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Protective Effects of Glabridin against Cytotoxicity and Oxidative Stress Induced by Doxorubicin in PC12 Cells

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This study was aimed at investigating effects of glabridin (Glab) isolated from *Glycyrrhiza glabra* (*G. glabra*) against cytotoxicity and oxidative stress induced by Doxorubicin (Dox), a chemotherapeutic agent with neurotoxic side effects, in PC12 cells. Glab was obtained from ethyl acetate extract of licorice roots by using solid phase extraction (SPE) and preparative HPLC. To evaluate whether Glab protects PC12 cells from Dox-induced cytotoxicity, we examined the direct cytotoxic effect of Dox on PC12 cells in the presence and absence of Glab at different time interval. Dox-induced cytotoxicity in a concentration-dependent manner. The IC_{50} value was 20.1 μM for 24 h exposure. However, 24 h pretreatment of cells with Glab protected cells from Dox-induced cytotoxicity. For evaluation of the effect of Glab on Dox-induced oxidative stress, the generation of reactive oxygen species (ROS) and also the glutathione (GSH) level were assayed. Adding Dox to PC12 cells caused a significant increase in ROS level (164%). Pretreatment with Glab (2.5μM) decreased significantly intracellular ROS levels to 86±4.3% in PC12 cells. Moreover, Dox decreased the GSH level by more than 40% of the control. Pretreatment with 2.5 and 5 μM of Glab increased significantly the GSH level to 77±3.3% and 86±3.8%, respectively. Taken together our observation indicated that Glab has the protective effect against cytotoxicity induced by Dox in the PC12 cells. The results highlighted that Glab may exert neuroprotective effects through its antioxidant actions.

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Introduction

Oxidative stress plays an effective role in the pathogenesis of many diseases including cancer, diabetes, schizophrenia, Alzheimer's disease, Parkinson's disease as well as contributing to the aging process [1-4]. Plants produce a variety of antioxidants against molecular damages caused by ROS. Phenolic compounds compose the major class of plant-derived natural antioxidants. Among the various phenolic compounds, the flavonoids are perhaps the most important group [5,6]. Flavonoids are components of a wide variety of edible plants such as fruits, vegetables, and grains and are an integral part of the human diet. They are able to scavenge free radicals and to prevent oxidation. The root extracts of *Glycyrrhiza glabra* L. (*G. glabra*) (Fabaceae) have been exhibited antioxidant activity and several antioxidant flavonoids, such as glabridin [(R)-4-(3,4-dihydro-8,8-dimethyl)-2H,8H-benzo[1,2-b:3,4-b′]dipyran-3yl)-1,3-benzenediol] (Glab), have been isolated from this plant [7]. Glab (Figure 1) is a prenylated isoflavonoid that for the first time in 1976 was isolated from the roots of *G. glabra* L. (licorice) [8-10]. It is the major flavonoid of licorice root that the content of it in the plant root varies between 0.07–0.8%w/w [8,11].Glab has exhibited a wide variety of pharmacological activities. It has beneficial effects on the skin through an inhibitory effect on melanogenesis and inflammation [12, 13]. The other biological effects of this compound include antibacterial activity against *Helicobacter pylori* and methicillin-resistant *Staphylococcus aureus*, antifungal, hypolipidemic, hypoglycemic, antiatherosclerotic, estrogenic, cytotoxic and anti-proliferative activity against human breast cancer cells [8, 12-17]. This flavonoid has protective effects on low-density lipoprotein oxidation and skin carcinogenesis and also has revealed cardio-protective, neuroprotective and nephroprotective activities [13, 16, 18, 19]. It has been shown that Glab protects mitochondrial functions from oxidative stresses and prevents the oxidative DNA fragmentation and the activation of apoptosis-associated proteins in human keratinocytes [14,20]. According to the observed effects of Glab, today this compound is widely used in the pharmaceutical, food and cosmetic industries [15].

Doxorubicin (Dox) is one of the most potent broad-spectrum antitumor anthracycline antibiotics, widely used to treat a variety of cancers [21, 22]. On the other hand, the clinical use of Dox has been restricted because of its serious side effects and toxicities in the nonspecific organs [21, 23]. Dox exerts its toxic effects on various organs and cells such as heart, liver, lung, brain, kidney, testis and blood cells [21, 24-26]. The mechanisms by which Dox causes its cytotoxic effects have not been fully understood [27]. It is believed that oxidative stress and the formation of free radicals (especially ROS) play a crucial role in the mechanism of Dox toxicity [22,24,28,29] .However, other various mechanisms have been suggested, such as the formation of iron complexes, perturbation of calcium homeostasis, intercalation into DNA, interaction with topoisomerases, disruption of mitochondrial functions and damage to cell membranes [24,27,30]. In our previous studies, we demonstrated that Dox is able to induce oxidative stress and apoptosis in PC12 cells, as an appropriate in vitro model for assessment of neural cell death [31]. So, according to antioxidant and protective effects and radical scavenging activity of Glab, in the current study, we examined the protective effect of Glab isolated from *G. glabra* on neural cell death induced by Dox in PC12 cells.

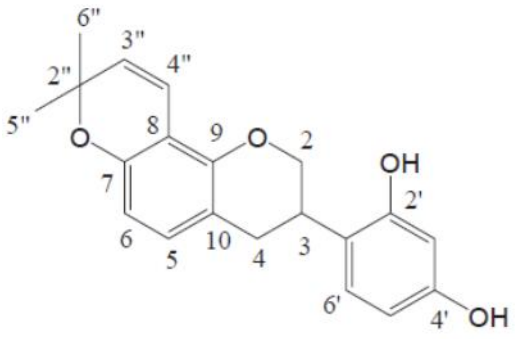

Fig. 1. Chemical structure of glabridin.

Materials and Methods

Materials

All solvents (n-hexane, ethyl acetate and methanol) were purchased from Merck (Germany). Also, HPLC-grade methanol and Dox hydrochloride were prepared from Merck (Germany) and Sigma-Aldrich (United States), respectively. Deuterated chloroform (CDCI3, 99.8% D atom) was purchased from Acros Organics (United States). Sep-Pak Silica® cartridge (10g/60ml) was prepared from Waters (United States). PerfectSil Target ODS-3® HPLC column (250×4.6 mm, 5 µm) and VertiSep® GES C-18 HPLC column (250×21 mm, 10 µm) were from Knauer (Germany) and Vertical (Thailand), respectively. 3-(4,5-dimethylthiazol-2yl)-2,5–diphenyltetrazolium bromide (MTT), dichlorfluorescein-diacetate (DCF-DA), and glutathione (GSH) level detection kit were purchased from Sigma Aldrich (St Louis, MO, USA).

Preparation of Plant Material

The roots of *G. glabra* were collected from farms around the city of Islamabad-Gharb in Kermanshah province (Iran). To identify and verify the scientific name of the plant, shoots and roots were harvested in July and November respectively. The plant was authenticated by Herbarium of the Ferdowsi University of Mashhad (voucher specimen number 25947).

Preparation of Crude Extract

Fifty grams of dried powder of licorice roots (*G. glabra* L.) was extracted on the stirrer with 500 mL ethyl acetate for 24 h at room temperature. After filtration, the filtrate was collected and the residue was extracted again (two times) with the same volume of fresh solvent. The combined extracts were evaporated to dryness on a rotary evaporator under reduced pressure below 40˚C.

Solid Phase Extraction (SPE)

SPE of Glab from crude extract was performed in a Sep-Pak Silica® cartridge (10g/60ml) packed with silica gel. Two grams of crude extract was dissolved in 4mL of ethylacetate-hexane (1:4 v/v). The SPE cartridge was conditioned with 150mL of hexane and 150mL of ethylacetate-hexane (1:4, v/v) successively. Then 4mL of the sample solution was loaded on the cartridge. After loading, the cartridge was sequentially rinsed with 150mL of ethylacetate-hexane (1:4, 1:2, 1:1, 1:0,v/v) solutions. Four fractions were collected and all fractions were separately dried below 40˚C under vacuum by a rotary evaporator. The residues were dissolved in methanol by sonication and filtered through a 0.22 µm membrane and then were analyzed by analytical HPLC before injecting to preparative HPLC.

HPLC Analysis of Fractions

In order to identify the faction that has the most content of Glab, all fractions were analyzed by an analytical method using reversed-phase HPLC-PDA. The instrument used was Knauer HPLC system equipped with a Smartline 1000 quaternary pump version 7603, manager 5000 version 7602, UV detector 2600 version 7605, dynamic mixing chamber version 1119-1 and

Chrom Gate HPLC software 3.1.7. Samples were injected by using a rheodyne injector fitted with a 20 µL loop. The analytical separation was performed on aC18 column (250×4.6 mm, 5 µm). Detection was carried out by UV at 225 nm. The solvent system consisted of 65% (v/v) methanol in water for 30 min, then the percentage of methanol was raised to 100% (v/v) over 2 min and run isocratically for 20 min to purge the column. The flow rate was 0.8 ml/min. After analyzing all the fractions, a fraction that had the highest content of Glab, was selected for purification of Glab.

Purification of Glab

Glab was purified by reversed-phase preparative HPLC from the fraction of 1:2 ethylacetate-hexane which had the highest content of Glab. Preparative HPLC separation was carried out on a Knauer HPLC system. The preparative HPLC column applied in this work was a C-18 column (250×21 mm, 10 µm). The separation was performed using gradient elution (0-200 min) with a flow rate of 4 ml/min and a detection wavelength of 225 nm. The solvent system consisted of 65% (v/v) methanol in water for 120 min, then the percentage of methanol was raised to 100% (v/v) over 5min and run isocratically for 75 min. Glab was collected between 88 and 96 min. The solvent was dried at 40˚C under vacuum on a rotary evaporator to obtain a purified compound. The purity of Glab was assessed using analytical HPLC by peak area calculation. Finally, the structure of Glab was characterized on the basis of UV, ^{1}H- and ^{13}C-NMR spectral data.

Cell Culture Conditions

PC12 cells (NCBI Code: C153) were obtained from Institute of Pasture (Iran). The PC12 cells maintained in Dulbecco's modified Eagle's medium (DMEM-F12) with 10% (V/V) heat-inactivated fetal bovine serum (FBS), penicillin G (100 U/mL) and streptomycin (100 mg/mL) at 37˚C in 95% CO2 humified incubator. The medium was changed every 2-3 days and sub-cultured when the cell population density reached to 70–80% confluence. Cells were seeded at an appropriate density according to each experimental design.

Cell Viability Assay

Cellular toxicities of Dox and Glab were evaluated in PC12 cells using MTT method. Four sets of experiments were performed at standard culture conditions: (1) untreated control cells, (2) cells were treated with different concentrations of Dox (0-200 μM), (3) cells were treated with different concentrations of Glab (0–50 μM), and (4) cells were pretreated with different concentrations of Glab for 1, 24 and 48 h then medium was changed and cells were treated with IC_{50} concentration of Dox for another 24 h. The viability of cells was analyzed using MTT methods. Briefly, after treatment, 20 μL of a 5 mg/mL MTT solution was added to each well. After 3 h incubation, the medium was carefully aspirated and the purple formazan crystals were solubilized with 100 μL DMSO. Optical density was measured at 570 nm (reference wavelength 630 nm) in a microplate reader (Bio-Tek, ELX 800, USA). The absorbance of the untreated culture was set at 100%.

Determination of Intracellular ROS

Intracellular ROS levels were examined using DCF-DA. DCF-DA is a non-fluorescent lipophilic ester that easily crosses the plasma membrane. Into the cytosol, the acetate group is rapidly removed by unspecific esterases. The oxidation of this molecule to the fluorochrome DCF results in green fluorescence. The intensity of this fluorescence is generally considered to reflect the level to which ROS are present [12]. After seeding for 24 h, PC12 cells were washed with PBS buffer (pH 7.4). The cells pretreated with Glab (1.25, 2.5

and 5 μM) for 24 h were then treated with IC_{50} concentration of Dox for an additional 24 h. After washing with PBS, the cells were incubated with 20μL DCF-DA at 37°C for 30 min. The percentage of DMSO in solution did not exceed 0.5%. After incubation, cells were lysed with Triton X-100. The fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 528 nm using a fluorescence microplate reader (BioTek, H1M, USA).

Measurement of total glutathione (GSH)

PC12 cells (1×10^5 cells/ml) were treated with different concentration of Glab then IC_{50} concentration of Dox was added to cells. After 24 h the GSH (reduced form GSH + oxidized form GSSG) was determined according to the user's manual for the Sigma Glutathione Colorimetric Detection Kit (USA). Briefly, cells (5×10^5 cells) were centrifuged at 700 ×g for 5 min at 4°C and the supernatants were removed. The pellets were washed with ice-cold PBS, lysed in 80 μl ice-cold Glutathione Buffer and incubated on ice for 10 min. Then the samples were dissolved with 5% 5-sulfosalicylic acid (SSA, 20 μl) and centrifuged at 8000 ×g for 10 min. The supernatants (20 μl) were incubated in 160 μl of the reaction mix at room temperature for 10 min and then substrate solution (20 μl) was added. Next, the mixture was incubated for a further 10 min. Finally, absorbance was measured at 415 nm using a microplate reader. The standard curve was obtained from the absorbance of the diluted GSH standard that was incubated in the mixture as in samples. The results of the samples were referred to those of a standard curve of GSH.

Statistical Analysis

Each experiment was performed at least three times and the results were presented as mean ± SEM. One-way analysis of variance (ANOVA) followed by Turkey's test was used to compare the differences between means. A probability value of $P<0.05$ was considered to be statistically significant.

Results

Purification of Glab

Chromatogram obtained from preparative HPLC has been shown in Figure 2. Glab (figure 1) was purified as a yellow to light brown amorphous solid with purity 95.8% (by HPLC, Figure 3). Information obtained from spectroscopic studies of the purified Glab is summarized as follows:
UV λ_{max}(Ethanol): 226.5nm, 280.5nm. ^{1}H NMR (300MHz, $CDCl_3$): δ 1.44*s* (3H, H-5″), 1.45*s* (3H, H-6″), 2.84*dd* (J=15.34Hz, J=4.21Hz, 1H, H-4), 2.97*dd* (J=15.64Hz, J=10.53Hz, 1H, H-4), 3.49*m* (1H, H-3), 4.02*t* (J=9.93Hz, 1H, H-2), 4.37br*d* (J=9.33Hz, 1H, H-2), 5.58*d* (J=9.93Hz, 1H, H-3″), 6.40*d* (J=8.12Hz, 2H, H-6, H-5′), 6.41*s* (1H, H-3′), 6.67*d* (J=9.93Hz, 1H, H-4″), 6.83*d* (J=8.42Hz, 1H, H-5), 6.94br*d* (J=7.82Hz, 1H, H-6′). ^{13}C NMR (75MHz, $CDCl_3$): δ 27.5 (C-5″), 27.7 (C-6″), 30.5 (C-4), 31.6 (C-3), 70.1 (C-2), 75.7 (C-2″), 103.2 (C-3′), 107.9 (C-5′), 108.8 (C-6), 110.0 (C-8), 114.5 (C-10), 116.9 (C-4″), 120.0 (C-1′), 128.4 (C-6′), 129.0 (C-3″), 129.2 (C-5), 149.7 (C-9), 151.8 (C-7), 154.5 (C-2′), 155.2 (C-4′).
The structure of purified compound was confirmed by comparing the spectral data obtained from purified compound with literature data of Glab [7, 9, 32, 33].

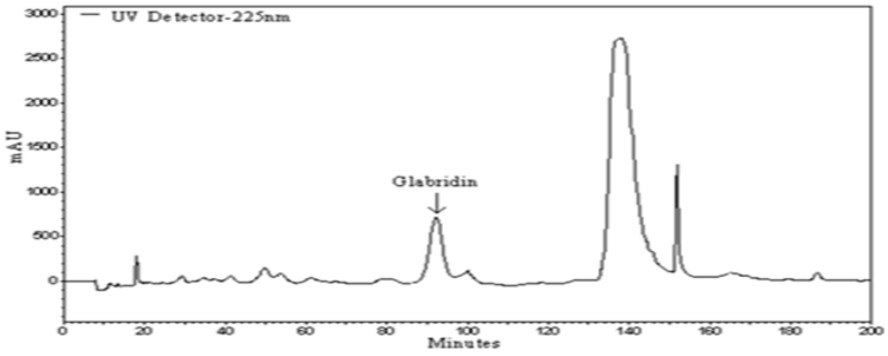

Fig. 2. Preparative HPLC chromatogram of the fraction of 1:2 ethyl acetate-hexane (column: C-18 250×21 mm, 10 µm; mobile phase: 65% (v/v) methanol in water for 120 min, then the percentage of methanol was raised to 100% (v/v) over 5 min and run isocratically for 75 min; flow rate: 4 ml/min; detection wavelength: 225 nm.

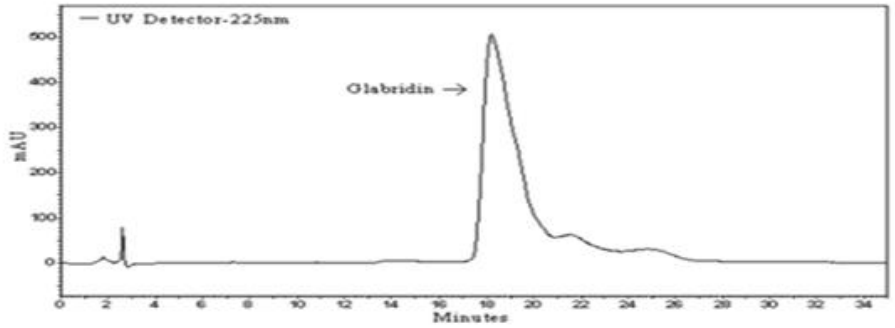

Fig. 3. Analytical HPLC chromatogram of the purified Glab (column: ODS 250×4.6 mm, 5 µm; mobile phase: methanol/water 70/30; flow rate: 0.8 ml/min; detection wavelength: 225 nm).

Cell viability after exposure with Dox and Glab alone

The viability of PC12 cells was evaluated after 24 h exposure to different concentrations of Dox. Cell viability was evaluated by the MTT method. As shown in Figure 4a, Dox-induced cytotoxicity was dose dependent. The IC_{50} value (mean ± SEM) was 20.1 ± 0.018 μM for 24 h exposure. In order to set Glab at concentrations which are non-toxic to cells but could prevent Dox-induced cytotoxicity, we also examined the effects of different concentrations of Glab on cell viability in PC12 cells. Figure 4b clearly revealed that 24 h treatment with Glab, had no cytotoxic effect at the concentrations up to 5 µM, while 24 h exposure to 8 µM of Glab decreased viability of cells to 50%.

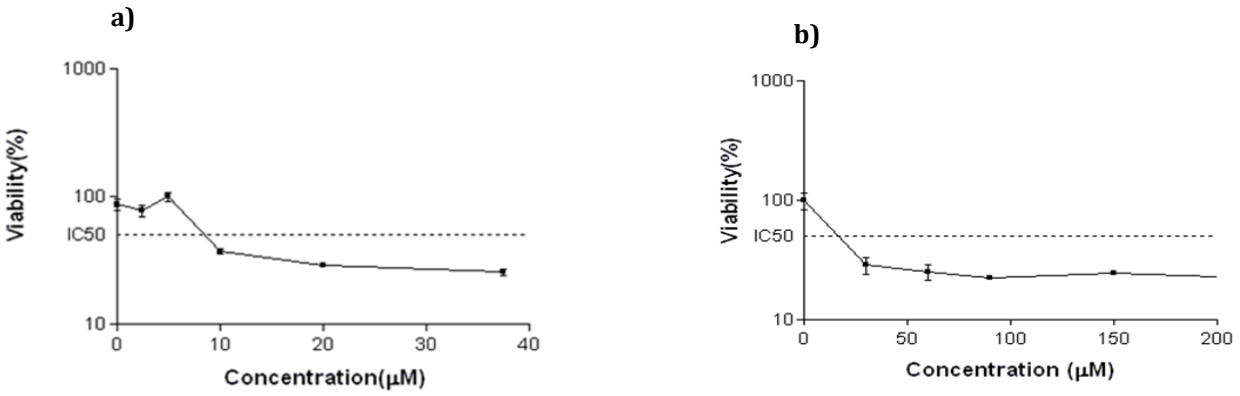

Fig. 4. The effects of a) Glab and b) Dox on PC12 cell viability. The cell viability was determined by MTT assay as described in material and methods. Data are expressed as the mean ± SEM of three separate experiments (N=6).

Effect of Glab pretreatment on Dox-induced cell death

For evaluation of the effect of Glab pretreatment on Dox-induced cytotoxicity, PC12 cells were pretreated for different time interval with non-toxic concentrations of Glab (1.25, 2.5 and 5), then the medium was changed and cells were treated with IC50 concentration of Dox for another 24 h. As shown in the figure 5c compared to Dox-treated cells, the 24 h pretreatment with Glab (1.25, 2.5 and 5 μM) increased significantly PC12 cell viability. Pretreatment with one and six hours had no notable protective effect against cytotoxicity induced by Dox (5a and b). Therefore 24 h pretreatment with Glab was chosen for further studies.

Effect of Glab on ROS Induced by Dox in PC12 Cells

In order to measure oxidative stress induced by Dox, fluorescent dye DCF-DA was used to measure ROS generation. As anticipated adding Dox to PC12 cells caused a significant increase in ROS level (164%). Next, we investigated the inhibitory effect of different concentration of Glab on ROS production in the presence of Dox. Pretreatment with Glab (2.5 μM) isolated from *G. glabra* decreased significantly intracellular ROS level to 86±4.3% in PC12 cells (Figure 6).

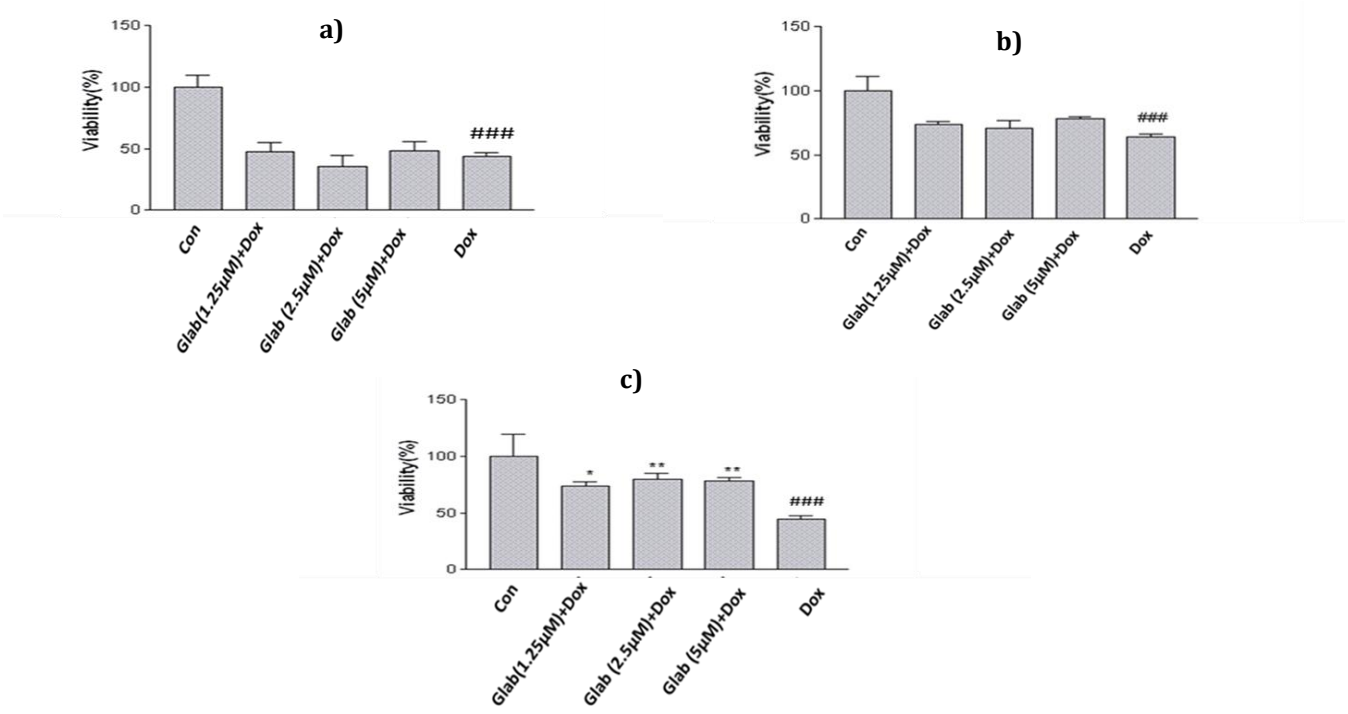

Fig. 5. The effect of a) 1 h, b) 6 h and c) 24 h pretreatment with different concentrations of Glab on Dox (20.1 μM) induced cytotoxicity in PC12 cells. The cell viability was determined by MTT assay as described in material and methods. Data are expressed as the mean ± SEM of three separate experiments (n= 6). ### p<0.001 vs. Control, **p<0.01, *p<0.05 vs. Dox treated cells.

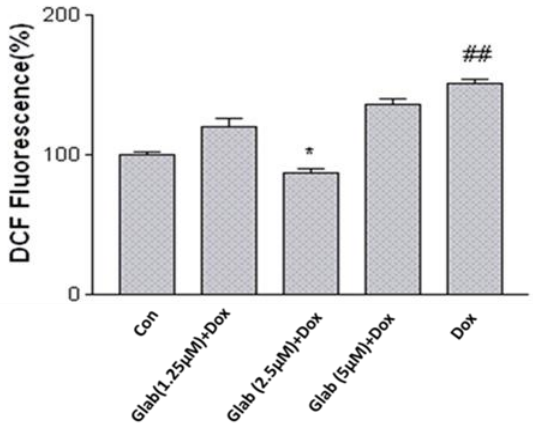

Fig. 6. The effect of Glab pretreatment on DOX-induced ROS generation. Cells were pretreated with three different concentrations of Glab for 24 h followed by incubation with IC_{50} concentration of Dox (20.1 μM) for another 24 h. Data are expressed as the mean ± SEM of three separate experiments (n = 4). ## p<0.01 vs. Control, * p<0.05 vs. Dox treated cells.

Effect of Glab on intracellular GSH levels

To examine whether Glab prevents GSH depletion, we measured the levels of intracellular GSH. Figure 7 shows the effect of Dox on intracellular GSH levels in the presence and absence of Glab. This figure clearly reveals 24 h exposure with 20.1 μM of Dox decreased the GSH level by more than 40% of the control in PC12. Pretreatment with 2.5 and 5 μM of Glab increased significantly the GSH level to 77±3.3 and 86±3.8%, respectively.

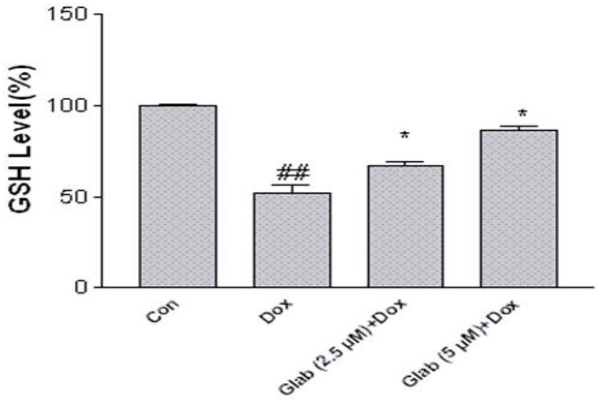

Fig. 7. The effect of Glab pretreatment on Dox-induced GSH depletion. Cells were pretreated with three different concentrations of Glab for 24 h followed by incubation with Dox (20.1 μM) for another 24 h. Data are expressed as the mean ± SEM of three separate experiments (N=4). ## $p<0.01$ vs. Control, * $p<0.05$ vs. Dox treated cells.

Discussion

Plant-derived bioactive compounds known as phytochemicals are rich in antioxidant and free radical scavenging properties [34]. Many research studies have been carried out to identify plants with significant antioxidant potential by analyzing their radical scavenging activities using both in-vitro and in-vivo systems [35]. Glab is known for antioxidant properties and acts as a free radical scavenger by inhibiting lipid peroxidation and oxidative DNA damage. Additionally, it has been proposed as a candidate to treat cardiovascular disease involving oxidative stress [36]. Dox, a well-known chemotherapeutic drug, can induce considerable oxidative stress inside the cell. This stress can lead to a number of unwanted side effects on nonspecific organs such as the heart and brain. In the current study, we aimed to assess the neuroprotective activity of Glab on Dox-induced cytotoxicity and oxidative stress in PC12 cell line as a widely accepted model of neuronal cells [31]. The obtained result showed that Glab had no cytotoxic effect on PC12 cells at concentrations below 5 μM. Dox, as has been previously reported, was able to induce oxidative stress and cytotoxicity in PC12 cells [31,37-38]. In agreement with our previous studies, evidence from the current investigation suggested that oxidative stress plays a significant role in Dox-induced cell injury in this model. DOX significantly increased intracellular ROS, and also decreased GSH level which may eventually lead to cytotoxicity in PC12 cells.

Previous studies demonstrated that Dox treatment induces lipid peroxidation in the brain, heart, liver, lung, and kidney tissues [38,39]. Moreover, in the another study, it has been showed that Dox increases the brain mitochondria permeability to Ca^{2+} thereby predisposing brain cells to oxidative stress and death [40]. When the protective effect of Glab on Dox-induced cytotoxicity was examined, it was observed that the pretreatment of PC12 cells with sub-toxic concentrations of Glab markedly protected the PC12 cells from Dox-induced cytotoxicity. GSH is one of the endogenous antioxidants that protect cells against the deleterious effects of ROS [41]. We suggested that a marked increase in the GSH level prevent the accumulation of free radicals inside PC12 cells thus reducing oxidative stress. Therefore, this result indicated that Glab may protect the PC12 cells against cytotoxicity induced by Dox by preventing increased oxidative stress. The present findings corroborate similar finding by XQ et al who evaluated the effect of Glab on staurosporine-induced damage in cultured rat cortical neurons. Their findings indicated that Glab had a neuroprotective effect via inhibition of superoxide production and modulation of multiple pathways associated with apoptosis [42].

Conclusion

In summary, our findings imply that Glab isolated from *G. glabra* attenuates cytotoxicity and oxidative stress injury induced by Dox in PC12 cells. However, further studies are necessary to determine neuroprotective mechanisms before definite conclusions can be drawn.

Acknowledgment

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Conflict of Interests

Authors certify that there is no actual or potential conflict of interest in relation to this article.

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