Pharmaceutical Sciences
June 2019, 25(2), 132-137
doi: 10.15171/PS.2019.20
https://ps.tbzmed.ac.ir/

Research Article

Investigation of Some Medicinal Plants Inhibitory Effect on NO Production in Oxidative Stressed PC12 Cells

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Article Info

Article History:
Received: 4 January 2019
Revised: 26 January 2019
Accepted: 27 January 2019
ePublished: 30 June 2019

Keywords:
-Nitric Oxide
-PC12
-*Astragalus jolderensis*
-*Convolvulus commutatus*
-*Salvia multicaulis*
-Griess assay

ABSTRACT

Background: Nitric oxide and reactive nitrogen species play an important role in various pathological conditions like cancer, inflammation and neurodegeneration. As plants and natural compounds have a great potency of discovering lead compounds which might affect NO production during inflammation and various pathologies, we examined the effects of three medicinal plants native to Iran, on NO production during oxidative stress in PC12 cells.

Methods: In this study, cell death and NO levels were measured by MTT and by Griess assay, respectively. Oxidative stress was induced by hydrogen peroxide and extracts of *Astragalus jolderensis*, *Convolvulus commutatus* and *Salvia multicaulis* were used as pretreatment in oxidative stressed PC12 cells.

Results: *A. jolderensis* extract significantly suppressed NO production in 150 and 200 µg/ml concentrations and *C. commutatus* extract in all concentration inhibited NO production in stressed PC12 cells. In addition, the extract of *S. multicaulis* inhibited NO production during stress at all concentrations above 50 µg/ml. Besides, the extract of *S. multicaulis* showed protective effect at lower doses in stressed cells.

Conclusion: According to the results, *S. multicaulis* inhibited NO production and protected cells from oxidative stress. Hence, *S. multicaulis* is a good candidate for further *in vitro* and *in vivo* investigations. *A. jolderensis* and *C. commutatus* also suppressed NO production during stress. Therefore, they could be noticed in experiments that centralize on the inhibition of NO production and drug discovery studies in the field of neurodegenerative and chronic inflammatory diseases.

Introduction

Nitric oxide (NO) is a gaseous free radical molecule with a short half-life that is produced by three types of nitric oxide synthase (NOS) enzymes including neuronal, endothelial and inducible isoforms. NO in role of an intra and extracellular signaling molecule extensively participates in the cardiovascular, immune and nervous systems.[1,2] In various pathological conditions, large amounts of NO produced by inducible isoform, provides a major source of reactive nitrogen species (RNS). Among RNS, extensively studied, peroxynitrite can damage many vital cellular molecules including DNA, lipids, and proteins. In addition, RNS promotes apoptosis through oxidative/nitrosative stress (ONS) either dependent or independent from mitochondria.[3-5] Furthermore, ONS plays a critical role in various diseases like Alzheimer and other neurodegenerative diseases, liver disease, asthma and cancer.[2,3,6-8]

Over the last decades, many drug discovery researches have focused on compounds that inhibit NO production in ONS and herein the plant extracts, as expected, are valuable sources for such compounds due to their antioxidant and anti-inflammatory properties. In addition, plant derived secondary metabolites are favorite compounds for drug discovery because they contact several molecular targets and are able to react with multiple enzymes, envisaging that a compound which modestly interacts with several related pathways is more potent and less toxic as a therapeutic mean.[9]

Lamiaceae, one of the largest flowering plant family, is widely distributed through the world and has many endemic species in Middle East countries including Iran. Generally, the plants in the Lamiaceae family have phytochemicals such as terpenes, flavonoids, and phenolic acids and have a special importance in Ethnopharmacology and phytotherapy.[10] *Salvia* is the

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largest genus of the Lamiaceae family and its species have complex and rich diversity. Furthermore, studies show that active compounds from *Salvia spp*. have antimicrobial, antioxidant and anti-inflammatory effects.[11,12] In a recent study, the effects of salvianolic acid A, tanshinone I, and tanshinone IIA from *S. miltiorrhiza* was investigated in allergic asthma with *in vivo* and *in vitro* methods. From the results, authors suggested that salvianolic acid A and tanshinone IIA may be potential anti-allergic therapeutics.[13] *S. multicaulis* has phytoconstituents mainly in flavonoids and phenolic class of compounds like other related genus. Furthermore, there are several reports of diterpenoid and triterpenoid isolation from this plant.[14-17]

Noteworthy, *Astragalus* and *Convolvulus* are other species which have been considered in drug discovery studies. *Astragalus* L. from the Leguminosae family is a genus widely distributed throughout Europe, Asia and North America. Moreover, most of phytochemicals found in this genus are pharmacologically active and mainly belong to the polysaccharide and the saponin classes. The remarkable pharmacological properties of *Astragalus* spp. are hepatoprotective, immunostimulant and antiviral effects, and recent researches revealed promising positive effects of various *Astragalus* species in oxidative stress and inflammation.[18-21] In addition, researches revealed that *Astragalus* polysaccharides from *Astragalus melittin* protects against injuries of coxsackievirus B3-induced myocardial damage and inflammation.[22]

Besides, the Convolvulaceae family, especially the genus *Convolvulus*, has a large number of medicinal plants and some species like *C. arvensis*, *C. austro-aegypticus*, *C. pilosellifolius* are widely used in folklore medicine of Asia and Africa. Additionally, *C. pluricaulis* has neuropharmacological effects such as anti-stress, anxiolytic and antidepressant. This genus generally contains flavonoids and alkaloids.[23-25] A study on the flower flavonoid patterns of *Convolvulus* L. populations from Markazi Province, Iran, showed that kaempferol, hesperidin and naringenin were the most abundant flavonoids, respectively and *C. commutatus* had the highest flavonoid content among other species.[26]

Hence, in the present study we evaluated the effect of above mentioned medicinal plants on NO production during oxidative stress in PC12 cells.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA solution, penicillin/streptomycin 100 units, dimethylsulfoxide (DMSO) and all other fine chemicals were obtained from Merck, Germany. 3-(4,5 dimethylthiazole-2-yl)-2,5-dimethyl tetrazolium bromide (MTT), Griess reagent [1:1 mixture (v/v) of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 5% H3PO4] and trypan blue were purchased from Sigma (St Louis, MO).

Plant materials

Three plants including *Astragalus jolderensis* B. Fedtsch. (Leguminosa) and *Convolvulus commutatus* Boiss. (Convolvulaceae) were collected in May 2014 from Golestan province and *Salvia multicaulis* Vahl. (Lamiaceae) was collected in October 2010 from Semnan province of Iran. All plants were identified by a qualified botanist at Traditional Medicine Research Center (TMRC), Shahid Beheshti University of Medical Sciences, Tehran, Iran. A voucher specimen of each species is deposited at respective herbarium.

Preparation of extracts

Aerial parts of *C. commutatus* and *S. multicaulis* and roots of *Astragalus jolderensis* were shade dried, powdered and macerated in methanol. Maceration lasted for 72 h and a fresh solvent was replaced the extract every 24 h. All extracts were collected and concentrated by rotary evaporator Heidolph 4000 (Schwabach, Germany) at room temperature, to remove all solvent residuals.

Cell culture & treatment

PC12 (rat pheochromocytoma) cells obtained from Pasteur Institute (Tehran, Iran), were grown in DMEM enriched by 10% FBS, supplemented with 100 unit/ml penicillin and 100 mg/ml streptomycin and maintained at 37 ˚C in a humidified atmosphere (90%) containing 5% CO_2.

All extracts were dissolved in dimethyl sulfoxide (DMSO), to make stock solutions. Regarded concentrations of extract and hydrogen peroxide (H_2O_2) were made with DMEM prior to use. Experiments were performed in serum-free DMEM to avoid rapid H_2O_2 degradation by antioxidants and interference with Griess assay by FBS.[27,28]

Cytotoxicity Assay

The extracts' cytotoxicity was determined by MTT method.[29] Briefly PC12 cells were incubated after seeding in 96-well plates (35000 cells/well) for 24 h. Next the medium was freshened and treated with different concentrations of each extract (3.125-100 µg/ml) and incubated for 24 h. Negative control wells were treated with 1% (v/v) DMSO in equal volume of medium. At next step the supernatant was removed and MTT salt was diluted with fresh medium to 0.5 mg/ml and added to cultured plate. After 24 h of incubation, the culture supernatant was removed and the formazan crystals were dissolved in DMSO and the absorbance of wells were measured by ELISA plate reader from Fisher Scientific Company (Ontario, USA) at 570 nm. The cell viability was exhibited as the percentage of mean absorbance of negative control wells, which was considered as 100% viability.

Griess assay

A general procedure was used to determine NO production in stressed PC12 cells.[30] Concisely after 24 h incubation of cells in 96-well plate, the medium was

freshened and the extracts (50-300 µg/ml) were added. The negative control wells were treated with 1% (v/v) DMSO. After 24 h of incubation, the medium was removed and FBS free DMEM contains H_2O_2 (1.5 mM) was added, except for the negative control groups. In the last step, equal volume (100µl) of Griess reagent and supernatant of each well was mixed and incubated for 15 min in dark and subsequently the absorbance of the wells was determined at 540 nm with ELISA plate reader. After the Griess assay, the cell viabilities were determined with the MTT assay, too.

Statistical analysis

All experiments were repeated three times and data were represented as the mean ± SEM. The groups means were compared with one-way analysis of variance (ANOVA), followed by Dunnett's and Sidak's posttests. All statistical analysis was carried out by GraphPad Prism software version 6.01 from GraphPad Software Inc. (San Diego, CA, USA).

Results

Cytotoxicity of the extracts in PC12 cells

According to the MTT test, none of the extracts up to 100 µg/ml reached out the 50% of cell viability (Figure 1). Therefore, the IC_{50} (the half maximal inhibitory concentration) could not be calculated for any of extracts.

The extracts effects on NO production during oxidative stress

The effects of extracts on NO production were determined with Griess assay after inducing oxidative stress (1.5 mM H_2O_2 for 24h) as described previously. As seen in Figure 2, there was a statistical significant difference between stressed control and control groups which treated with DMSO 1% ($p<0.001$) and NO production was increased to 123.18±5.27 percent of control group which assumed to be 100%. *A. jolderensis* extract significantly suppressed NO production in 150 and 200 µg/ml concentrations ($p<0.001$). More effectively, *C. commutatus* extract resulted in 94.8±2.00, 95.55±1.60, 98.68±2.19 and 103.68± 2.39 percent of control group at 50, 100, 150 and 200 µg/ml concentrations, respectively.

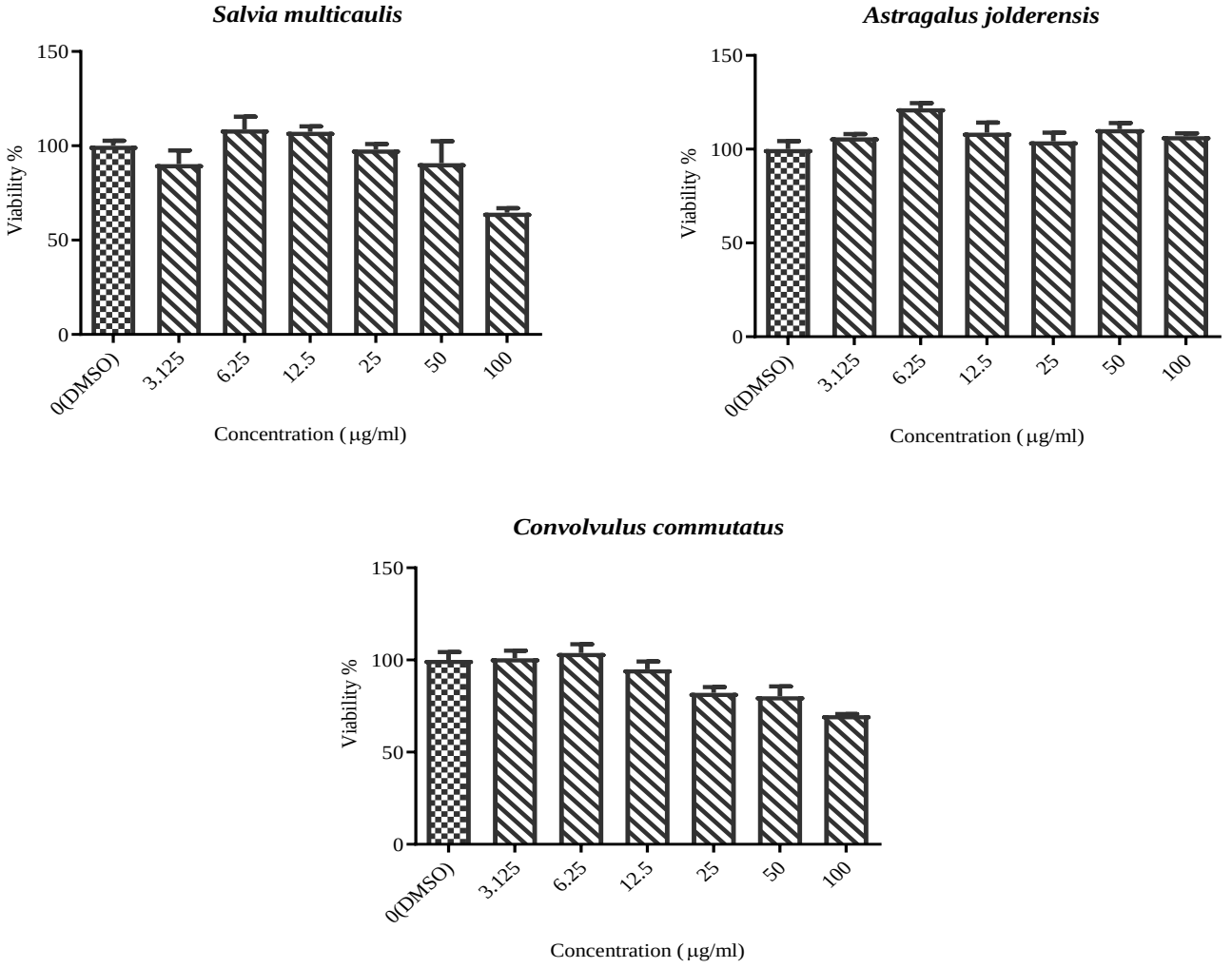

Figure 1. The viability percent of PC12 cells that treated with various concentration of tested plants for 24 h. Data were expressed as percentage of control group mean absorbance (% Viability) and represent as mean ± SEM (n = 6).

Hence *C. commutatus* extract in all concentrations inhibited NO production in stressed PC12 cells ($p<0.001$). Besides methanol extract of *S. multicaulis* prevented NO increasing in stressed cells at 100 and 150 µg/ml concentrations more than in 20 µg/ml ($p<0.001$ versus $p<0.01$).

Cytoprotective effects of extracts in H_2O_2-induced oxidative stress

As aforementioned, after carrying out the Griess test, MTT test was performed in sequence to determine whether the extracts protect cells from oxidative stress alongside the inhibition of NO production or exacerbate the viability of cells. Results (Figure 3) showed the statistically significant difference between control and stressed-control group viability percent ($p < 0.001$), which confirmed the oxidative stress was successfully induced. Methanol extracts of *A. jolderensis* and *C. commutatus* had no effects on cell survival in stress condition. In contrast, *S. multicaulis* extract well protected cells during stress, thus the cell viability percent reached to 92.66±1.18 and 99.77±2.60 percent of the control group at 50 and 100 µg/ml, respectively ($p < 0.001$).

Discussion

Our results showed that the selected medicinal plants have promising effect on NO inhibition in stressed cells. Methanol extract of *A. jolderensis* did not exert any positive or negative effect in cytoprotection during stress condition, but it inhibited NO production at higher doses (150 and 200 µg/ml).

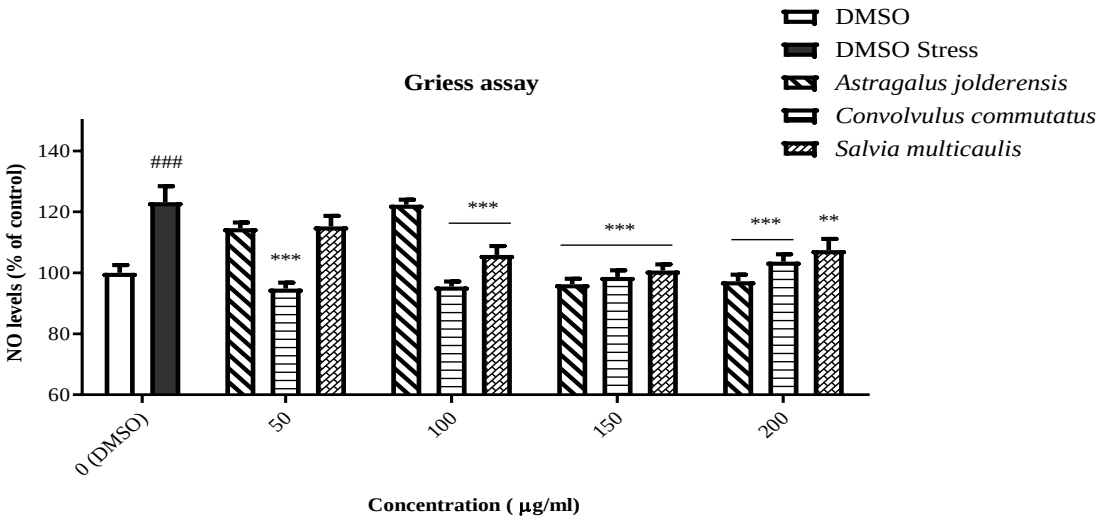

Figure 2. The effect of total extracts on NO production was assessed in PC12 cells that treated with H_2O_2 1.5 mM for 24 h. Data were expressed as percentage of control group mean absorbance (% of control) and represent as mean ± SEM (n = 6). $^{###}$ and *** $p < 0.001$ (** $p < 0.01$) compared to control and stressed-control group, respectively.

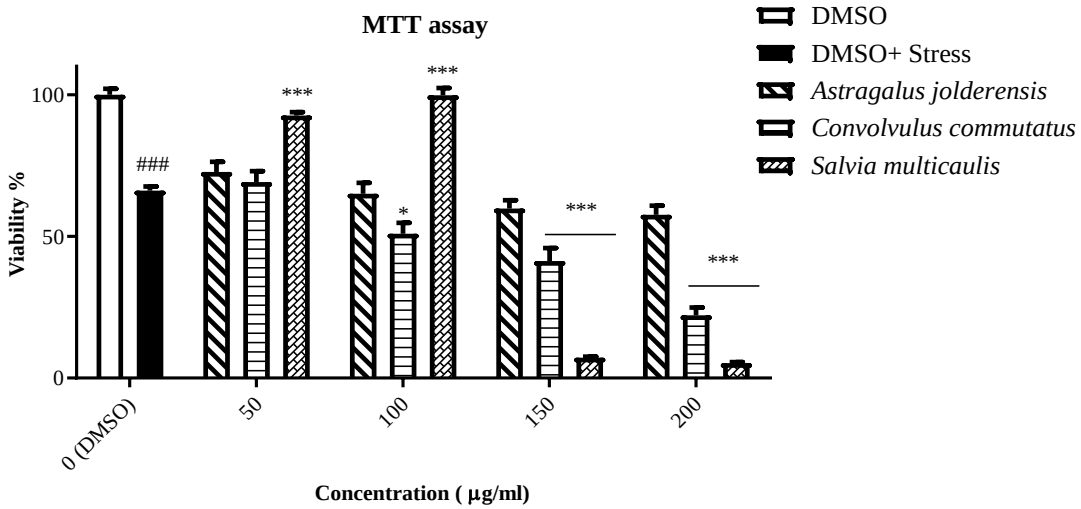

Figure 3. The viability percent of PC12 cells that pre-treated with various concentration of tested plants for 24 h and afterward treated with H_2O_2 1.5 mM for another 24 h. Data were expressed as percentage of control group mean absorbance (% Viability) and represent as mean ± SEM (n = 6). $^{###}$ and *** $p < 0.001$ (* $p < 0.05$) compared to control and stressed-control group, respectively.

Although, biological effects of this species has not been previously investigated, studies showed promising result either *in vivo* or *in vitro* on another species named *A. membranaceus*. Some of those studies were conducted with extracts enriched by specific class of compounds such as flavonoids, saponins or glycosides, therefore evaluation of the fractions of *A. jolderensis* seems to be valued.[18-20,31-33]

In addition, *C. commutatus* extract inhibited NO production in stressed cells at all implied concentrations. Cytotoxic effect was not detected in the unstressed cells either in the present or in the previous study on this extract, however, it lowered the cell viability during the stress condition.[28] Consequently, *C. commutatus* extract might be effective in other biological studies related to the suppression of NO production.

According to our results, extract of *S. multicaulis* inhibited NO production best at higher doses, and protected cells at lower doses. A study investigated effect of novel C20-norabietane diterpenoids which were isolated from *Salvia officinalis* via bioassay-guided fractionation, on lipopolysaccharide (LPS)-induced NO production in RAW264 cells. Accordingly, they exerted NO inhibitory effect and Salofficinoids G was the most potent compound among them [34]. Moreover, antioxidant and anti-inflammatory activity of ethyl acetate and methanol extracts of *S. multicaulis* were observed in previous studies.[35-37]

Conclusion

Studies showed that NO might be associated with many pathophysiological responses including chronic inflammation, neurodegeneration and cancers. On the other side, plants and natural compounds are in the center of studies to find lead compounds which are effective on NO production and therefore related to pathophysiological responses.[38] In that regard, we studied the effect of three herbal extracts, *A. jolderensis*, *C. commutatus* and *S. multicaulis*, on NO production during oxidative stress in PC12 cells.

As discussed, *A. jolderensis* and *C. commutatus* effectively inhibited NO production in stressed PC12 cells and the methanol extract of *S. multicaulis*, showed cell protective effect in addition to inhibition of NO. As aforementioned, previous studies on related species of this plants produced anti-inflammation effects and they could be noticed for further phytochemical and pharmacological researches. From the cell protective effect of *S. multicaulis*, it is a good candidate for further experiments that centralize on the inhibition of NO production and drug discovery studies in the field of neurodegenerative and chronic inflammatory disease.

Conflict of interests

The authors claim that there is no conflict of interest.

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