JoP Journal of Pharmacopuncture
Journal of Pharmacopuncture 2022;25(3):216-223
pISSN: 2093-6966 • eISSN: 2234-6856
https://doi.org/10.3831/KPI.2022.25.3.216

Original Article

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Neuroprotective Effect of Root Extracts of *Berberis Vulgaris* (Barberry) on Oxidative Stress on SH-SY5Y Cells

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Received April 21, 2022
Reviewed May 6, 2022
Accepted June 7, 2022

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Objectives: Oxidative stress plays a key role in chronic and acute brain disorders and neuronal damage associated with Alzheimer disease (AD) and other neurodegeneration symptoms. The neuroprotective effects of berberine and *Berberis vulgaris* (barberry) root extract against apoptosis induced by hydrogen peroxide (H_2O_2) in the human SH-SY5Y cell line were studied.
Methods: The methanolic extraction of barberry root was performed using a maceration procedure. Oxidative stress was induced in SH-SY5Y cells by H_2O_2, and an MTT assay was applied to evaluate the neuroprotective effects of berberine and barberry root extract. The cells were pretreated with the half maximal inhibitory concentration (IC_{50}) of each compound (including berberine, barberry root extract, and H_2O_2), and the anti-apoptotic effects of all components were investigated using RT-PCR.
Results: The SH-SY5Y cell viability increased in both groups exposed to 75 and 150 ppm barberry extract compared with that in the H_2O_2-treated group. The data showed that exposing SH-SY5Y cells to 30 ppm berberine significantly increased the cell viability compared with the H_2O_2-treated group; treatment with 150 and 300 ppm berberine and H_2O_2 significantly decreased the SH-SY5Y cell viability and was associated with berberine cytotoxicity. The mRNA levels of Bax decreased significantly under treatment with berberine at 30 ppm compared with the control group. A significant increase in Bcl-2 expression was observed only after treatment with the IC_{50} of berberine. The expression level of Bcl-2 in cells exposed to both berberine and barberry extracts was also significantly higher than that in cells exposed to H_2O_2.
Conclusion: The outcomes of this study suggest that treatment of SH-SY5Y cells with barberry extract and berberine could suppress apoptosis by regulating the actions of Bcl-2 family members.

Keywords: apoptosis, oxidative stress, sh-sy5y cells, berberine, barberry, alzheimer disease

INTRODUCTION

Alzheimer disease (AD) is known as a destructive neurodegenerative disorder with an invariable course that decreases quality of life in the elderly, especially those older than 65 years [1]. Elderly dementia caused by AD is also the most common symptom in people who silently develop memory problems, cannot meet their most basic needs, and suffer from physical/mental malfunction or cognitive impairment [1].

Neuronal structure degeneration or trauma may lead to oxi-

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dative stress, which is seen in diseases such as AD, Parkinson disease (PD), Huntington disease, amyotrophic lateral sclerosis, and cerebral ischemic stroke.

Highly reactive molecules, anions, and free radicals (such as superoxide anions, hydrogen peroxide [H_2O_2], and hydroxyl radicals, which are known as reactive oxygen species [ROS]) cause oxidative stress and cellular and/or DNA damage; all of these depend on the exposure time and potency, which may lead to cell death or survival with related mechanisms [2].

H_2O_2 is not highly reactive by itself, but in the vicinity of enzymes, multivalent metals, and other catalyzing agents, reactions with sulfur compounds such as thiols can occur, causing a change to sulfonic acid and its anion form, i.e., sulfonate (SO^-).

Exposure to H_2O_2 results in reduced viability and mitochondrial membrane depolarization in SH-SY5Y cells, which may lead to apoptotic and necrotic cell death as detected by staining the cell with annexin V–fluorescein isothiocyanate (FITC) and propidium iodide (PI); the amount of apoptosis can be detected by flow cytometry [3, 4].

The cell cycle is an extremely regulated procedure with multiple checks to ensure a homeostatic balance between cell multiplication and death in the presence of adequate environmental signals [5]. These results indicate that therapeutic strategies aiming to prevent cell apoptosis could be a rational choice to control diseases. However, the removal of ROS and free radicals is perhaps one of the most efficient defense mechanisms of the body to retain the oxidative-antioxidant ratio and balance [6].

Berberine, a plant alkaloid used in traditional and Ayurvedic medicine, has been identified in several genera, such as *Berberis*, *Coptis*, and *Hydrastis*, in different parts of plants, including roots, rhizomes, leaves, and stem bark. Many species of the *Berberis* genus, such as *B. vulgaris*, *B. aquifolium*, and *B. aristata*, have a high content of berberine.

In traditional medicine, berberine has broad-spectrum antimicrobial effects against protozoans, bacteria, fungi, helminths, chlamydia, and viruses [7-9]. Berberine alkaloids have some benefits and pharmacological effects on improving cancers and other diseases. Recently, various studies have shown the potential neuroprotective properties of berberine, such as improved survival, progression, and function of brain neurons and protection of these cells [10]. Berberine has also been shown to fight neurodegeneration in related diseases (including AD, PD, and Huntington disease) by increasing toxically misfolded and aggregated protein clearance in brain neuronal cells via autophagy induction [11].

Growing evidence shows that berberine can reduce oxidative stress in the brain and central nervous system (CNS) [12]. Several lines of evidence have evidentially demonstrated that berberine can control and treat age-related dementia by regulating neurotransmitter function, suppressing oxidative stress, decreasing neuronal inflammation, affecting metabolism, and acting on other multi-target pathways. Hence, berberine may act as a neuroprotective agent in AD pathogenesis and may improve oxidative stress via its antioxidative potential, suggesting that it is a possible means for AD treatment [13, 14]. Several types of research have also investigated the benefits of berberine's significant antioxidant effects in neurodegeneration [11].

Studies have indicated that the alkaloid content of *B. vulgaris* (barberry) root extract is greater than that of other parts of the plant. Some alkaloids, such as berlambine and bervulcine, were found only in the roots of the plant. Regarding traditional medicine and documents about barberry root extract and its effects, this study was designed to evaluate the neuroprotective effects of barberry root extract and berberine (the main alkaloid of the plant) on human neuroblastoma SH-SY5Y cells.

MATERIALS AND METHODS

1. Chemicals

DMEM/F12, L-glutamine, fetal bovine serum (FBS), and penicillin-streptomycin solution for cell culture experiments were purchased from Gibco BRL (Life Technologies, Paisley, Scotland). Berberine as chloride was purchased from Sigma-Aldrich (MO, USA). Solvents such as DMSO and methanol were purchased from Merck (Darmstadt, Germany).

2. Apparatus

An RNX plus kit MR7713C (CinnaGen Co., Tehran, Iran) was used to extract mRNA. A First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc., MA, USA) was chosen to synthesize complementary DNA (cDNA). A multi-well plate reader (Elx 800 Microplate Reader, Bio-TEK, Winooski, Vermont, USA) was used for the viability assay. PCR was performed using ABI real-time PCR equipment (AB Applied Biosystems, CA, USA) and GreenStar Master Mix (Amplicon, Denmark). A Biosystems 7500 real-time PCR System (Applied Biosystems, CA, USA) was used for the reaction analysis and related experiments.

3. Preparation of root extract

Barberry roots were collected at the full flowering stage during spring 2019. The plant roots were dried in air at room temperature, avoiding exposure to direct sunlight; after drying, the roots were ground. The powdered root was weighed at exactly 50 g and passed through a 350 mesh sieve to obtain a uniform particle size, and 150 mL of methanol was used to extract it using the maceration method at room temperature for 24 h. The extraction was filtered, and a rotary evaporator under vacuum conditions was used to concentrate the filtered solution.

4. Cell culture, treatment, and viability assay

The SH-SY5Y cell line was purchased from the Department of Cell Bank, Pasteur Institute of Iran (Tehran, Iran), cultured in DMEM/F12 (1/1) medium with L-glutamine, supplemented with 10% FBS and 1% penicillin-streptomycin, and maintained at 37℃ with 5% CO_2 and humid conditions. The MTT assay was applied to assess cell viability.

Cells at a density of 2×10^4 per mL were seeded in 96-well plates for 24 h, and treatment was performed with both commercial berberine and root extract at different concentrations of 15, 30, 75, 150, and 300 ppm in duplicate. Cell viability at different time intervals was assayed by adding 100 μL of MTT (5 mg/mL) to each well for 4 h before harvesting. After stopping the reaction with DMSO, the absorbance of wells was determined by a multi-well plate reader at 520 and 630 nm wavelengths.

The protective effect of the evaluated compounds against an oxidative impact was evaluated by pretreatment of the cells with the mentioned concentrations of the barberry root extract and commercial berberine for 24 h, followed by replacement with fresh medium containing 0.25 ppm H_2O_2 and incubation for another 3 h [13]. As abovementioned, the MTT assay was used to determine cell viability, and the results are expressed as the percentage ratio of surviving cells to control cells.

5. Real-time quantitative PCR

Cells at a density of 1.2×10^5 per well were seeded onto 6-well plates with the incubation conditions of 37℃, 5% CO_2, and humid air for 24 h and then exposed to the half maximal inhibitory concentration (IC_{50}) of both barberry extract and berberine for 24 h. Finally, cells were trypsinized and centrifuged at 3,000 rpm, 4℃ for 5 min. An RNX plus kit was applied to extract total mRNA from the pellet. All types of high-quality RNA were obtained for further determination by a method based on the extraction of RNA by guanidine salt and precipitation along with protein in the phenolic phase.

Optical density measurements of RNA at an OD ratio of A260/A280 with a value of 1.8 or above were determined for quantity and purity by a spectrophotometer, and the integrity was confirmed by running on a 1% agarose gel. A First Strand cDNA Synthesis Kit was used to synthesize cDNA from the total RNA and was stored at –20℃. Real-time PCR was performed in a total volume of 20 μL using a reaction mixture containing 20 ng of cDNA template, 10 μL of 2X GreenStar master mix, 200 nM of primers (forward and reverse), and PCR-grade water.

Bax and Bcl-2 genes as primers and β-actin as a reference gene were used as sequences for quantitative RT-PCR (Table 1).

The reaction was performed in a real-time PCR System with the following cycling conditions: enzyme activation at 95℃ for 3 min, initial denaturation at 95℃ within 40 cycles of 3 s, and 32 s of annealing/extension at 58℃. To ensure amplification specificity and to verify the specificity of each primer after PCR, a melting curve analysis was performed.

The comparative threshold cycle (Ct) method was used to determine the Ct number.

6. Cell preparation to examine the ROS level

After separating the cells from the flask and washing, the cell volume was extended to 400 μL with phosphate-buffered saline (PBS). The colors of dichloro-dihydro-fluorescein diace-

Table 1. The sequences of the primers and reference genes for the qRT-PCR

Gene	Sense	Antisense
Bax	5′-CCCGAGAGGTCTTTTTCCGAG-3′	5′-CCAGCCCATGATGGTTCTGAT-3′
Bcl-2	5′-CATGTGTGTGGAGAGCGTCAA-3′	5′-GCCGGTTCAGGTACTCAGTCA-3′
β-Actin	5′-TGCAGAGGATGATTGCTGAC-3′	5′-GAGGACTCCAGCCACAAAGA-3′

 https://doi.org/10.3831/KPI.2022.25.3.216

tate (DCFH-DA) and propidium iodide (PI) overlap; thus, four tubes of the sample, including a colorless tube, a tube containing DCFH-DA dye, a tube containing PI dye, and a tube containing both PI and DCFH-DA dye, were prepared to correct for and adjust this overlap. The first and third tubes were stored at 4℃, and the second and fourth tubes were incubated for 45 min at 37℃. Then, 1 mL of PBS solution was added to the tubes, and they were centrifuged at 1,500 rpm for 5 min. Finally, another 500 μL of PBS was added to the cell sediment. While reading the samples, 3 μL of PI dye was added to the third and fourth tubes.

7. Statistical analysis

Data from three individual tests performed in duplicate were analyzed and are presented as the mean ± SD. The relative expression of oqxA and B efflux pump genes was determined using the $2^{-\Delta\Delta C_T}$ method. The $2^{-\Delta\Delta C_T}$ value of different groups was calculated and statistically analyzed using SPSS version 16 (SPSS Inc., Chicago, Ill., USA) and REST 2009. One-way ANOVA was performed to evaluate statistical significance. p values less than 0.05 were considered statistically significant.

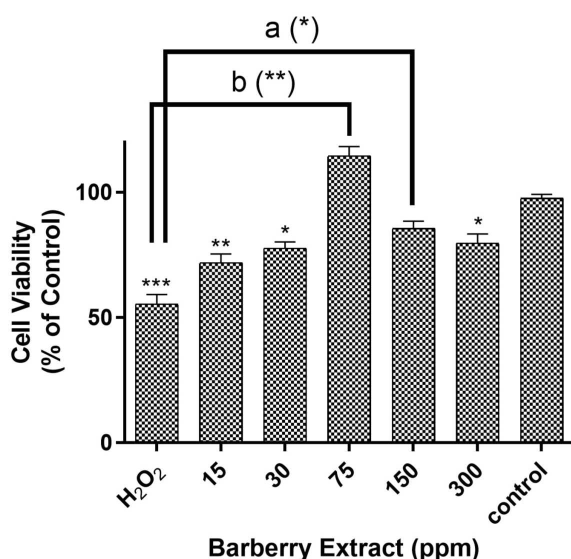

Figure 1. Effect of barberry extract on SH-SY5Y cells viability. The cells were treated with different concentrations of plant extracts (15, 30, 75, 150, and 300 ppm) and H_2O_2 (0.25 ppm). The treatment group at 150 ppm showed a protective effect against H_2O_2. Data are expressed as mean ± SD (*n* = 6). Values are statistically significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared to the control group. (a) and (b) show a significant increase in cell viability compared to the H_2O_2 group.

RESULTS

1. Effect of barberry extract on SH-SY5Y cell viability

The cells were treated with barberry root extract at different concentrations of 15, 30, 75, 150, and 300 ppm. The MTT assay was applied to assess cell viability. According to Fig. 1, SH-SY5Y cell viability was increased in all groups exposed to extract compared with the H_2O_2 group. This protective effect was significant at 75 and 150 ppm ($p < 0.01$ and $p < 0.05$, respectively), but, at 75 ppm, there was a proliferative effect compared with the control group; thus, 150 ppm was considered to be the IC_{50}.

2. Effect of berberine on SH-SY5Y cell viability

The cells were treated with berberine at different concentrations of 15, 30, 75, 150, and 300 ppm. The MTT assay was applied to assess cell viability. Our findings indicated that exposure to 15, 30, and 75 ppm berberine increased cell viability in the MTT assay. This protective effect was significant at 30 ppm ($p < 0.05$) compared with the H_2O_2 group; thus, it was selected as the IC_{50}. However, exposure to 150 and 300 ppm significantly decreased cell viability compared with the H_2O_2 group, which could be considered a toxic effect (Fig. 2).

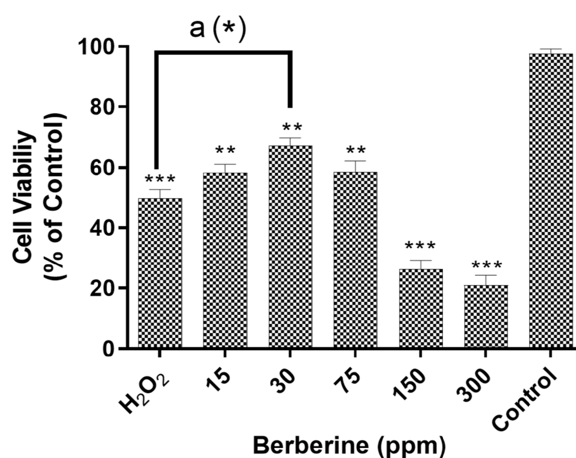

Figure 2. Effect of berberine on SH-SY5Y cells viability. The cells were treated with different concentrations of plant extracts (15, 30, 75, 150, and 300 ppm) and H_2O_2 (0.25 ppm). The treatment group at 30 ppm showed a protective effect against H_2O_2. Data are expressed as mean ± SD (*n* = 6). Values are statistically significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared to the control group. (a) shows a significant increase in cell viability compared to the H_2O_2 group.

3. Effect of berberine and barberry root extract on Bcl-2 and Bax expression

Bcl-2 and Bax expression levels were measured by real-time PCR in cells exposed to the IC_{50} of berberine, barberry root extract, and H_2O_2 to determine whether these components could increase the viability compared with H_2O_2 exposure.

As presented in Fig. 3, there was a significantly higher expression of Bax in the presence of H_2O_2 at 0.25 ppm than in the control group ($p < 0.001$). The exposure to the IC_{50} concentration of berberine and plant extract decreased the mRNA levels of Bax compared with those in the H_2O_2-treated group ($p < 0.001$). However, only treatment with the IC_{50} of H_2O_2 significantly decreased the mRNA levels of Bcl-2 ($p < 0.01$); we observed a significant increase in the expression of Bcl-2 using treatment with IC_{50} of berberine and barberry extract ($p < 0.01$). The expression level of Bcl-2 in cells exposed to both berberine and barberry root extract was also significantly higher than that in cells exposed to H_2O_2 ($p < 0.001$).

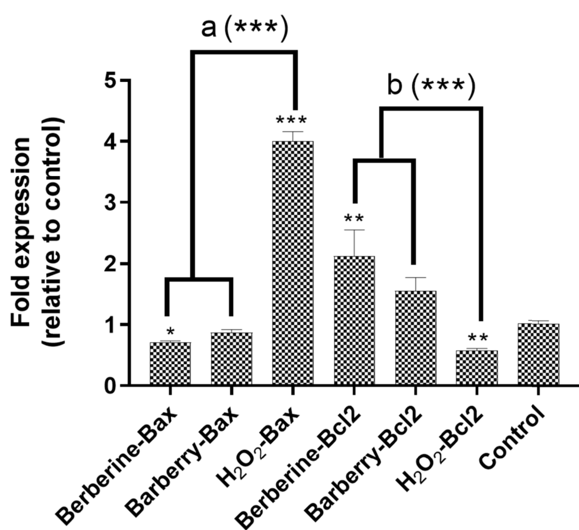

Figure 3. Expression levels of Bax and Bcl-2 in SH-SY5Y cells treated with IC_{50} concentration of berberine (30 ppm), barberry extract (150 ppm), and H_2O_2 (0.25 ppm). Data represent relative gene expression (target/β-actin) as mean ± SD of 5 experiments (n = 6). Values are statistically significant at *p < 0.05, **p < 0.01, and ***p < 0.001 compared to the control group. (a) and (b) show a significant decrease in Bax and an increase in Bcl2 gene expression levels in cells exposed to barberry extract and berberine compared to the H_2O_2 group.

4. Effect of berberine and barberry root extract on the ROS level

The ROS level in the groups of plant extracts and commercial berberine in comparison with H_2O_2 as a positive control is shown in Fig. 4. Our results demonstrated that both treatment groups, including the extract and commercial berberine treatment groups, were significantly different from positive and negative controls, and the ROS level decreased ($p < 0.001$ for both).

DISCUSSION

Neurodegenerative diseases, such as AD, are progressive diseases, causing serious complications in personal and financial aspects and affecting familiar relationships. Globally, millions of people have dementia, the most common form of which is AD. The total global societal cost of controlling and treating dementia is estimated to be $1 trillion per year [15, 16].

The most important factors are increasing age and the increasing incidence of disease, which is highly related to older age [17].

Some approved medications, such as donepezil, rivastigmine, galantamine, and memantine, have recently been intro-

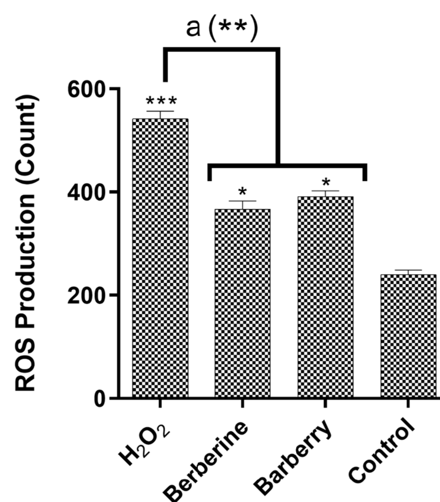

Figure 4. Evaluation of ROS levels at IC_{50} concentration of barberry extract (150 ppm) and berberine (30 ppm) compared to the H_2O_2 (0.25 ppm) and control groups. Data represent ROS production as mean ± SD (n = 6). Values are statistically significant at *p < 0.05, **p < 0.01, and ***p < 0.001 compared to the control group. (a) shows a significant decrease in ROS levels in cells exposed to barberry extract and berberine compared to the H_2O_2 group.

 https://doi.org/10.3831/KPI.2022.25.3.216

duced and investigated to promote AD [18-20]. Although these agents may be beneficial in maintaining memory and improving behavioral disorders, they have side effects. Accordingly, the present study was developed to assess the neuroprotective effects of barberry root extract (*B. vulgaris*) and berberine as natural compounds and phytochemical agents on human neuroblastoma SH-SY5Y cells due to their few side effects on the human body.

Researchers all over the world have agreed that oxidative stress plays a prominent role in neurodegeneration via a multifunctional metabolic pathway. A large body of evidence suggests that berberine may mitigate the negative effects of oxidative stress in the brain and CNS [21]. Our results from the MTT assay indicate that different concentrations of barberry root extract can improve cell viability when H_2O_2 is present. These results also show that low doses of berberine [15, 30, and 75 ppm] can increase cell viability compared with H_2O_2 treatment. However, barberry root extract was more effective in improving the viability of SH-SY5Y cells than berberine, suggesting a promising use of barberry extract in the treatment of neurological degenerative diseases such as AD.

According to the most recent published study, *B. vulgaris* and its derivatives (such as berberine) can treat senile dementia and protect neurons through regulating neurotransmitters, anti-apoptotic and antioxidative agents, and other multi-target pathways [22]. Berberine has been shown to have great potential against a variety of potential neurodegenerative diseases (such as AD and PD) and stroke [23] due to its beneficial antioxidant and anti-apoptotic properties, as evidenced by a previous study [24].

Plaques containing developed amyloid-β (Aβ) and hyperphosphorylated tau-consisting neurofibrillary tangles (NFTs) can be used to identify neurodegenerative disorders [25].

Luo et al. [26] found that berberine reduced malondialdehyde and ROS against Aβ-induced cell death generation in rat cortical neurons. Berberine also limited the neuroinflammation process, which consequently may delay oxidative stress in the brains of patients with AD. It is thought that the accumulation of Aβ, plaques, and NFTs in neuronal cells can cause inflammation and oxidative stress, resulting in a vicious cycle that can be broken by berberine.

However, the mechanism of action of berberine in controlling and suppressing the pathogenesis of neurodegenerative disease or in having a protective effect is not clearly defined. Further research in this area is necessary to fully elucidate the protective effects of berberine or, if needed, other related compounds such as barberry root extract in the pathogenesis of oxidative damage.

The roles of berberine and barberry root extract were investigated by determining the mRNA expression levels of Bax and Bcl-2 as apoptotic parameters in SH-SY5Y cell lines pretreated with berberine and barberry root extract and H_2O_2 by real-time PCR.

The RT-PCR results revealed that treatment with the IC_{50} of H_2O_2 increased the mRNA expression level of Bax and decreased that of the Bcl-2, indicating cell apoptosis. Our findings also showed that the IC_{50} of both barberry root extract and berberine significantly decreased the expression level of Bax and increased that of Bcl-2, indicating the protective and anti-apoptotic effects of these components on SH-SY5Y cells when H_2O_2 was present; however, when the studied components were compared, barberry extract at a concentration of 75 ppm was more effective than berberine, but at lower concentrations, no significant difference was observed.

The protective effects of berberine against oxidative stress, Aβ-induced toxicity, and H_2O_2-induced apoptosis suppression in SH-SY5Y cells may be due to a mechanism that inactivates p38 MAPK and reduces ROS.

Beta- and gamma-secretase enzymes are activated by phosphorylation of p38 MAPK. Berberine prevents the phosphorylation and activation of p38 MAPK, thereby preventing the activation of beta- and gamma-secretase enzymes. Therefore, the enzyme activity decreases [27].

Furthermore, berberine could decrease the ratio of Bax/Bcl-2 and reduce the release of cytochrome C, ultimately activating caspase 3. Therefore, a decrease in PARP cleavage and reduction in apoptosis induced by Aβ and H_2O_2 is accompanied by a reduction in caspase-3 activity. However, more studies are needed to clarify the exact mechanism [28].

CONCLUSION

In the neurodegenerative process, the oxidative stress induced by the H_2O_2 molecule should be considered one of the main targets for the treatment of related diseases. Based on our results, both berberine and barberry root extract may suppress the apoptotic pathway through various molecular mechanisms, but the effect of the plant extract was more significant than that of commercial berberine. This study revealed the effectiveness of the protection of *B. vulgaris* extract and its useful compounds

(such as berberine) or other alkaloids against H_2O_2-induced apoptosis in SH-SY5Y cells. *B. vulgaris* extract acts as a potential supplement or at least adjuvant therapy to prevent and control diseases such as AD or perhaps as a pretreatment to reduce disease progression. Studies on effective factors that target H_2O_2 and/or enzymes involved in apoptotic pathways may provide a way to treat neural cell damage.

ACKNOWLEDGMENT

The authors would like to thank the Food and Drug Control Laboratories, the Iran Food and Drug Administration, for their assistance in the preparation of chemicals, standards, and reagents, as well as for technical and scientific comments.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

FUNDING

This research did not receive any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

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