

Protective effects of fractions from *Artemisia biennis* hydro-ethanolic extract against doxorubicin-induced oxidative stress and apoptosis in PC12 cells

Mahdi Mojarrab¹, Mehran Mehrabi², Farahnaz Ahmadi¹, Leila Hosseinzadeh^{1*}

¹ Pharmaceutical Sciences Research Center, School of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, Iran

² Students Research Committee, Kermanshah University of Medical Sciences, Kermanshah, Iran

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ABSTRACT

Objective(s): This study was designed to indicate whether different fractions from *Artemisia biennis* hydroethanolic extract could provide cytoprotection against oxidative stress and apoptosis induced by doxorubicin (DOX) in rat pheochromocytoma cell line (PC12).

Material and Methods: Cell viability was determined by MTT assay. Also, activation of caspase-3 and superoxide dismutase were evaluated by spectrophotometry. Detection of reactive oxygen species (ROS) and measurement of mitochondrial membrane potential (MMP) were performed by flowcytometry.

Results: Treatment of PC12 cells with DOX reduced viability dose dependently. For evaluation of the effect of fractions (A-G) on DOX-induced cytotoxicity, PC12 cells were pretreated for 24 hr with the *A. biennis* fractions and then cells were treated with DOX. The fractions C and D increased PC12 cells viability significantly compared to DOX treated cells. Moreover, pretreatment with fractions C and D for 24 hr attenuated DOX-mediated apoptosis and the anti-apoptotic action of *A. biennis* fractions was partially dependent on inhibition of caspase 3 activity and also increasing the mitochondrial membrane potential (MMP). Selected *A. biennis* fractions also suppressed the generation of ROS and increased superoxide dismutase (SOD) activity.

Conclusion: Taken together our observation indicated that subtoxic concentration of aforementioned fractions of *A. biennis* hydroethanolic extract has protective effect against apoptosis induced by DOX in PC12 cell. The results highlighted that fractions C and D may exert cytoprotective effects through their antioxidant actions.

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Introduction

Oxidative stress results from an imbalance between the generations of oxygen derived radicals and the antioxidant potential of the organism (1). Reactive oxygen species (ROS) mediate most of the reactions leading to oxidative stress. The effects of ROS are exerted through reactions with a large variety of easily oxidizable cellular components such as NADH and NADPH (2). It has been reported that many anti-neoplastic agents induce oxidative stress in biological systems. This can have a considerable impact on the conditions and treatment outcome of patients undergoing chemotherapy (3).

Doxorubicin (DOX) is one of the most effective chemotherapeutic agents and has been used in cancer therapy for over thirty years (4). For a long time, DOX was considered to act as a pro-oxidative acting agent but an increasing amount of experimental data has shown that this anticancer drug can induce considerable oxidative stress inside

the cell (5, 6). This stress can lead to a number of unwanted side effects on nonspecific organs such as the heart and brain (7, 4). Despite the well-known side effects of DOX treatment on the heart, little is known about its effects in the brain. The penetration ability of DOX into the brain is low, but if the blood brain barrier is temporarily opened by use of mannitol, morphine, dexamethasone or ondansetron, it's penetration will be enhanced into the brain. Therefore the toxicity of DOX is expressed in a more diffuse manner with respect to injuries of the neurons in the cortex and subcortical nuclei of the brain (8). Furthermore, some evidence indicates that DOX reduces hippocampal neurogenesis and volume (9, 10).

Artemisia biennis Willd. (Compositae) is one of the 34 *Artemisia* species growing in Iran (11). Camphor has been reported as the main component of the essential oil of *A. biennis* from Iran (12). The major volatile compound identified

*Corresponding author: Leila Hosseinzadeh. Pharmaceutical Sciences Research Center, School of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, Iran. Tel: +98-833-4276480; Fax: +98-833-4276493; email: lhosseinzadeh@kums.ac.ir; lhosseinzadeh90@yahoo.com

in the aerial parts of *A. biennis* in Western Canada is (E)-beta-farnesene (13). Dichloromethane, ethyl acetate and ethanolic fractions of *A. biennis* were reported to exhibit cytotoxicity on the cervical cancer cell line (14) and potential antimalarial effect (15) as well as leishmanicidal activity (16), respectively while no sesquiterpene lactone has been detected in the terpenoid extract of the species (17). Leaf decoction of *A. biennis* has traditionally been used for gastric troubles and stomach pain in Western Himalaya (18).

In our previous study we evaluated the antioxidant activity and total phenolic content of fractions from hydro-ethanolic extract of *A. biennis* using cell free systems (19). The purpose of the present study was to determine whether the fractions derived from reversed - phase vacuum liquid chromatography of *A. bienni* hydroethanolic extract(A- G) could provide neuroprotection against cytotoxicity induced by DOX in the PC12 cell line as a widely accepted model of neuronal cells (20, 21). Efforts were also made to investigate the underlying mechanism of protection.

Materials and Methods

Reagents and chemicals

Fluorescent probe 2,7-dichlorofluorescein diacetate (DCF-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), Triton X-100, FBS and Rhodamine-123 were purchased from Sigma (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM-F12) was purchased from Gibco (Gibco, Grand Island, NY, USA). Caspase-3 Detection Kit was provided from Sigma. Super Oxide Dismutase Assay Kits were purchased from Cayman (Ann Arbor, MI, USA). LiChroprep® RP-18 (15-25 µm) were purchased from Merck (Darmstadt, Germany) and all the solvents used for extraction from Scharlau and Caledon (Sentmenate, Spain).

Plant materials

Aerial parts of *A. biennis* Willd. were collected from Zoshk (Razavi Khorasan province, Iran) in September 2010. Sample was identified by Dr Valiollah Mozaffarian (Research Institute of Forest and Rangelands, Tehran, Iran). The voucher specimen (No. 12570) has been deposited in the Herbarium of School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.

Preparation of extracts and fractions

The dried powdered aerial parts (80 g) of *A. biennis* were extracted with petroleum ether (40-60), dichloromethane, ethyl acetate, ethanol and ethanol-water (1:1 v/v) respectively (Sequential maceration with ca. 3× 0.8 l of each solvent). The extracts were filtrated with filter paper and dried using rotary evaporator at a reduced pressure at a

temperature below 45 °C to yield 4.2, 5.8, 0.4, 1.1 and 7.9 g of each extract, respectively. Five g of the most active extract (hydroethanolic) in cell free antioxidant assays (19) was subjected to a vacuum liquid chromatography (VLC) system (reversed-phase RP-18 (25-40 µm), 25 g) with H₂O containing increasing amounts of MeOH (5%, 10%, 20%, 40%, 60%, 80% and 100%) to give 2.2, 0.74, 0.21, 0.43, 0.36, 0.2 and 0.18 g of each fraction (A-G) respectively.

Cell culture and treatment

Rat pheochromocytoma-derived cell line PC-12 was obtained from Pasteur Institute (Tehran, Iran) and maintained at 37°C in a humidified atmosphere (90%) containing 5% CO₂. Cells were cultured in DMEM-F12 with 10% (v/v) heat-inactivated fetal bovin serum, 100 U/ml penicillin and 100 mg/ml streptomycin. The medium was changed every two days and cells were plated at an appropriate density according to each experimental scale.

Cell viability assay

Four sets of experiments were performed at standard culture conditions: (1) untreated control cells, (2) cells were treated with different concentrations of *A. biennis* (0-125 µg/ml), (3) cells were treated with different concentrations of DOX (0-33 µM), and (4) cells were pretreated with different concentrations of fractions for 24 hr, then medium was changed and cells were treated with IC₅₀ concentration of DOX for another 24 hr. Viability of PC-12 cells were measured using MTT method. Briefly, after treatment, the medium was removed and replaced by 200 µl of 0.5 mg/ml of MTT in growth medium and then the plates transferred to a 37 °C incubator for 3 hr. Then, the medium was removed, and the purple formazan crystals were dissolved in DMSO (200 µl/well). Absorbance was determined on an ELISA plate reader (Biotek, H1M) with a test wavelength of 570 nm and a reference wavelength of 630 nm to obtain sample signal (OD570-OD630). Morphological alterations and cell damage were investigated using a phase contrast inverted microscope (Motic, China) at 10x magnification.

Measurement of intracellular ROS

Intracellular ROS levels were examined using DCF-DA. DCF-DA reveals the intra cellular production of redox-active substances and has been used widely to investigate oxidative damage in intact cells. It 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 (8). Briefly cells pretreated with fractions C and D were then treated with DOX for an additional 24 hr. After washing with PBS, the cells were incubated with 20 μ l of DCF-DA at 37 °C for 30 min. After incubation, cells were lysed with Triton X-100. The fluorescence was measured by flowcytometry using a Partec™ cytometer (Germany) with standard Argon laser for 480-nm excitation and 530-nm band pass (FL1) filter.

Determination of superoxide dismutase (SOD) activity

SOD are metallo enzymes that catalyze the dismutation of superoxide anion to molecular oxygen and hydrogen peroxide and thus form a crucial part of the cellular antioxidant defense mechanism (22). The SOD activity was measured using commercial SOD assay kit Cayman (USA), following the manufacturer's protocol. Cayman superoxide dismutase assay kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine (23). One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radicals. Values were expressed as U/mg protein.

Measurement of mitochondrial membrane potential (MMP)

In this study MMP was measured by using rhodamine 123 fluorescent dye. Depolarization of MMP during cell apoptosis results in the loss of rhodamine 123 from the mitochondria and a decrease in intracellular fluorescence intensity (22). Cells were incubated with rhodamine 123 for 30 min at 37 °C. The fluorescence was measured by flow-cytometry using a Partec™ cytometer (Germany) with standard Argon laser for 488-nm excitation and 520-nm band pass (FL1) filter.

Determination of Caspase-3 activity

The caspase-3 activity was measured using commercial caspase-3 assay kit Sigma (USA), according to the manufacturer's protocol. The kit is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-val-Asp-p-nitroanilide (Ac-DEVD-pNA) by caspase 3, resulting in the release of the p-nitroaniline (pNA) moiety. Briefly, 1×10^6 cells were collected and lysed with 50 μ l of chilled lysis buffer and incubated on ice for 10 min. Cell lysates were centrifuged at maximum speed for 5 min at 4 °C, 10 μ l of cell lysate was combined with an equal amount of substrate reaction buffer containing a caspase-3 colorimetric substrate. This mixture was incubated for 2 hr at 37 °C, and then the pNA light emission was quantified using a microplate reader at 400 or 405 nm (BioTek, H1M.). Comparison of the absorbance of pNA from an apoptotic sample with an un-induced control allowed determination of the fold

increase in caspase-3 activity. The protein content was determined by the Bradford method using the bovine serum albumin as a standard.

Statistical analysis

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

Results

Effect of the fractions on cytotoxicity induced by DOX

Cells were treated with fractions of *A. biennis* hydroethanolic extract at concentrations between 0 and 125 μ g/ml. Fractions A to E had no significant effect on the PC12 cell viability up to 125 μ g/ml (relative MTT activity > 80%) (Figure 1b). Moreover, the viability of PC12 cells was evaluated after 24 hr of exposure to different concentrations of DOX using MTT method. Our results showed that DOX induced cytotoxicity in a concentration dependent manner. The mean IC₅₀ value \pm SEM for DOX was 2.5 ± 0.098 μ M (Figure 1a). Using the non-toxic concentrations of fractions, another experiment was performed to

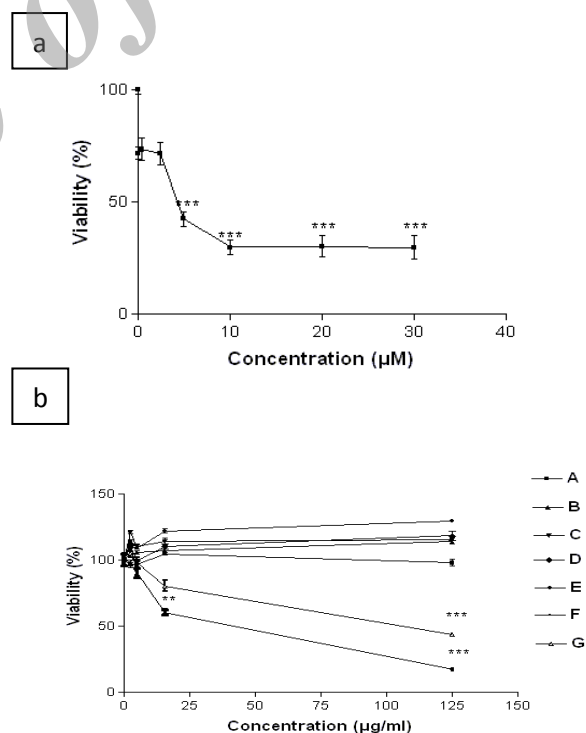


Figure 1. The effects of a) DOX (0-33.3 μ M) and b) A-G fractions (0-125 μ g/ml) of *A. biennis* hydroethanolic extract on the viability of the PC-12 cells. The cell viability was determined by MTT assay after 24 hr exposure as described in materials and methods. Data are expressed as the mean \pm SEM of three separate experiments. ** $P < 0.01$, *** $P < 0.001$ vs. Control

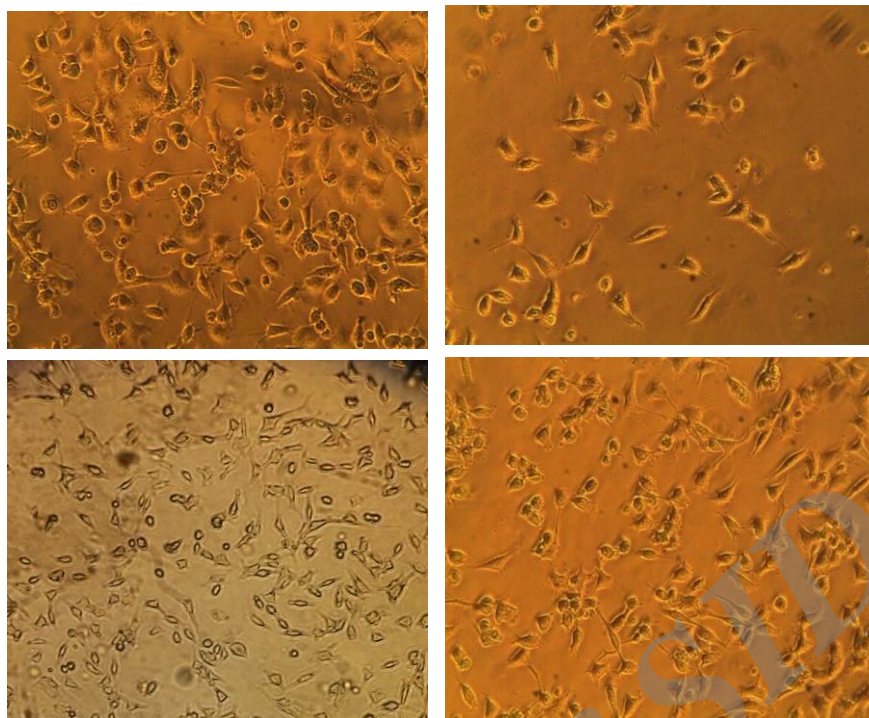


Figure 2. Representative photomicrograph shows morphological changes of the PC12 cells. Cells were pretreated with fractions C and D of aerial parts of *Artemisia biennis* hydroethanolic extract for (25 µg/ml) 24 hr before exposure to 2.5 µM of DOX and imaged by inverted phase contrast microscope

evaluate the effects of these fractions on cytotoxicity induced by DOX. As shown in the table 1, fractions C and D (25 µg/ml) proved to have outstandingly protective effects against DOX-induced toxicity in PC12 cells as demonstrated by the IC₅₀ values. The other fractions exhibited different sequences of potency (**B > E > A > F**). Moreover, after incubation with 2.5 µM of DOX the occurrence of various morphological abnormalities was recorded and a large number of cells became rounded and ongoing to detach from the flasks. Compared with the group treated with DOX alone, the cells number had increased in the groups that had been pretreated with fractions D and C (25 µg/ml). They showed no significant difference compared with control PC12 cells (Figure 2). Based on this information 24 hr pretreatment with fractions C and D from hydroethanolic extract of *A. biennis* was used for subsequent studies.

Effects of the selected fractions on DOX- induced mitochondrial membrane potential (MMP) collapse

MMP was evaluated using a cell permeable cationic fluorescent dye. Depolarization of mitochondrial membrane potential caused by the DOX-induced damage of the outer membrane resulted in the loss of the dye from the mitochondria and a decrease in intracellular fluorescence, so that DOX (2.5±0.098) significantly decreased MMP to 62.03 % of control in PC12 cells. Pretreatment of cells with

the active fractions was able to inhibit reduction of MMP induced by DOX (Figure 3).

Effect of the selected fractions on caspase-3 activity

Activation of caspase cascade is critical in the initiation of apoptosis in various biological systems. Caspase-3 has been shown to be an important regulator of apoptosis (24). The obtained results showed that DOX significantly increased caspase-3 activation in PC12 cells to 137.70±5.88% compared to the control cells. Pretreatment with fractions C and D decreased significantly caspase-3 activation to 81.16±3.64% and 71.42±2.91%, respectively (Figure 4).

Effect of the selected fractions on DOX-induced ROS generation

In order to measure oxidative stress induced by DOX, ROS accumulation was detected in PC12 cells by using the fluorescent probe DCF, which detects intracellular peroxides. As anticipated adding DOX to PC12 cells caused a significant increase in ROS level (163±4.05%). Therefore, PC12 cells are probably killed due to oxidative stress, since DOX increases intracellular ROS levels. Then we evaluated whether selected fractions protect PC12 cells from oxidative damage caused by DOX via lowering levels of ROS (Figure 5). As demonstrated in the Figure 4 the active fractions exerted excellent radical scavenging activity. Pretreatment of the cells with fractions C and D significantly reduced the ROS levels to 64.01 ±2.29% and 87.34±2.4%, respectively.

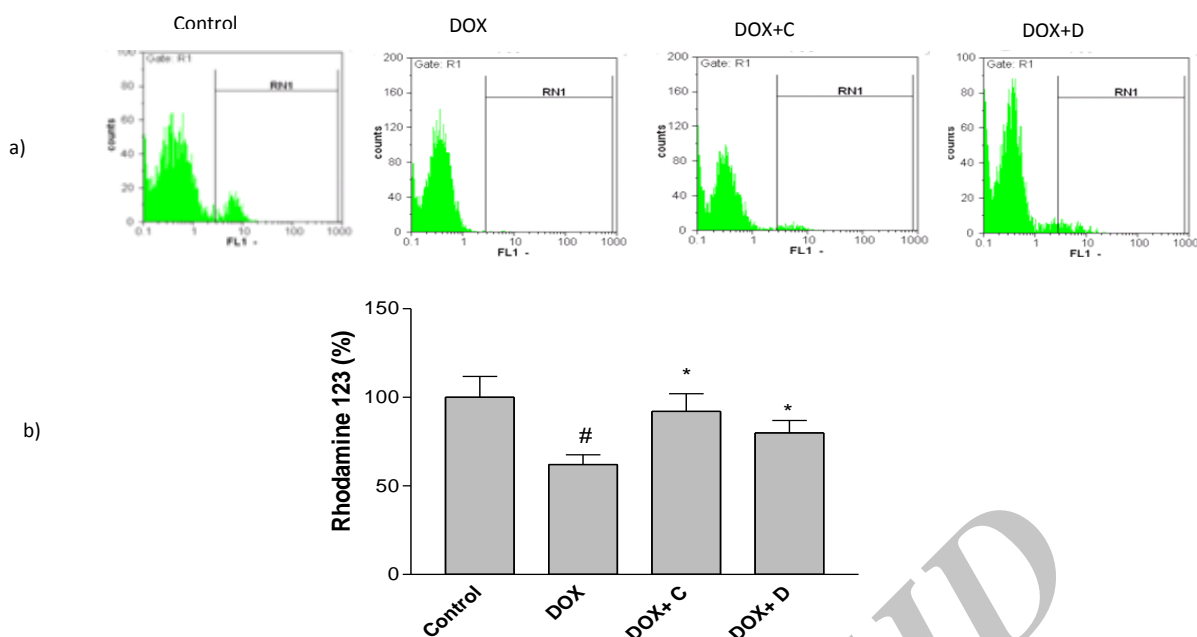


Figure 3. Effect of fractions C and D (25 $\mu\text{g/ml}$) of aerial parts of *Artemisia biennis* hydroethanolic extract on DOX (2.5 $\mu\text{g/ml}$) -induced mitochondrial membrane potential (MMP) collapse as detected by Rhodamine 123 using flowcytometry. a) Representative of flow cytometry with rhodamine 123 plots of PC12 cells in different groups. b) Column bar graph of mean cell fluorescent for rhodamine 123. Data are expressed as the mean \pm S.E.M of three separate experiments. # $P < 0.05$ vs. Control, * $P < 0.05$ vs. DOX treated cells

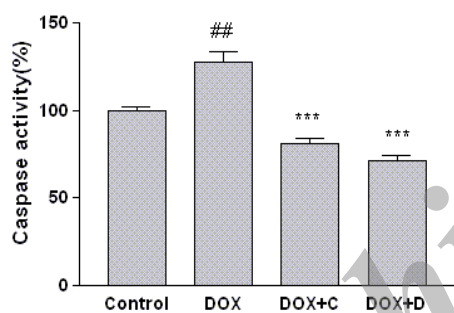


Figure 4. Effect of fractions C and D on caspase-3 activity. Cell pretreated with fractions (25 $\mu\text{g/ml}$) 24 hr before exposure to 2.5 μM of DOX. Caspase-3 activity was measured by colorimetric detection of p-nitroaniline and expressed as percent of control. Data are expressed as the mean \pm S.E.M of three separate experiments. ## $P < 0.01$ vs. Control, *** $P < 0.001$ vs. DOX treated cells

Effect of the selected fractions on the activity of SOD

Reactive oxygen species (ROS) has important role in the activation of apoptosis pathway. SOD as one of the components of intrinsic antioxidant defense system, is responsible for dissemination of superoxide anion radicals (22). Therefore, we next tested the activity of SOD in the PC12 cells. As shown in Figure 5, after exposure to DOX, the activity of SOD decreased significantly ($P < 0.05$) when compared with the control. Treatment with two selected fractions markedly increased the activity of SOD when compared with DOX-incubated cells under our experimental conditions. This result demonstrates

that fractions C and D improve the activity of SOD leading to suppression of apoptosis of PC12 cells in the presence of DOX (Figure 6).

Discussion

Evidence from the current investigation suggested that DOX could decrease the cell viability in the PC12 cells. DOX significantly increased intracellular ROS, and also inhibited SOD activity which may eventually lead to cytotoxicity in PC12 cells. Additionally, our results indicated that DOX-induced cytotoxicity is mainly executed by apoptosis. The involvement of mitochondria in DOX-induced apoptosis was investigated by evaluating the loss of the mitochondrial membrane potential. Results indicated that the stimulation of the intrinsic mitochondrial apoptotic pathway by ROS causes an increase in outer membrane permeabilization and mitochondria-to-cytosol trans-location of cytochrome *c*, AIF, or Smac/Diablo which trigger caspase-dependent or caspase-independent cytosolic signaling events (25). Caspases play an important role in the apoptotic process via two pathways: the death receptor pathway and the mitochondrial pathway. Regardless of the pathway involved, caspase-3 acts as an apoptotic executor. Free radical-mediated oxidative stress has been suggested as a main mechanism causing DOX-induced apoptosis in the brain (22). Previous studies show that DOX treatment promotes lipid peroxidation in the brain, heart, liver, lung, and kidney tissues (3, 26). A similar study using rat brain mitochondria demonstrated the role of oxidative

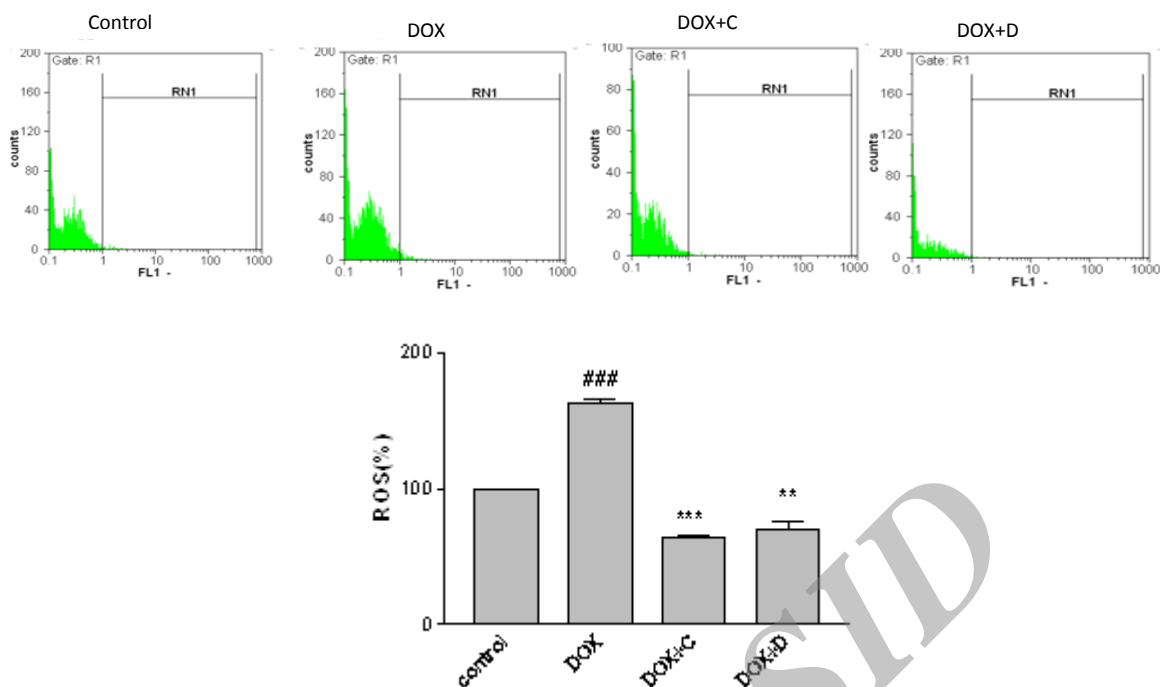
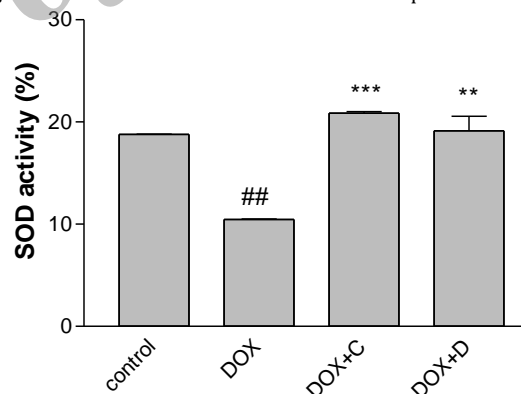


Figure 5. Effect of fractions C and D of aerial parts of *Artemisia biennis* hydroethanolic extract on DOX-induced ROS overproduction as detected by DCF using flowcytometry. Cells were pretreated with fractions C and D (25 $\mu\text{g}/\text{ml}$) 24 hr before exposure to 2.5 μM of DOX. a) Representative of flowcytometry with DCF-DA plots of PC12 cells in different groups. b) Column bar graph of mean cell fluorescent for DCF-DA. Data are expressed as the mean \pm SEM of three separate experiments. ### $P < 0.001$ vs. Control, ** $P < 0.01$, *** $P < 0.001$ vs. DOX treated cells

stress in DOX-induced toxicity and suggested that DOX treatment increases the brain mitochondria permeability to Ca^{2+} thereby predisposing brain cells to degeneration and death (7). In another study, the administration of DOX decreased MMP, disturbed the balance of Bcl-2 family protein, increased cytochrome c release and ultimately led to apoptotic cell death in the brain tissue (27). Furthermore, Oz and Ilhan (2006) reported that DOX-treated rats undergoing co-treatment with melatonin, a free radical scavenger and antioxidant compound, showed lower levels of lipid peroxidation in the kidney, lung, liver, and brain (28). Accordingly, evidence from the current investigation suggested that ROS plays a significant role in DOX-induced apoptosis in this model. When the protective effect of seven fractions on DOX-induced cytotoxicity in PC12 cells was examined, it was observed that the pretreatment of PC12 cells with sub-toxic concentration of fractions C and D markedly protected the cells from DOX-induced cytotoxicity. A marked increase in the SOD activity and significant reduction of intracellular ROS indicated that fractions C and D may protect the PC12 cells from oxidative injury by preventing increased oxidative stress. Moreover, our results indicated that selected fractions prevent DOX-induced collapse of mitochondrial membrane potential in PC12 cells. It is not

Figure 6. Effect of fractions C and D of aerial parts of *Artemisia biennis* hydroethanolic extract on the activity of SOD. Cells were pretreated with fractions C and D (25 $\mu\text{g}/\text{ml}$) 24 hr before exposure to 2.5 μM of DOX. SOD activity was measured using a colorimetric assay kit, and activity was represented as the percent inhibition of the superoxide anions. ## $P < 0.01$ vs. Control, ** $P < 0.01$, *** $P < 0.001$ vs. DOX treated cells



biennis hydroethanolic extract on the activity of SOD. Cells were pretreated with fractions C and D (25 $\mu\text{g}/\text{ml}$) 24 hr before exposure to 2.5 μM of DOX. SOD activity was measured using a colorimetric assay kit, and activity was represented as the percent inhibition of the superoxide anions. ## $P < 0.01$ vs. Control, ** $P < 0.01$, *** $P < 0.001$ vs. DOX treated cells

surprising that in our experiments, pretreatment with potent fractions was also associated with the inhibition of downstream apoptosis signaling pathways, ultimately reducing effector caspase-3 activity. It was previously reported that the fraction D of *A. biennis* hydroethanolic extract had the highest total phenolic content compared with other fractions. The same fraction showed the most potent antioxidant effects using free radical scavenging methods while chelating activity of fraction C for

ferrous ions was superior to that of fraction D (19). Naturally occurring phenolic compounds are regarded as potential neuroprotective agents. New isolated phenolic compounds from the rhizomes of *Gastrodia elata* exhibited potent neuroprotective activity against the H₂O₂-induced PC12 cell damage (29). Two well-known flavonoids, baicalein and daidzein, have been reported to exert neuroprotective effects against β -amyloid (A β) via inhibitory effects on *in-vitro* A β aggregation and A β -induced cytotoxicity in PC12 neuronal cells (30). Isolated coumarin glycosides from the stems of *Hydrangea paniculata* presented neuroprotective activity against the serum deprivation-induced PC12 cell damage (31). In summary, our findings, which agree with previous results for the same samples (19), imply that some fractions of the hydroethanolic extract of *A. biennis* attenuate oxidative stress injury and apoptosis induced by DOX in the PC12 cells. However, further studies are necessary to isolate and elucidate the structures of the active components and to determine explicit neuroprotective mechanisms before definite conclusions can be drawn.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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