Avicenna Journal of Phytomedicine (AJP)

Original Research Article

Protective effect of *Viola tricolor* and *Viola odorata* extracts on serum/glucose deprivation-induced neurotoxicity: role of reactive oxygen species

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Article history:
Received: May 10, 2015
Received in revised form: Jun 11, 2015
Accepted: Jun 20, 2015
Vol. 6, No. 4, Jul-Aug 2016, 434-441.

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Keywords:
PC12
Reactive oxygen species
Viola tricolor
Viola odorata

Abstract

Objective: Oxidative stress plays a key role in the pathophysiology of brain ischemia and neurodegenerative disorders. Previous studies indicated that *Viola tricolor* and *Viola odorata* are rich sources of antioxidants. This study aimed to determine whether these plants protect neurons against serum/glucose deprivation (SGD)-induced cell death in an *in vitro* model of ischemia and neurodegeneration.

Methods and Material: The PC12 neuronal cells were pretreated for 4 hr with 1 to 50 µg/ml of *V. odorata* or *V. tricolor* hydroalcoholic extracts followed by 24 hr incubation under SGD condition. Cell viability was evaluated by 4,5-dimethyl-2-thiazolyl-2,5-diphenyl-2H-tetrazolium bromide assay. The level of intracellular reactive oxygen species (ROS) was quantitated by flow cytometry using 2',7'- dichlorofluorescin diacetate as a probe.

Results: SGD condition led to significant decrease in cell viability ($p<0.001$). Pretreatment with both *V. tricolor* and *V. odorata* extracts reduced the SGD-induced cytotoxicity. SGD resulted in a significant increase in intracellular ROS production ($p<0.001$). Both extracts at concentrations of 25 and 50 µg/ml could reverse the increased ROS production ($p<0.05$).

Conclusion: Results of the present study showed that *V. tricolor* and *V. odorata* protect neuronal cells against SGD-induced cell death, at least in part, by their antioxidant activities. Further studies on the possible application of these plants in prevention or treatment of cerebral ischemia and neurodegenerative diseases seem to be warranted.

Please cite this paper as:
Mousavi SH, Naghizade B, Pourgonabadi S, Ghorbani A. Protective Effect of *Viola tricolor* and *Viola odorata* Extracts on Serum/Glucose Deprivation-Induced Neurotoxicity; Role of Reactive Oxygen Species. Avicenna J Phytomed, 2016; 6 (4): 434-441.

Introduction

In spite of remarkable progress in prevention and treatment of cerebral ischemia, it still remains a leading cause of death and incapacitation among the aged population (Amantea et al., 2009). It has been well known that reactive oxygen

species (ROS) are involved in ischemia-induced neuronal cell damage as well as neurodegenerative disorders (Amantea et al., 2009; Behl and Moosmann, 2002). Therefore, a promising approach to neuroprotection is the use of antioxidants, which suppress the effects of ROS (Ochiaia et al., 2004). Recently, there has been an increasing interest toward the use of herbal antioxidants in the prevention and treatment of ischemic and neurodegenerative cell damage.

Viola tricolor and *Viola odorata* (Family Violaceae) are common horticultural plants grown in Iran. *V. tricolor* has been reported to have a number of medicinal features including antioxidant (Piana et al., 2012; Vukics et al., 2008a), anti-inflammatory (Toiu et al., 2009), anti-microbial (Witkowska-Banaszczak et al., 2005), sedative (Ghorbani et al., 2012), and anti-cancer (Mortazavian and Ghorbani, 2012; Mortazavian et al., 2012; Sadeghnia et al., 2014) activities. Traditionally, *V. odorata* has been used to treat anxiety (Keville, 1991), insomnia, and hypertension (Duke et al., 2002). Pharmacological studies have shown that this plant has also diuretic, laxative (Vishal et al., 2009), and antioxidant (Ebrahimzadeh et al., 2010) properties.

This study has attempted to determine whether *V. tricolor* and *V. odorata* extracts protect neurons against serum/glucose deprivation (SGD)-induced cell death. SGD condition is a suitable *in vitro* model for revealing molecular mechanisms involved in neuronal damage following ischemia and investigating neuroprotective agents for management of ischemia-induced brain injury (Hillion et al., 2005; Asadpour et al., 2014; Ghorbani et al., 2015; Woronowicz et al., 2007).

Materials and Methods

Cell line and Reagents

The rat pheochromocytoma cell line (PC12) was obtained from Pasteur Institute (Tehran, Iran). 2, 7-dichlorofluorescin diacetate (DCFH-DA) and 4,5-dimethyl-2-thiazolyl-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma (St Louis, MO, USA). High glucose Dulbecco's modified Eagle's Medium (DMEM, 4.5 g/L), glucose-free DMEM, penicillin-streptomycin solution, and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) was bought from Merck (Darmstadt, Germany).

Preparation of extracts

The aerial parts of *V. tricolor* and *V. odorata* were separately dried, powdered, and extracted with 70% ethanol in a Soxhlet apparatus for 48 hr. The hydroalcoholic extracts were then concentrated on water bath and kept at -20 ºC until use.

Cell culture

PC12 cells were cultured in high-glucose DMEM containing 10% (v/v) FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin under a humidified atmosphere containing 5% CO_2 at 37°C. For cell viability assay, the cells were seeded in 96-well culture plates overnight and then were pretreated with different concentrations (1-50 µg/ml) of *V. odorata* or *V. tricolor* extracts for 4 hr. Thereafter, the cells were exposed to SGD condition for 24 hr by replacing the standard culture medium with a glucose-and FBS-free DMEM containing antibiotic.

MTT assay

Cell viability was determined by MTT assay as described previously (Hajzadeh et al., 2007; Ghorbani et al., 2014). In brief, at the end of incubation under SGD condition, the MTT dye was added to the cell media at final concentration of 0.5 mg/ml. Then, the cells were incubated in a humidified atmosphere containing 5% CO2 at 37°C. After 2 hr, the resulting formazan was solubilized using DMSO and the absorbance was measured at 545 nm.

Measurement of Reactive Oxygen Species

The level of intracellular ROS was determined as described previously with minor modifications (Wang et al., 1999; Ochiaia et al., 2004). Cells were seeded into 24-well culture plate (10^5 cells/well) and pretreated with extracts as described above. After 4 hr of SGD insult, the cells were incubated with 10 µM DCFH-DA at 37ºC for 30 min in the dark. The fluorescence intensity of 2', 7'-dichlorofluorescein (the oxidation product of DCFH-DA) was measured by flow cytometry using 485-nm excitation and 530-nm emission wavelengths. Flow cytometry Analysis was performed using WinMDI software.

Statistics

One-way analysis of variance and Bonferroni's post hoc test were used for data analysis. All results were expressed as mean ± SEM. $p<0.05$ was considered statistically significant.

Results

Effects of *V. tricolor* and *V. odorata* extracts on cell viability

SGD condition significantly reduced cell viability as compared to control condition (100 ± 8.2% *vs* 10±2.4%, $p<0.001$). Pretreatment with 5, 25, and 50 µg/ml of *V. tricolorr* significantly increased the percentage of viable cells to 36.2 ± 1.2% ($p<0.05$), 44.4 ± 5.8% ($p<0.01$), and 45.8 ± 7.6% ($p<0.01$), respectively (Figure 1). Similarly, *V. odorata* at concentrations of 5, 25, and 50 µg/ml was able to increase the percentage of viable cells to 32.4 ± 5.3% ($p<0.05$), 34.5 ± 1.8% ($p<0.01$), and 36.7 ± 3.7% ($p<0.01$), respectively (Figure 2).

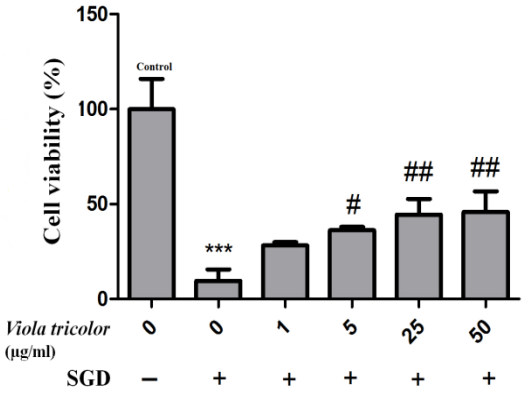

Figure 1. Effect of *Viola tricolor* on viability of PC12 cells under serum/glucose deprivation (SGD) condition. The cells were pretreated for 4 hr with *V. tricolor* extract and then exposed to SGD for an additional 24 hr. The cell viability was expressed as the percentage of cells cultured in high-glucose medium (control). The data presented are means ± SEM of three independent experiments (n = 3). *** $p<0.001$ SGD compared to control. # $p<0.05$, ## $p<0.01$, and ### $p<0.001$ compared to concentration of 0 µg/ml in SGD condition.

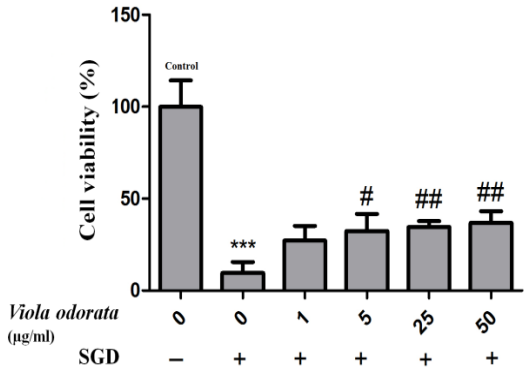

Figure 2. Effect of *Viola odorata* on viability of PC12 cells under serum/glucose deprivation (SGD) condition. The cells were pretreated with *V. odorata* extract for 4 hr and then exposed to SGD for an additional 24 hr. The cell viability was expressed as the percentage of cells cultured in high-glucose medium (control). The data presented are means ± SEM of three independent experiments (n = 3). *** $p<0.001$ SGD compared to control. # $p<0.05$, ## $p<0.01$, and ### $p<0.001$ compared to concentration of 0 µg/ml in SGD condition.

Effects of *V. tricolor* and *V. odorata* extracts on ROS production

Production of intracellular ROS in PC12 cells was significantly increased after 4 hr of SGD insult, as compared to that in cells cultured under control condition (11.2 ± 0.2% versus 0.45 ± 0.15%, p<0.001). Pre-incubation with 25 and 50 µg/ml of *V. tricolor* extract significantly decreased the SGD-induced ROS accumulation to 6.65 ± 0.65% (p<0.05), and 1.05 ± 0.75% (p<0.01), respectively (Figures 3 and 4). Similarly, the presence of 5, 25 and 50 µg/ml of *V. odorata* extract in cell medium reduced the ROS content from 11.2 ± 0.2% to 1.5 ± 0.7% (p<0.001), 0.9 ± 0.6% (p<0.001) and 1.05 ± 0.05 (p<0.001), respectively (Figures 5 and 6).

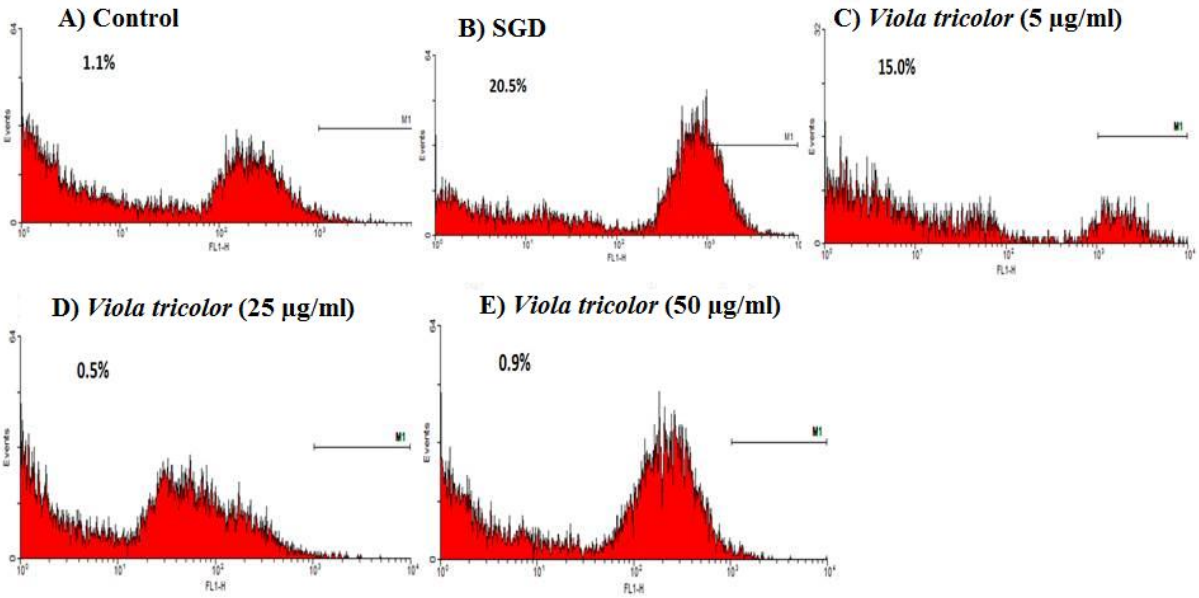

Figure 3. Flow cytometry for measuring ROS production in PC12 cells pretreated with *Viola tricolor*. A: control cells; B: Cells under serum/glucose deprivation (SGD) condition; C-E: pretreatment with 5, 25, and 50 µg/ml *Viola tricolor*, respectively, followed by 4 hr exposure to SGD.

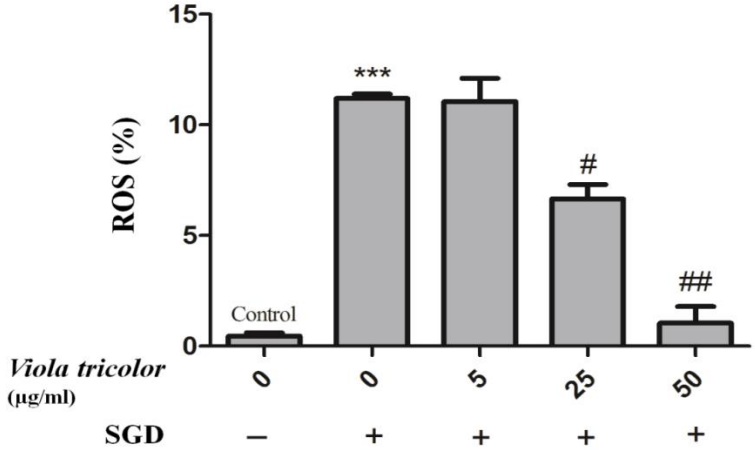

Figure 4. Effect of *Viola tricolor* on the level of intracellular reactive oxygen species (ROS) in PC12 cells under serum/glucose deprivation (SGD) condition. The cells were pretreated with *V. tricolor* extract for 4 hr and then exposed to SGD for an additional 24 hr. The values represent 5 independent experiments. *** p<0.001 SGD compared to control; # p<0.05 and ## p<0.01 compared to concentration of 0 µg/ml in SGD condition.

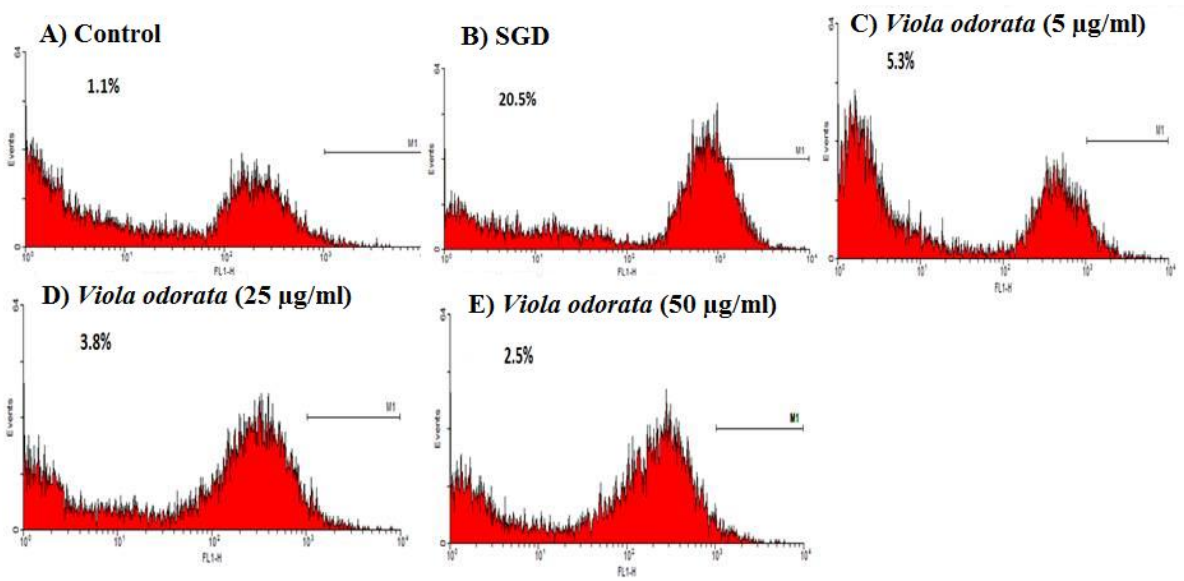

Figure 5. Flow cytometry for measuring ROS production in PC12 cells pretreated with *Viola odorata*. A: control cells; B: Cells under serum/glucose deprivation (SGD) condition; C-E: pretreatment with 5, 25, and 50 µg/ml *Viola odorata*, respectively, followed by 4 hr exposure to SGD.

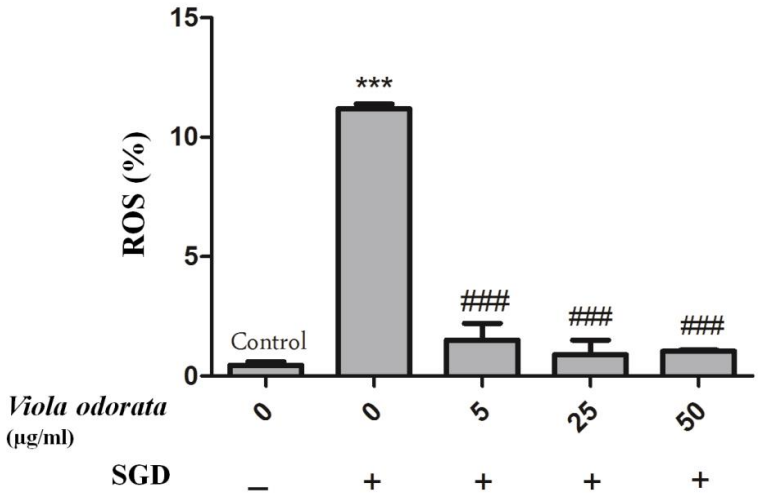

Figure 6. Effect of *Viola odorata* on the level of intracellular reactive oxygen species (ROS) of PC12 cells under serum/glucose deprivation (SGD) condition. The cells were pretreated with *V. odorata* extract for 4 hr and then exposed to SGD for an additional 24 hr. The values represent 5 independent experiments. *** $p<0.001$ SGD compared to control; ### $p<0.001$ compared to concentration of 0 µg/ml in SGD condition.

Discussion

Oxidative stress is a key deleterious factor in neuronal cell damage during brain ischemia which is also involved in other neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, and traumatic brain injury (Manzanero et al., 2013; Navarro-Yepes et al., 2014). In acute ischemia, the increased level of ROS can cause oxidative damage to cellular macromolecules including lipids, proteins, and nucleic acids. Polyunsaturated fatty acids of lipid membranes are especially vulnerable to ROS-induced lipid peroxidation. The oxidation of these fatty acids rises the hydrophilic nature of the membrane resulting in alteration in fluidity and permeability. Also, the function of membrane bound receptors and enzymes is inhibited (Fisher et al., 2001; Manzanero et al., 2013). Therefore, utilization of novel antioxidant agents might be a good therapeutic approach against neuronal damage during brain ischemia (Gilgun-

Sherki et al., 2002; Love et al., 1999; Navarro-Yepes et al., 2014). In ischemia, restriction of blood flow results in deficiency of oxygen, glucose and serum growth factors leading to neuronal damage (Broughton et al., 2009). Deprivation of cultured neurons from serum and glucose provides a reliable *in vitro* method for studying pathological process of cerebral ischemia and for development of new agents for management of ischemia (Hillion et al., 2005; Asadpour et al., 2014; Ghorbani et al., 2015; Woronowicz et al., 2007). In this study, we utilized the SGD-induced insult in PC12 cells and showed that hydroalcoholic extracts of *V. tricolor* and *V. odorata* are able to inhibit neuronal damage under SGD condition. SGD condition led to a 90% decrease in cell viability, which was comparable to that observed previously (70-90% cell death) (Alinejad et al., 2013; Sadeghnia et al., 2012; Forouzanfar et al., 2013; Mousavi et al., 2010). Also, consistent with previous reports, SGD condition resulted in an enhancement of intracellular ROS level (Ghorbani et al., 2015; Forouzanfar et al., 2013). Pretreatment with *V. tricolor* and *V. odorata* effectively blocked the SGD-induced ROS production, indicating that an inhibition of intracellular ROS generation might be involved in the neuroprotective effects of *V. tricolor* and *V. odorata*. Consistent with our findings, Ebrahimzadeh et al. (2010) evaluated the Fe^{2+}-chelating ability and 1,1-diphenyl-2-picryl hydrazyl radical-scavenging activity of *V. odorata* methanolic extract and demonstrated that it has antioxidant and free radical scavenging properties. Also, it has been reported that the crude extract of *V. tricolor* flowers shows much better antioxidant capacity than ascorbic acid (Piana et al., 2012). The antioxidant effect of these plants may be due to their flavonoid compounds such as rutin and violantin (Piana et al., 2012; Vukics et al., 2008a; Vukics et al., 2008b; Ebrahimzadeh et al., 2010; Gonçalves et al., 2012). For example, the antioxidant properties of rutin have been demonstrated in both *in vitro* and *in vivo* studies (Yang et al., 2008; Sadeghnia et al., 2013). Also, in a middle cerebral artery occlusion animal model of brain ischemia, it has been shown that pretreatment with rutin attenuates ischemic neural apoptosis by increasing endogenous antioxidant enzymatic activities (Khan et al., 2009).

In conclusion, the results of the present study showed that *V. tricolor* and *V. odorata* protect neuronal cells against SGD-induced cell death through their antioxidant mechanisms. Further studies on the possible application of these plants in prevention and/or treatment of cerebral ischemia and neurodegenerative diseases seem to be warranted.

Acknowledgement

This study has been financially supported by a grant (No. 921057) from Research Affairs of Mashhad University of Medical Sciences, Mashhad, Iran. This study was a part of an MSc thesis. The authors would like to appreciate Ms. Aghaee for her assistance in the preparation of *V. tricolor* and *V. odorata* extracts. Also, we are grateful to Mr. Malaeke for his assistance in flow cytometry.

Conflict of interest

The authors have no conflict of interests to declare.

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