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Original Article

The Effect of *Rosmarinus officinalis L* Extract on the Inhibition of High Glucose-Induced Neurotoxicity in PC12 Cells: an In Vitro Model of Diabetic Neuropathy

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Abstract

Background and Aim: Diabetes mellitus and continued hyperglycemic condition prompt serious diabetic complications such as diabetic neuropathy. Numerous studies have shown the involvement of oxidative stress and high glucose-induced cell death in the progress of diabetic neuropathy. *Rosmarinus officinalis L* has been suggested in the literature as an anti-diabetic herbal medicine that contains potent antioxidant components. Therefore, the neuroprotective effect of *Rosmarinus officinalis L* (RE) extract was investigated in glucose-induced neurotoxicity via pheochromocytoma (PC12) cells as an appropriate in vitro model of diabetic neuropathy.

Materials and Methods: Cell viability was determined using MTT assay. Cleave caspase-3 and Bax: Bcl2 ratio, as biochemical parameters of cellular apoptosis, were measured by western blotting analysis.

Results: Our data showed that a 4-fold elevation in the glucose in the medium significantly reduced cell viability ($P < 0.01$) and increased caspase-3 activation ($P < 0.01$) as well as Bax: Bcl2 ratio ($P < 0.05$) in PC12 cells after 24 h. Incubation of high glucose medium cells with 60µg/ml RE extract decreased high glucose-induced cell toxicity and prevented caspase-3 activation and Bax: Bcl2 ratio.

Conclusion: It could be concluded that RE extract is effective in the protection against hyperglycemia-induced cellular toxicity. This could be relevant to the prevention of apoptosis. Moreover, the results suggest that RE has the therapeutic potential to attenuate diabetes complications such as that neuropathy.

Keywords: Rosmarinus officinalis, hyperglycemia, cell toxicity, apoptosis, diabetic neuropathy, PC12 cells

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Introduction

Diabetes mellitus is one of the most frequent metabolic disorders that causes various central and peripheral nervous system complications such as neuropathy (1). However, the exact mechanisms for the pathogenesis of glucose neurotoxicity remain unclear. Moreover, current therapeutic approaches are not capable of bringing about complete reliefs in the neuropathic complications such as loss of sensation, pain, and motor weakness (2). Uncured Diabetic side effects are typically related to a sustained exposure to high level glucose concentrations; consequently, chronic hyperglycemia is mostly considered a key pathogenic factor of diabetic neuropathy and tissue damage (1,2). Many of basic and clinical studies suggest that oxidative stress caused by hyperglycemia plays an important role in the pathogenesis of neurotoxicity (1,3). Oxidative stress is stimulated by glucose through a combination of free radical production and decreased free radical scavenging. Hydrogen peroxide is produced by the action of superoxide dismutase on superoxide, and it has produced in the mitochondria by elevated oxidative metabolism of glucose (3,4). A large number of researchers in this field have indicated that elevated cellular oxidative stress induced by hyperglycemia stimulates several glucose metabolism pathways which are associated with the progress of the neuropathy. These include protein kinase C (PKC) activation, NAD(P)-redox imbalances, sorbitol and fructose accumulation, activation of nuclear enzyme poly (ADP-ribose) polymerase, increased hexosamine pathway, superoxide overproduction and reduced levels of crucial antioxidative enzymes (2,3).

Apoptosis could be suggested as a potential mechanism for high glucose-induced neural dysfunction and cell death in both in vitro and in vivo studies (5–8). Apoptotic cell death can result in the formation of several forms of chemical and physiological inducers of oxidative stress. For example, hydrogen peroxide can induce apoptosis in various cell types, and this effect can be prohibited by the employment of antioxidant reagents (9,10). Studies have indicated potent properties of natural products or their active components and antioxidant therapy for the prevention and/or treatment of chronic diseases such as neurodegenerative and cardiovascular disorders (11). In particular, natural herbal products have been shown to prevent neuronal death and apoptosis in some neurodegenerative disorders (12,13). Rosemary (*Rosmarinus officinalis L.*) is a perennial herb from Lamiaceae family, typical of the Mediterranean region, known as a medicinal herb due to its high antioxidant activity, which is used in traditional medicine for the treatment of diabetes mellitus (14).

Therefore, the present study was planned to investigate the possible effects of *Rosmarinus officinalis* leaf extract in an in vitro model of diabetic neuropathy. In the present study, we have evaluated the effect of RE extract on glucose induced neurotoxicity, and the potential anti-apoptotic effect of RE extract on high glucose treated pheochromocytoma (PC12) cells.

Materials and Methods

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), heat-inactivated horse serum (HS), penicillin–streptomycin solution and trypsin EDTA were purchased from Gibco BRL (USA). Culture dishes were obtained from SPL Life Sciences Inc. (South Korea). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide (MTT) were acquired from Sigma (USA). D-(+)-glucose powder were acquired from Merck Chemicals (Darmstadt, Germany). Primary monoclonal anti-β-actin and primary polyclonal anti-caspase 3 antibodies were purchased from Cell Signaling Technology, Inc. (USA). Primary monoclonal anti-Bcl-2 and primary polyclonal anti-Bax antibodies were obtained from Santa Cruz Biotechnology, Inc. (USA).

Preparation of Extract

RE extract was prepared in Razi Herbal Medicines Research Center (Lorestan, Iran). The healthy leaves were dried under shaded conditions to avoid the disruption of chemical properties. About two hundred grams of the dried Rosemary leaves were grounded into fine powder. The powder was extracted 3 times with ethanol/water (70:30). The collective aqueous extract was filtered, the filtrate was concentrated to dryness under reduced pressure in a rotary evaporator, and then the resulting aqueous extract (about 10 g)

was freeze-dried.

Chromatographic Conditions

A HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of a quaternary pump (LC-10ATvp), UV-Vis detector (SPD-M10Avp), vacuum degasser, and system controller (SCL-10Avp) was used. A manual injector with a 20 µL sample loop was used for loading the sample. A Class VP-LC workstation was used to acquire and process chromatographic data. An RP C18 analytical column (Shim-Pack VP-ODS, 250 × 4.6 mm id, 5 µm particle size; Shimadzu) was also used.

A: 0.1 % orthophosphoric acid in water (v/v), B: 0.1 % orthophosphoric acid in Methanol (v/v) was the mobile phase. Before preparation, the solvents were degassed separately using an EMD Millipore Corp. Vacuum pump (No. XF5423050). The UV detector was set at 330 nm. The chromatograms were run for 15 min at a flow rate of 1 mL/min at 30 ℃.

Cell Culture

PC12 cells were obtained from the cell culture lab of Kerman Neuroscience Research Center (Kerman, Iran). The cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum, 5% heat-inactivated horse serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37 ºC in a humidified atmosphere (90%) containing 5% CO2. Growth medium was renewed 3 times a week. Differentiated PC12 cells were plated at the density of 5000 per well in a 96 micro plate well for the cell viability assay. Control cells were grown in DMEM with 25mM glucose and the other cells (high glucose-treated) were grown in DMEM with 100mM glucose. For protein extraction, cells were grown in a 6 plate well and allowed to attach and grow for 24 h. Then the cells were incubated with medium by 100 mM glucose and different concentration of RE extract for 24 h.

Cell Viability Assay

The cell viability was explored with MTT assay. This method is based on the reduction of 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. MTT (0.5 mg/ml in final solution) was added to the 96-well plates and the cells were permitted to incubate for 2 h at 37°C. After removing the medium resulting formazan, which was solubilized with 100 µl Dimethyl sulfoxide (DMSO) per weld, the absorption was determined at 570 nm by an automatic microplate reader (Eliza MAT 2000, DRG Instruments, GmbH). Results were expressed as percentages of control.

Western Blot Analysis

The western blotting analysis was used to detect caspase-3 activation in PC12 cells. In brief, cultured cells were collected by trypsin-EDTA (0.5%), followed by two washes with cold PBS and lysed in the in cold lysis buffer (10mM Tris–HCl (pH 7.4), 1mM EDTA, 0.1% SDS, 0.1% Na deoxycholate, 1% NP-40; 2 µg each of the protease inhibitors aprotinin, leupeptin, and pepstatin A; and 0.5 µmol/l PMSF) and incubated on ice for 30 min. The homogenate was centrifuged twice at 14000 rpm at 4°C for 20 min. The resulting supernatant was removed to clean tubes as the whole cell fraction. Protein concentrations of each fraction were measured with the Bradford method (Bio-Rad, Germany). The protein samples from each fraction were separated via 10% SDS/PAGE and subsequently transferred to PVDF membrane for Western blotting. The PVDF membranes were probed with caspase 3 rabbit monoclonal antibody (1:1000 overnight at 4 ºC), Bax and Bcl-2 (1:1000) for 3 h at room temperature and subsequently exposed to secondary HRP-conjugated IgG. Antigen-antibody complexes were then visualized by ECL system and exposed to Lumi-Film chemiluminescent detection film (Roch, Germany). Beta-actin immunoblotting (1:1000) was used to control the loading. Photographs were digitized and the band intensity was quantified using ImageJ software.

Statistical Analyses

Data are expressed as mean ± SEM and analysis was performed using SPSS V20 software. The difference in cell viability (mean MTT assay) between groups was determined by one-way ANOVA, followed by the Tukey test. The values of caspase 3, Bax, Bcl2 and beta-actin band density were expressed as tested cleaved caspase-3/beta-actin ratio for each sample. The averages for different groups were compared by one-way ANOVA, followed by the Tukey test. A probability level of $P<0.05$ was considered significant.

Results and Discussion

HPLC

The amount of rosmarinic acid in its extract was

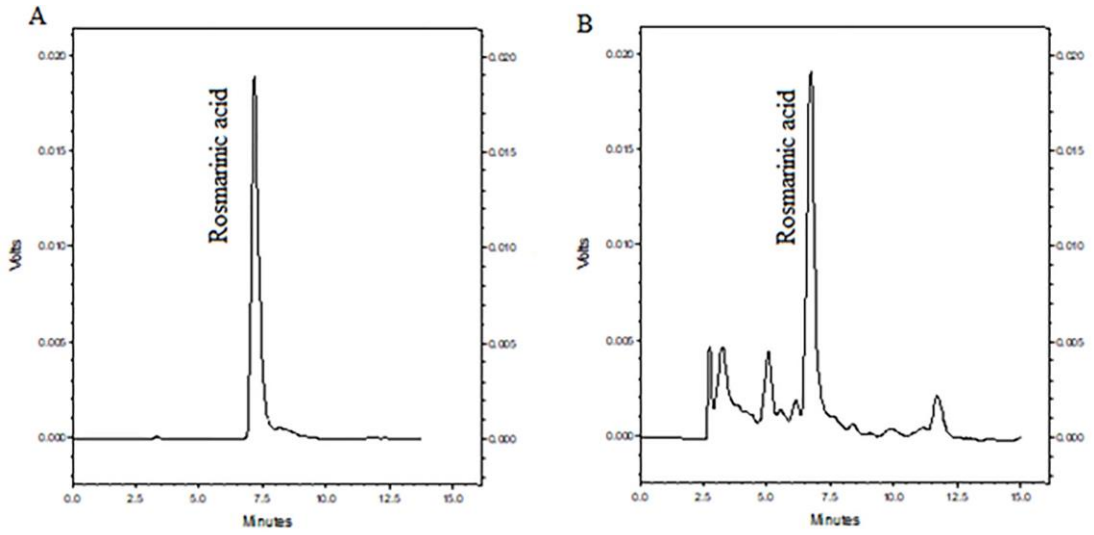

Figure 1. HPLC chromatograms of Rosmarinic acid standard sample (A) and *Rosmarinus officinalis L* extract (B) sample.

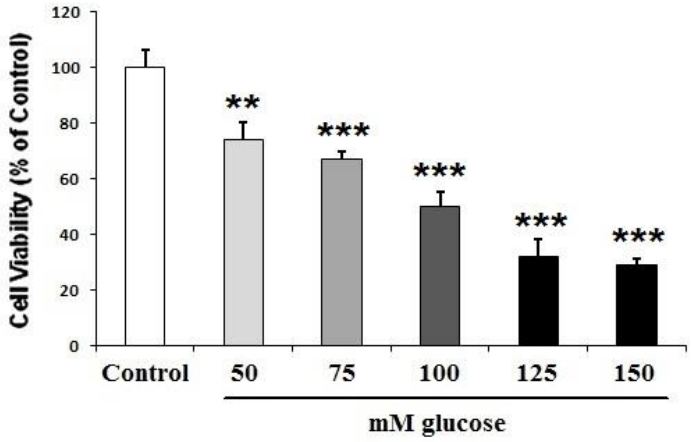

Figure 2. Effect of glucose on PC12 cell viability. Cell viability was determinate by MTT assay. PC12 cells exposed to increasing levels of glucose concentration. Data are expressed as mean±SEM; n = 5–6 wells for each group; ** P<0.01 and *** P<0.001 compared to control cells.

reported 4.5%. (Fig. 1).

Analysis of Cell Viability

Since the optimal glucose concentration for PC12 cell cultures is 25mM, we simulated in vitro hyperglycemia by increasing the medium glucose level at the concentrations of 50, 75, 100, 125 and 150mM for 24 h. PC12 cells exposed to increasing levels of glucose at 50, 75, 100, 125 and 150mM exhibited toxicity, reaching a maximal effect in 100mM glucose which resulted in 50.33±5.18% of relative cell viability. This glucose concentration was selected for further study as representative of hyperglycemic conditions that can hurt the PC12 cells and assess the protective effects of the RE extract. (Fig. 2). In addition, 24 h treatment of cultured PC12 cells with different doses of RE extract did not show toxic effects (Fig. 3).

As shown in Fig. 4, RE extract in a dose of 60 µg/ml noticeably prohibited high glucose-induced toxicity in PC12 cells after 24 h, whereas, RE extract could not prevent cell damage in other concentrations (Fig. 4).

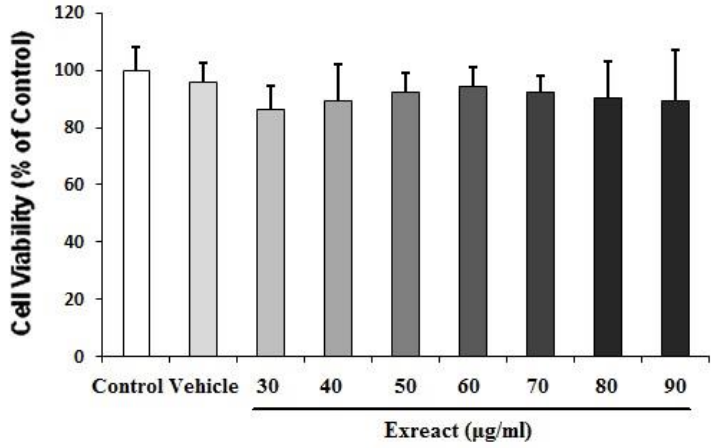

Figure 3. Effects of various doses of RE extract for 24 h on PC12 cells viability. Data are expressed as mean±SEM; n = 5–6 wells for each group.

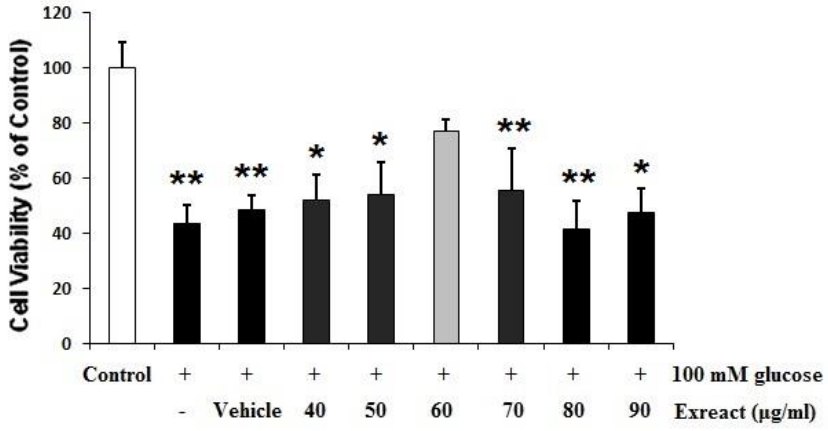

Figure 4. Effects of various doses of RE extract on high glucose-treated PC12 cell viability. High-glucose medium reduced cell viability and RE extract (60 µg/ml) protected the PC12 cells against high-glucose-induced cell death. Data are expressed as mean ± SEM; n = 5–6 wells for each group; * P<0.05 and ** P<0.01 compared to high glucose-treated cells.

The Effect of Hyperglycemic Condition on the Levels of Caspase-3 Activation and Bax: Bcl2 Ratio as an Apoptotic Parameter

To evaluate the potential parameter of apoptosis, we examined caspase-3 activation by western blotting analysis. The PC12 cells were divided into control, high glucose media and high-glucose media plus diverse concentrations of RE extract for 24 h. As shown in fig. 5, 24 h incubation with glucose at 100 mM enhanced the expression of procaspase-3 protein compared to the control and vehicle groups. The caspase-3 activation was increased in glucose treated cells after 24 h. In high glucose media PC12 cells that were treated with 60 µg/ml RE extract, no significant change was observed in procaspase-3 protein expression compared to the control group (Fig. 5). Furthermore, there was a significant increase in the Bax: Bcl-2 protein ratio in high glucose treated PC12 cells. The increased Bax: Bcl-2 ratio was attenuated in PC12 cells treated with 60 mg/kg RE (Fig. 6).

It is widely acknowledged that diabetes and hyperglycemic condition elevate the formation of harmful ROS and decrease the cellular antioxidant defense system (3,6). Furthermore, many studies have indicated that acute or continuous oxidative-stress play central or contributing roles in the progress of different chronic diseases such as diabetic neuropathy that is related to neural complication (1,2). Moreover, an

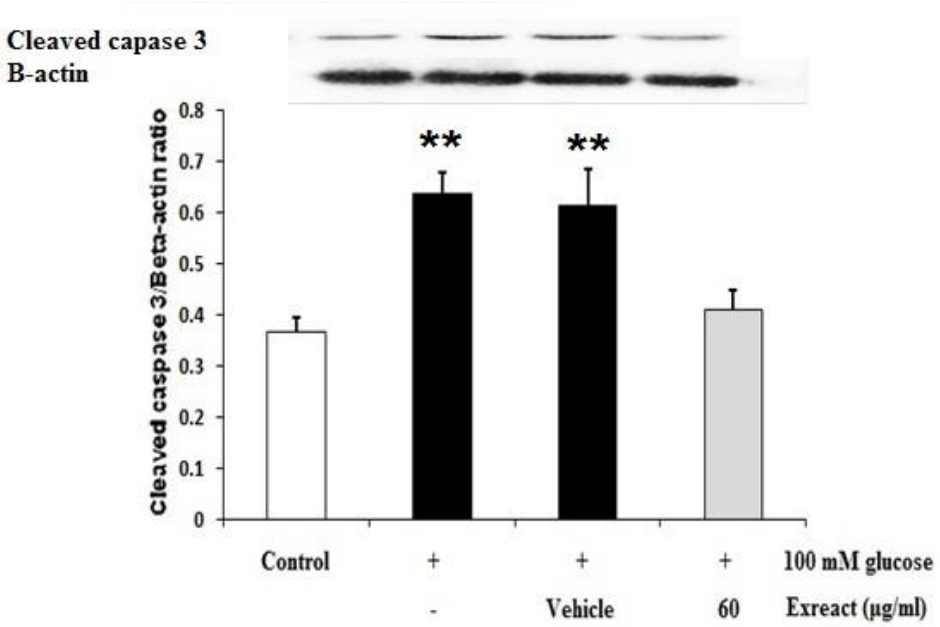

Figure 5. The activation of the caspase-3 protein in PC12 cells exposed to high glucose medium and high-glucose plus 60 µg/ml of RE extract for 24 h which was determinate by Western blot. Each value in the graph is the mean±SEM band intensity ratio for each group. Beta-actin was used as an internal control. **P < 0.01 compared to control cells.

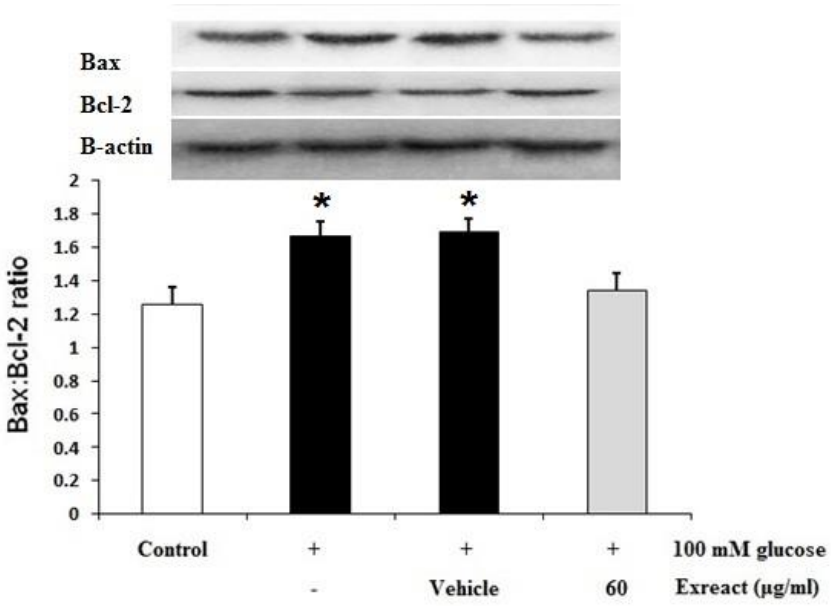

Figure 6. Western blot analysis of Bax: Bcl2 ratio in PC12 cells exposed to high glucose medium and high-glucose plus 60 µg/ml of RE extract for 24 h. Each value in the graph is the mean±SEM band intensity ratio for each group. Beta-actin was used as an internal control. *P < 0.05 compared to control group.

increase in caspase activation has been formerly illustrated in high-glucose conditions (15).

Our data in this study revealed that the glucose-induced toxicity in cultured PC12 cells was mediated through the apoptosis. Furthermore, our study indicated that the presence of 60 µg/ml RE extract could successfully decrease the high glucose-induced suppressed apoptosis.

A variety of researches published in scholarly journals have indicated that oxidative stress can cause cell

death through apoptosis (16–18). There are numerous kinds of chemical and physiological inducers of oxidative stress which can cause apoptosis. For example, in several different cell types, hydrogen peroxide can induce apoptotic cell death (9,18). The mechanism by which oxidative stress induces apoptosis has not been completely elaborated so far. Nevertheless, several pathology situations can result from the oxidative stress-induced apoptotic signaling that follows ROS elevation and/or antioxidant diminutions (16). Furthermore, caspase-3 activation and Bab: Bcl2 ratio elevation through oxidative stress have significant roles in the promotion of glucose toxicity (3,4).

In recent years, the demonstration of antioxidative and neuroprotective characters of natural herbal products has drawn intensive interest. It is notable that several natural herbal products have been shown to prevent the death and apoptosis of neuronal in several neurodegenerative diseases (5,6,19–21). As mentioned above, oxidative stress is one of the important issues in tissue damage such as neuronal structure injury in diabetes mellitus subjects (2). In line with these studies, our results showed that the hyperglycemic condition could lead to significant increases in caspase-3 activation and Bax: Bcl2 ratio (as a main biochemical factor of apoptosis) (Fig. 4 and 5). In addition to the beneficial antioxidant effect of the natural product, some natural inhibitors of apoptosis suppress high-glucose-induced cell toxicity (8). In recent years, much attention has been directed to herbal polyphenolic compounds because they can assist a number of antioxidative mechanisms and stimulate protective enzymes, hence they are effective against oxidative cell damage (20). Rosmarinus officinalis L. is a habitual plant from Lamiaceae family. In folk medicine, the aerial portions of rosemary are primarily used as analgesics in renal and abdominal colic pains and rheumatic disorders (22). In line with these studies, our study showed that RE extract treatment had a suppressing effect on the biochemical factors of apoptosis and eventually prevented high-glucose-induced apoptosis in PC12 (Fig. 4 and 5). It has been reported that this plant possesses considerable antioxidant activity, and its extract exhibited significant radical-scavenging properties probably due to the high concentration of polyphenolic components such as rosmarinic and caffeic acids (23). Moreover, rosemary has several important biological characteristics, such as antidiabetic, antioxidant and antidepressant effects (14,24). Altogether, the analgesic effects of rosemary may be on the neuronal cells apoptosis reduction. The neuroprotective properties of these extracts, at least in part, can be due to the presence of the great amount of phenolic and flavonoids compounds and their antioxidant effects beside the free radical-scavenging property of this plant.

Conclusion

In conclusion, this study suggests that aqueous extract of *R. officinalis*, protects PC12 cells against high glucose-induced toxicity. The mechanisms of these effects might be due, at least in part, to the reduction of neuronal apoptosis.

Acknowledgment

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Conflict of Interest

The authors declare that they have no conflict of interest.

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