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Eerdun Wurile protects neuron and promotes neurite outgrowth through regulation of apoptotic gene expression in PC-12 cells

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Eerdun Wurile (EW) is one of the most widely used traditional Mongolian medicines for stroke recovery. Previous studies revealed that EW regulates brain gene expression in a rat model of middle cerebral artery occlusion (MCAO). However, the fraction of active components and the specific genes regulated by such fractions have not been elucidated clearly. The study shows that the extracts of EW regulate the expression of genes involved in oxidative stress and apoptosis in rat pheochromocytoma (PC-12) cells. Hydrogen peroxide (H_2O_2)-induced cell death was reversed by EW extracts, and reactive oxygen species (ROS) production was reduced, while superoxide dismutase (SOD) activity as well as catalase (CAT) activity increased significantly. Moreover, the expression of Bcl-2, PARP and NF-kB p65 was upregulated by EW extract, while Bax was downregulated. Similarly, caspase 9 and Jnk was remarkably downregulated by EW extracts. Significantly, EW extracts promoted the neurite outgrowth of PC-12 cells. Our data collectively suggested that EW contains active fractions that regulate the expression of genes involved in oxidative stress and cell apoptosis, which may contribute to the neural protection effect of EW.

Key words: Eerdun Wurile, Gene expression, antioxidant, PC-12 cell, apoptosis.

INTRODUCTION

Stroke is the second major cause of death worldwide (Donnan et al., 2008; Wang et al., 2017). Stroke related disability rate is considerably high, which can lead to numbness, incontinence, as well as speech and vision loss. Prompt restoration of blood supply is the major principle for stroke therapy (Wang et al., 2017), and the thrombolysis medicine (tissue plasminogen, tPA) remarkably decreases the rate of disability (Saver, 2006). Eerdun Wurile (EW) is one the most effective traditional Mongolian medicine used in clinic for stroke recovery. Treatment of stroke with EW significantly alleviates stroke symptoms including limb numbness, slurred speech, and

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alexia (Hua et al., 2014; Tian, 2011). The remarkable therapeutic effects and negligible side effects of EW are due to the natural products used for the formulation of EW, including fruits of *Terminalia chebula Retz*, *Amomum tsaoko*, *Gardenia jasminoides Ellis*, seeds of *Myristica fragrans*, *Abutilon theophrasti*, *Melia toosendan*, *Cassia obtusifolia*, flowers of *Sieb Carthamus tinctorius*, and roots of *Glycyrrhiza uralensis Fis*, and *Saussurea costus*. These plants contain bioactive molecules such as isoliquiritigenin and diphenylheptanes, which have been proven to have protective effects in rat middle cerebral artery occlusion (MCAO)-induced ischemic stroke model (Zhan and Yang, 2006) through protection from nerve injury and post-stroke recovery (Zhang et al., 2016; Han et al., 2017).

It was discovered that EW can regulate the gene expression in the peri-ischemic center, resulting in significant upregulation of growth factors including TGF-β, Igf1, and Igf2, which may contribute to nerve tissue repair and growth. EW treatment in a rat middle cerebral artery occlusion (MCAO) model also induced significant upregulation of microglia markers, including Iba-1, CX3CR1, CD68 and CSF1R, which may have resulted from anti-inflammatory polarization of microglia upon EW treatment (Gaowa et al., 2018; Qiburi et al., 2020). We hypothesize that the active molecules in EW also protect nerve tissue through direct intervention with neurons. To prove this, we treated neuron cells with various extracts of EW, and investigated the protection effects of the extracts on the cells treated with H_2O_2 as an oxidative inducer. This data suggest that EW extracts reduce oxidative stress induced cell death and ROS production, and increase SOD activity and CAT activity, and lead to the protection of rat pheochromocytoma (PC-12) cells from oxidative stress-induced cell death.

MATERIALS AND METHODS

Chemicals and instruments

Eerdun Wurile (internal medicine number M14010080, batch number 20180225) was obtained from National Mongolian Pharmaceutical Preparation Center, International Mongolian Hospital, Inner Mongolia, China. Voucher specimens have been deposited in the Virtual Herbarium of Inner Mongolia Medical University, Hohhot, China. TRIZOL reagent was purchased from Invitrogen (Carlsbad, CA, USA). PrimeScript™ RT Master Mix (Perfect Real Time) and TB Green™ Premix Ex Taq™ Ⅱ (Tli RNaseH Plus) were purchased from Takara Biomedical Technology Co., Ltd. (Beijing, China). Absolute ethanol, n-butanol, ethyl acetate and petroleum ether were purchased from Tianjin Fengchuan Chemical Reagent Technologies Co., Ltd. (Tianjin, China). HPLC grade water was prepared by Milli-Q® Direct 8 Water Purification System from Millipore (Burlington, MA, USA).

Extraction of samples

EW powder (0.2 g per flask) were extracted under reflux with 20 ml of either distilled water (WA), absolute ethanol (EE), n-butanol (BE), ethyl acetate (EAE) or petroleum ether (PE) for 24 h at 37°C. The extracts were evaporated with a rotary evaporator under reduced pressure to remove the solvent which was dissolved in DMSO and stored at 4°C.

Cell culture and cytotoxicity assay

PC-12 (rat pheochromocytoma cell line) was purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Pudong, Shanghai, China). PC-12 cells were cultured at 37°C, 5% CO_2 in a DMEM supplemented with 10% FBS (HyClone) and 1% 100 units/ml penicillin and 100 μg/ml streptomycin. PC-12 cells were seeded in 24-well plates at 5×10^4 cells per well in 500 μl of culture medium and reached 70-80% for experimental use. To evaluate cellular toxicity of EW extracts, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was carried out. PC-12 cells were seeded in a 96-well plate (Corning-Coaster, Tokyo, Japan) at a density of 10000 cells/well in 100 μl DMEM medium and the cells were incubated for 24 h. The cells were then treated with various concentrations of EW extracts in 100 ml of DMEM medium. After the treated cells were incubated for 24 h, 50 μl of 1X MTT solution was added to each well, and incubated at 37°C for 4 h. After the viable cells with active metabolism convert MTT into a purple colored formazan product, the cell medium was removed, and 100 μL of DMSO was added to dissolve the formazan product. The quantity of formazan is measured by recording changes in absorbance at 570 nm, using a plate reading spectrophotometer (FilterMax F5, Molecular Devices, USA).

ROS production, CAT activity and SOD activity

Reactive oxygen species (ROS) production, Catalase (CAT) activity and Superoxide dismutase (SOD) activity were measured using kits (Nanjing Jiancheng, Nanjing, China) according to the manufacturer's instruction.

Real-time polymerase chain reaction (PCR)

The total RNA was extracted from PC-12 cells, using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The expression of mRNA was measured using PrimeScript™ RT Master Mix (Perfect Real Time) for RT-qPCR and TB Green™ Premix Ex Taq™ (Tli RNaseH Plus) for quantitative PCR. Primers were purchased from Takara Biotechnology Co., Ltd (Beijing, China). The sequences of primers used for RT-qPCR were listed in Table 1.

Statistical analysis

Statistical significance was determined using one-way analysis of variance (ANOVA) with a Dunnett's multiple comparisons test. *P* values of < 0.05 were considered statistically significant. All the results were expressed as mean ± SEM. The analysis was performed using GraphPad Prism 7.0.

RESULTS AND DISCUSSION

To determine neuroprotective effects of EW, the components of EW were extracted according to the polarity of the chemicals using five different solvents. The

Table 1. The sequences of primers used for RT-qPCR.

Gene	Primer sequences
Bcl-2	Forward: GTACCTGAACCGGCATCTG;
	Reverse: GGGGCCATATAGTTCCACAA
Bax	Forward: CGAGCTGATCAGAACCATCA
	Reverse: GGGGTCCCGAAGTAGGAA
AKT	Forward: CCATCATTCTTGAGGAGGAAGT
	Reverse: GACGTAGCCATTGTGAAGGAG
P38	Froward: CGAGTCCAAAACCAGCATC
	Reverse: GAACTTCGCAAATGTATTTATTGGT
Jnk	Forward: TGACAGACGGCGAAGAGA
	Reverse: GCAGCCGTCTCCTTTAGGT
PARP1	Forward: CCAGCAGAAGGTCAAGAAGAC
	Reverse: ACCTCCATGCTGGCCTTT

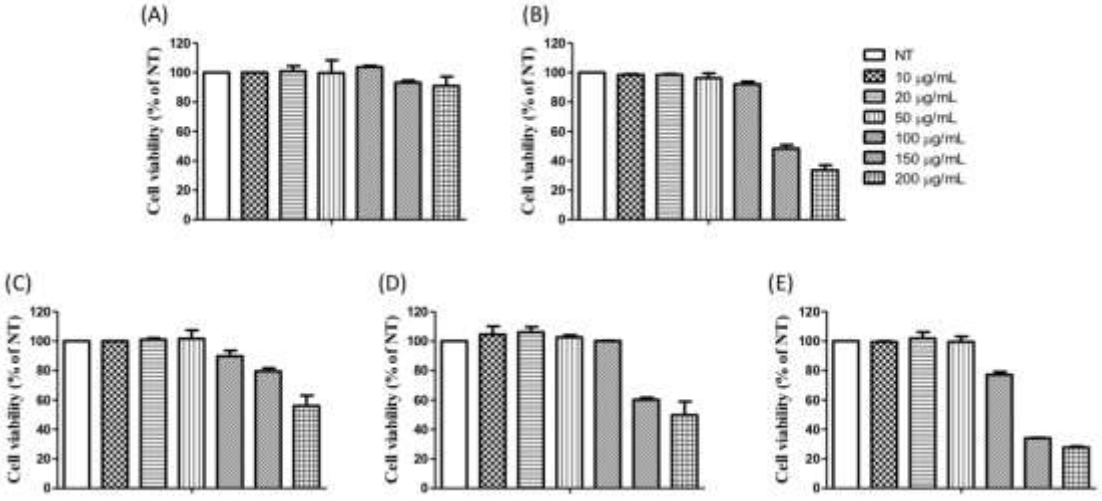

Figure 1. Cell viability assay of EW extracts. PC-12 cells were treated with various concentrations of EW extracts, and cell viability was measured by MTT assay 24 h after the treatment. (A) water extract; (B) ethanol extract; (C) n-butanol extract; (D) ethyl acetate extract; (E) petroleum either extract.

yield of the extracts increased with the increase of the polarity of the solvent used in the extraction process, with water yielding the highest amount of the chemical mixture and petroleum ether providing the least amount of solid products. Meanwhile, the PE extracts showed considerable cytotoxicity on PC-12 cells, with 30% cell viability at a concentration of 150 μg/mL. The water extract of EW showed negligible cytotoxicity on PC-12 cells (Figure 1). The IC_{50} of the extracts were as following: water extract (400 μg/ml), ethanol extract (160 μg/mL), n-butanol extract (220 μg/mL), ethyl acetate extract (200 μg/mL), and petroleum either extract (80 μg/ml).

Treatment of PC-12 cells with hydrogen peroxide (H_2O_2) induced serious damage and cell death, with only 40% cell viability 24 h after treatment (250 μM H_2O_2). Subsequent treatment of the H_2O_2-treated cells with EW extracts remarkably increased the cell viability, demonstrating that EW extracts can rescue the neurons undergoing oxidative stress. While all extracts promoted cell survival after H_2O_2 treatment, WE and EE showed slightly higher potential for quenching the oxidative stress in neurons (Figure 2).

H_2O_2 treatment induces upregulation of reactive oxygen species (ROS) in neurons, which mimics the oxygen glucose depravation (OGD) condition. After acute ischemic stroke, the production of ROS rapidly increases in the infarct center, which immediately overwhelms defensive antioxidant, leading to autophagy, apoptosis, and necrosis (Rodrigo et al., 2013). To assess whether or not EW extracts quench ROS or downregulate ROS production, we first treated PC-12 cells with H_2O_2 and

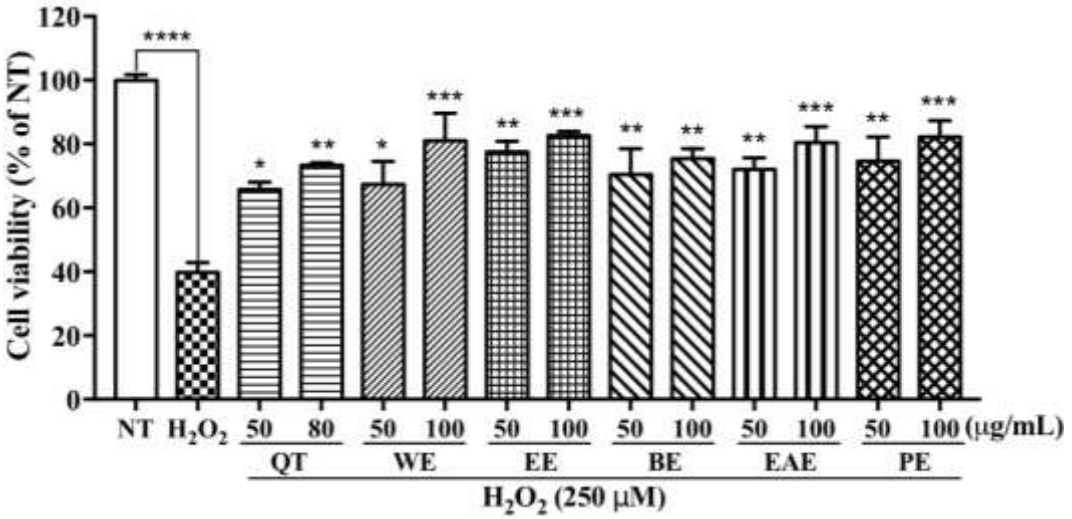

Figure 2. EW extracts rescue hydrogen peroxide (H_2O_2)-induced oxidative injury and cell death. PC-12 cells pre-treated with H_2O_2 (250 μM) were treated with positive control (quercetin, QT) or various EW extracts. Cell viability was measured 24 h after the treatment. The symbol "*" ($p < 0.05$), "**" ($p < 0.01$), "***" ($p < 0.005$) and "****" ($p < 0.0001$) indicates significant differences compared with the NT group.

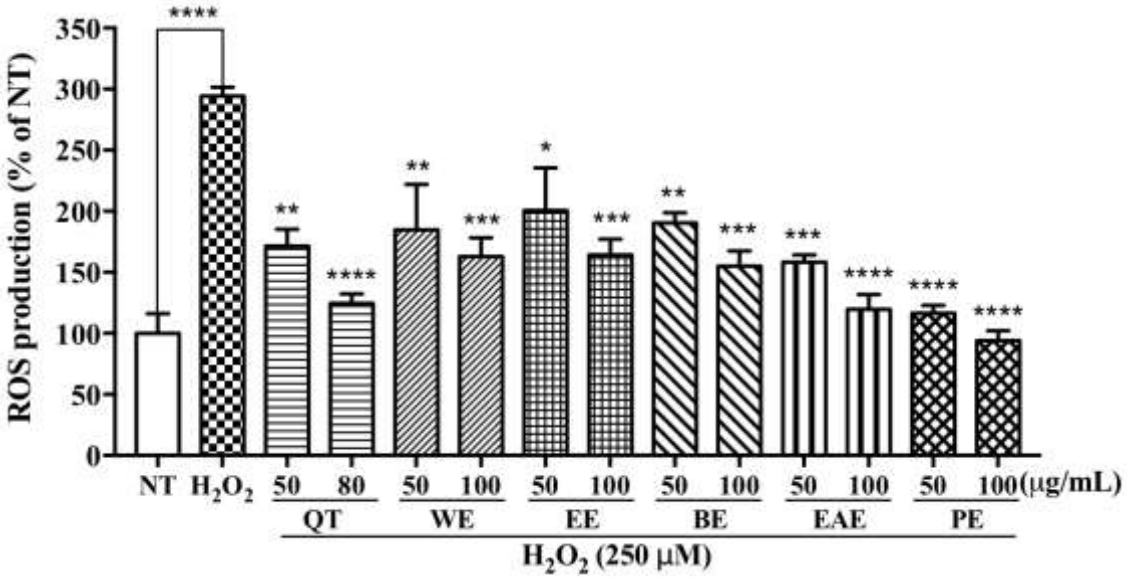

Figure 3. EW extracts reduce hydrogen peroxide (H_2O_2)-induced ROS production. PC-12 cells pre-treated with H_2O_2 (250 μM) were treated with positive control (quercetin, QT) or various EW extracts. ROS production was measured 24 h after the treatment. The symbol "*" ($p < 0.05$), "**" ($p < 0.01$), "***" ($p < 0.005$) and "****" ($p < 0.0001$) indicates significant differences compared with the NT group.

subsequently exposed the cells to different extracts of EW at different concentrations. As shown in Figure 3, treatment of PC-12 cells with H_2O_2 significantly upregulated the ROS production. The positive control (QT) reduced ROS dose dependently. All EW extracts decreased ROS production, with PE extract showing most potent inhibitory zeffect of ROS production (Figure 3), indicating that EW contains active chemicals that ether downregulate the expression of genes involved in ROS production, or directly quench ROS, which is beneficial for neural protection after stroke hit.

Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide (O2−), which is a by-product of oxygen metabolism that causes cell damage

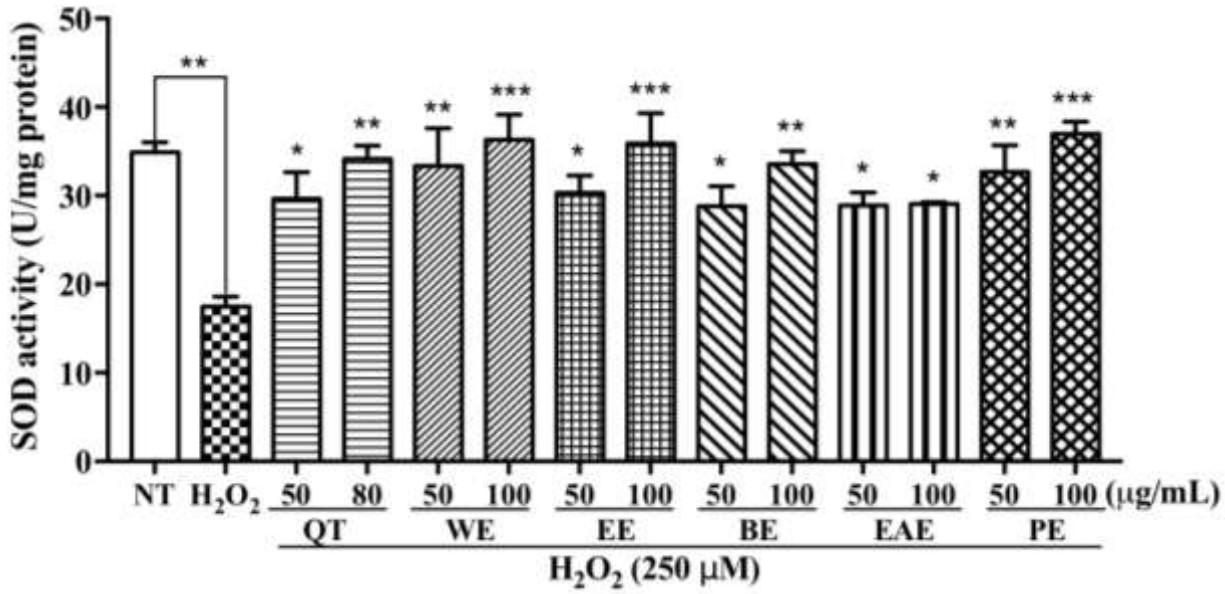

Figure 4. EW extracts increase SOD activity after hydrogen peroxide (H_2O_2) treatment. PC-12 cells pre-treated with H_2O_2 (250 μM) were treated with positive control (quercetin, QT) or various EW extracts. SOD activity was measured 24 h after the treatment. The symbol "*" ($p < 0.05$), "**" ($p < 0.01$), "***" ($p < 0.005$) and "****" ($p < 0.0001$) indicates significant differences compared with the NT group.

(Hayyan et al., 2016). SOD is an important antioxidant defense by being a major free radical scavenging system, and it has powerful anti-inflammatory activity. The SOD activity is decreased in the serum of acute cerebral ischemic injury, and replacement of antioxidant activity is beneficial for stroke patients (Spranger et al., 1997). To assess the impact of EW on SOD activity in neurons, SOD deficient cell model was first established by treating PC-12 cells with H_2O_2. Then, the cells were treated with various EW extracts, and the SOD activity measured. Treatment with H_2O_2 significantly decreased the SOD activity, which is rescued by QT. All EW extracts exhibited the ability to enhance SOD activity. Cells treated with WE, EE and PE extracts showed the best SOD activity (Figure 4), demonstrating that EW can replace SOD activity decreased by H_2O_2.

Catalase catalyzes the decomposition of H_2O_2 to water and oxygen, which protects cells from oxidative damage by ROS. CAT activity in the cell decreases when the cell is exposed to H_2O_2. To measure the influence of EW extracts on CAT activity, PC-12 cells were sequentially treated with H_2O_2 and various EW extracts, respectively, and after 24 h, CAT activity was measured. EW extracts rescued the decrease of CAT activity induced by H_2O_2. PC-12 cells treated with PE extract showed highest CAT activity (Figure 5).

Since EW extracts can efficiently reduce ROS production, and increase SOD and CAT activity, we next analyzed the viability of H_2O_2 treated PC-12 cells using live/dead cell double staining using calcein AM and propidium iodide staining kit. The kit which contains Calcein-AM (stain viable cells) and Propidium Iodide (PI) (stain dead cells) solutions can stain viable and dead cells simultaneously. As shown in Figure 6, PC-12 cells treated with EW extracts efficiently maintained the cell viability (green channels) after H_2O_2 treatment, which showed red signal in PI channel. To investigate the underlining mechanism for antioxidant effect of EW extracts, the expression of genes involved in cell apoptosis were measured. As shown in Figure 7, the decreased expression of Bcl-2, PARP and NF-kB (p65) upon H_2O_2 treatment was reversed by the treatment of EW extracts, with EE and PE extracts showing the best upregulating effects of these genes. The upregulation of PARP can significantly facilitate DNA damage repair pathway (Pascal, 2018). On the other hand, the Bax was significantly downregulated by BE, EAE and PE extracts. Bax interacts with the mitochondrial voltage-dependent anion channel (VDAC), and increases the opening of VDAC, leading to the loss in membrane potential and the release of cytochrome c (Oltvai et al., 1993).

Caspase 9 is crucial to the apoptotic pathway in many tissues including in the nervous system. During ischemic stroke, neuronal apoptosis leads to the release of Caspase 9 from the mitochondria and accumulating of Caspase 9 in the nuclei of neurons in hippocampus (Krajewski et al., 1999). PE extract of EW significantly downregulates the expression of Caspase 9 in H_2O_2 treated PC-12 cells (Figure 8), while upregulating Akt and p38, implicating potential anti-inflammatory and anti-apoptotic effect of EW. Neurite outgrowth from PC12 cells is a well characterized model of neuron differentiation

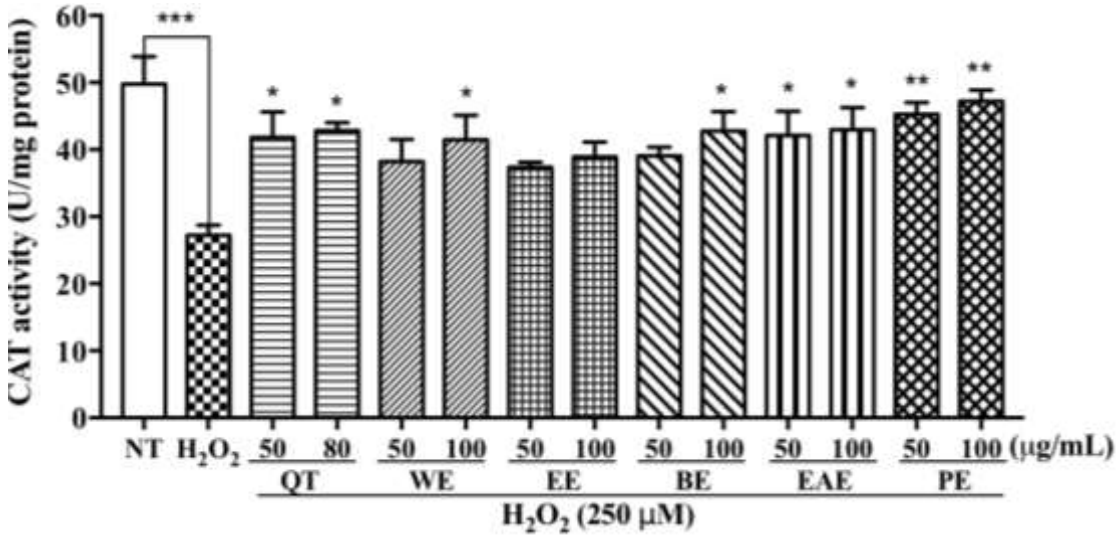

Figure 5. EW extracts enhance CAT activity after hydrogen peroxide (H_2O_2) treatment. PC-12 cells pre-treated with H_2O_2 (250 □M) were treated with positive control (quercetin, QT) or various EW extracts. CAT activity was measured 24 h after the treatment. The symbol "*" ($p < 0.05$), "**" ($p < 0.01$), "***" ($p < 0.005$) and "****" ($p < 0.0001$) indicates significant differences compared with the NT group.

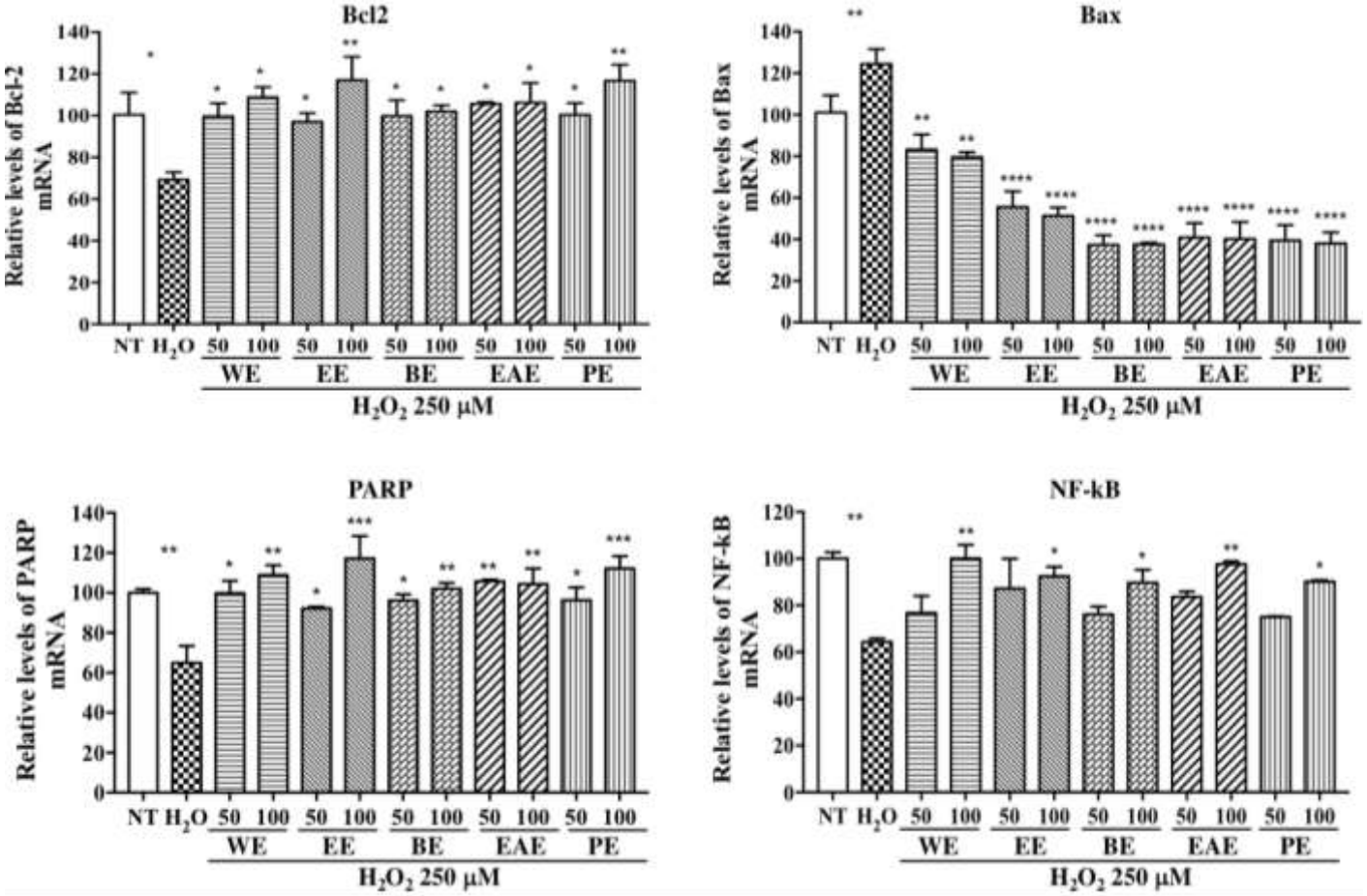

Figure 6. EW extracts regulate the expression of genes involved in apoptosis. PC-12 cells pre-treated with H_2O_2 were treated with positive control (quercetin, QT) or various EW extracts. Gene expression level was measured by RT-qPCR 24 hours after the treatment. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.0001$.

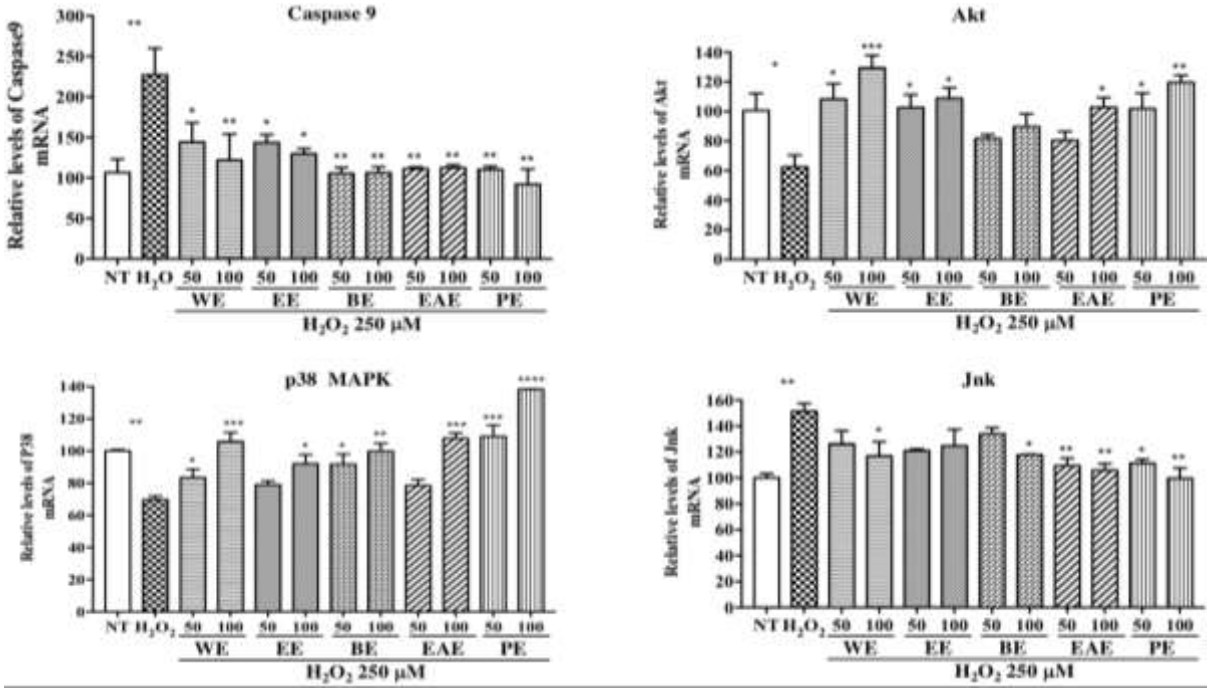

Figure 7. EW extracts regulate the expression of genes involved in apoptosis. PC-12 cells pre-treated with H_2O_2 were treated with positive control (quercetin, QT) or various EW extracts. Gene expression level was measured by RT-qPCR 24 h after the treatment. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.0001$.

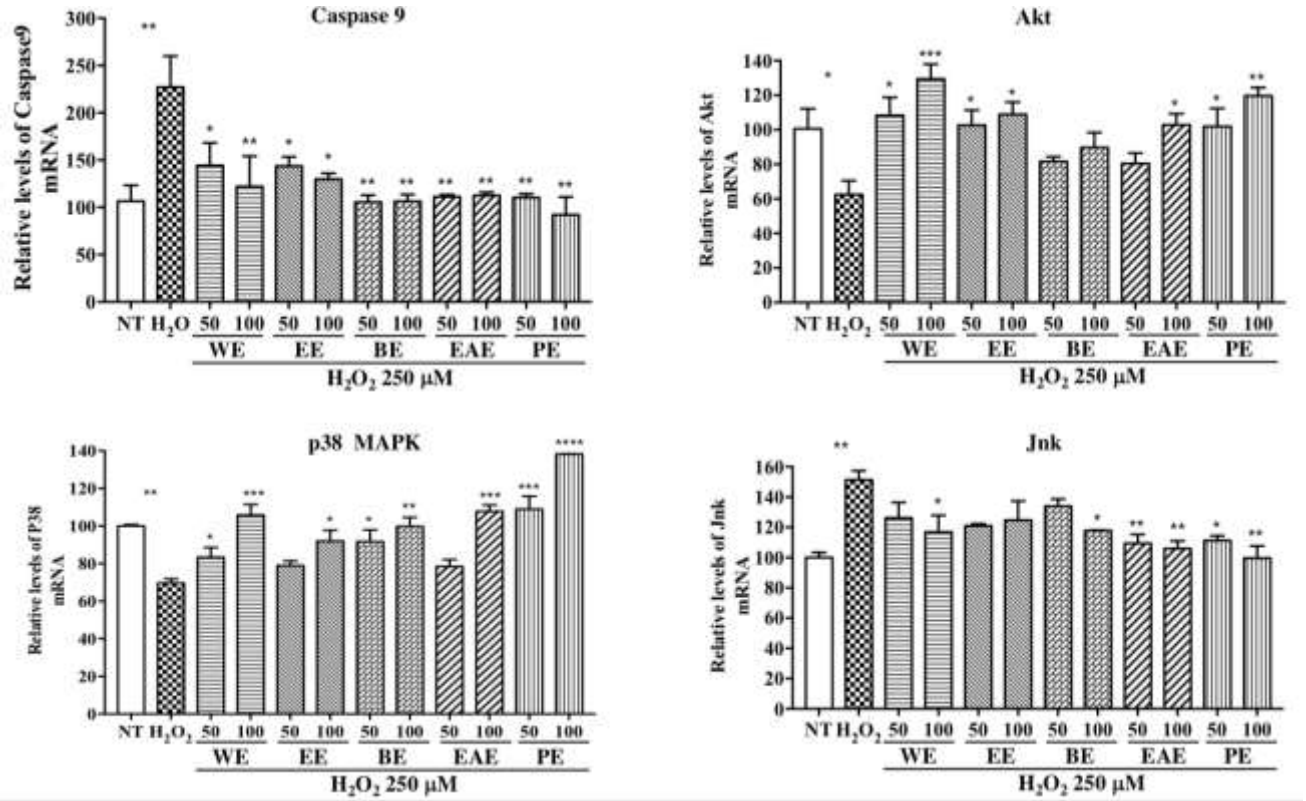

Figure 8. EW extracts regulate the expression of genes involved in apoptosis. PC-12 cells pre-treated with H2O2 were treated with positive control (quercetin, QT) or various EW extracts. Gene expression level was measured by RT-qPCR 24 hours after the treatment. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.0001$.

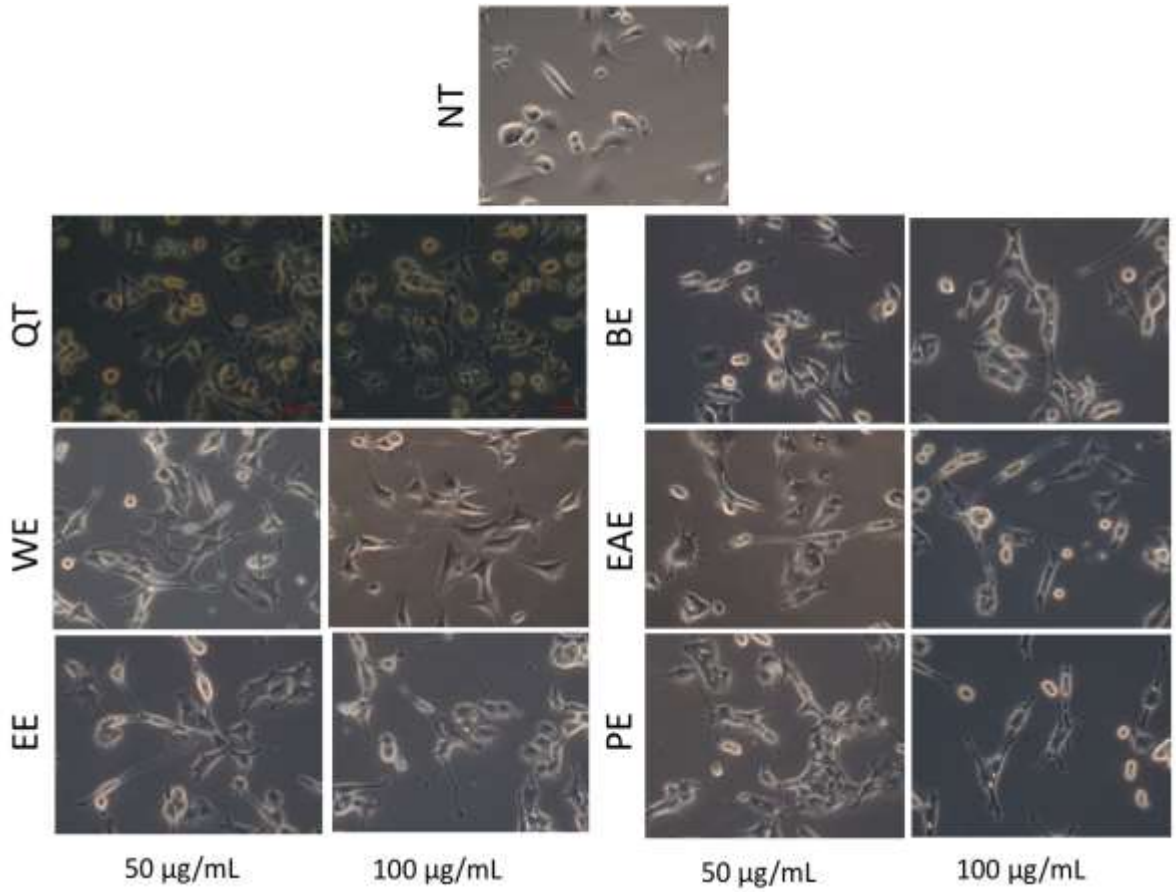

Figure 9. EW extracts stimulate neurite outgrowth. PC-12 cells were treated with positive control (quercetin, QT) or various EW extracts. Cell morphology was observed under microscope 24 hours after the treatment. Magnification: X40

(Burstein et al., 1982). To assess whether or not EW extracts stimulate neurite outgrowth, PC-12 cells were treated with various EW extracts at two different concentrations, and observed the cell morphology under microscope. At a concentration of 100 µg/ml, PE extract significantly enhanced neurite outgrowth of PC-12 cells (Figure 9). The growth of neurite is stimulated by neurotrophic factors, such as nerve growth factor (NGF). Our previous studies evidenced that EW upregulates the expression of neurotrophic factors such as IGF1 and IGF2. PE extract has the most potent stimulatory effect for neurite growth, which implicates that PE extract may contain the growth stimulating bioactive molecules.

Conclusion

This study shows that the extracts of EW reverse hydrogen peroxide (H_2O_2)-induced cell death, reduce ROS production, and increase SOD as well as CAT activity. EW extracts upregulated the expression of Bcl-2, PARP and NF-kB p65, and downregulated Bax, Caspase 9 and Jnk. Moreover, EW extracts enhanced the neurite outgrowth of PC-12 cells. Collectively, the data suggests that the active fractions of EW may contribute to the neural protection effect of EW through regulating the expression of genes involved in apoptosis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

FUNDING

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ABBREVIATIONS

EW, Eerdun Wurile; **H_2O_2,** hydrogen peroxide; **ROS,** reactive oxygen species; **SOD,** superoxide dismutase; **CAT,** catalases: **tPA,** tissue plasminogen; **MCAO,** middle cerebral artery occlusion; **WA,** water extract of EW; **EE,** absolute ethanol extract of EW; **BE,** n-butanol extract of EW; **EAE,** ethyl acetate extract of EW; **PE,** petroleum ether extract of EW.

REFERENCES

Donnan GA, Fisher M, Macleod MM, Davis SM (2008). Stroke. Lancet 371:1612-1623.

Wang W, Jiang B, Sun H, Ru X, Sun D, Wang L, Wang L, Jiang Y, Li Y, Wang Y (2017). Prevalence, Incidence, and Mortality of Stroke in China: Results from a Nationwide Population-Based Survey of 480 687 Adults. Circulation 135:759-771.

Saver JL (2006). Time is brain--quantified. Stroke 37:263-266.

Hua LXZ, Relebagen H, Bai L, Sun L, Ma C (2014). Effect of Erdun Wurile on Serum Components of Ischemic Stroke Patients. Chinese Journal of Experimental Traditional Medical Formulae 20:201-204.

Tian L (2011). Observation of Clincal Therapeutic Effects of Erdun Wurile. Chinese Journal of Ethnomedicine and Ethnopharmacy 8:10-11.

Zhan C, Yang J (2006). Protective effects of isoliquiritigenin in transient middle cerebral artery occlusion-induced focal cerebral ischemia in rats. Pharmacological Research 53:303-309.

Zhang TT, Lu CL, Jiang JG (2016) Neuroprotective and Anti-Inflammatory Effects of Diphenylheptanes from the Fruits of Amomum tsaoko, a Chinese Spice. Plant Foods and Human Nutrition 71:450-453.

Han SY, Hong ZY, Xie YH, Zhao Y, Xu X (2017) Therapeutic effect of Chinese herbal medicines for post stroke recovery: A traditional and network meta-analysis. Medicine (Baltimore) 96:e8830.

Gaowa S, Bao N, Da M, Qiburi Q, Ganbold T, Chen L, Altangerel A, Temuqile T, Baigude H (2018). Traditional Mongolian medicine Eerdun Wurile improves stroke recovery through regulation of gene expression in rat brain. Journal of Ethnopharmacology 222:249-260.

Qiburi Q, Ganbold T, Bao Q, Da M, Aoqier A, Temuqile T, Baigude H (2020). Bioactive components of ethnomedicine Eerdun Wurile regulate the transcription of pro-inflammatory cytokines in microglia. Journal Ethnopharmacology 246:112241.

Rodrigo R, Fernandez-Gajardo R, Gutierrez R, Matamala JM, Carrasco R, Miranda-Merchak A, Feuerhake W (2013). Oxidative stress and pathophysiology of ischemic stroke: Novel therapeutic opportunities. Formerly Current Drug Targets-CNS and Neurological Disorders) 12(5):698-714.

Hayyan M, Hashim MA, AlNashef IM (2016). Superoxide Ion: Generation and Chemical Implications. Chemical Reviews 116:3029-3085.

Spranger M, Krempien S, Schwab S, Donneberg S, Hacke W (1997). Superoxide dismutase activity in serum of patients with acute cerebral ischemic injury. Correlation with clinical course and infarct size. Stroke 28:2425-2428.

Pascal JM (2018). The comings and goings of PARP-1 in response to DNA damage. DNA Repair (Amst) 71:177-182.

Oltvai ZN, Milliman CL, Korsmeyer SJ (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74:609-619.

Krajewski S, Krajewska M, Ellerby LM, Welsh K, Xie Z, Deveraux QL, Salvesen GS, Bredesen DE, Rosenthal RE, Fiskum G (1999). Release of caspase-9 from mitochondria during neuronal apoptosis and cerebral ischemia. Proceedings of the National Academic Science USA 96:5752-5757.

Burstein DE, Blumberg PM, Greene LA (1982). Nerve growth factor-induced neuronal differentiation of PC12 pheochromocytoma cells: Lack of inhibition by a tumor promoter. Brain Research 247:115-119.