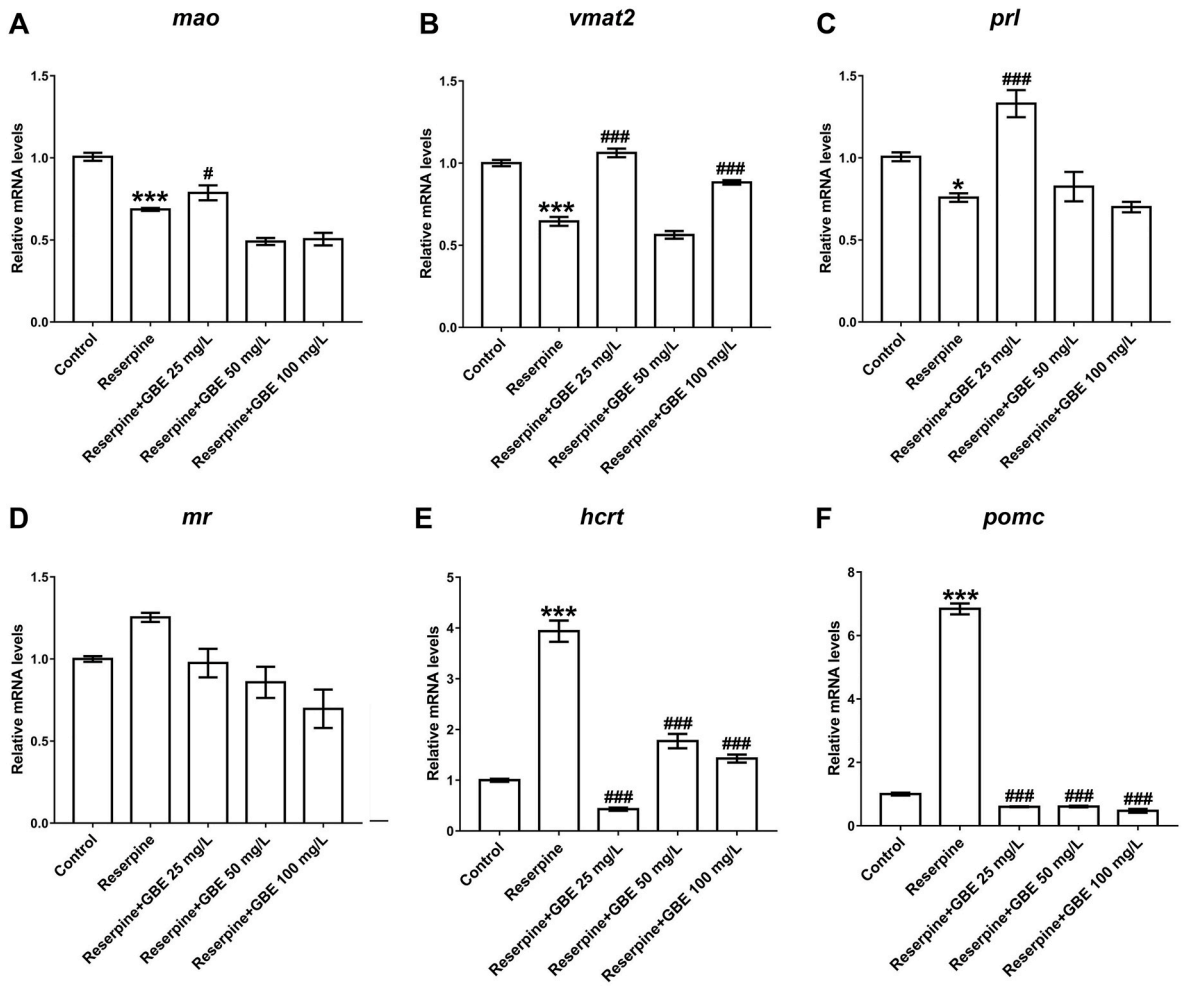

Fig. 7. Effect of GBE on the expression of depression-related genes.

were reported from *G. elata* as evident from network pharmacology. Furthermore, GBE ameliorated depression-like symptoms, including increase in the exploratory behavior and regulation of depression-related genes in zebrafish depression model. The ameliorative effect of GBE on depression might be associated with the inhibition of RTN4R-related and apoptosis pathways. Our findings increased the current understanding about the anti-depressant effect of *G. elata* and unraveled its putative underlying mechanism.

CRediT authorship contribution statement

Rongchun Wang: Investigation, Formal analysis, Visualization, Writing – original draft. **Qingyu Ren:** Investigation, Formal analysis, Visualization. **Daili Gao:** Formal analysis, Visualization. **Yam Nath Paudel:** Formal analysis. **Xia Li:** Formal analysis, Writing – original draft. **Lizhen Wang:** Formal analysis. **Pengyu Zhang:** Visualization. **Baokun Wang:** Formal analysis. **Xueliang Shang:** Conceptualization, Resources. **Meng Jin:** Conceptualization, Supervision, Methodology, Formal analysis, Writing – review & editing, All authors read and approved the final manuscript.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2022.115018.

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