



Evaluation of Neuroprotective Effect of *Althaea Officinalis* Flower Aqueous and Methanolic Extracts against H₂O₂-Induced Oxidative Stress in PC12 Cells

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Abstract

This study was conducted to evaluate the possible antioxidant activity and neuroprotective effects of aqueous and methanolic extracts of *Althaeaofficinalis* flowers against H₂O₂-induced oxidative stress in PC12 cells. The antioxidant potential of extracts was evaluated by radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. For cytoprotective activity, the cells were pretreated with different concentrations (12.5, 25, 50, 100, 200 and 400 µg/ml) of the extracts for 24h and then incubated with H₂O₂ (480 µM) for 3 h. In co-treatment protocol, cells were simultaneously treated with H₂O₂ (480 µM) and the same concentrations of extracts, used in pretreatment protocol. Percentage of viability was measured using MTT assay. The aqueous and methanolic extracts did not show strong DPPH radical scavenging activity (IC₅₀ value of 128 and 255 µg/ml respectively) in comparison with ascorbic acid (IC₅₀ value of 6.1 µg/ml). The cytoprotection study revealed that neither the methanolic, nor the aqueous extracts at tested concentrations could protect the cells against H₂O₂-induced cytotoxicity compared to H₂O₂ alone, in either co-treatment or pretreatment experiments. Despite reporting the antioxidant activity of *A. officinalis* L. flowers, it seems that such a negligible cytoprotective activity may be related to some other factors. On the other hand, the presence of moderate antioxidant activity does not guarantee the protective activity against oxidative stress.

Keywords: *Althaeaofficinalis*, Neuroprotective, DPPH assay, Oxidative stress, PC12 cell line.

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Cite this article as: Arab A, Yazdian-Robati R, Rezaei-Seresht H, Ehtesham-Gharaee M, Soltani F, Evaluation of Neuroprotective Effect of *Althaea Officinalis* Flower Aqueous and Methanolic Extracts against H₂O₂-Induced Oxidative Stress in PC12 Cells. Iranian Journal of Pharmaceutical Sciences, 2017, 13 (1): 49-56

1. Introduction

Reactive oxygen species (ROS) including superoxide anion (O₂^{•-}), hydroxyl radical (•OH), singlet oxygen O₂ and hydrogen peroxide (H₂O₂) are highly reactive molecules or molecular fragments. They are produced continuously in all aerobic organisms. In addition to high reactivity, ROS are able to react with non-radicals, leading to the formation of new radicals [1]. Oxidative stress is outcome of overproduction of free radicals and/ or reduction in activity of antioxidant defenses against free radicals [2]. Oxidative stress can result in oxidative damage of biomolecules such as lipids, proteins and DNA, leading to many chronic and degenerative diseases in human. In brain and neuronal tissues, ROS are produced as by-products of excitatory amino acids and neurotransmitters metabolism. They can attack glial cells and neurons which are highly sensitive to free radicals, leading to neuronal damage [3]. It has been demonstrated that cells become more sensitive to oxidative stress by aging, which makes human more vulnerable to the neurodegenerative diseases such as Alzheimer. Several studies indicated that an antioxidant rich diet can reduce the deleterious effects of

oxidative stress and improve the signs of neurodegeneration [4]. Vitamin C, vitamin E, carotene and coenzyme Q are the most famous antioxidants. The antioxidant activity of plants may be related to the wide variety of free radical scavengers such as nitrogen bearing compounds, phenols, terpenoids and vitamins (3).

Marshmallow (*Althaea officinalis* L.), belongs to Malvaceae family, is growing in southern Europe and now is cultivated all over the world. It has been known as a medicinal plant since ancient times [5]. Different properties of this plant and its secondary metabolites have been reported in several studies. For instances, the anti-tussive effects of rhamnogalacturonan (heteropolysaccharide) extracted from the root [6], treating irritated mucous membranes [7], antioxidant activity of glucuronoxyl an against lipid peroxidation [8], antibacterial activity [9], anti-inflammatory activity (acute and chronic inflammation), anti-ulcerogenic activities, inhibition of platelet aggregation, increase of serum HDL cholesterol level [10] and protective effects against experimental parkinsonism model have been investigated so far [11].

To our knowledge, there is no study on probable neuroprotective activities of *A. officinalis* L. against oxidative stress. Accordingly, the aim of this study was to assess the cytoprotective effects of *A. officinalis* L against H₂O₂-induced oxidative stress on PC12 (rat pheochromocytoma) cell line as a proper model for studying neuronal cell damage due to oxidative stress [12]. In

addition, antioxidant activity of *A. officinalis* L was evaluated by DPPH method.

2. Materials and Methods

2.1. Chemicals and Reagents

H₂O₂ and MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] were purchased from Sigma Aldrich (St Louis, MO, USA). PC12 cells were obtained from Pasteur Institute of Iran. Cell culture medium, fetal bovine serum (FBS), and penicillin/streptomycin were prepared from Gibco (Grand Island, NY, USA). All other chemicals were of analytical grade or complied with the standards required for cell culture experiments.

2.2. Plant Materials and Preparation of Extracts

A. officinalis was collected from Sarakhs, near Mashhad (Razavi Khorasan Province, north-east of Iran) in August 2013 and identified by School of Pharmacy Herbarium. The collected plant parts were air-dried in darkness at room temperature (25 °C). The substance was 10g of filtered, dried aerial parts which were powdered and extracted with absolute methanol (3×50ml) by shaking for 48h at room temperature. Then the extracts were filtered and evaporated by using rotary evaporator below 40°C. The obtained plant extract was used for the next stages of testing.

2.3. DPPH Scavenging Activity

The antioxidant capacity of the plant extract was evaluated by using the 2, 2-diphenylpicrylhydrazyl (DPPH) assay (13). In

brief, a 1.5 ml aliquot of each extract in methanol at 12.5, 25, 50, 100, 200 and 400 µg/ml was added to 1 ml of 0.1 mM DPPH in methanol. The mixture was shaken for 1 min and allowed to stand in dark for 60 and 90 min at room temperature. The absorbance was measured at 517 nm. Ascorbic acid was used as positive control. The percent inhibition of free radical formation (I %) was calculated as; $I\% = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100$; where; “A blank” is the absorbance of the control reaction (containing all reagents except the extract), and “A sample” is the absorbance of the mixture containing the extract. The SC₅₀ (defined as the concentration required scavenging 50% of the free radicals) was calculated from plotting a graph of inhibition percentage against extract concentration. Determinations were carried out in triplicate.

2.4. Cell Culture

PC12 cell line was cultured in DMEM medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and FBS (10 %) at 37 °C in a humidified atmosphere of 5 % CO₂ and 95% air.

2.5. Effects of *A. Officinalis* extracts on Cell Viability

The crude methanolic and aqueous extracts were dissolved in the minimum amount of DMSO in a way that the final concentration of DMSO was less than 0.5%, when diluted with culture medium. The solutions were added to the culture medium to obtain different concentrations (12.5, 25, 50, 100, 200 and 400 µg/ml) of extracts. To assess cell viability,

MTT assay was used, which is based on the conversion of MTT dye to formazan by mitochondrial dehydrogenase in live cells (14). In brief, cells (4000 cells/well) seeded in 96-well plate were treated with different concentrations of extracts and then incubated for 3 and 24 h. After removing the medium, MTT solution (5 mg/ml) was added, and the cells were incubated at 37 °C for 4 h. The medium was removed and insoluble formazan product was dissolved in DMSO. The absorbance was measured at 570 nm and 670 nm in an ELISA reader. The percentage of cell viability for each concentration was calculated.

2.6. IC_{50} Determination of H_2O_2

In order to determine the IC_{50} of H_2O_2 for cytoprotective studies, cells (4000 cells/well) seeded in 96-well plate were treated with different concentrations of H_2O_2 (0-600 μ M) for 24 h and then cell viability was assessed by the MTT method as described previously.

2.7. Evaluation of Cytoprotective Activities of Extracts

To study protective effects of extracts against H_2O_2 -induced cytotoxicity, either co-treatment or pre-treatment protocol was applied. According to the co-treatment protocol, the seeded cells were incubated with various concentrations of methanolic or aqueous extracts (12.5, 25, 50, 100, 200 and 400 μ g/ml) and H_2O_2 (IC_{50} concentration) for 3 h. The cell viability was determined by MTT assay as described earlier. For pre-treatment test, the cells were treated with different

concentrations of extracts for 24 h and then, H_2O_2 (IC_{50} concentration) prepared in fresh medium was added and incubated for 3 h. After that, the MTT assay was conducted as described above.

2.8. Statistical Analysis

Statistical analyses were performed by InStat software. Differences between groups were evaluated by means of one-way analysis of variance (ANOVA) followed by the Dunnett's test. Values were expressed as mean \pm standard error of the mean (mean \pm SEM). Statistical significance was accepted at the $p < 0.05$ level.

3. Results and Discussion

In this study, probable neuroprotective effects of *A. officinalis* flower aqueous and methanolic extracts against H_2O_2 -induced oxidative stress were investigated in PC12 cells using MTT assay. In addition, the antioxidant activity was investigated by DPPH method.

Based on the antioxidant assay data (data not shown), although the aqueous and methanolic extracts could scavenge the DPPH radicals at SC_{50} value of 128 and 255 μ g/ml respectively, but in comparison with ascorbic acid (SC_{50} value of 6.1 μ g/ml) such activities were negligible.

The IC_{50} of H_2O_2 for PC12 cells were obtained 480 μ M by MTT assay (data not shown) and this concentration was selected for further studies. According to figure 1, aqueous and methanolic extracts (12.5-400 μ g/ml) did not exhibit any cytotoxic effects compared to

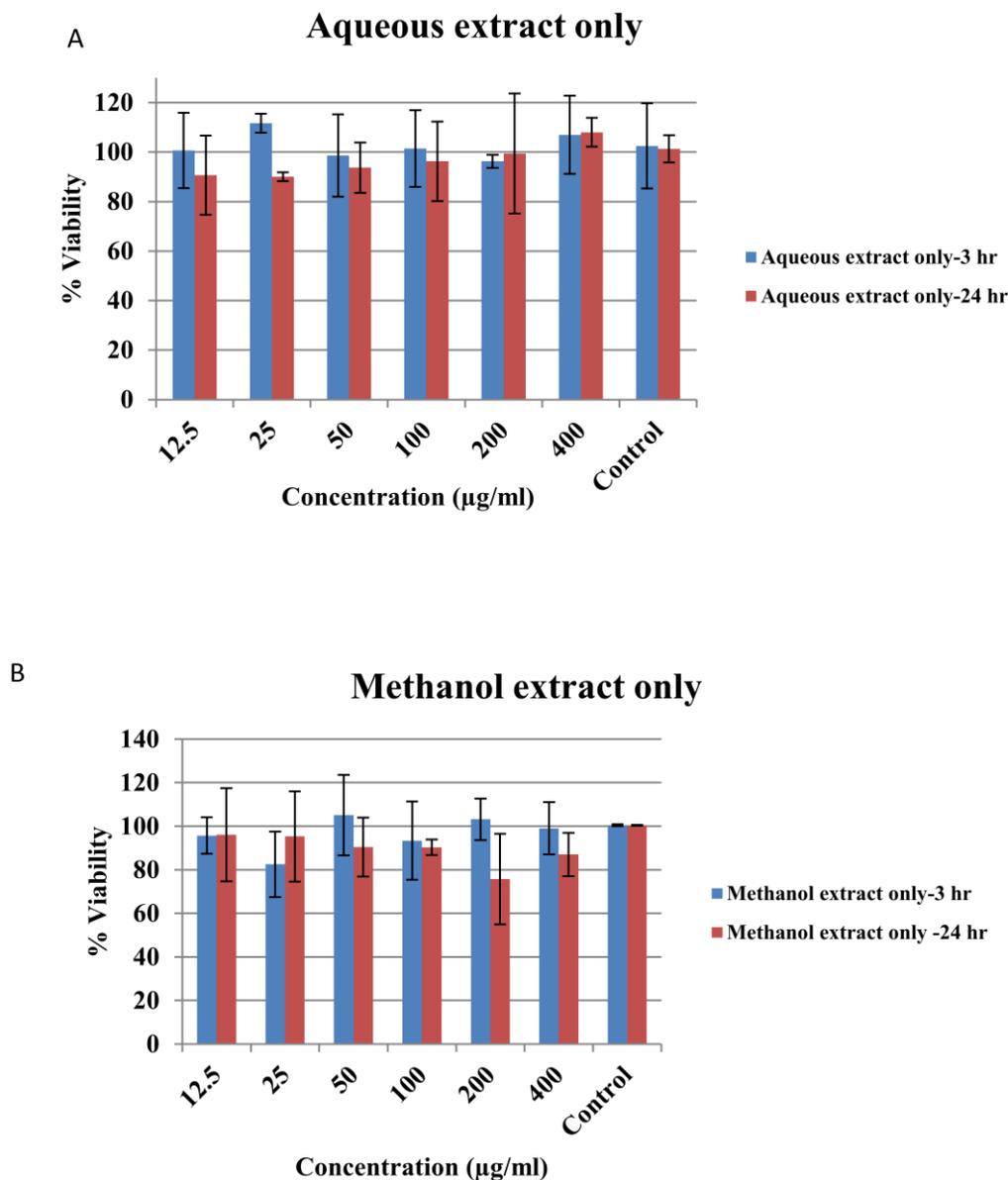


Figure 1. Effects of *A. officinalis* flower extracts on the viability of PC12 cells. A) Aqueous extract B) Methanolic extract for 3h and 24 h.

the negative control ($p > 0.05$). Based on the obtained results, different tested concentrations of extracts (methanolic and aqueous) could not protect the cells against H_2O_2 -induced cytotoxicity compared to H_2O_2 alone, in either co-treatment or pre-treatment experiments (Figure 2).

Oxidative stress, known as a consequence of overproduction of ROS and/or reduction of antioxidant level, can lead to promote cell death and injury (15). In the present study, we investigated the antioxidant activity of methanolic and aqueous extracts of *A. officinalis* by DPPH method as well as possible neuroprotective effects of these

extracts on PC12 cells. Several pharmacological effects of this plant such as anti-complement, anti-tussive, anti-viral, anti-fungal and anti-microbial activities have been reported (16). Ethanolic extract of marshmallow flower (*A. officinalis* L.) at concentrations of 50, 100 and 250 µg/ml showed 85.5%, 91.2%, and 96.4% anti-oxidation effect on linoleic acid emulsion respectively, in comparison with 100 µg/ml of

butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and α-tocopherol which which exhibited 94.5%, 99.1%, and 80% inhibitory effect on linoleic acid peroxidation, respectively. Moreover, at the same concentrations, ethanolic extract of marshmallow flower exhibited effective reducing power, superoxide anion radical scavenging, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and metal

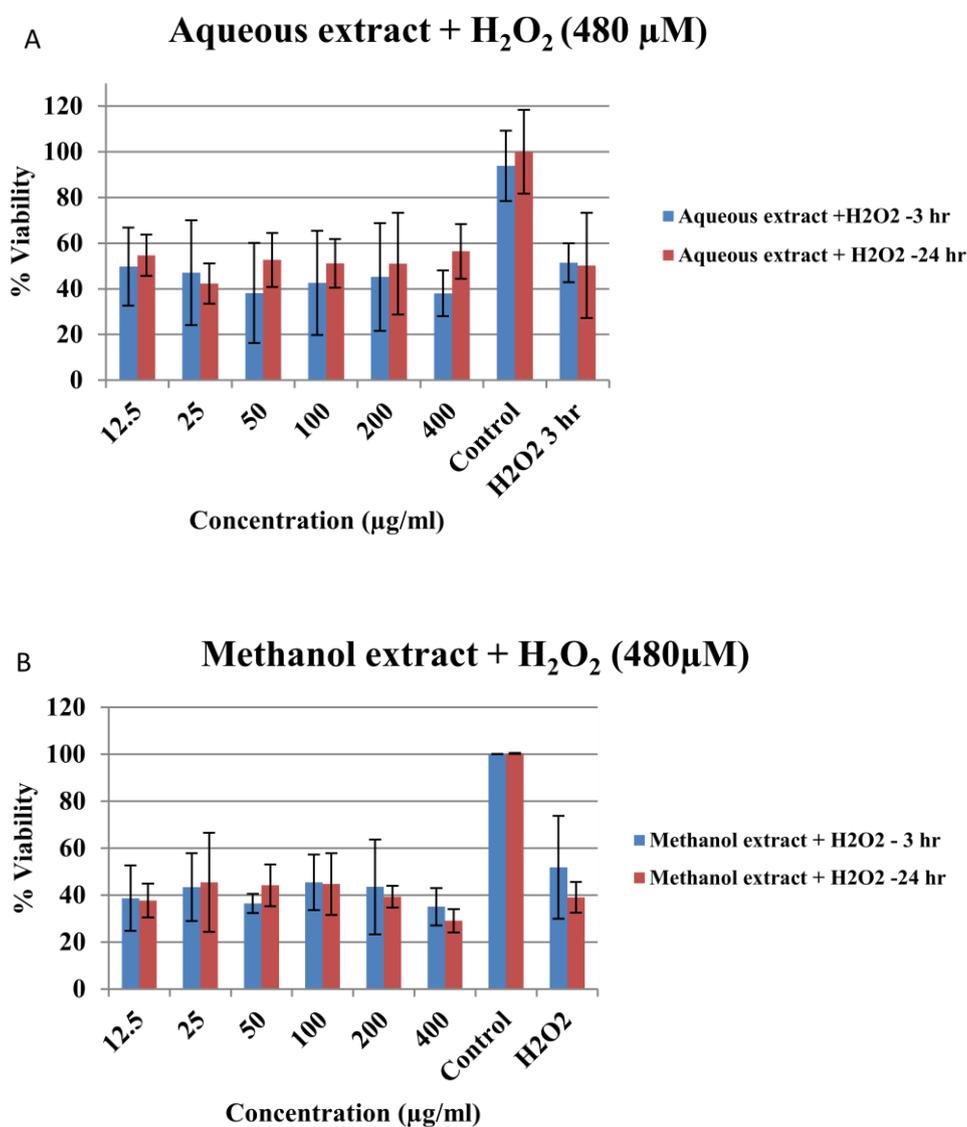


Figure 2. Effects of A) aqueous extract and B) methanolic extract of *A. officinalis* flower on H₂O₂-induced cytotoxicity in PC12 cells in pre-treatment (24h) and co-treatment experiments (3h).

chelating activities (17).

Antioxidant activity of aqueous and methanolic extracts of *A. officinalis* was determined by DPPH assay. This method is based on the reduction of DPPH to DPPH₂ by accepting a hydrogen atom from the scavenger molecule which determined by changing color from purple to yellow with absorbance drop at 517 nm (18). It was shown that the antioxidant activity of *A. officinalis* L. flowers depends on the intensity of flower color. Results of CUPRAC assay and ferric iron reducing assay in order to evaluate antioxidant activity of *A. officinalis* L. flowers showed that reddish pink flowers possessed the highest antioxidant activity compared to the white and pink ones (19). Anthocyanins which provide the color of most flowers, fruits, and vegetables are known as potent antioxidant substances. There is a probable correlation between the anthocyanin level in the deep color of plants and their antioxidant capacity and also biological effects related to antioxidant potency (19). According to the obtained data, the methanolic extract showed higher antioxidant capacity than aqueous extract. This may be described by the difference in the total phenolic and flavonoid content (20). However, such a scavenging activity was not as potent as ascorbic acid. Our cytoprotection results showed that methanolic and aqueous extracts of *A. officinalis* were not able to protect the cells against H₂O₂-induced oxidative stress using either concurrent or pre-treatment protocols. It seems that a weak radical scavenging property of extracts, at least at the tested concentrations, may not protect the cells against oxidative stress. Individual

phytochemicals may be responsible for neuroprotective effects, if so, it might be interesting to evaluate their activity as pure compounds rather than in the extracts which included low level of active compounds and also potential antagonists.

4. Conclusion

In conclusion our preliminary *in vitro* assay showed that the methanolic and aqueous extracts of *A. officinalis* L. white flowers at tested concentrations were not able to protect PC12 cells against stress oxidative. However, further *in vitro* studies either by using pure substances of this plant or the other colors of flowers is needed to clarify if the plant has neuroprotective effects or not.

Acknowledgments

This study was financially supported by Research Council of Sabzevar University of Medical Sciences, Iran.

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