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Original Article

Protective effect of *Rheum turkestanikum* root against doxorubicin-induced toxicity in H9c2 cells

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ABSTRACT

Doxorubicin is a chemotherapy drug but its clinical using is limited because of its cardiotoxicity. Reactive oxygen species play an important role in the pathological process. The aim of this study is to evaluate the protective effect of *Rheum turkestanicum* Janisch., Polygonaceae, against doxorubicin-induced apoptosis and death in H9c2 cells. The cells were incubated with different concentrations of *R. turkestanicum* extract and *N*-acetylcysteine as positive control for 2 h, followed by incubation with 5 μM doxorubicin for 24 h. Cell viability and apoptotic induction were determined by using MTT and PI assays, respectively. The level of reactive oxygen species and lipid peroxidation was measured by fluorimetric methods. Doxorubicin significantly decreased cell viability which was accompanied by an increase in ROS production and lipid peroxidation. Pretreatment with *R. turkestanicum* increased the viability of cardiomyocytes and could decrease lipid peroxidation and reactive oxygen species generation. Also, *R. turkestanicum* attenuated apoptotic induction. *N*-acetylcysteine at 100 μM reduced the levels of reactive oxygen species and lipid peroxidation. But, treating H9c2 cells with *N*-acetylcysteine did little to protect H9c2 cells from doxorubicin-induced cell death. *R. turkestanicum* exerts protective effect against oxidative stress-induced cardiomyocytes damage. Our findings showed that *R. turkestanicum* could exert the cardioprotective effects against doxorubicin-induced toxicity partly by anti-apoptotic activity. Also, *N*-acetylcysteine prevented oxidative stress via reduction of reactive oxygen species and lipid peroxidation. *N*-acetylcysteine induced less protective effects than *R. turkestanicum* extract against doxorubicin-induced cytotoxicity.

Introduction

Doxorubicin (DOX) as an effective chemotherapy drug has serious side effects such as dilated cardiomyopathy and congestive heart failure (Turakhia et al., 2007). Therefore, using this drug must be limited. The mechanisms of DOX-induced cardiotoxicity are not completely understood, but most evidences indicate that the generation of reactive oxygen species (ROS) is involved (Bryant et al., 2007). Increased level of ROS leads to protein and lipid peroxidation, DNA damage and irreversible cell damage (Ghorbani et al., 2015; Asadpour et al., 2014). ROS can directly impair contractile function, activate hypertrophy signaling pathways, stimulate cardiac fibroblast proliferation and induce extracellular matrix remodeling (Takimoto and Kass, 2007; Tsutsui et al., 2011). Excessive ROS also cause accumulation of intracellular Ca^{2+} in cardiac cells which increases the mitochondrial permeability and leads to the release of cytochrome c into the cytoplasm and the following apoptotic cascades (Pacher et al., 2001). Interestingly, some natural foods have been reported to contain substantial amounts of antioxidants and free radical scavenging agents. These compounds diminish some side effects of chemotherapeutic agents on normal cells by reducing their genotoxicity (Bryant et al., 2007). H9c2 myoblasts, a rat embryonic cell line, which has the ability to differentiate between a skeletal or cardiac muscle phenotype, can be instrumental in understanding DOX cytotoxicity in different stages (Branco et al., 2012). The recent studies have shown that some of the herbs act against oxidative injury-related cardiotoxicity. *Rheum* species, Polygonaceae, have a long history as medicinal plants in traditional Chinese medicine. The main active ingredients of the *Rheum* species are a series of anthraquinones, dianthrones, glycosides and tannins. The anthraquinone derivatives include emodin, rhein, chrysophanol, physcion, alizarin, citreorosein, and aloe-emodin (Dorsey and Kao, 2007). *R. turkestanicum* Janisch. is a plant that grows widely in central Asia and also in northeast of Iran. Traditionally, people use roots of *R. turkestanicum* as an antidiabetic and anti-hypertensive as well as anticancer agent

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(Dorsey and Kao, 2007). Recent studies have shown other species of *Rheum* such as *R. undulatum* is containing anti-oxidant compounds. Rhapontigenin and rhaponticin are isolated from *R. undulatum* scavenge ROS, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, and hydrogen peroxide (H_2O_2) (Zhang et al., 2007). Also, these compounds decrease membrane lipid peroxidation and cellular DNA damage, which are the main targets of oxidative stress-induced cellular damage (Zhang et al., 2007). The recent study showed some of isolated anti-oxidant compounds from Rheum species such as *R. emodi* protected H9c2 cells against H_2O_2 (Chai et al., 2012). In this study, the protective effect of hydro-alcoholic extract of *R. turkestanicum* on DOX-induced cardiotoxicity was evaluated for first time.

Material and methods

Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), thiobarbituric acid (TBA), 2,7-dichlorofluorescin diacetate (DCFH-DA), propidium iodide (PI), *N*-acetylcysteine (NAC), sodium citrate and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). High-glucose Dulbecco's Modified Eagles Medium (DMEM), penicillin-streptomycin and fetal bovine serum were purchased from Gibco. Trichloro acetic acid (TCA) and malondialdehyde bis-(dimethyl acetal) (MDA) were obtained from Merck (Darmstadt, Germany).

Preparation of extracts

The root of *Rheum turkestanicum* Janisch., Polygonaceae, was collected from Chenar, a village in Zavin Rural District, Kalat County, Razavi Khorasan Province, Iran. The plant was identified by M.R. Joharchi, from Ferdowsi University of Mashhad Herbarium. Voucher specimen (No. 21377) was deposited in Ferdowsi University of Mashhad Herbarium. Dried *R. turkestanicum* root (20 g × 3) was ground into fine powder and then 50 g of this powder was subjected to extraction with 70% ethanol in a Soxhlet apparatus for 48 h. The hydro-alcoholic extract was then dried on a water bath and kept frozen in less than −18 °C for the following use. The yield of extract was 19% (w/w). The extract was dissolved in dimethylsulfoxide (DMSO) to a final concentration of 50 mg/ml before being used in cytotoxicity and apoptosis assays.

Cell culture

Rat heart cell line H9c2 was obtained from American Type Culture Collection (ATCC CRL-1446) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were cultured in DMEM supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin. For the experiments, they were seeded in 96-well and 24-well culture plates for MTT/ROS and MDA assays, respectively. For apoptosis assay, cells were seeded at 100,000/well in a 24-well plate. All treatments were carried out in triplicate. The cells were pretreated with extract (6–200 μg/ml) for 2 h and then incubation was continued in the presence of the extract with 5 μM doxorubicin for 24 h. Also, NAC was applied as positive control at 10 and 100 μM.

Cell viability

The cell viability was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay as described previously (Mosmann, 1983; Malich et al., 1997). Briefly, MTT solution in phosphate-buffered saline (5 mg/ml), was added to each well at final concentration of 0.05%. After 3 h, the formazan precipitate was dissolved in DMSO. The absorbance at 570 and 620 nm (background) was measured using a StatFAX303 plate reader. The experiment was carried out in triplicate.

Lipid peroxidation assay

The level of lipid peroxidation was estimated by measuring MDA which is the end product of lipid peroxidation. At the end of incubation, the cells were scraped and centrifuged at 13,000 × *g* at 4 °C for 30 min (Buege and Aust, 1978). Then, 400 μl of TCA (15%) and 800 μl of TBA (0.7%) were added to 500 μl of cell samples. The mixture was vortexed and then heated for 40 min in a boiling water bath. Then, 200 μl of the sample was transferred to 96-well plate and the fluorescence intensity was read with excitation/emission of 480/530 nm. The experiment was carried out in triplicate.

Measurement of reactive oxygen species

Intracellular ROS level was evaluated using a fluorescent probe, DCF-DA (Wu and Yotnda, 2011). At the end of incubation, the cells were treated (30 min) with DCFH-DA (10 μM) at 4 °C in the dark. Then, the fluorescence intensity was detected with excitation/emission of 485/530 nm. The experiment was performed in triplicate.

Apoptosis

Apoptotic cells were detected by using PI staining of the treated cells followed by flow cytometry to detect the so-called sub-G1 peak. Briefly, H9c2 cells were cultured overnight in a 24-well plate and pretreated by *R. turkestanicum* for 4 h and then treated with DOX for 24 h. Floating and adherent cells were then harvested and incubated at 4 °C overnight in the dark with 500 μl of a hypotonic buffer (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100). The experiment was carried out in triplicate (Nicoletti et al., 1991).

Statistics

All data were expressed as mean ± SEM. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tamhane's T2 post hoc test. Differences were considered significant at $p < 0.05$.

Results

Effect of DOX on cell viability

Incubation of the cells with different concentrations of DOX (2.5–20 μM) showed that the cell viability reduced in a dose dependent manner (Fig. 1).

Effect of R. turkestanicum *extract on cell viability*

Incubation with 6 μM DOX significantly decreased cell viability to 55 ± 4.5% of control ($p < 0.001$). Pretreatment with 12–200 μg/ml of *R. turkestanicum* could increase the viability of H9c2 cells to 71 ± 0.68% ($p < 0.05$), 74.6 ± 2.5% ($p < 0.01$), 79 ± 1.3% ($p < 0.001$), 84 ± 3.6% ($p < 0.001$) and 88 ± 1.3% ($p < 0.001$) of control, respectively (Fig. 2). However, at dose of 6 μg/ml, *R. turkestanicum* was not able to protect H9c2 cells against DOX-induced cytotoxicity. Also pretreatment of cells with NAC at doses of 10 and 100 μM, protected cells against DOX (5 μM) at dose of 100 μM (69 ± 1.8%, $p < 0.05$) (Fig. 2).

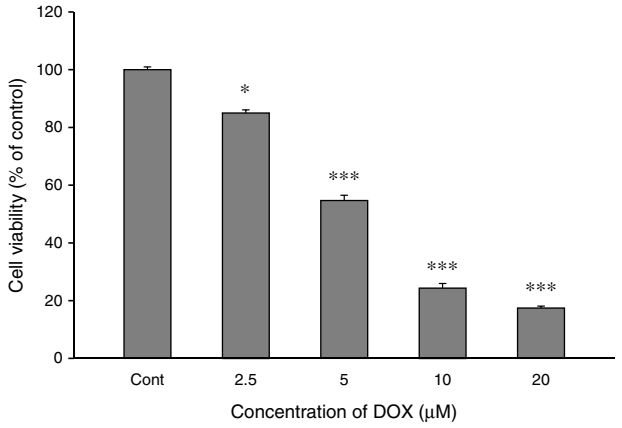

Fig. 1. Dose–response curve of DOX on viability of H9c2 cells. The cells were treated with different concentrations (2.5–20 μM) of DOX for 24 h. The cell viability was quantitated by MTT assay. Results are mean ± SEM from three independent experiments. $^{***}p < 0.001$, $^{*}p < 0.05$ versus control.

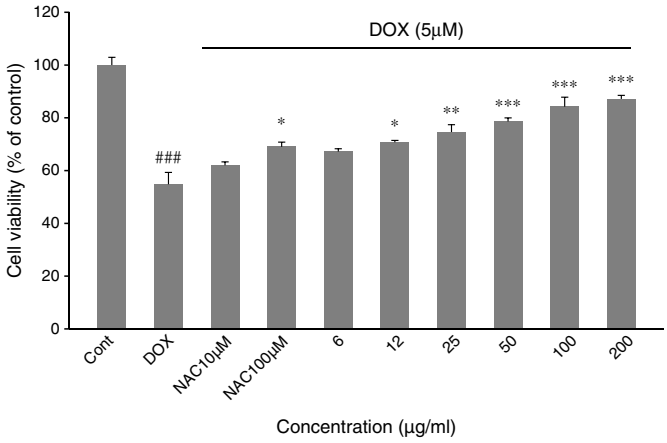

Fig. 2. Effect of *Rheum turkestanicum* extract and NAC (positive control) on viability of H9c2 cells. The cells were pretreated with different concentrations (25–200 μg/ml) of the extract for 2 h, then exposed to doxorubicin (DOX) for 24 h. The cell viability was quantitated by MTT assay. Results are mean ± SEM from three independent experiments. $^{###}p < 0.001$ versus control, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus DOX.

Effect of R. turkestanicum *on ROS production*

As expected, DOX caused a significant increase in the level of ROS in H9c2 cells (217 ± 4.6% of control, $p < 0.001$). The extract at concentrations of 25, 50, 100 and 200 μg/ml was able to decrease intracellular ROS level to 186 ± 5.2% ($p < 0.01$), 171 ± 4.6% ($p < 0.001$), 146 ± 1.1% ($p < 0.001$) and 132 ± 2.6% ($p < 0.001$) of control, respectively (Fig. 3). Also, NAC at dose of 100 μM significantly decreased ROS production (165 ± 2.3%, $p < 0.001$) (Fig. 3).

Effect of R. turkestanicum *extract on MDA level*

The level of lipid peroxidation was evaluated by measuring of MDA level, which is the end product of lipid peroxidation. As shown in Fig. 4, exposure of the cells to DOX resulted in a significant increase of MDA level (168 ± 6.5%, $p < 0.001$) as compared to control cells cultured in the absence of DOX (100 ± 1.3%). The content of MDA was significantly decreased in the cells pretreated with 25 μg/ml (137 ± 1.5%, $p < 0.01$), 50 μg/ml (129 ± 2.1%, $p < 0.001$), 100 μg/ml (117 ± 3.3%, $p < 0.001$), or 200 μg/ml (110 ± 4.1%, $p < 0.001$) of *R. turkestanicum*. Also, NAC

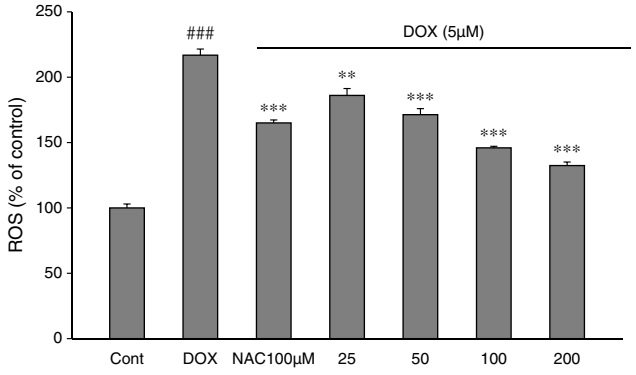

Fig. 3. The effects of *Rheum turkestanicum* extract and NAC (positive control) on intracellular ROS level of under doxorubicin treatment in H9c2 cells. The cells were pretreated with different concentrations (25–200 μg/ml) of the extract for 2 h, then exposed to doxorubicin (DOX) for 24 h. Results are mean ± SEM from three independent experiments. $^{###}p < 0.001$ versus control, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus DOX.

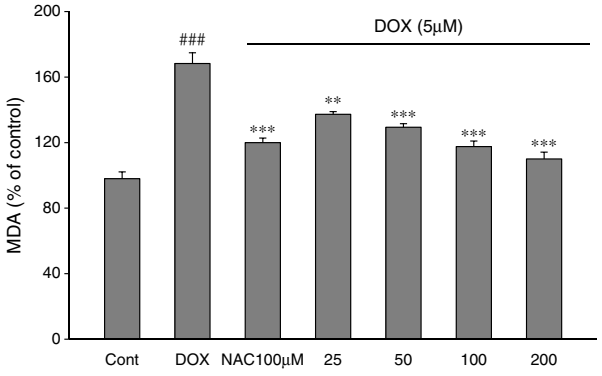

Fig. 4. The effects of *Rheum turkestanicum* extract and NAC (positive control) on MDA content under doxorubicin treatment in H9c2 cells. The cells were pretreated with different concentrations of the extract (25–200 μg/ml) for 2 h, then exposed to 5 μM doxorubicin and incubated for 24 h. Results are the means ± SEM from three independent experiments. $^{###}p < 0.001$ versus control, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus DOX.

at dose of 100 μM significantly decreased MDA content (120 ± 2.8%, $p < 0.001$) (Fig. 4).

Effect of R. turkestanicum *on apoptotic cell death*

The proportion of apoptotic cells was measured with PI staining of DNA fragmentation by flow cytometry. The extract induced a sub-G1peak (one of the reliable biochemical markers of apoptosis) in flow cytometry histogram of treated cells compared to control (Fig. 5).

Discussion

Our findings showed doxorubicin increased cell death, ROS generation, MDA production and induced apoptotic cell death. *R. turkestanicum* decreased cytotoxic effects of doxorubicin in cardiomyoblast cells. The extract increased cell viability, reduced ROS and MDA production. Also, NAC as positive control, protected the cells against DOX via reduction of ROS and MDA levels.

DOX, as an anthracycline antibiotic, has been used in cancer therapy for three decades. However, the clinical use of DOX is limited because of it increased risk of cardiotoxicity (Wencker et al., 2003; Xin et al., 2009). In addition, DOX affect on specific enzymes, transporters, and metabolic pathways in the cardiac muscle. The

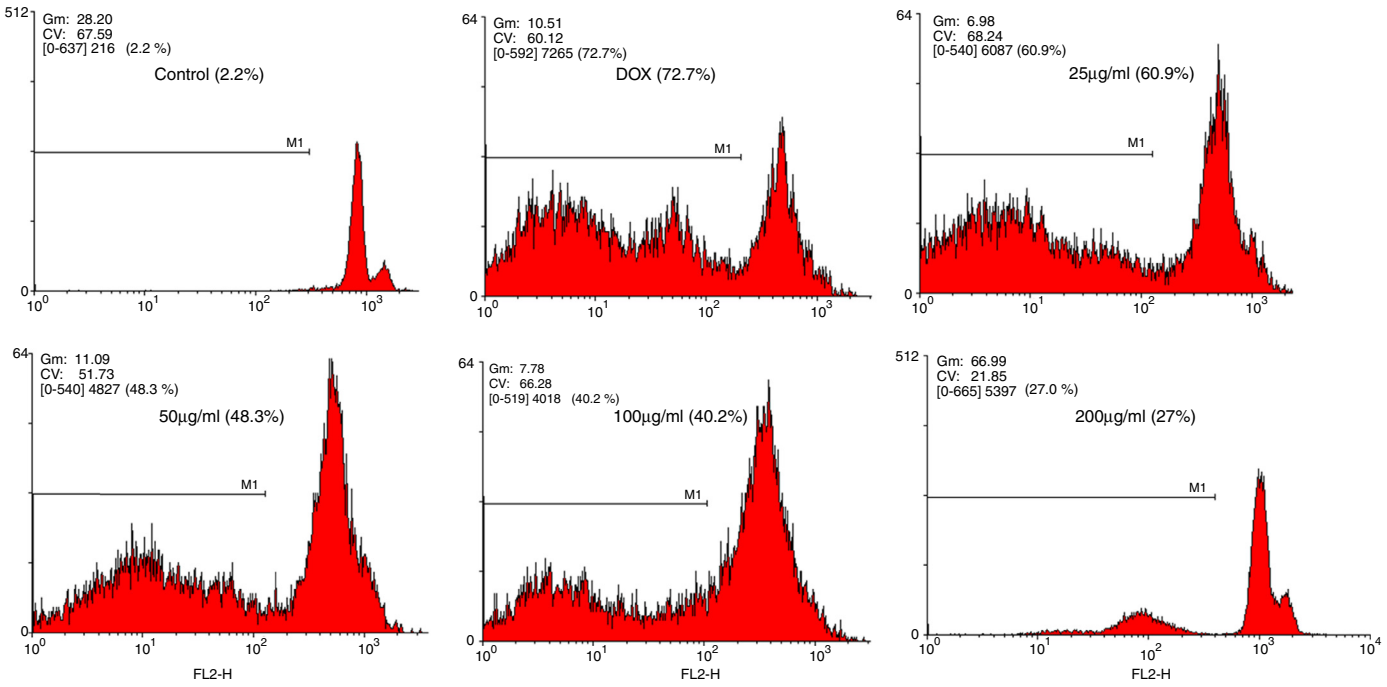

Fig. 5. The effect of the *R. turkestanicum* extract on apoptosis under doxorubicin treatment in H9c2 cells using PI staining and flow cytometry. Results are the means ± SEM from three independent experiments. $^{###}p < 0.001$ versus control, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus DOX.

accumulation of these defects may ultimately result in irreversible cardiac failure (Tokarska-Schlattner et al., 2006). The main mechanism of DOX-induced cardiotoxicity is production of ROS as a derivative of the DOX metabolism (Liu et al., 2009; Zhang et al., 2009). However, superoxide radicals are involved in other ROS and generate hydroxyl radical and hydrogen peroxide (Bast et al., 2007). Cardiac tissue is sensitive to oxidative damage because of its high oxidative metabolism and low antioxidant defenses in this organ compared with others (Pinto et al., 1986; Shug, 1987). In the present study H9c2 cells were used as a pharmacological model to evaluate the potential cardioprotective effect of *R. turkestanicum* against doxorubicin. The results showed that *R. turkestanicum* has protective effect on H9c2 cells against DOX-induced oxidative stress. H9c2 cells are morphologically similar to immature embryonic cardiomyocytes. Considering that these cells preserve electrical and hormonal signal pathways found in adult cardiac cells (Sheng et al., 2010), they are a useful model for studying oxidative stress-induced cardiomyocyte damage (Winstead et al., 2005). In this model, DOX significantly increased lipid peroxidation and induced the apoptotic rate. These changes are similar to the DOX-induced deleterious effects on normal cardiac cells which lead to the loss of cardiomyocytes viability. According to current reports, generating ROS by DOX causes mitochondrial damage, which may lead to cardiomyocyte apoptosis, necrosis, or death. The studies have shown, some natural substances, such as curcumin, naringenin-7-*O*-glucoside, and plantainoside D prevented DOX-induced mitochondrial injuries in cardiomyocytes (Kim et al., 2007; Hosseinzadeh et al., 2011). In our study, we used DCFH-DA as an intracellular method of ROS detection. The results demonstrated that *R. turkestanicum* can significantly reduce the intracellular ROS production by DOX in H9c2 cells. We also measured lipid peroxidation (MDA formation) in H9c2 cells. The reduction of MDA content suggested that *R. turkestanicum* can attenuate the oxidative stress induced by DOX in H9c2 cells. Also results showed NAC as potent anti-oxidant protected cells against DOX via reduction of oxidative-stress. The recent studies have shown NAC inhibited ROS production, lipid peroxidation, and restored antioxidant enzyme activities but had modest effect on the protection of DOX-induced cardiac cell death as compared to other natural sources of antioxidants (Shi et al., 2009). NAC induced less protective effects than *R. turkestanicum* extract against DOX-induced cytotoxicity. However protective effects of extract may be mediated via mechanisms similar to NAC.

Conclusion

This study shows that *R. turkestanicum* is a novel potent protective agent against DOX-induced cardiotoxicity, the protective effects may be due to its antioxidant properties. This effect is correlated with reducing oxidative stress and inhibition of apoptosis induction. But more investigations are needed to elucidate the probable underlying mechanisms of these therapeutic effects.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgement

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