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ORIGINAL ARTICLE

Protective Effects of *Elaeagnus angustifolia* L. Leaves against H_2O_2-Induced Oxidative Damage in Rat Schwann Cells (RSC-96) through Regulation of PI3K/Akt Signaling Pathway

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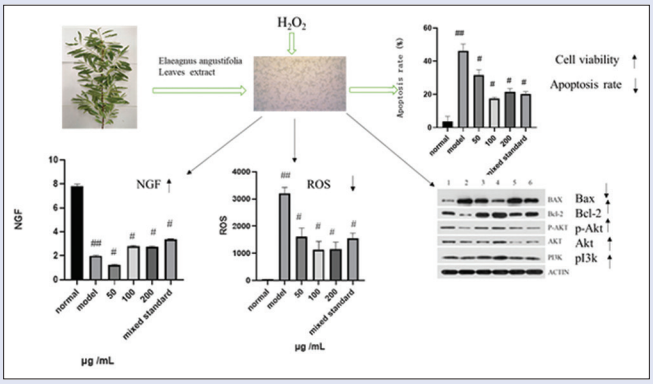
ABSTRACT

Background: As a special medicinal plant in Xinjiang province, *Elaeagnus angustifolia* L. plays an important role in windbreak and sand fixation. Its leaves are widely used in the field of traditional Chinese medicine treatment, mainly including diarrhea, dysentery, coronary heart disease and arrhythmia. Objective: In this study, our primary goal was to understand the mechanism of action of *Elaeagnus angustifolia* L. leaves (EALs) in protecting Rat Schwann cells (RSC-96) against H_2O_2-induced oxidative stress. **Materials and Methods:** The study material, EALs, was collected from Xinjiang Province in China. RSC-96 cells were stimulated by H_2O_2. Cell Counting Kit-8 assay was used in the detection of cell viability, and the cellular apoptosis and reactive oxygen species (ROS) levels were assessed by flow cytometry. The level of nerve growth factor (NGF) was assayed through enzyme-linked immunosorbent assay, and the expression of p-Akt BAX, PI3K, Bcl-2, and Akt was analyzed by Western blot. In addition, we detected the expression level of NGF. **Results:** According to our results, its levels were increased after stimulation with H_2O_2, but the levels of ROS and apoptosis were found to be reduced. Simultaneously, after induction with H_2O_2, the expression of Bcl-2, PI3K, p-Akt, and Akt was increased due to the presence of EALs extract, but the expression level of BAX was reduced. According to our results, EALs protects RSC-96 cells from hydrogen peroxide stress and its antiapoptotic and antioxidant effects are mainly mediated through PI3K/Akt signaling pathway. **Conclusion:** EALs extracts protected RSC-96 cells against H_2O_2-induced oxidative stress by exerting antioxidative and antiapoptotic effects through PI3K/Akt signaling pathways.

Key words: *Elaeagnus angustifolia L.* leaves, PI3K/Akt, RSC-96

SUMMARY

- EALs extract could protect the viability of RSC-96 cells stimulated by H_2O_2, increase the expression of NGF, and decrease the level of ROS and apoptosis.
- EALs extract exerts its anti-apoptotic and anti-oxidation effects mainly through PI3K/Akt signaling pathway.
- EALs extract can protect RSC-96 cells from the stimulation of hydrogen peroxide.



Abbreviations used: EALs: Elaeagnus angustifolia L. leaves; RSC-96: rat Schwann cells; CCK-8: Cell Counting Kit-8; ROS: reactive oxygen species; NGF: nerve growth factor; p-Akt: Phosphorylated-Akt; BAX: BCL2-Associated X; PI3K: phosphoinosmde-3-kinase; Bcl-2: B-cell lymphoma-2

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INTRODUCTION

Elaeagnus angustifolia L. mainly grows in North America, Southwest China, and subtropical Europe. It is commonly referred to as Russian olive, white willow, wild olive, or fragrant willow.[1] It is known as "treasure tree" of saline-alkali land and sand wasteland. Its branches, leaves, fruits, and flowers have important pharmaceutical value. In traditional Iranian medicine, the dried *E. angustifolia* L. leaves (EALs) are used to control bleeding and accelerate wound healing.[2]

We previously reported that EALs primarily contain peptides, tannins, saponins, flavonoids, steroids, triterpenoids, and other ingredients and that their water extract is composed of ferulic acid, ellagic acid, and gallic acid.[3] The content of total flavonoids in EALs was 18.75 ± 0.11 mg/g. The optimal extraction condition for flavonoids is to reflux with 70% alcohol twice for 1.5 h each time, and the ratio of material to liquid is 1:12 (m/V, g: ML).[4] The amount of the three-aforementioned organic acids is different for different stages of the growth and from different regions based on high-performance thin-layer chromatography.[5] The level of these three organic acids is approximately 0.590 mg/g.

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An increasing number of antioxidants isolated from plants which have been identified to support wellness and health, address cellular redox homeostasis in imbalance. The imbalance of cellular redox homeostasis is also one of the causes of disease.[6-8] The water extract of EALs demonstrates good antioxidant activity.[9] In this study, the protective effect of EALs on H_2O_2-induced oxidative damage in rat Schwann cells (RSC-96) was studied and the possible mechanism of its protective effect was explored. In this study, we aimed to provide experimental basis for the development and utilization of EALs in Xinjiang Province in China.

MATERIALS AND METHODS

Chemicals, reagents, and antibodies

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 2.5% trypsin solution, and phosphate-buffered saline (PBS) were purchased from Thermo Fisher Scientific Company. H_2O_2 was obtained from Damao Chemical Reagent Factory. Cell Counting Kit-8 (CCK-8) was obtained from Dalian Meilun Biotech Co., Ltd. (Dalian, China). Bicinchoninic acid (BCA) protein assay reagent, BAX, horseradish peroxidase (HRP)-conjugated secondary antibody, Bcl-2, β-actin, PI3K, p-Akt, and Akt were purchased from Wuhan Service Biotechnology Co., Ltd. (Wuhan, China). Superoxide dismutase enzyme-linked immunosorbent assay (ELISA) kits were purchased from the Shanghai ZhuoCai Biotechnology Co., Ltd. Reactive oxygen species (ROS) testing kit and the Annexin V/FITC kit were obtained from the BD Biosciences. The reagents used in other experiments were of analytical grade.

Plant material and extraction

EALs were collected from Xinjiang Province Urumqi Carp Mountain. The dried leaves were identified as those belonging to genus *Elaeagnus* of Elaeagnaceae by Li Yong-he, who is a Chief Physician in the Autonomous Region Chinese Traditional Medicine Hospital. Briefly, 100 g of EALs was extracted with water twice and underwent reflux extraction to obtain 1 mg/mL EAL water extract.

Cell culture and treatment

RSC-96 cells were obtained from Cell Bank of Chinese Academy of Science (Shanghai, China) and were maintained in DMEM. Simultaneously, 5 mmol/L glucose, 10% FBS, and 1% penicillin/streptomycin were added to the medium, respectively. Finally, cells are cultured in an environment with a temperature of 37°C and 5% carbon dioxide.

Cell viability assay

CCK-8 was used to determine cell viability. The cells were grown for 24 h after plating on a 96-well plate. EAL water extracts at the final concentrations of 50, 100, 200, 600, 1000, 1400, 1800, and 2000 μg/mL were added into respective wells. The culture plates were incubated at 37°C for 20 h, and the viability of the cells was analyzed. Finally, cell viability was analyzed.

After the medium was removed, 10% CCK-8 solution was added to the cells and incubated at 37°C for 1 h. Then, the optical density (OD) was measured with an enzyme marker.

The experiments were repeated thrice, and then, the cell viability is determined by the following formula, $(A_{experiment} - A_{blank})/(A_{control} - A_{blank})$ a 100%, A stands for OD.

Standard product (ferulic acid + quercetin, 2 μg/mL) and water extracts (50, 100, and 200 μg/mL) were added to the incubation medium of RSC-96 cells and treated with 400 μmol/mL H_2O_2 for 6 h. Then, the cell viability was determined by CCK-8 kit.

Apoptosis and reactive oxygen species assays by flow cytometry

After 24 h of culture, we can observe the adherent growth of the cells and continue to culture for 24 h under this condition. EAL was added to the cells at a concentration of 50, 100, and 200 μg/mL. Then, 500 μmol/L H_2O_2 was added to each well and incubated for 20 h. Then, the cells (1×10^4) were harvested and washed with PBS. Flow cytometry (FCM) was used to detect the state of apoptosis. In addition, FCM was used to detect ROS levels in cells. Each experiment was repeated thrice.

Determination of nerve growth factor in RSC-96 cells by enzyme-linked immunosorbent assay

The effect of EALs on nerve growth factor (NGF) of RSC-96 cells induced by H_2O_2 was determined by ELISA. Briefly, RSC-96 cells (10,000 cells/well) were plated in a 96-well plate and incubated for 24 h. Then, EAL water extracts (50, 100, and 200 μg/mL) and mixed standard (ferulic acid + quercetin 2 μg/mL) were added to the cells and incubated at 37°C for 20 h. Subsequently replace the solution in each well with a H_2O_2 solution with a concentration of 500 μmol/L. Before testing, rinse the residual H_2O_2 solution in each well with PBS solution. NGF was tested in accordance with kit instructions. The average of three experiments was obtained.

Western blot assay

To analyze the protein levels using BCA kit, the cellular proteins were extracted and separated using 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 4°C. The separation was performed overnight, and the gel was incubated with p-Akt, BAX, Pl3k, and Bcl-2 primary antibody. Then, the separated protein bands were transferred onto polyvinylidene difluoride membrane. The membrane was cleaned and incubated at room temperature for 4 h with the HRP-conjugated secondary antibody. Chemiluminescence method can be used to detect protein bands, and β-actin can be used as an internal reference.

Statistical analysis

The data were analyzed by analysis of variance or *t*-test with GraphPad Prism 5 software(USA, Graphpad software), and the values are expressed as mean ± standard deviation. SPSS 21.0 (IBM Inc., Chicago, IL, USA) was mainly used to calculate Chi-squared value. $P < 0.05$ indicates that the differences are statistically significant.

RESULTS

Protective effects of *Elaeagnus angustifolia* L. leaf water extract on the cell viability of H_2O_2-stimulated RSC-96 cells

In this study, the cell viability of RSC-96 cells was evaluated using CCK-8. Figure 1a shows that EAL water extract (range: 50–600 μg/mL) did not show any toxicity in normal cells. Therefore, we selected a concentration range of 50–200 μg/mL for further experiments. Compared with the normal group, the cells incubated in EAL water extract (200 μg/mL) showed significant differences ($P < 0.05$). Figure 1a shows the results. In addition, the cell viability was significantly decreased after stimulation of the cells with H_2O_2 ($P < 0.01$). Furthermore, 50 and 100 μg/mL EAL water extract and mixed standard (ferulic acid and quercetin) demonstrated increased activity of RSC-96 cells after induction with H_2O_2. The cell survival rate for 100 μg/mL EAL water extract ($P < 0.01$) or for mixed standard was significantly different ($P < 0.05$) compared with model group. Our results showed that EALs can protect the activity of injured cells after they are damaged by H_2O_2. Figure 1b shows the results.

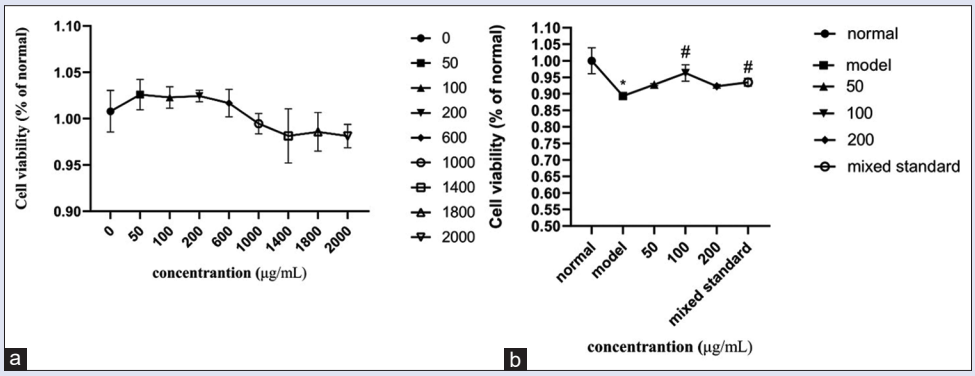

Figure 1: RSC-96 cells stimulated by H_2O_2 can protect the vitality of cells under the action of *Elaeagnus angustifolia* L. leaf water extract. (a) *Elaeagnus angustifolia* L. leaf water extract effect on the cell viability of RSC-96. The concentration range of RSC-96 cells protected by *Elaeagnus angustifolia* L. leaf water extract ranged from 50 to 2000 μg/mL which was last for 20 h. Average standard deviation ($n = 3$) is directly used to express the above experimental values, where $^{\#}P < 0.05$ versus normal group. (b) *Elaeagnus angustifolia* L. leaf water extract effect on the activity of RSC-96 cells induced by hydrogen peroxide. The concentration of *Elaeagnus angustifolia* L. leaf water extract was selected from 50 μg/mL to 200 μg/mL, and the processing time lasted for 20 h. Furthermore, hydrogen peroxide with a concentration value of 400 μg/mL was used for cell-specific processing, which lasted for 6 h. Cell Counting Kit-8 was used to measure cell activity. The above values are expressed as mean standard deviation ($n = 3$). Compared with normal group, $^{*}P < 0.01$; compared with model group, $P < 0.05$

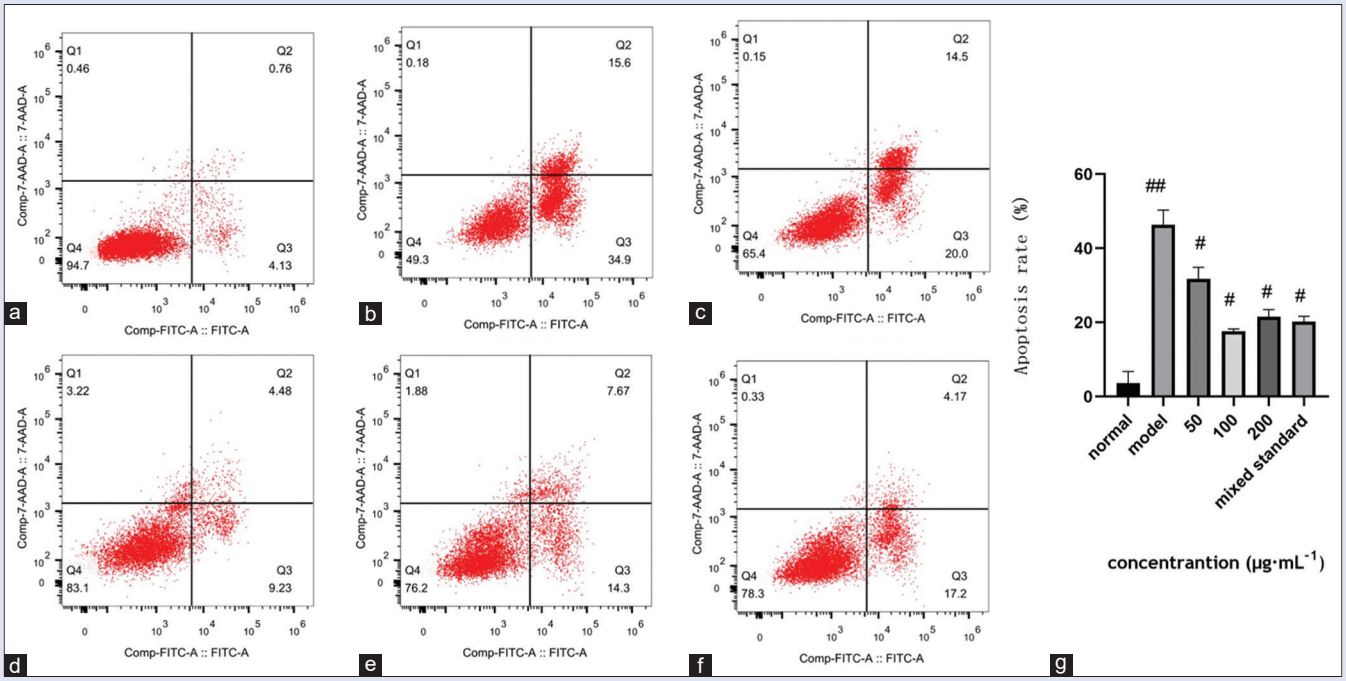

Figure 2: The effect of *Elaeagnus angustifolia* L. leaf water extract effects on apoptosis of RSC-96 cells stimulated by H_2O_2 (a) Normal group. (b) Model group. (c) 50 μg/mL *Elaeagnus angustifolia* L. leaf water extract group. (d) 100 μg/mL *Elaeagnus angustifolia* L. leaf water extract group. (e) 200 μg/mL *Elaeagnus angustifolia* L. leaf water extract group. (f) 2 μg/mL mix standard. (g) The apoptosis rate of cells is different in different groups of cells. When RSC-96 cells were treated within 24 h using *Elaeagnus angustifolia* L. leaf water extracts with concentrations of 50, 100, and 200 μg/mL, it can be observed that apoptosis can be detected by flow cytometry within 20 h. When H_2O_2 concentration is 500 μg/mL, average standard deviation ($n = 3$) is used to express the experimental value, which is $^{\#\#}P < 0.01$ versus normal group and $^{\#}P < 0.01$ versus model group

Effects of *Elaeagnus angustifolia* L. leaf water extract on the H_2O_2-induced apoptosis of RSC-96 cells

The effect of EAL water extract on apoptosis of RSC-96 cells stimulated by H_2O_2 was evaluated using Annexin V-FITC/7-AAD fluorescent double staining cell kit. Figure 2 shows the percentage of apoptosis. According to the results, compared with RSC-96 cells in the control group, the percentage of apoptosis increased significantly after induction of the cells using H_2O_2 ($P < 0.01$). However, water extracts (50, 100, and 200 μg/mL) significantly reduced the rate of apoptosis ($P < 0.01$).

Elaeagnus angustifolia L. leaf water extract changes reactive oxygen species levels in H_2O_2-stimulated RSC-96 cells

The measurement of ROS is achieved using FCM to observe the intensity of the fluorescent probe 2',7'-Dichlorodi-hydrofluorescein diacetate

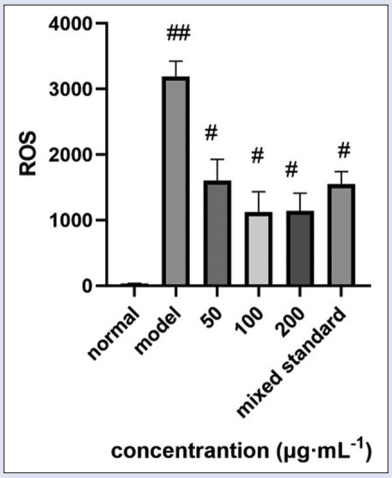

Figure 3: The reactive oxygen species level of RSC-96 cells stimulated by hydrogen peroxide was influenced using *Elaeagnus angustifolia* L. leaf water extract. By setting up different experimental groups, RSC-96 cells were treated with hydrogen peroxide fore 1 day and the cells were treated with hydrogen peroxide (500 μmol/L) for 20 h. Flow cytometry was used to detect intracellular reactive oxygen species levels. All values were expressed as mean ± standard deviation (n = 3). $^{\#\#}P < 0.01$ versus normal group; $^{\#}P < 0.01$ versus model group

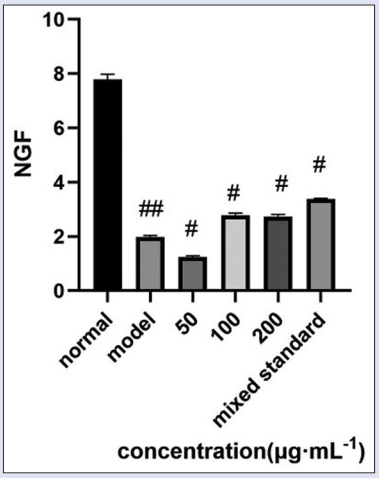

Figure 4: Effect of *Elaeagnus angustifolia* L. leaf water extract on the nerve growth factor of RSC-96 cells. The above values are expressed as average standard deviation (n = 3). $^{\#\#}P < 0.01$ versus normal group; $^{\#}P < 0.01$ versus model group

(DCFH-DA). Figure 3 shows the specific results. Compared with normal cells, the ROS level of model cells showed a significant increase in the ROS level ($P < 0.01$). However, EAL water extracts significantly reduced ROS expression in RSC-96 cells in a dose-dependent manner ($P < 0.01$). Our results show that EAL water extracts can significantly reduce ROS levels and protect cells against oxidative injury.

Effect of *Elaeagnus angustifolia* L. leaf water extract on nerve growth factor of RSC-96 cells

Effect of EAL water extract on the expression of NGF in RSC-96 cells induced by H_2O_2 was determined by ELISA. Compared with that of the model group, NGF expression increased with the increase in the concentration of the extract in the experimental group ($P < 0.01$). According to the results, EALs increased the secretion of NGF by RSC-96 cells. Figure 4 shows the results.

The effects of water extracts on the expression of BAX, Bcl-2, PI3K, Akt, and p-Akt proteins in RSC-96 cells

In this study, we aimed to analyze the protective effect of EAL water extracts in RSC-96 cells stimulated with H_2O_2 and its antiapoptotic mechanism of action. According to Figure 5, compared with the normal group, BAX protein expression in RSC-96 cells showed a significantly increased trend after H_2O_2 treatment, whereas Bcl-2 protein expression showed a decreasing trend ($P < 0.01$). The expression level of BAX protein ($P < 0.01$) differed with different concentrations of EAL. EAL (50 and 100 μg/mL) significantly increased Bcl-2 expression levels in RSC-96 cells after stimulation with H_2O_2 ($P < 0.01$). According to the results, PI3K, p-Akt, and Akt showed a significant decrease, compared with normal group after induction with H_2O_2 ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively). However, EAL water extracts at 50 and 100 μg/mL concentration significantly increased the expression levels of PI3K and p-Akt protein ($P < 0.01$). Compared with model cells, EAL water extract increased Akt expression ($P < 0.01$) but only at 100 μg/mL concentration.

DISCUSSION

In this study, we successfully induced oxidative stress in RSC-96 cells with H_2O_2. According to the results, the water extract of EAL can significantly inhibit the apoptosis of RSC-96 cells induced by H_2O_2.

In the experiment, the protective effects of ferulic acid, ellagic acid, gallic acid, and quercetin against H_2O_2-induced oxidative damage in RSC-96 cells were investigated. Ferulic acid and quercetin had superior effects, and their mixture was, therefore, used as the mixed standard group. The protective effect of the mixed standard group in the RSC-96 cells was not as good as that of EAL water extract (100 μg/mL). Ferulic acid and quercetin present in the EAL water extract showed protective effect in RSC-96 cells induced by H_2O_2. However, other active components in EAL water extract have not yet been found, which requires further research and exploration.

Oxidative stress can induce neuronal apoptosis. After H_2O_2-induced cell injury, the antioxidant effect of PI3K/Akt pathway is one of the primary cell protection mechanisms, which is the protective mechanism of central and peripheral neurons.[10-11] It is critical to activate the survival and apoptosis of neurons, which can be promoted or inhibited by the activation of PI3K/Akt pathway.[12] Due to the effect of BAX, the release of Bcl-2 protein in cells is inhibited and the antiapoptotic effect is also affected. Therefore, BAX can continuously release cytochrome C in cytoplasm.[13] Bcl-2 has pro-survival, antioxidant, antiapoptotic, and cytoprotective functions. Akt is the key medium of PI3K activation signal. Serine/threonine kinase can regulate Bcl-2 and BAX in cells to ensure the survival rate of neurons.[14-15] After PI3K activation, Akt phosphorylation (p-Akt) occurs in cells. Therefore, this result can be used as an indicator of cell activity.[16] In this study, EAL water extract upregulated the expression of PI3K, p-Akt, Akt, and Bcl-2 protein but downregulated the expression of BAX protein in RSC-96 cells induced by H_2O_2. Our results demonstrate that EALs show remarkable antiapoptotic and protective effects against the H_2O_2-induced oxidative injury by regulating the signaling of PI3K/Akt.

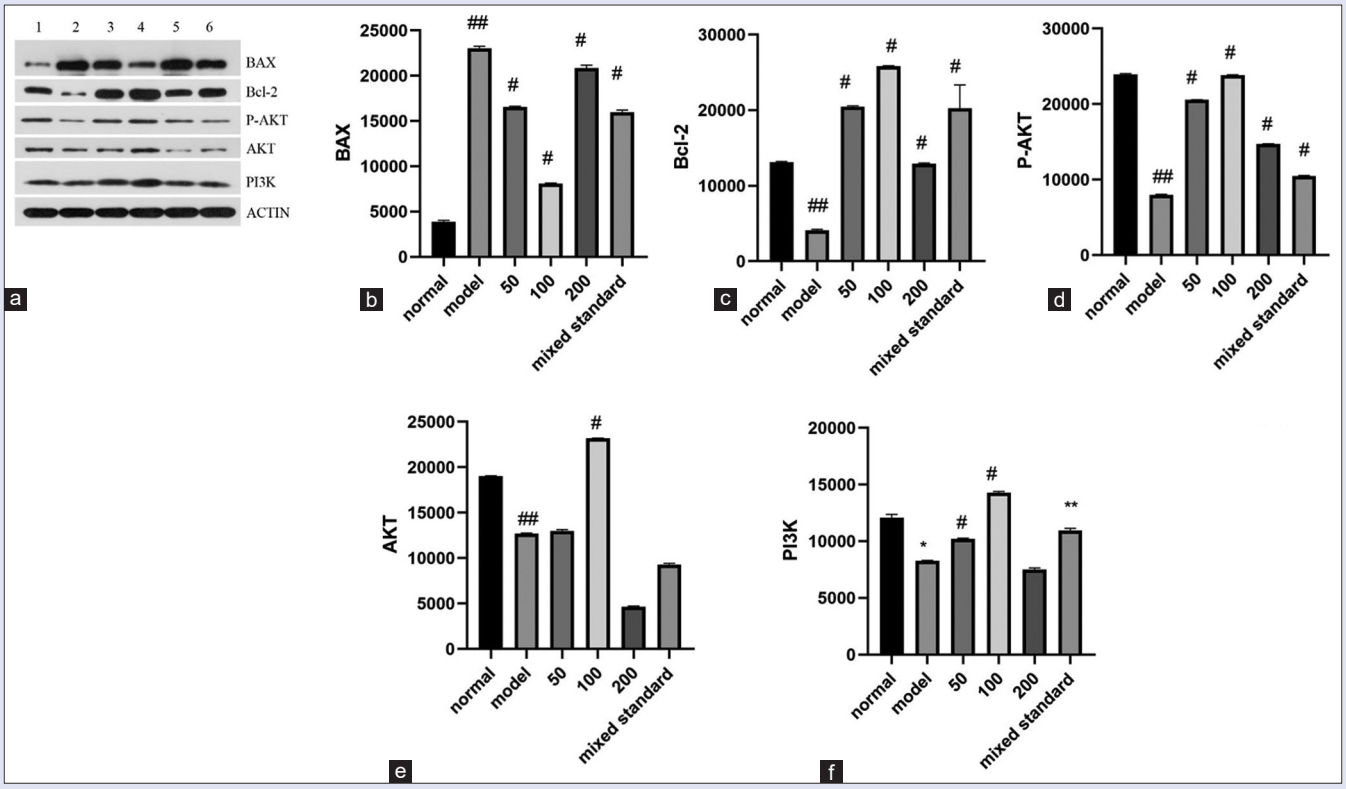

Figure 5: When hydrogen peroxide is used to stimulate cells, the water extract of *Elaeagnus angustifolia* L. leaves will affect the expression of BAX, Bcl-2, p-AKT, Akt, and PI3K. Within 24 h, the cells were treated with different concentrations of *Elaeagnus angustifolia* L. leaf water extract (50, 100, and 200 μg/mL). The expression of BAX, Bcl-2, PI3K, Akt, and p-Akt was defected after 20 h after hydrogen peroxide treatment. Meanwhile, the control group was selected as β-actin. Average standard deviation ($n = 3$) can be used to express the above experimental data. $^{\#\#}P < 0.01$ versus normal group; $^{\#}P < 0.01$ versus model group; $^{**}P < 0.05$ versus model group (a)Western blot assay results; Relative expression quantity histogram(b)BAX protein (c) Bcl-2 protein (d) p-Akt protein(e) Akt protein(f) PI3K protein

CONCLUSION

EALs protect RSC-96 from H_2O_2-induced oxidative stress. RSC-96 cells are closely related to peripheral nerve cells. This property is closely related to the antioxidant activities of EALs. This study helps in developing the antioxidant activity of EALs in Xinjiang Province and provides experimental basis for the development and utilization of EALs.

Financial support and sponsorship

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Conflicts of interest

There are no conflicts of interest.

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