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Original Article

Protective effect of CoQ10 and *Artemisia sieberi* combination on PC12 cells model of 6-hydroxydopamine induced toxicity

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Abstract

Introduction: Parkinson's disease (PD) is a progressive neurodegenerative disease that affects motor function. The etiology of PD is unknown and routine therapies temporarily relieve the symptoms. Neuroprotective based therapies preserve the remaining neurons and prevent the progression of PD. *Artemisia sieberi* has anti-cancer and neuroprotective effects. The CoQ_{10} also is an antioxidant that has proven anti-inflammatory and antioxidant properties. In order to study the effect of Artemisia and CoQ_{10} on the PD cellular model, the present research was designed.

Methods: PC12 cells were treated with different concentrations of 6-hydroxydopamine. Then the cells divided into the control (cells were not treated), DMSO group and experimental groups treated with the different concentrations of *Artemisia sieberi* extracts, CoQ_{10} and combination of them for 24h. The viability of the cells, reactive oxygen species (ROS) generation and p53 expression were evaluated.

Results: Artemisia at a concentration of 200µg/ml and CoQ_{10} at a concentration of 75µg/ml significantly increased cell viability in the treated groups after 24h. Their combination showed better and more significant results compared to each alone. Hoechst staining showed significantly reduced apoptosis in treated cells. ROS generation reduced in the treated groups with better results for the combination-treated groups. The same results acquired for the expression of P53 in the treated cells.

Conclusion: Regarding the results of both Artemisia and CoQ_{10}, it could be concluded that they act synergistically with possible similar pathways. Although the Artemisia itself showed significant results, it seems that the combination method might have more therapeutic effects.

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Keywords:
Parkinson's cell model;
Artemisia sieberi;
CoQ_{10};
Apoptosis

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Introduction

Parkinson's disease (PD) is an age-dependent neurodegenerative disease that results in the death of dopaminergic neurons in the substantia nigra and

reduced dopamine levels lead to a series of symptoms, especially motor disorders (Henchcliffe and Beal, 2008). PD affects 1% of people over the age of 65, and the incidence of this disease in males is more than females (Hirtz et al., 2007). Due to increased oxidative factors in the environment, the risk of developing neurodegenerative diseases is increasing (Cameán et al., 2013). The treatment of PD still remains unsuccessful; consequently, taking appropriate therapies or prophylactic procedures is necessary to prevent or postpone the onset of it. On the other hand, pharmacological and conservative treatments can only relieve the symptoms of the disease (Lipski et al., 2011). Surgery, cell therapy and gene therapy are also very risky and costly. The progressive nature of the disease and the development of severe disabilities in the late stages of the disease and the increasing prevalence of PD has become an important medical problem, which has not yet been treated with effective therapies or methods of prevention. In studies conducted with antioxidant and anti-apoptotic herbs, it has been proven that the neuroprotective based therapies maintain the remaining neurons and stop the progression of the disease (Singh et al., 2007). Among all the therapeutic methods, traditional medicine has recently achieved remarkable results based on the use of herbal medicines. The side effects and cost of using herbal drugs are lower than those of chemical origin. Recently, lots of attention has been paid to the role of various plants with therapeutic applications. Artemisia, known in Iran as the Artemisia (*Artemisia sieberi*) plant, is from the family of the Asteraceae (Gordanian et al., 2014) herbaceous plant, which is native Siberian species in the north. The use of the Artemisia plant for the treatment of some diseases goes back to ancient times. Artemisia family is used as the most important medicinal plant in the treatment of many diseases, including malaria, diarrhea, etc. in the world. These plants have antioxidant properties, are used in pharmaceutical applications. The medicinal properties of this plant are related to its evaporating essences, which include a range of active chemicals, such as terpenes (monoterpenes, sesquiterpenes, diterpenes, artemisinin) as well as flavonoids and polyphenols. The importance of the Artemisia genus is due to the presence of a sesquiterpene called artemisinin, which is used to treat malaria. In addition to artemisinin, another compound called β-caryophyllene, which has anti-inflammatory and anti-cancer properties and can be used as a local anesthetic. Artemisia also has antispasmodic and anticonvulsant effects (Gordanian et al., 2014). The study of the protective effects of artemisinin on oxidative stress and brain damage, as well as cerebral ischemic, the damage was carried out by Bora and Sharma (2010). According to their observations, pre-treatment with Artemisia extract reduces the tissue- injury induced by cerebral infarction. Muto et al. (2003) studied the toxic dose of Artemisia, they showed some changes in hematological and biochemical properties in mice exposed to Artemisia. By analyzing the antioxidant compounds in *Artemisia sieberi*, Mojarab et al. (2009) showed that the flavonoids and anthocyanin in *Artemisia sieberi* are high and the high levels of these compounds are effective in reducing the oxidative stress. By investigating *Artemisia vulgaris*, Lee et al. (2000) reported effective phenolic compounds in the plant and the inhibitory effect on monoamine oxidase. The use of natural antioxidants plays an important role in preventing the formation of free radicals and preventing diseases. Among well-known antioxidants with therapeutic effects, the Q_{10} coenzyme as a mitochondrial enzyme plays an important role in the electron-transfer of the respiratory cell cycle, which has attracted enormous interest. The CoQ_{10} interferes with the protection of membrane and cellular molecules as a therapeutic agent in certain diseases, especially neurodegenerative diseases. Jameie et al. (2014) reported the combined therapeutic effects of low-level laser therapy and CoQ_{10} on the treatment of neuropathic pain in rats. Regarding the role of oxidative stress in PD and the neuroprotective and antioxidant properties of Artemisia and CoQ_{10}, the present research was designed to evaluate the effects of CoQ_{10} and Artemisia combination treatment on a cellular model of PD induced by 6-hydroxydopamine (6-OHDA) in PC12 cell line.

Materials and methods

Reagents

The PC12 cell line was purchased from the Iran pastor institute. FBS and DMEM (Gibco), 6-OHDA (Sigma), dimethyl sulfoxide (Sigma), Hochest 33342 (Sigma), dichlorofluorescein (DCF, Sigma) and P53

(Abcam) were used in this study.

Artemisia extraction

Extracting the *Artemisia sieberi* plant was carried out by soaking. The 10g of *Artemisia sieberi* plant was soaked with 200ml of 80% ethanol for 2 days. The contents were filtered by filter paper. The solution was transferred to a rotary machine to remove the ethanol solvent. The solution was rotated at 40°C and 279rpm. The concentrated extract appeared as a dark-colored paste fluid in the bottom of the balloon and was placed in a foam machine at a temperature of 40-30°C after extraction to dry completely.

Cell culture

PC12 cells were cultured in flasks containing RPMI medium and 10% FBS, 100U/ml penicillin, and 100µg/ml streptomycin under 5% CO2 and 95% humidity at 37°C temperature. Once a monolayer is formed, it passages and then after three passages were used for experiments.

Treatment

In this study, the cells were treated with different concentrations of 6-OHDA dissolved in DMSO (25, 50, 75, 100 and 125µM) for 24h. The experimental groups consisted of control (without treatment), 6-OHDA (received 75µM 6-OHDA), DMSO (received DMSO) and experimental groups with different concentrations of *Artemisia sieberi* extracts (200, 400, 600, 800 and 1000µg/ml), CoQ_{10} concentrations (25, 75, 100, 150, 200 and 250µg/ml) and their combinations (CoQ_{10} 75+ Artemisia200, CoQ_{10} 100+ Artemisia400 and CoQ_{10} 150+ Artemisia600) at 24h.

Viability assay

The 5000 cells were cultured in 96-well plates and placed in an incubator for 1 day. After the cells adhered to the plate, all wells were treated with 6-OHDA for 24h except for negative control and the sham group. Then the treatment was performed with CoQ10, *Artemisia sieberi* and combination then incubated. After incubation time, 100µl MTT (5mg/ml in PBS to a final concentration of 0.5mg/ml) was added to the wells and plate incubated for 2-4h, then 100µl DMSO was added and the plate was read by an ELISA reader (BioTek, ELx800, USA) at 570nm wavelength. The following formula was used to convert optical absorption into the percentage of viable cells:

$$\%\ \text{viable cells} = \frac{(abs_{sample} - abs_{blank})}{(abs_{control} - abs_{blank})} \times 100$$

Reactive oxygen species (ROS)

This technique was used to evaluate the production of free radicals. The cells were cultured in 6-well plates and then washed once with PBS after 24h. Then, 0.4mg of DCF powder in 1ml DMSO was dissolved and diluted with PBS. Subsequently, subsequently, 1ml DCF was added to the wells in the dark for 30min. After washing with 1ml PBS, it was read with a microplate reader fluorescence measurements were made using a citation 3 imaging reader (BioTek Instruments) set to 37°C. Measurements were made using a 485/20 excitation and a 528/20 emission filter pair and a PMT sensitivity setting of 55.

Hoechst staining

To Hoechst stain preparation (0.2mg of powder stain in 1ml distilled water) was dissolved and filtered through the syringe filter. All procedure was done in the dark. The cells were first cultured in a 24-well plate. The number of cells in each well was considered to be 30,000 cells and after treatment with the mentioned concentrations, the culture medium was evacuated and the cells were washed 2 times with PBS. The cells were then fixed with 4% paraformaldehyde for 20-30min and brought out from fixative. The cells were again rinsed with PBS and stained with Hoechst. The detection of the apoptotic cell nucleus was evaluated by an inverted fluorescence microscope model (IM-3FL4) at 357nm excitation and 447nm emission wavelengths, respectively. The treatment was repeated three times, for each group 5 slides were prepared and each slide was counted into 5 zones (Shah et al., 2014).

Western blotting

PC12 cells were cultured in 96 well plates (3.0×105/well) and treated with CoQ10 and Artemisia combination and alone. After incubation, cells were collected and lysed in a buffer containing 50mM HEPES (pH 7.4), 150mM NaCl, 0.1% Triton X-100, 1.5mM MgCl2, 1mM EDTA, 2mM sodium orthovanadate, 4mM sodium pyrophosphate, 100mM

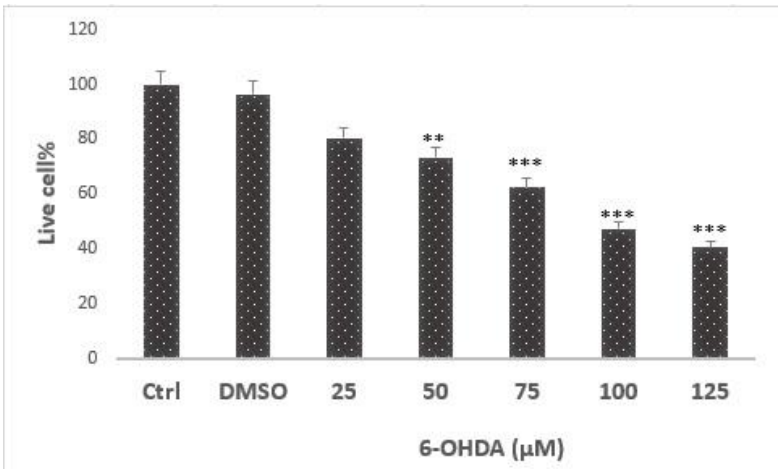

Fig.1. The viability of the PC12 cells treated by 6-hydroxydopamine (6-OHDA) after 24h. All experiments were performed in triplicate and data as mean±SEM. $^{***}P<0.001$, $^{**}P<0.01$ compared to the control groups.

NaF and protease inhibitor mixture (1:500; Sigma-Aldrich) for cell lysates. Then by 10% SDS-polyacrylamide gel (Invitrogen) electrophoresis was performed and transferred onto polyvinylidene fluoride membranes. The membranes were subsequently probed with antibodies, including rabbit polyclonal P53 (ab226419) and mouse monoclonal β-actin antibody (1:10,000; ab6276) from Abcam (Cambridge, MA, USA). The p53 antibody was added to the membrane. A secondary antibody was added onto the membrane within 24h so that the entire surface of the membrane was covered. The membrane container and secondary antibodies were placed on a slow-moving pad at ambient temperature for an hour. After the incubation time, the secondary antibodies were removed and then washed by TBS buffer for 3 to 10min and bands were evaluated. Densitometry analyses of bands were adjusted against β-actin, which functioned as a loading control. The percentage increase or reduction in protein expression levels was estimated by comparison to DMSO control. Experiments were performed in triplicate, separately (Li et al., 2005).

This research has been approved by the ethics committee of Neuroscience Research Center of Iran University of Medical Sciences with code 946 / P1/ 94

Statistical analysis

The results were analyzed by SPSS software using One Way ANOVA. To evaluate the normal distribution of data, the Kolmogorov-Smirnov test was used and to evaluate the equivalence of variances, the Levin test was also used. To compare the mean of the groups, Tukey's post hoc test was used and a $P<0.05$ was considered statistically significant.

Results

Viability assay results

The viability outcomes of the various concentrations of 6-OHDA (25, 50, 75, 100 and 125µM) after 24h are shown in Figure 1. Consequently, 75µM showed a 50% significant decrease in the vital capacity of the cells ($P<0.01$). Thus, an effective concentration of 75µM was considered for the induction of Parkinson's. Also, the results of this test determined the suitable time for the function of 6-OHDA on the cell line at 24h. Therefore, this dose and time were used to continue the study.

The viability of cells 24h after incubation with 6-OHDA and various concentrations of CoQ_{10} is shown in Figure 2. All CoQ_{10} 75, 100, 200 and 250µM concentrations showed a significant increase compared with the 6-OHDA cells ($P<0.001$). The number of live cells at the 75µM concentration was a more significant increase compared to other concentrations.

The viability results of Artemisia treatment in 6-OHDA treated cells showed that all concentrations had a significant positive correlation with the control group ($P<0.01$). The 200µg/ml concentration of Artemisia showed a more significant increase in the number of living cells compared to 6-OHDA cells and other concentrations ($P<0.01$). Also, the results showed that there was no significant difference between the 1000µg/ml concentration compared to the 6-OHDA cells, but decreased significantly ($P<0.05$) compared to the control and DMSO (Fig. 3).

The results of the combined concentrations of

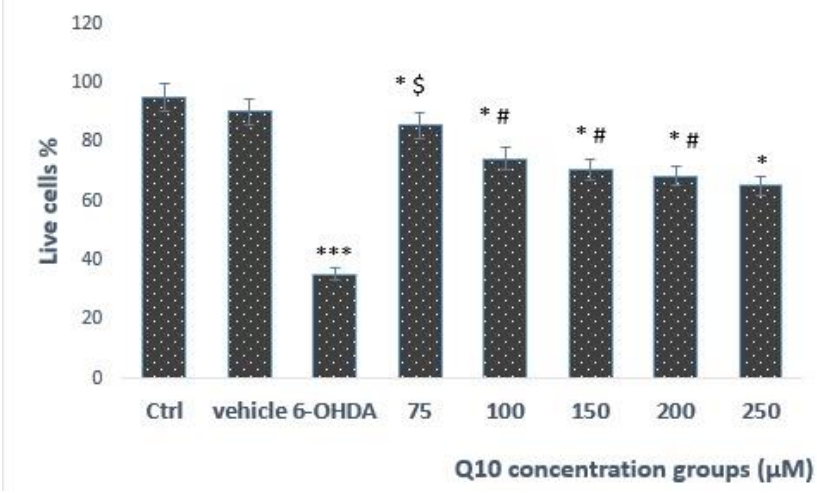

Fig.2. The viability of 6-hydroxydopamine (6-OHDA) treated cells with different concentrations of Q10 after 24h. All experiments were performed in triplicate and data as mean±SEM. $^{***}P$<0.001 significant decreased with control; $^{\#}P$<0.001 significant increased with the 6-OHDA; $^{\$}P$<0.01 significant differences with the other concentrations.

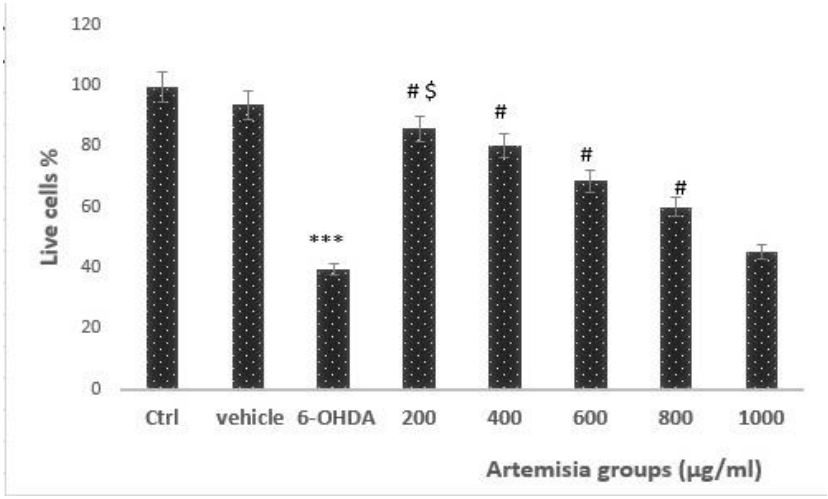

Fig.3. Comparison of the viability of 6-hydroxydopamine (6-OHDA) treated cells with different concentrations of *Artemisia sieberi* after 24h. All experiments were performed in triplicate. $^{\#}P$<0.01 significant increase compared with the 6-OHDA cells; $^{\$}P$<0.05 significant increase between 200 with other concentrations; $^{***}P$<0.001 significant decreases of treated cells compared with the DMSO and control.

Artemisia sieberi and CoQ_{10} are shown in Figure 4. The combined concentration of (CoQ_{10}75+ Artemisia200), (CoQ_{10} 100+ Artemisia400) and (CoQ_{10} 150+ Artemisia600) significantly increased the number of live cells compared to 6-OHDA cells (P<0.05). Also, the CoQ_{10} 75+ Artemisia200 concentration significantly increased (P<0.001) compared to the other concentrations. The results of other concentrations were not dose-dependent and results showed the highest concentration had no significant effect.

A comparison of the effective concentration of CoQ_{10}, Artemisia and their combination on cell viability is shown in Figure 5. The combined treated cells significantly increased the number of living cells compared to CoQ_{10} and Artemisia treated cells alone (P<0.001). In addition, there was no significant difference between CoQ_{10} and Artemisia.

Hoechst staining results

To evaluate the nuclear density, Hoechst staining was used. According to the MTT assay results, the 200µg/ml concentrations of *Artemisia sieberi*, 75µM concentration of CoQ_{10}, and combination of CoQ_{10}+ *Artemisia sieberi* were used. As it is shown in Figures 6, the apoptotic cells observed with the brilliant nuclei in 6-OHDA group cells more than antioxidant treatment groups. The percentage of apoptotic cells are shown in Figure 6A. The percentage of apoptotic cells in all oxidant treatment groups was significantly

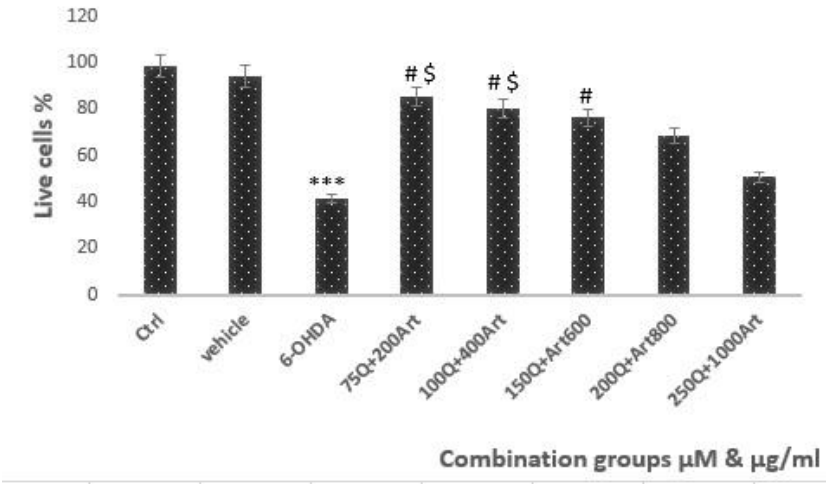

Fig.4. The viability of 6-hydroxydopamine (6-OHDA) treated cells under Artemisia and CoQ_{10} treatments. All experiments were performed in triplicate. $^{***}P$<0.001 significantly decreased viability compared to 6-OHDA cells; $^{\$}P$<0.05 significant differences at concentration of (100 +400), (75+ 200), with other concentrations; $^{\#}P$<0.001 significant increased with 6-OHDA cells.

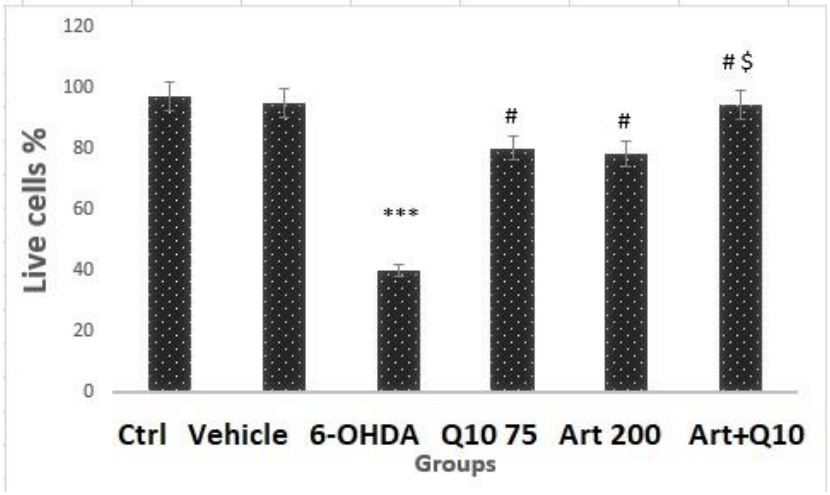

Fig.5. Comparison of the effective combination concentration in the viability of the 6-hydroxydopamine (6-OHDA) cells. All experiments were performed in triplicate and the data were considered as Mean±SEM. $^{***}P$<0.001 significance decreased with the 6-OHDA; $^{\#}P$<0.01 significantly increased the Artemisia200 compared with the 6-OHDA; $^{\$}P$<0.05 the significance of the combination of 75 µm CoQ_{10} and *Artemisia sieberi* 200µg/ml treated cells compared each one alone.

lower than the 6-OHDA. Also, there was a significant decrease in the combination group in comparison to the CoQ_{10}, and Artemisia groups (P<0.01), and the Artemisia showed a significant decrease of apoptotic cells compared to the Q_{10} group (P<0.05, Fig 6B).

ROS assay results

The evaluation ROS production showed that a combination of two antioxidants significantly inhibited the production of ROS compared to 6-OHDA treated cells and each antioxidant treatment alone (P<0.01). The ROS production in Artemisia200 treated cells was significantly decreased in comparison with the CoQ_{10} 75µM treated cells (P<0.05, Fig. 7).

Results of P53 protein expression

The expression of P53 according to Figure 8 showed that in the 6-OHDA group significantly increased compared to the control group (P<0.001). The expression of P53 in the treated cells significantly decreased compared the 6-OHDA cells but, in the combination of two antioxidants, more significantly decreased compared with the 6-OHDA cells and each one alone (P<0.001). Also, this data showed the P53 expression between the Q_{10} and Artemisia groups was not significant.

Discussion

Parkinson's disease is a neurodegenerative disease

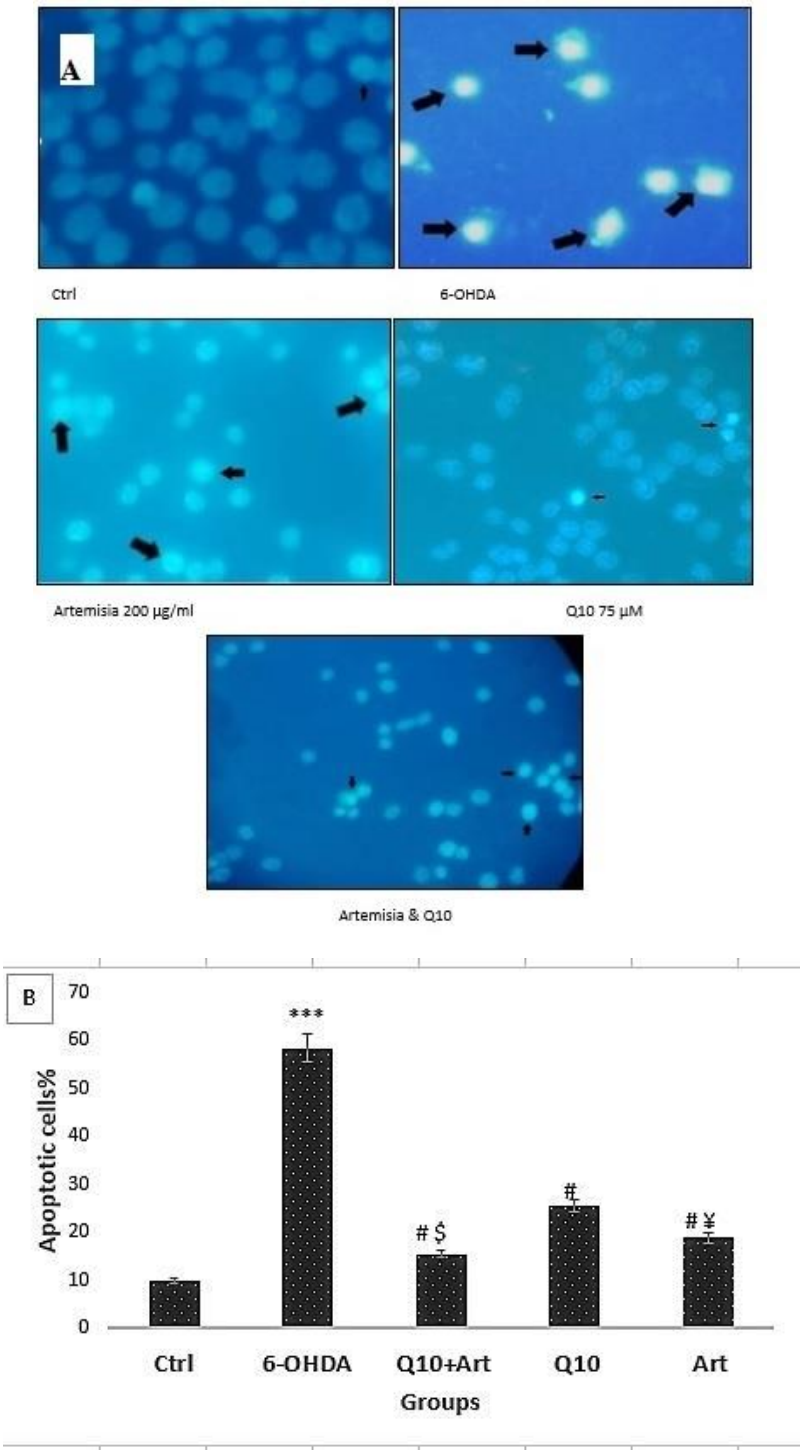

Fig.6. A: Hoechst staining of the treated cells using a fluorescence microscope at 400x magnification with three repetitions. The arrows represent apoptotic nuclei. **B:** The comparison of apoptotic cells resulted in Hoechst 33342 staining (Mean±SEM). $^{***}P<0.001$ significantly increase the apoptotic cells in the 6-hydroxydopamine (6-OHDA) cells compared with the control group; $^{\#}P<0.01$ the significantly decreased compared with 6-OHDA; $^{\$}P<0.05$ the significantly decreased compared to the Q10 and Artemisia treated cells; $^{¥}P<0.05$ significantly decreased compared with the Q10 treated cells.

that affects motor, cognitive and emotional functions (Henchcliffe and Beal, 2008). The exact etiology of PD is still unknown and the role of oxidative stress in the pathophysiology of PD paid more attention recently. It is generally accepted that oxidative stress leads to mitochondrial dysfunction that in turns causes DNA damage and ultimately neuronal death. Despite advances in pathogenesis and pathophysiology of PD, routine treatments are mostly symptomatic. During recent years, many types of research focused on the effectiveness of antioxidant agents with different origins. Among them, the CoQ10

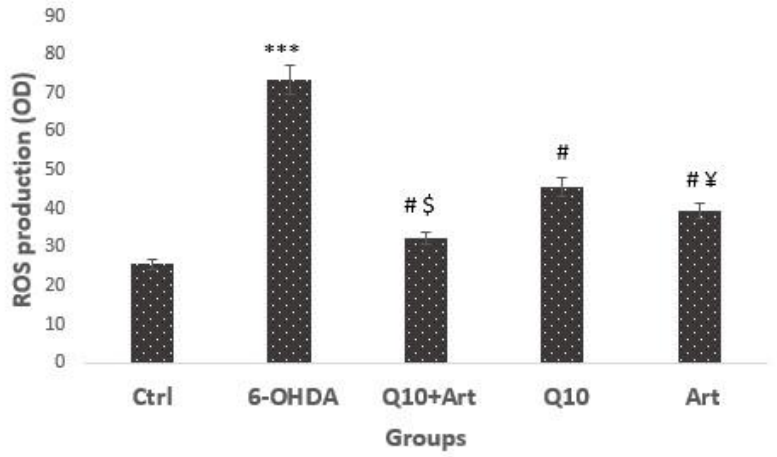

Fig.7. Represented ROS production between groups. All experiments were performed in triplicate and the data were considered as Mean±SEM. $^{***}P$<0.01 significant increase 6-hydroxydopamine (6-OHDA) compared with the control; $^{\#}P$<0.01 significantly decrease antioxidants treated cells in comparison with the 6-OHDA cells; $^{\$}P$<0.05 significantly decrease in the combination group with two treatment groups; $^{¥}P$<0.05 significant decrease compared with CoQ10.

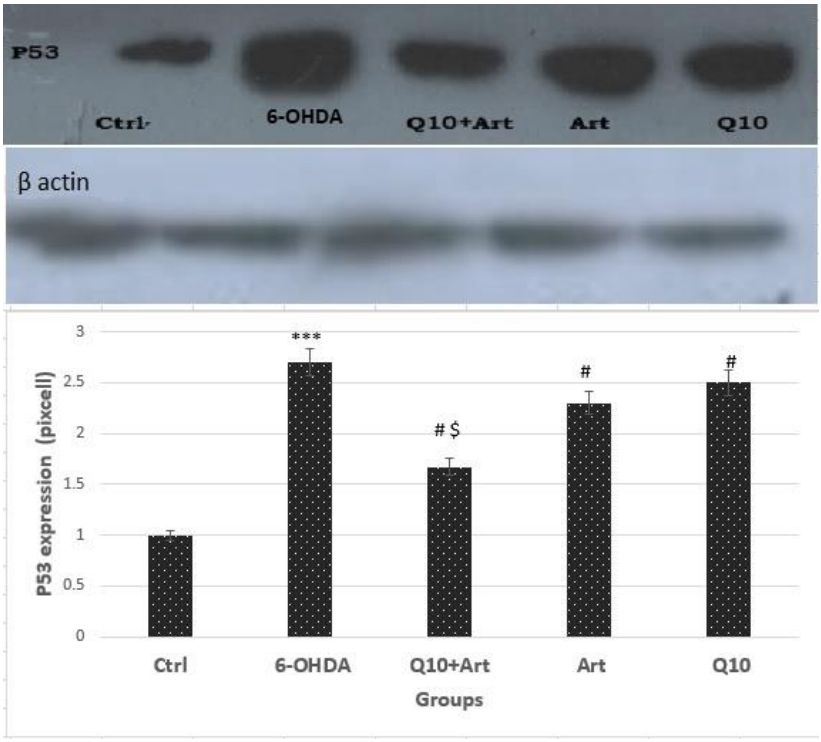

Fig.8. Represented the expression of p53 protein by western blotting assay (three times). $^{***}P$<0.001significantly increased compared with the control group; $^{\#}P$<0.01 significantly decreased compared to 6-hydroxydopamine (6-OHDA) cells; $^{\$}P$<0.05 significance between groups.

has received more attention. Loganathan et al. (2013) showed the dose-dependent protective effects of CoQ10 on MDA-MB-468 and BT549 cell lines. Seifried et al. (2007) reported the protective effect of CoQ10 on a variety of cells, they also showed that CoQ10 has the potential to reduce Parkinson's and Alzheimer's symptoms by reducing oxidative stress. Additionally, herbal extracts with antioxidant properties seem to be useful in the treatment of certain neurodegenerative diseases. In the present study, 75μM dose of CoQ10 was the most effective dose increasing viability in the PD cellular model compared to the other concentration. Li et al. showed that 100μm of CoQ10 could protect the SHSY5Y cells against the beta-amyloid neurotoxin peptide and inhibit cell death (Li et al., 2005) and they described CoQ10 as a compound promoting antioxidant defense capacity and the activity of antioxidant

enzymes is able to (Li et al., 2016). Moreover, da Silva Machado et al. (2013) reported that 0.1, 0.5 and 1µg/ml concentration of CoQ10 prevent DNA damage against neurotoxin cisplatin in PC12 cells. Regarding Artemisia, there are some reports that indicate its antioxidant properties. Shoaib et al. (2015) showed that the *Artemisia Macrocephala* is able to inhibit the activity of butyrylcholinesterase and acetylcholinesterase enzymes, which are effective to treat certain neurodegenerative diseases. Choi et al. (2013) extracted the phenolic compounds in the hydro alcoholic extract of the *Artemisia persica* and reported the high antioxidant capacity of these compounds. In another study by Poiata et al. (2009) showed that the aqueous extract of the Artemisia Species *Afra Jacy* reduced malondialdehyde, while increased superoxide dismutase and glutathione peroxidase, which inhibit the oxidative effect of hydrogen peroxide. Zeng et al. (2014) in their study reported that DSF-52 an extract from *Artemisia Argyi* affects the microglia cells and inhibits the inflammatory and nitric oxide the response of these cells in neurodegenerative diseases. Hong and Lee (2009) showed that ethyl acetate in *Artemisia capillaris*, as ROS inhibitor, not only protects V79 cell line against hydrogen peroxide but also increases the activity of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase as well as prevent lipid peroxidation. Kaur et al. (2012) reported also the same property in *Artemisia scoparia,* they had attention to the essential oils of *Artemisia scoparia* as a strong ROS-inhibiting defense mechanism that inhibits the hydrogen peroxide activity. Also, in the research in 2019 the protective different ethyl acetate and total flavonoids extracts of *Artemisia ciniformis* against H202 toxicity evaluated. Their results showed the ethyl acetate extract had the most effective on suppressing the toxicity of H2O2 and A. *ciniformis* is a potential choice for preventing different neurodegenerative diseases (Hosseinzadeh et al., 2019). The results of our study of using CoQ10 and *Artemisia sieberi* separately are in line with the aforementioned studies and confirmed their antioxidant and anti-apoptotic properties. Although the species of Artemisia that we used in our study are different from the species used by other researchers, it seems that all the species of Artemisia have the same effective extraction with almost similar properties. Additionally, our results showed better effects of CoQ10 than Artemisia. This finding might be due to using the total extract of Artemisia instead of its effective component, Artemisinin. Thus, it seems the better results for the combination use of Artemisia and CoQ10 are related mainly to CoQ10 activity than Artemisia. It is also possible that CoQ10 and Artemisia act synergistically via similar or different pathways. Beneficial clinical effects of CoQ10 showed in many studies, these effects of CoQ10 could happen via different mechanisms. Among this mechanism, immune modulation and enzymatic rebalance properties of CoQ10 have received more attention. Soleimani et al. (2014) reported the effect of CoQ10 on the ratio of TH1/TH2 in the experimental autoimmune encephalomyelitis model of multiple sclerosis. Fuller et al. (2006) reported that CoQ10 could suppress the increased production of certain inflammatory mediators such as IL-6. In addition to the mentioned mechanism, it has been shown that CoQ10 exerts/has synergistic effects when combined with certain drugs in patients with cancer (Premkumar et al., 2007). It has been generally accepted that CoQ10 not only produces sub-cellular energy but also acts as an antioxidant that prevents lipid peroxidation and scavenges superoxide anions. CoQ10 can be diffused within the biological membrane and it can leak out the inner membrane of mitochondria (Fuller et al., 2006). N-acetyl cysteine, a known antioxidant, can block the effects of TNF-α in HeLa cells. It acts via interaction with kinases involved in the cellular signaling pathway (Cammer, 2002). Unlike CoQ10, the exact action mechanisms of Artemisia are still unknown. In order to answer the questions regarding the effects of Artemisia and its mechanisms, we are going to examine the role of its effective extract known as Artemisinin in upcoming projects. However, we believe that Artemisia also acts in a similar way to the CoQ10.

Conclusion

Based on our findings, the combination of agents with antioxidant and anti-apoptotic properties with consideration of the using concentration could have better therapeutic results. More studies need to show exact mechanisms of action.

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Conflict of interest

The authors declare no conflict of interest.

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