

The Protective Effect of Different Extracts of Three *Artemisia* Species against H₂O₂-Induced Oxidative Stress and Apoptosis in PC12 Neuronal Cells

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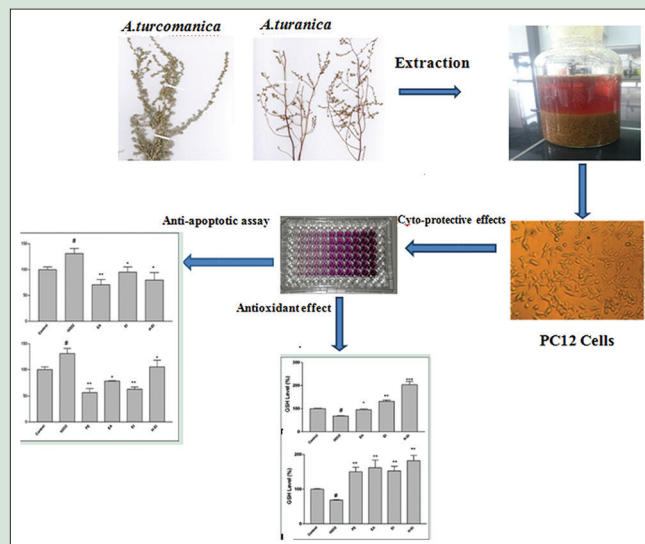
ABSTRACT

Background: Oxidative stress causes cell damage and is involved in many neurological diseases. The antioxidant properties of plant materials for the maintenance of health and protecting against different diseases stimulated scientist to investigate different herbs. Different *Artemisia* species have exhibited antioxidant activity. This study aims to investigate whether different *Artemisia* species could protect the PC12 cells against oxidative stress mediated by H₂O₂. **Methods:** For this purpose, different extracts of three *Artemisia* species (*Artemisia aucheri*, *Artemisia turanica*, and *Artemisia turcomanica*) were prepared using petroleum ether, dichloromethane, ethyl acetate, ethanol, and Water: Ethanol mixture (1:1 volume ratio). The protective effect of the prepared extracts against H₂O₂-induced cytotoxicity and reactive oxygen species production were compared. The effect of treatment of PC12 cells with different extracts on total glutathione (GSH) level, caspase-3 activity, and mitochondrial membrane potential were also compared. **Results:** The *A. aucheri* extracts could not rescue the PC12 cells from oxidative stress consequences. The *A. turanica* and *A. turcomanica* extracts were found potent in suppressing the toxicity and apoptosis of PC12 cells mediated by H₂O₂ and significantly antagonized the H₂O₂-induced GSH depletion. The hydroethanolic and ethyl acetate extracts of *A. turanica* and the petroleum ether and hydroethanolic extracts of *A. turcomanica* more efficiently suppressed cytotoxicity and loss of GSH caused by H₂O₂. **Conclusion:** This study shows the protective effects of *Artemisia* extracts on PC12 cell line and suggested that these species could be also considered as promising neuroprotective agents in treatment of different neurodegenerative diseases.

Key words: *Artemisia aucheri*, *Artemisia turanica*, *Artemisia turcomanica*, oxidative stress, PC12

SUMMARY

- *Artemisia turanica* and *Artemisia turcomanica* extracts were found to potentially exert neuroprotective effect on PC12 cells. The results exhibited that the cytoprotective potential and anti-apoptotic mechanism of these species is not the same for different extracts, and suggested that based on the type of species and the type of solvents used in extraction, both intrinsic and extrinsic pathways could be included in the anti-apoptotic mechanism of these species.



Abbreviations Used: GSH: Glutathion. ROS: Reactive Oxygen Species. GSSG: Glutathione disulfide. DCF-DA: 2',7'-Dichlorofluorescein diacetate. FBS: Fetal Bovin Serum. MMP: Mitochondrial Membrane Potential. H-Et: Hydro-ethanolic. DCM: Dichloromethane. PE: Petroleum Ether. Et: Etanolic. EA: Ethyl Acetate.

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INTRODUCTION

Oxidative stress occurs when excessive production of free radicals or reactive oxygen species (ROS) completely defeats the antioxidant defense system or when there is a significant decrease or lack of antioxidant defense.^[1,2] Oxidative stress is known as an inducer of several diseases and causes damage of biomolecules such as proteins, lipids, amino acids, and nucleic acids. It could be resulted from different factors such as stress, environmental pollutants, numerous drugs, and different diseases.^[1,3,4] It possesses a vital role in the pathogenesis of many diseases such as Alzheimer's disease (AD), Parkinson's disease, atherosclerosis, cancer, chronic obstructive diseases, rheumatoid arthritis, diabetes, schizophrenia, periodontal disease and sickle cell anemia, and also contributes to the aging process.^[2,3] Plants

can produce a variety of antioxidants against ROS-mediated molecular damages. Regarding the established carcinogenicity of the synthetic antioxidant, it is necessary to find naturally occurring antioxidants.

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The genus *Artemisia* is one of the largest genera in the tribe *Anthemideae* (family *Asteraceae*) with about 500 species, which could be found widely in the temperate zone of Asia, Europe, and North America.^[5] About 43 *Artemisia* species grows in different areas of Iran.^[6] In the Iranian traditional medicine, *Artemisia* species (locally known as “Dermaneh”) have been widely used to treat various diseases such as constipation, intestinal disorders caused by parasites,^[7] headache,^[8] and epilepsy.^[9] Moreover, several studies proved the notable bioactivities of the *Artemisia* species, such as cytotoxicity,^[10-14] antimalarial,^[15-17] and antifungal effects.^[18] On the other hand, there are many studies reporting the high content of polyphenols, coumarins, and flavonoids in the *Artemisia* species, which made them probably potent for the antioxidant objectives.^[19-21] Oxygenated derivatives of geraniol have been isolated from *Artemisia aucheri*.^[22] 1,8-cineole, verbenol, camphor, and linalool were reported to be the major constituents of its essential oil in different studies.^[23-26] 1,8-cineole, chrysanthenone, and davanone were reported as the major constituents of essential oil of *Artemisia turanica* aerial parts.^[27] While a high amount of α -thujone was observed in the leaf essential oil.^[28-30] Some sesquiterpenoid lactones and a monoterpene were isolated from hydromethanolic extract of aerial parts of *Artemisia turcomanica*.^[31] 1,8-cineole was identified as the major volatile in the aerial parts of the species,^[12,32] while the notable presence of *cis*-Chrysanthenyl acetate in the leaf and stem essential oil of the species was reported in other study.^[33]

This stimulated us to investigate the protective effect of different extracts of *A. aucheri*, *A. turanica*, and *A. turcomanica* against hydrogen peroxide-induced oxidative stress and apoptosis in the PC12 cell line. This cell line is an accepted model of the neural cells and has been extensively used in neuroprotective studies.^[3]

MATERIALS AND METHODS

Reagents

Petroleum ether (40–60), dichloromethane, ethyl acetate, methanol, and ethanol were purchased from Merck, Germany. Dimethyl sulfoxide (DMSO), 2',7'-Dichlorofluorescein diacetate (DCF-DA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Triton X-100, and Rhodamine 123 were supplied from Sigma-Aldrich (St Louis, MO, USA). Trypsin-ethylenediaminetetraacetic acid was procured from Bon Yakhteh, Iran. Fetal bovine serum (FBS) was purchased from Gabon, USA.

Preparation, authentication, and extraction of the *Artemisia* species

The aerial parts of *A. aucheri*, *A. turanica*, and *A. turcomanica* were collected from Chahar Bagh region (Golestan province, Iran), Torbate Jam (Khorasan Razavi province, Iran), and Bojnord (North Khorasan province, Iran) in autumn 2011, respectively. The plants were authenticated in Agricultural and Natural Resources Research Center of Golestan (Golestan Province, Gorgan, Iran) and Herbarium of Mashhad University of Medical Sciences (Khorasan Razavi province, Iran), respectively. The voucher specimens (with the identification numbers of 22,383, 12,572, and 12,573, respectively) have been deposited in the herbarium.

The maceration method was used for extraction. For this purpose, 50 g of the shade-dried samples were milled and then were extracted with petroleum ether (40–60), dichloromethane, ethanol, ethanol-water (1:1 v/v), and ethyl acetate (Sequential maceration with ca. 3 L \times 0.5 L of each solvent).

The extracts were filtrated and dried using rotary evaporator (Heidolph, Germany) under a reduced pressure condition and at low

temperature ($\leq 45^\circ\text{C}$). The dried extracts were kept at -20°C for further investigation.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

In this study, rat pheochromocytoma-derived cells (PC12 cell line) were prepared from Pasteur Institute of Iran (Tehran, Iran). The Dulbecco's modified Eagle's medium (DMEM-F12) supplemented with FBS (10% v/v) and 1% penicillin/streptomycin (100 U/mL: 100 U/mL) was used for cell grows at 37°C in a humidified incubator containing 5% CO_2 . The PC12 cells were seeded in a 96-well culture plate while the number of cells in each plate was $15\text{--}20 \times 10^3$ cell/well. In this study, different concentrations of each extract were prepared by dissolving the dried extract in DMSO. At appropriate time intervals, the medium was replenished with 0.5 mg/mL MTT solution and plates were further incubated for 3 h at 37°C . After the incubation period, and to solubilize the formazan crystals, 100 μL of DMSO was added to each well. The optical density at 570 nm (OD570) was detected using an Eliza microplate reader (BioTek Instruments, USA).^[34] Cell viability percentage was determined by dividing the optical density of treatment group to that of control group at 570 nm, multiplying by 100. The IC50 value was obtained considering the concentration in which 50% of cells were killed. All the MTT assays were conducted in triplicate in this study.

Determination of intracellular reactive oxygen species

The DCF-DA indicator was performed to determine the overall intracellular ROS generation. This indicator is lipophilic and nonfluorescent dye which could be fluorescent (in the form of DCF molecules) on the reaction with ROS (in this study generated by H_2O_2).^[35] The amount of fluorescence in cells could be an indicator for their amount of free radicals formed. To carry out the intracellular ROS study, at the end of treatment 100 μL DCF-DA was added to the cells followed by incubation at 37°C for 30 min. After the incubation period, cells were lysed with Triton X-100, and the amount of their fluorescence is measured at a wavelength in range of 488–510 nm using fluorescence microplate reader (BioTek, H1M).

Measurement of total glutathione

The assessment of total glutathione (GSH) was carried out based on the user's manual for the Cayman GSH Colorimetric Detection Kit (USA) to obtain total GSH (reduced form GSH + oxidized form Glutathione disulfide). The cultured PC12 cells (with the density of $15\text{--}20 \times 10^3$ cell/well) were treated with different extracts of *Artemisia* species and subsequently treated with H_2O_2 (150 μM) for 24 h at 37°C . A group of cells received only H_2O_2 and had not been treated with extracts. At the end of the experiments, PC12 cells were rinsed with phosphate-buffered saline (PBS) and were centrifuged for 10 min at 200 rpm. The supernatant was replaced and pellets washed with ice-cold PBS, lysed in 80 μL ice-cold GSH Buffer and incubated on ice for 10 min. Then, 30 μL of 5-sulfosalicylic acid 5% was added to the cells. The samples were centrifuged at 4000 rpm for 10 min. Next, the supernatant was incubated in 160 μL of the reaction mixture at the room temperature for 5 min and 50 μL of substrate solution was added. After 10 min incubation, the absorbance of obtained solution, at 415 nm, was measured using a microplate reader (BioTek, H1M).

Caspase-3 activity assay

Caspase-3 activity was determined using the sigma colorimetric caspase kit. For this purpose, the PC12 cells were seeded in 6-well tissue culture

plates and incubated for 24 h. Then, different extracts of *Artemisia* species (selected after MTT study) were added to the wells and incubated for the next 24 h and then H_2O_2 was added to the treated cells and incubated for further 4 h. A group of cells treated only with H_2O_2 and had not received any of extracts. Thereafter, the treated cells were allowed to sediment by centrifugation at 1300 rpm for 5 min and were lysed in 15 μ L of the cell lysis buffer included with the kit. To extract the protein content of cells, lysates were then centrifuged at 13,000 rpm and 4°C for 15 min. The substrate reaction buffer containing caspase-3 substrate was added to the supernatant and incubated for 2 h at 37°C. The absorbance was then measured at 405 nm using a plate reader (BioTek, H1M).^[36]

Measurement of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was assessed using Rhodamine 123, as fluorescent dye.^[3] Briefly, cells were seeded in 6-well tissue culture plates and incubated for 24 h, and then, the selected extracts were added to wells. After 24 h, the IC_{50} concentration of H_2O_2 was added to the cells and incubated for another 4 h. At the end of the treatment, 15 μ L Rhodamine 123 (20 μ M) was added to wells and plate was incubated for 40 min at 37°C. After the incubation period, cells were lysed with 1 mL Triton X-100 and the amount of their fluorescence is measured at a wavelength in the range of 488–510 nm using fluorescence microplate reader (BioTek, H1M).

Statistical analysis

In the present study, all the experiments were conducted in triplicate and the reported values were represented as the mean value \pm standard

error of the mean. One-way analysis of variance using Tukey's test was performed to compare the results. The statistical significance of variations could be confirmed at $P < 0.05$.

RESULTS

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay results

The cell viability assay was used to determine the PC12 cell viability after exposure of different *Artemisia* extracts and investigate whether the nontoxic concentrations of *Artemisia* extracts can reduce the toxicity of H_2O_2 against PC12 cells [Figures 1 and 2]. To set extracts (0–50 μ g/mL) at concentrations which are nontoxic to cells but could prevent H_2O_2 -induced cytotoxicity, we first examined the effects of different concentrations of extracts on cell viability. The results of the cell viability percentage after treatment with different extracts are demonstrated in Figure 1a-c. The extracts had no significant effect on the viability of PC12 cells up to 12.5 μ g/mL (relative MTT activity >80%). According to the results, nontoxic concentrations (6.25 and 12.5 μ g/mL) were selected to be performed for the cytoprotective study against H_2O_2 -induced cytotoxicity. The concentration of H_2O_2 was also chosen based on a preliminary MTT assay and the IC_{50} concentration (6.25 μ M) was chosen for the remained analyses.

The comparison between the cell viability percentages of H_2O_2 -treated cells and those pretreated with 6.25 μ g/mL of different *A. turanica* extracts reveals that all of these extracts (except dichloromethane extract) possessed the significant protective effect against H_2O_2 -induced cytotoxicity [Figure 2a]. The most potent *A. turanica* extract at selected concentration (6.25 μ g/mL) against H_2O_2 -induced cytotoxicity was

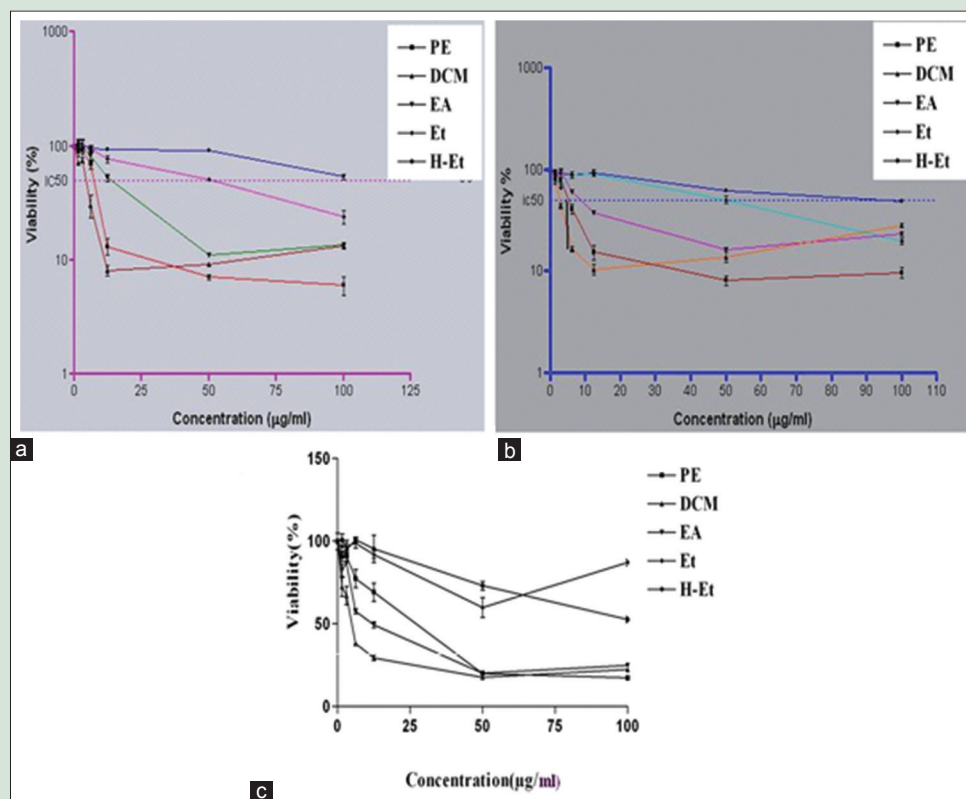


Figure 1: The viability percentage PC12 cells in the presence of different concentrations of the extracts of (a) *Artemisia turanica*, (b) *Artemisia Aucheri*, and (c) *Artemisia turcomanica*. The cell viability was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after 24 h and exposure as described in materials and methods. Data are expressed as the mean \pm standard error of the mean of three separate experiments

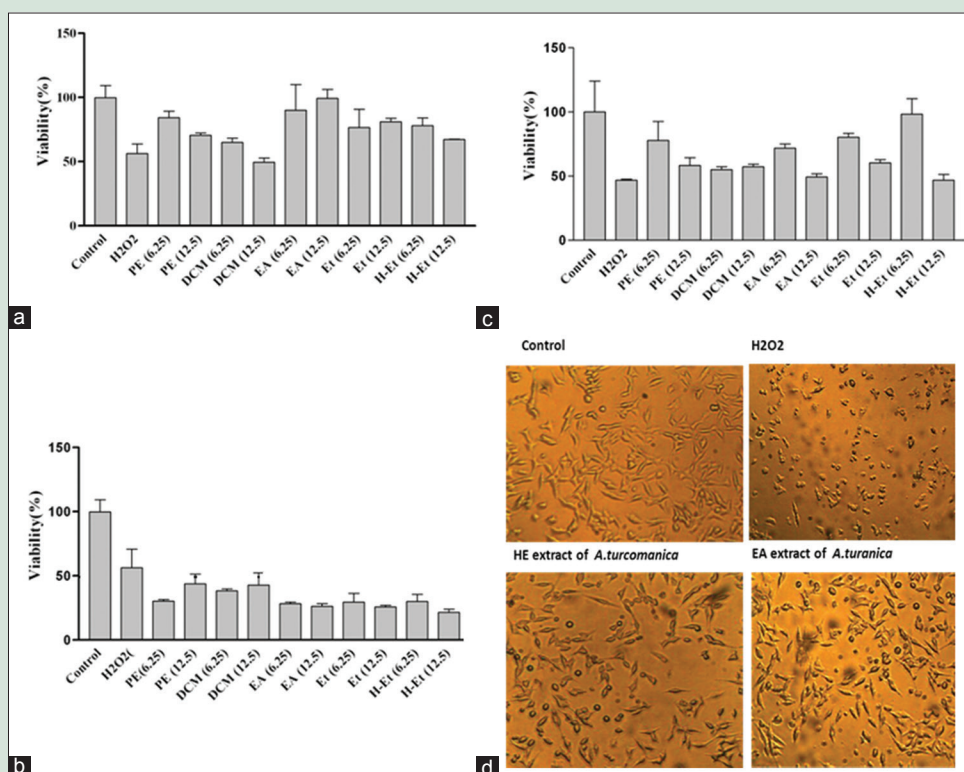


Figure 2: The effect of pretreating the PC12 cells with different selected *Artemisia turanica* (a), *Artemisia aucheri* (b), and *Artemisia turcomanica* extracts (c) on H_2O_2 -induced cytotoxicity in PC12 cells; and (d) Representative photomicrograph showing morphological changes of the PC12 cells. Cells were pretreated with potent extracts (hydroethanolic extract of *Artemisia turcomanica* and ethyl acetate extract of *Artemisia turanica*) for 24 h before exposure to H_2O_2 and imaged by inverted phase contrast microscope. Data are expressed as the mean \pm standard error of the mean of three separate experiments

the ethyl acetate one with 24% increase in the percentage of viability. The petroleum ether, hydroethanolic, and ethanolic extracts were the next potent ones, which increased the cell viability up to 28, 22, and 20%, respectively. The dichloromethane extract could not significantly inhibit the cytotoxic activity of H_2O_2 , and even the viability percentage decreased in the presence of 12.5 $\mu\text{g}/\text{mL}$ of this extract, exhibiting the fact that the extract potentiated the H_2O_2 -induced cytotoxicity toward PC12 cells. The possible cytoprotective effect of *A. turcomanica* extracts (6.25 $\mu\text{g}/\text{mL}$) was also investigated, and it was observed that all the extracts possessed the cytoprotective effect on the H_2O_2 -induced cells [Figure 2c]. The most potent extract of *A. turcomanica* was the hydroethanolic extract, resulting 52% increase in cell viability, following by the ethanolic, petroleum ether, and ethyl acetate extracts. The least potent *A. turcomanica* extract against H_2O_2 -induced toxicity was the dichloromethane extract, which could not significantly cause cytoprotective effect on PC12 cells. According to the low cytoprotective activity of dichloromethane extract of *A. turcomanica* and potentiating activity of the same extract of *A. turanica* on the cytotoxicity, the aforementioned extract of both species were found not promising, and this stimulated the authors to omit these extracts from further investigations. It should be noted that all of the *A. aucheri* extracts were also toxic and potentiated the cytotoxic effect of H_2O_2 on PC12 cells [Figure 2b].

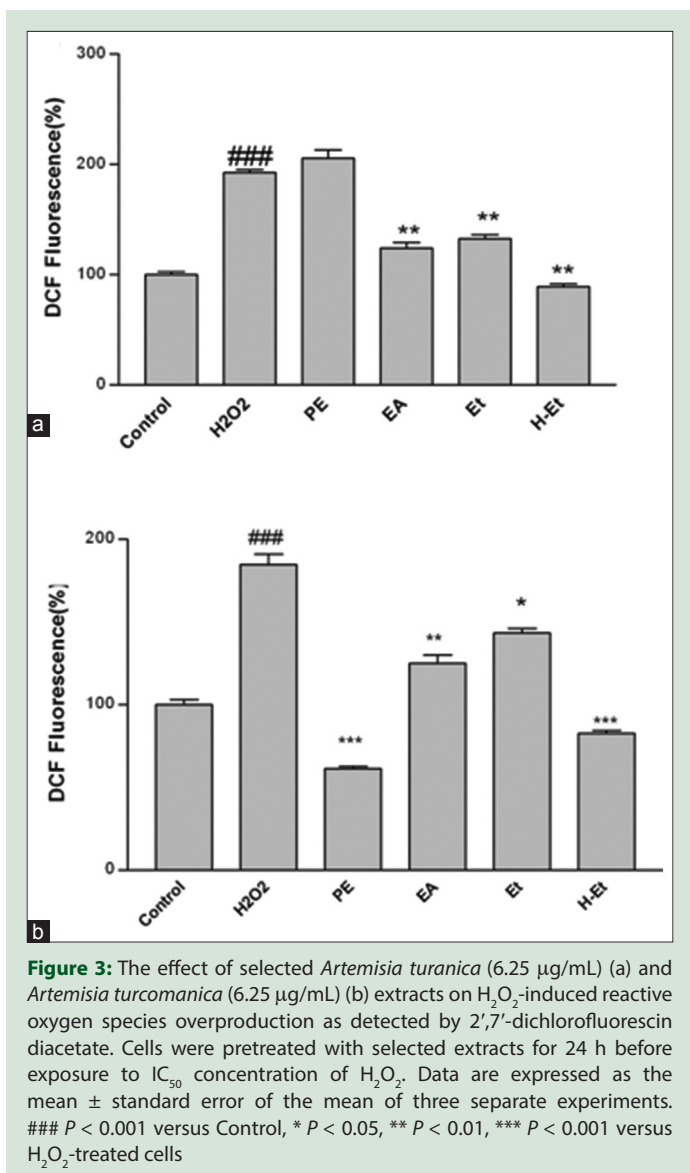
Effect of selected extracts on the H_2O_2 -induced intracellular reactive oxygen species

The intracellular ROS accumulation was assessed for different groups of treated cells (control, H_2O_2 -treated, and extract

pretreated- H_2O_2 -treated cells). As expected, H_2O_2 -treated cells showed significant increase in the ROS level ($92\% \pm 6.3\%$) which was due to the induction of free radicals and could cause the death of cells [Figure 3]. The cell groups pretreated with different extracts (6.25 $\mu\text{g}/\text{mL}$) possessed the desired radical scavenging activity and decreased the ROS level significantly. The petroleum ether extract of *A. turanica* was the only sample without radical scavenging activity. This extract was omitted from considerations in the remained analyses. Among the *A. turanica* extracts, the hydroethanolic extract possessed the best radical scavenging activity and decreased the ROS level up to 103%. The next ones were the ethyl acetate and ethanolic extracts, respectively. Pretreating the PC12 cells with *A. turcomanica* extracts also significantly reduced the ROS level, and the most significant potential on the reduction of free radicals was attributed to the petroleum ether extract, resulting in 123.3% reduction in the ROS level. The ethanolic extract of *A. turcomanica* possessed the least radical scavenging activity among these four extracts.

Effect of selected extracts on the total glutathione level

Oxidative stress causes the generation of toxic metabolites (such as H_2O_2) and superoxides, and causes the oxidation of GSH to GSSH, and consequently depletion of GSH in the cells. To examine the effect of pretreatment on the total GSH level of cells, the H_2O_2 -treated cells and corresponding pretreated cells were compared in case of the level of intracellular GSH [Figure 4]. A glance at figure exhibits that pretreatment with the selected extracts reduces the GSH level in comparison to control cells and H_2O_2 -treated ones. The most effective extracts, notably inhibited



depletion of GSH, were the hydroethanolic extracts of *A. turanica* and *A. turcomanica*. These extracts increased the GSH level of cells up to 135.2 and 114%, respectively.

Effect of selected extracts on the caspase-3 activity

Caspase-3 is a sensitive biomarker of the-mediated cell death through mitochondria-dependent apoptotic pathway.^[37] We speculated that *Artemisia* extracts can reduce apoptosis induced by oxidative stress in PC12 cells. To address this issue, the caspase-3 activity of different groups of cells (control, H₂O₂-treated, and extract pretreated-H₂O₂ treated PC12 cells) was compared. As shown in Figure 5, it is obvious that the amounts of cleaved caspase-3 increased on the application of H₂O₂, confirming that oxidative stress, induced by H₂O₂, could activate apoptosis in the performed PC12 cells. To evaluate the ability of different *Artemisia* extracts against apoptosis, cells were pretreated with the selected extracts. The results indicated that pretreatment of cells with all the selected extracts suppressed H₂O₂-induced apoptosis through decreasing the activity of caspase-3. After evaluating and comparing the quantities of the caspase-3 in pretreated cells, it was found that the most effective extracts on the inhibition of

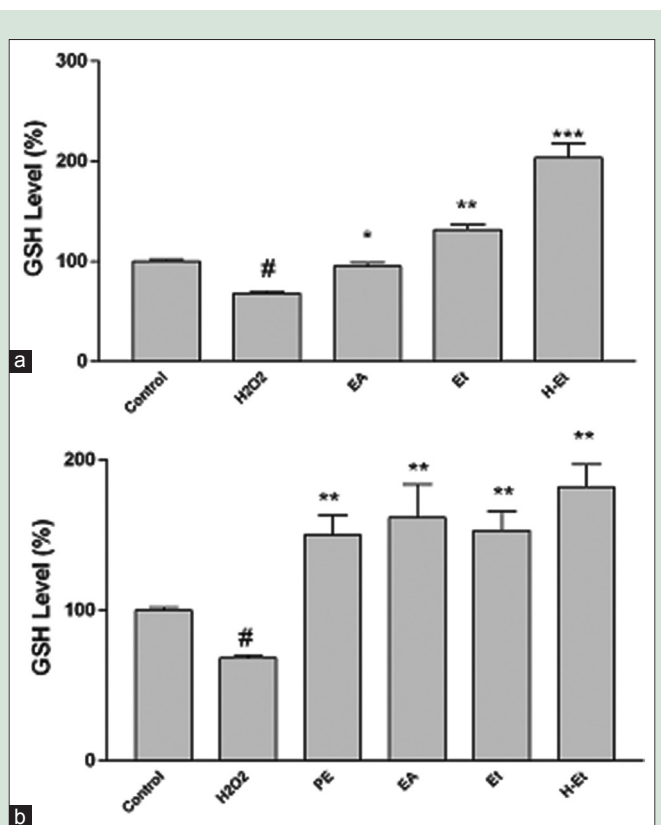


Figure 4: The effect of selected *Artemisia turanica* (6.25 µg/mL) (a) and *Artemisia turcomanica* extracts (6.25 µg/mL) (b) extracts on the glutathione level. Cells were pretreated with selected extracts for 24 h before exposure to IC₅₀ concentration of H₂O₂. Data are expressed as the mean ± standard error of the mean of three separate experiments. # P < 0.05 versus Control, * P < 0.05, ** P < 0.01, *** P < 0.001 versus H₂O₂ treated cells

H₂O₂-induced apoptosis were the ethyl acetate extract of *A. turanica* and the petroleum ether extract of *A. turcomanica* (63.2 and 74.15% reduction in activity).

Effect of selected extracts on the mitochondrial membrane potential

To examine whether the *Artemisia* extracts could stabilize the MMP and consequently attenuate the cell death, the MMP was determined [Figure 6]. It was observed that induction of H₂O₂ decreased the MMP significantly (about 27% decrease in Rhodamine 123 fluorescence). Pretreatment of cells with the ethyl acetate extract of *A. turanica* promoted the inhibition of MMP reduction, induced by H₂O₂, while the other extracts of this species were not potent in inhibition of MMP reduction and even potentiate this effect. Among the selected *A. turcomanica* extracts, the potential of inhibition of H₂O₂-induced MMP reduction was observed only in ethanolic and hydroethanolic extracts. The ethanolic extract was the most potent extract in inhibition of the effect of H₂O₂ on the MMP.

DISCUSSION

Free radical is taken into account as one of the major risks damaging the brain cells. This is because of the high oxygen turnover of brain and high content of polyunsaturated fatty acids in neuronal membranes which are potential targets for lipid peroxidation.^[38] Oxidative stress can induce

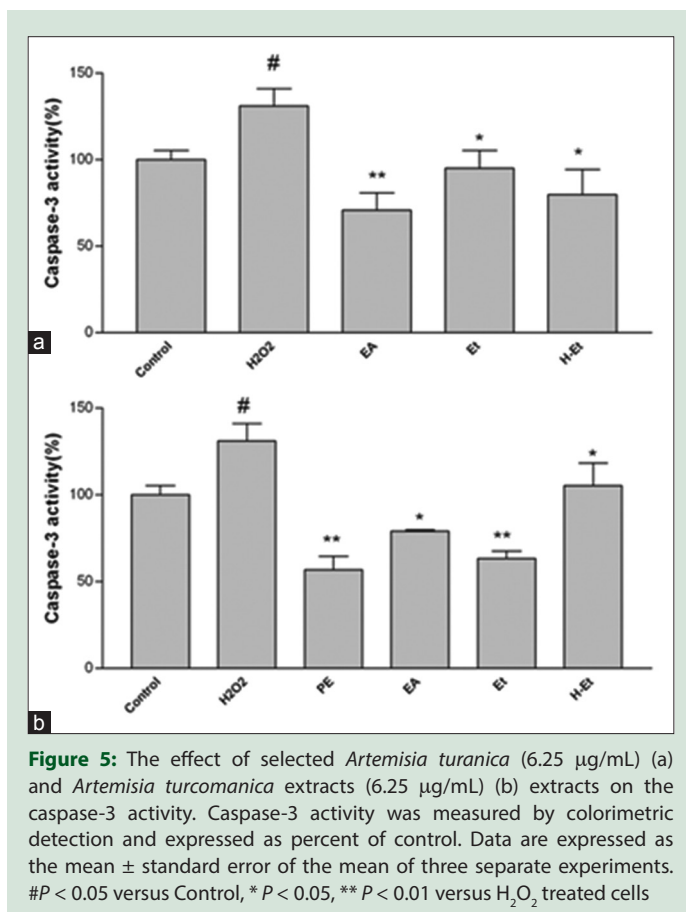


Figure 5: The effect of selected *Artemisia turanica* (6.25 µg/mL) (a) and *Artemisia turcomanica* extracts (6.25 µg/mL) (b) extracts on the caspase-3 activity. Caspase-3 activity was measured by colorimetric detection and expressed as percent of control. Data are expressed as the mean ± standard error of the mean of three separate experiments. #*P* < 0.05 versus Control, **P* < 0.05, ***P* < 0.01 versus H₂O₂ treated cells

the death in neuronal cells and is implicated in extensive neurological disorders such as AD, epilepsy, multiple sclerosis, and Parkinson's disease.^[39] Hydrogen peroxide is one of the main ROS, commonly known to elevate oxidative stress and consequently apoptosis or necrosis of PC12 cells.^[40] The results of the present study exhibited that hydrogen peroxide caused production of ROS in PC12 cells. On the other hand, it caused reduction of up to 53% in the cell viability. The preliminary tests were conducted to assess the effect of different *Artemisia* extracts on the toxicity induced by H₂O₂. Based on these results, the noneffective extracts were identified and omitted from further studies. All the *A. aucheri* extracts were found noneffective in inhibition of induced cytotoxicity and were omitted from the next studies. Although the antioxidant activity and free-radical scavenging ability of this species has been reported in former investigations,^[41-43] the extracts were found not beneficial in protecting PC12 cells against H₂O₂-induced oxidative stress. The extracts resulted from the other two species, except the dichloromethane extracts, were found to be cytoprotective. The dichloromethane extracts were also omitted from the remained analyses. The results also declared that all the selected extracts (except the petroleum ether extract of *A. turanica*) possessed the protective effect against H₂O₂-induced ROS production. Among these selected extracts, the protective effect of hydroethanolic extract of *A. turanica* and petroleum ether extract of *A. turcomanica* were rather significant. Apoptosis has a principle role in the progressive loss of structure or function of the neurons. Increasing evidence has demonstrated that natural products can protect neurons against the neurotoxicity involved in the different neurodegenerative disorders by inhibiting the apoptosis.^[44] It has been reported that various subgroups of polyphenols (flavonoids, phenolic acids, stilbenes, and lignanes) and terpenes perform their anti-Parkinsonian effect through suppressing

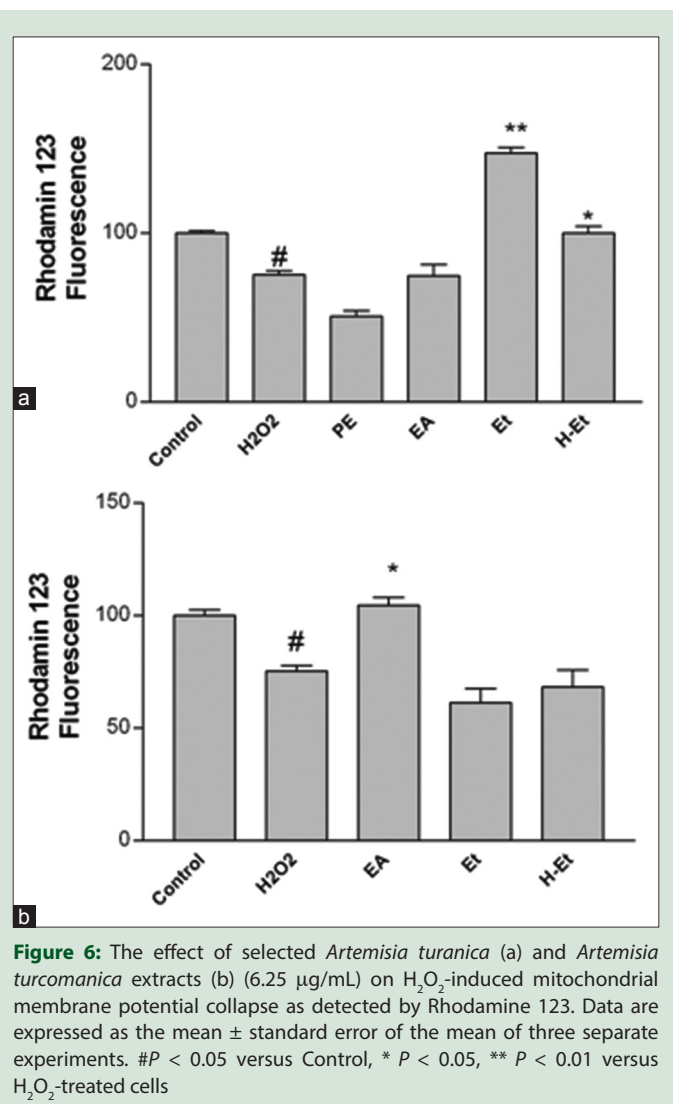


Figure 6: The effect of selected *Artemisia turanica* (a) and *Artemisia turcomanica* extracts (b) (6.25 µg/mL) on H₂O₂-induced mitochondrial membrane potential collapse as detected by Rhodamine 123. Data are expressed as the mean ± standard error of the mean of three separate experiments. #*P* < 0.05 versus Control, **P* < 0.05, ***P* < 0.01 versus H₂O₂-treated cells

apoptosis through the reduction of Bax/Bcl-2, caspase-3, -8, and -9, and α-synuclein accumulation.^[45]

Therefore, to further elucidate the mechanisms, by which these compounds protect PC12 cells from H₂O₂-induced cell death, we examined key parameters involved in the apoptosis. The mitochondria play a crucial role in activating apoptosis in mammalian cells. Permeabilization of the mitochondrial membrane causes bioenergetics failure and permits the release of soluble molecules from the outer space of the mitochondria to the cytosol. This ultimately leads to cytochrome c release and caspase-9 activation. Mature caspase-9 activates additional caspase-9 molecules as well as caspase-3 in turn for the execution of cell apoptosis. Caspase-3 is the most prevalent caspase in the cells,^[42] which acts as an apoptotic executor in the apoptotic process through two pathways: the death receptor pathway and the mitochondrial pathway.^[43] According to the results, in the present study, H₂O₂-mediated apoptotic activity and increased remarkably the caspase-3 activity. Among the *A. turanica* extracts, the ethyl acetate, ethanolic, and hydroethanolic extracts inhibited the H₂O₂-induced apoptosis, significantly. Based on this fact, the effect of extracts on MMP of cells was also determined. The MMP results in association with the results of caspase-3 activity could be interpreted for the sake of finding the possible pathway for

inhibition of apoptosis. While the ethyl acetate extract considerably elevated the MMP, ethanolic, and hydroethanolic extracts had no effect on the MMP. Hence, it could be concluded that ethyl acetate extract inhibited the H₂O₂-induced apoptosis through intrinsic mitochondrial pathway, while ethanolic and hydroethanolic extracts inhibited the apoptosis probably through extrinsic pathways. On the other hand, a comparison between the effects of different extracts of *A. turcomanica* on caspase-3 activity and MMP identified that the petroleum ether, ethyl acetate, and ethanolic extracts reduced the activity of caspase-3, and inhibited the apoptosis of PC12 cells, mediated by H₂O₂. For finding out the apoptosis inhibition pathway, the MMP results were also compared. It was identified that the petroleum ether and ethyl acetate extracts reduced MMP even more than H₂O₂, while the ethanolic and hydroethanolic extracts could increase the potential of mitochondrial membrane. It exhibited that the petroleum ether and ethyl acetate extracts of *A. turcomanica* protected PC12 cells against apoptosis probably through extrinsic pathways, while the ethanolic extract possessed the potential of inhibition of apoptosis through intrinsic mitochondrial pathway. Since the hydroethanolic extract of *A. turcomanica* could not affect the caspase-3 activity, it probably inhibited apoptosis through an alternative pathway, independent from caspase-3.

In this study, different solvents were utilized for the extraction of bioactive components from *Artemisia* species. Based on the polarity and different properties of these solvents, the composition of different extracts and protective activity against H₂O₂-induced oxidative stress might be various.^[43]

The hydroethanolic and ethyl acetate extracts of *A. turanica* were more potent than other extracts of this species in protective PC12 cells against injury induced by H₂O₂. There are reports that suggest ethyl acetate extract as a promising source of antioxidant and neuroprotective molecules.^[46-48] Hydroethanolic extract was the most active one in inhibition of H₂O₂-induced dysfunctions and exhibited the highest potential in suppression of ROS generation and reduction of GSH. Other studies on neuroprotective potential of hydroethanolic extracts of the species in the same genus have shown the highest total phenolic content in comparison with other extracts.^[49,50]

Among the extracts of *A. turcomanica*, the petroleum ether and hydroethanolic extracts of *A. turcomanica* were found to be the most effective extracts in the protection of PC12 cells. The petroleum ether extract was found the most potent extract in reducing the ROS level and inhibiting the cell apoptosis by reduction of caspase-3 activity. It should be noted that the composition of petroleum ether extract of *A. turcomanica* might be rather close to that of essential oil of the species. 1,8-cineole, as the major component of the essential oil,^[12,33] is known to be a potent monoterpenoid in the protection of PC12 cells against oxidative stress and suppression of cytotoxicity.^[51,52] In the recent study, it has been showed that 1,8-cineole is able to protect PC12 cells against oxidative stress and apoptosis induced by H₂O₂.^[52]

CONCLUSION

In conclusion, in the present study, it was found that H₂O₂-induced oxidative stress could be well inhibited by preincubation of PC12 cells with the extracts of *A. turanica* and *A. turcomanica* species. The extracts were able to rescue PC12 cells from H₂O₂-induced cell death and significantly antagonize the H₂O₂-induced GSH depletion in PC12 cells. Thus, based on the results of this study, the selected extracts of *Artemisia* species might be useful candidates for further study on the treatment of neurological diseases because of their ability to protect neurons against cellular damage.

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Conflicts of interest

There are no conflicts of interest.

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