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Original Article

Protective effects of protocatechuic acid against doxorubicin- and arsenic trioxide-induced toxicity in cardiomyocytes

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Abstract

Background and purpose: Some chemotherapeutic drugs are associated with an increased risk of cardiotoxicity in patients. Protocatechuic acid (PCA) is a phenolic acid with valuable cardiovascular, chemo-preventive, and anticancer activities. Recent studies have shown the cardioprotective effects of PCA in several pathological conditions. This investigation aimed to assess the possible protective effects of PCA on cardiomyocytes against toxicities caused by anti-neoplastic agents, doxorubicin (DOX), and arsenic trioxide (ATO).

Experimental approach: H9C2 cells were exposed to DOX (1 μM) or ATO (35 μM) after 24 h pretreatment with PCA (1-100 μM). MTT and lactate dehydrogenase (LDH) tests were used to define cell viability or cytotoxicity. Total oxidant and antioxidant capacities were evaluated by measuring hydroperoxides and ferric-reducing antioxidant power (FRAP) levels. Expression of the TLR4 gene was also quantitatively estimated by real-time polymerase chain reaction.

Findings/Results: PCA showed a proliferative effect on cardiomyocytes and significantly enhanced cell viability and reduced cytotoxicity of DOX and ATO during MTT and LDH assays. Pretreatment of cardiomyocytes with PCA significantly decreased hydroperoxide levels and elevated FRAP value. Moreover, PCA meaningfully decreased TLR4 expression in DOX- and ATO-treated cardiomyocytes.

Conclusions and implications: In conclusion, antioxidant and cytoprotective activities were found for PCA versus toxicities caused by DOX and ATO in cardiomyocytes. However, further *in vivo* investigations are recommended to assess its clinical value for the prevention and treatment of cardiotoxicity induced by chemotherapeutic agents.

Keywords: Arsenic trioxide; Cardiomyocytes; Cardiotoxicity; Doxorubicin; Protocatechuic acid.

INTRODUCTION

Cancer is a prominent reason of mortality worldwide, accounting for almost 10 million deaths in 2020 (1). The use of anti-neoplastic drugs significantly reduces the progression of cancers and prevents their recurrence in many patients. Doxorubicin (DOX) is an agent from anthracyclines used to treat a variety of solid tumors such as breast cancer, sarcoma, leukemia, and lymphoma with beneficial effects in preventing metastasis (2). However, this chemotherapeutic drug is associated with a risk of cardiotoxicity in patients after acute or cumulative doses. Inflammatory and oxidative injuries to various macromolecules such as DNA, proteins, and lipids, and impairment in mitochondrial and cell membrane functions due to the production of large amounts of reactive oxygen species, iron-DOX complexes, and cytokines have been described as some of the important pathological mechanisms in DOX-induced cardiotoxicity (3,4). DOX also triggers toll-like receptors (TLRs) including TLR2 and TLR4 in cardiomyocytes which respond to various signals and contribute to cardiac damage.

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The signaling pathways of TLRs stimulate various transcription factors, like nuclear factor-κB (NF-κB), consequently persuading the production of pro-inflammatory cytokines and interferons, and finally resulting in cardiomyopathy and the development of heart failure due to cell damage and death (5). Arsenic trioxide (ATO), mainly used in the management of acute or refractory promyelocytic leukemia, is another chemotherapeutic agent which induces cardiac toxicity through oxidative stress, abnormalities in calcium signaling and mitochondrial function, and activating several stress and death pathways (6).

Several chemo-protective drugs such as statins, dexrazoxane, erythropoietin, inhibitors of cyclooxygenase, and antagonists of angiotensin and beta-adrenergic receptors have been suggested as the prophylactic strategy for the management of cardiotoxicity caused by chemotherapy (7). In recent years, the role of antioxidants and natural compounds has been considered for protective effects against cardio-toxic drugs. Protocatechuic acid (PCA) or 3, 4, dihydroxybenzoic acid is a phenolic agent with various pharmacological activities. The anti-inflammatory, neuroprotective, anti-fibrotic, chemo-preventive, and anticancer properties have been established for this antioxidant compound (8,9). Some clinical investigations have shown anti-skin aging and antimicrobial effects for the topical use of PCA (10,11).

PCA has also shown cardioprotective effects in hypertensive hearts, ischemia/reperfusion, cardiac hypertrophy, atherosclerosis, type 1 diabetes mellitus, and in cardiotoxicity induced by an environmental toxin called 2,3,7,8-tetracholorodibenzo-p-dioxin (TCDD) (12-17). These supportive effects of PCA on cardiac tissue occur through inhibiting oxidative stress, improving heart function, hampering NF-κB activity and mitochondrial dysfunction, increasing anti-apoptotic proteins, reducing the expression of apoptotic markers, hypertrophic factors, and cell adhesion molecules (12-17). Moreover, it has been reported that PCA hinders the inflammatory process by inhibiting TLR4 and dependent pathways (18).

Regarding the beneficial cardiovascular properties of PCA, this *in vitro* study was planned to examine the possible protective effect of PCA on toxicities induced by DOX or ATO in cardiomyocytes.

MATERIALS AND METHODS

Chemicals

PCA was obtained from Cayman Chemical Co. (USA; Cat No. 14916-25) as a synthetic material with ≥ 98% purity. DOX was prepared from EBEWE Pharma GmbH Nfg KG Co. (Austria; Cat No. 118529), and ATO from Bahar Sabz Talaie Pharmacy (Iran; Global Trade Item No. 06262749061436). Fetal bovine serum (FBS) was purchased from Biosera Co. (France). Dulbecco's modified eagle's medium (DMEM) was obtained from BioIdea Co. (Iran). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay kit was purchased from Alfa Aesar GmbH & Co KG (Germany). The assay kits for the determination of total oxidant capacity and ferric-reducing antioxidant power (FRAP) were prepared by Hakiman Shargh Research Co. (Iran). The lactate dehydrogenase (LDH) release assay kit was from Kiazist (Iran). BIOFACT^{TM} total RNA Prep kit was purchased from BioFact Ltd. (Korea), the cDNA synthesis kit from Yekta Tajhiz Azma Co. (Iran), and the Quantitect SYBR Green master mix kit from Qiagen (Germany). All other chemicals were from Merck Co., Germany.

Cell culture

Rat H9C2 cardiomyocytes were procured from Iran Cell Bank and were cultivated in a high glucose medium of DMEM enhanced with FBS (10%) and antibiotics (penicillin-streptomycin, 1%) under 95% humidified atmosphere and 5% CO_2 at 37 °C.

MTT assay

For the determination of the effect of PCA, DOX, and ATO on H9C2 cell viability, an MTT kit was used (19). In Brief, 1×10^5 cells per mL were cultivated in every well of a plate and incubated for 24 h. Next, cells were exposed to PCA (1-100 µM), DOX (0.25-4 µM), or ATO (5-60 µM) separately for an additional 24-h period. After emptying the media and washing it out with phosphate-buffered saline (PBS), an MTT reagent was added, and cardiomyocytes

were preserved for 3 h. Dimethyl sulfoxide (DMSO, Sigma-Aldrich) was then used for dissolving the formazan crystals and the absorbance of the solution in each well was finally detected by a microplate reader/spectrophotometer (BioTek Instruments, USA) at 570 nm.

For evaluation of the cytoprotective activity of PCA against DOX or ATO toxicities, cardiomyocytes were first pretreated with PCA (1-100 µM) for 24 h. Since management of proven cardiomyopathy may not ensure complete recovery, preventive strategies are now suggested for reducing cardiotoxicity caused by chemotherapy (7). Therefore, pretreatment of cardiomyocytes with PCA was used in the present study.

After changing the medium and washing out with PBS, cells were exposed to DOX (1 µM) or ATO (35 µM) for an additional 24 h. The rest of the experiment was done as above. The untreated cells were considered as a negative control. Experiments were performed in triplicate and repeated three times. The viability of samples was assessed using the following equation:

$$\text{Cell viability (\%)} = \frac{\text{OD test} - \text{OD blank}}{\text{OD negative control} - \text{OD blank}} \times 100 \quad (1)$$

where OD stands for optical density.

LDH assay

For evaluation of the effect of PCA against DOX- and ATO-induced cytotoxicity, LDH releases assay kit was used following the manufacturer's instructions (19). The released LDH in collected culture media which reflects a direct measurement of the dead fraction of cells was quantified by microplate reader/spectrophotometer at 560 nm. To measure supreme LDH release or high control, some cardiomyocytes were exposed to the PermiSolution reagent. For estimation of unprompted LDH release or low control, cells were preserved with culture media. Cytotoxicity was calculated according to the following equation:

$$\text{Cytotoxicity (\%)} = \frac{\text{Test sample absorption} - \text{Low control absorption}}{\text{High control absorption} - \text{Low control absorption}} \times 100 \quad (2)$$

Total oxidant capacity assay

Assessment of hydroperoxides concentration as an indicator of total oxidant capacity was performed using a commercial kit based on the FOX-1 (ferrous ion oxidation by xylenol orange) technique (20). In this assay, the supernatant of the cardiomyocytes after being pretreated with different concentrations of PCA and then exposed to DOX or ATO was mixed with FOX-1 solution. After 30 min maintenance in a dark place at room temperature, absorbance was recorded at 540 nm on a spectrophotometer. An H_2O_2 standard curve was used for the calculation of hydroperoxides concentration.

Total antioxidant capacity assay

For the determination of total antioxidant capacity, a ferric-reducing antioxidant power (FRAP) assay was performed using a commercial kit in which the ferric-tripyridyltriazine complex is reduced to ferrous iron (21). After pretreatment of cardiomyocytes with different concentrations of PCA and then incubation with DOX or ATO, the supernatant of each well was incubated with FRAP solution for 40 min in an incubator (40 °C). Then, absorbance was read at 570 nm using a spectrophotometer. FRAP value was estimated as $FeSO_4$ equivalents using its standard curve.

TLR4 gene expression assay

After 24 h incubation, extraction of total RNA was done from H9C2 cells by BIOFACT™ total RNA Prep kit following the manufacturer's protocol. Concentrations of RNA and their purity was confirmed on a NanoDrop system spectrophotometer at 260/280 nm. Then, a cDNA synthesis kit was used for the conversion of RNA to cDNA. To estimate the expression of the TLR4 gene, quantitative real-time polymerase chain reaction (RT-PCR) was executed on StepOne™ RT-PCR System (USA) using Quantitect SYBR Green master mix kit as stated previously (22). Each cycle of amplification was as follows: denaturation at 95 °C for 15 min and 45 cycles at 95 °C for 20 s, 60 °C for 30 s, and finally, 72 °C for 30 s.

Primers were synthesized by Sina Clon Co. (Iran) and normalization of TLR4 gene expression was done based on the endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression *via* the $2^{-\Delta\Delta Ct}$ procedure. The sequences of primer were as follows: TLR4 forward 5'-CACATAGCAGATGTTCCTAG-3', TLR4 reverse 5'-CCAAAGCTGATATCCTCTC-3', GAPDH forward 5'- CTC CCG CTT CGC TCT CTG-3', and GAPDH reverse 5'-TCCGTTGACTCCGAC CTTC-3'.

Statistical analysis

Results were stated as mean ± SEM and one-way analysis of variance (ANOVA) with Tukey posthoc test was used for statistical evaluation *via* SPSS software (version 25). Values with $P < 0.05$ reflected the significant level.

RESULTS

Effects of PCA, DOX, and ATO on H9C2 cells viability

The probable cytotoxicity of PCA on H9C2 cells was assessed by the MTT test. There was no inhibitory result after 24 h treatment with PCA (1-100 µM) on H9C2 cells viability (Fig. 1). Remarkably, PCA at its highest concentration (100 µM) showed the proliferative effect on H9C2 cells ($P < 0.05$).

Exposure to DOX and ATO significantly reduced the viability of cardiomyocytes after 24 h with IC_{50} values of 1.07 µM and 35.09 µM, respectively (Fig. 2A and B). These concentrations were used to examine the protective properties of PCA on cardiomyocytes.

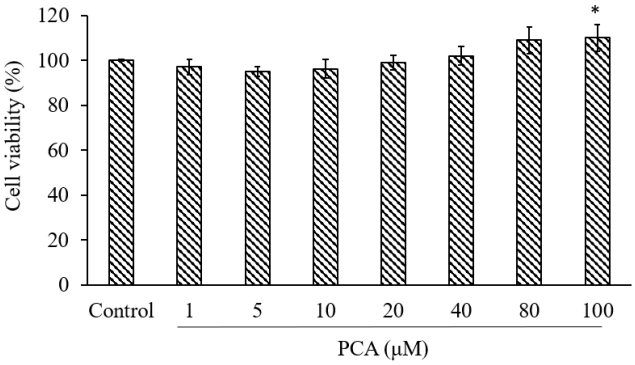

Fig. 1. Effect of PCA on H9C2 cell viability determined by MTT assay. Cells were incubated with PCA for 24 h. Values are means ± SEM from three independent experiments in triplicate. $^{*}P < 0.05$ Indicate significant differences versus the control group (untreated cells). PCA; Protocatechuic acid.

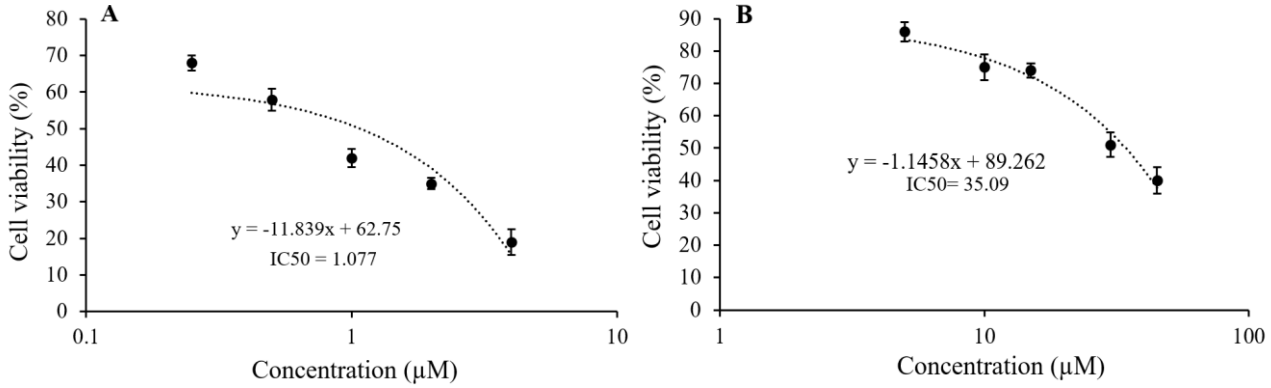

Fig. 2. Effect of (A) doxorubicin and (B) arsenic trioxide on H9C2 cell viability determined by MTT assay. Cells were incubated with doxorubicin or arsenic trioxide for 24 h. Values are means ± SEM from three independent experiments in triplicate.

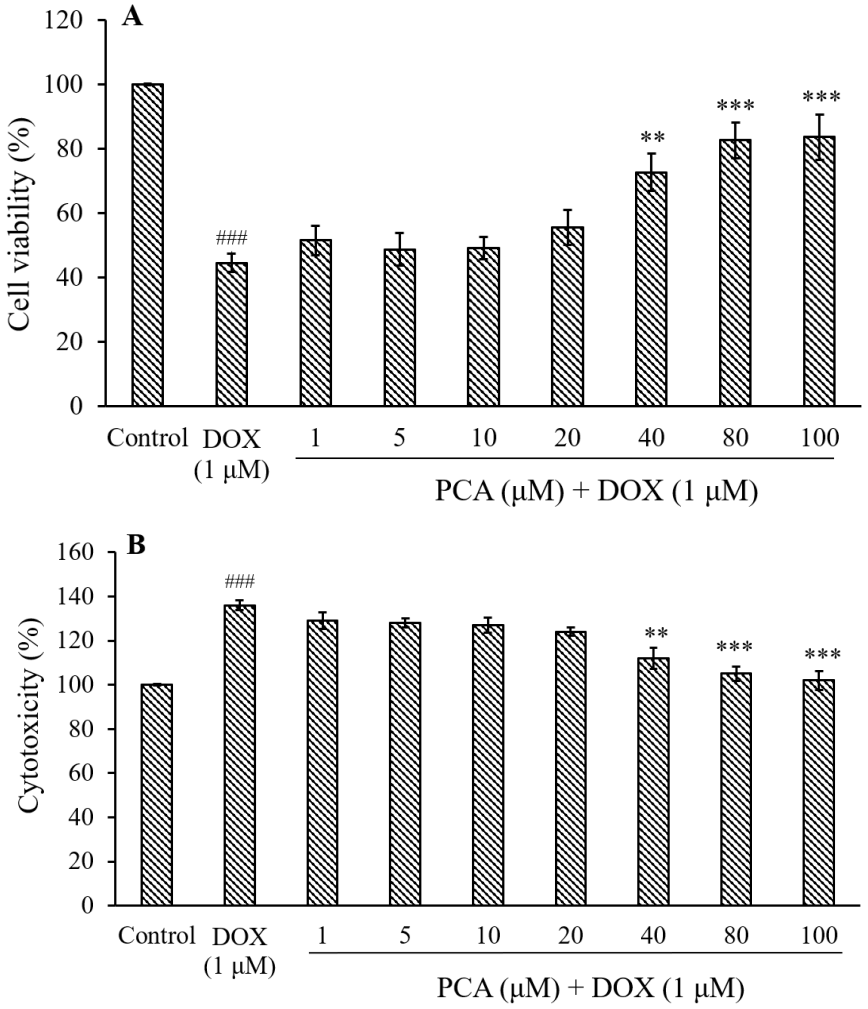

Fig. 3. Effect of PCA on H9C2 cell viability in DOX-induced toxicity determined by (A) MTT assay or (B) LDH assay. Cells were incubated with DOX (1 μM, 24h) after pretreatment with different concentrations of PCA for 24 h. Values are means ± SEM from three independent experiments in triplicate. $^{###}P < 0.001$ Indicates significant differences in comparison with the control group (untreated cells); $^{**}P < 0.01$ and $^{***}P < 0.001$ versus DOX. PCA; Protocatechuic acid; DOX, doxorubicin.

Effects of PCA against DOX- and ATO-induced cytotoxicity

MTT and LDH methods were used for the evaluation of the cytoprotective effects of PCA against toxicity caused by DOX and ATO in H9C2 cells. For this mean, cells were first pretreated with PCA for 24 h and then exposed to DOX or ATO for another 24-h period. Exposure to DOX-induced 55.4% and 36.2% cell death in MTT and LDH assays, respectively. PCA showed cytoprotective activities at concentrations of 40-100 μM against DOX-induced cardiomyocyte toxicity (Fig. 3A and B).

After incubation of cells with ATO, cell death was observed as 45.7% and 42.0% in MTT and LDH assays, respectively. Pretreatment of H9C2 cells with PCA markedly reduced the cytotoxicity of ATO at the concentrations of 20-100 μM in the MTT test and at the concentrations of 10-100 μM in the LDH test (Fig. 4A and B).

Effect of PCA on total oxidant capacity

Figure 5 shows the effects of PCA on hydroperoxides concentration as a measure of total oxidant capacity in H9C2 cells after exposure to DOX or ATO. The hydroperoxides concentration was meaningfully raised after incubation of cells with DOX and ATO when compared to the untreated cells. Incubation of cardiomyocytes with PCA significantly decreased the hydroperoxides levels at the concentrations of 80 and 100 μM compared to the DOX or ATO groups.

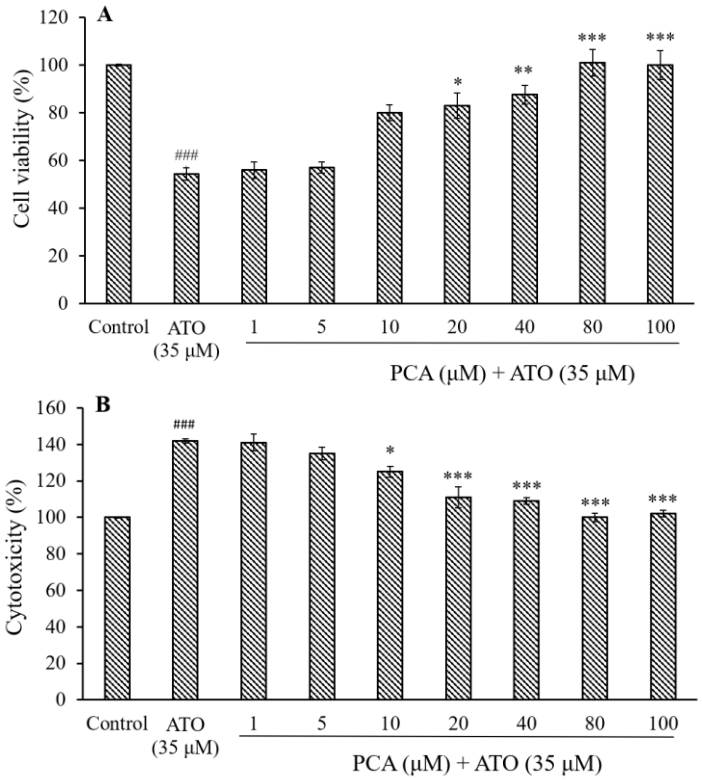

Fig. 4. Effect of PCA on H9C2 cell viability in ATO-induced toxicity determined by (A) MTT assay or (B) LDH assay. Cells were incubated with DOX (1 µM, 24 h) after pretreatment with different concentrations of PCA for 24 h. Values are means ± SEM from three independent experiments in triplicate. $^{###}P < 0.001$ Indicates significant differences in comparison with the control group (untreated cells); $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus ATO. PCA, Protocatechuic acid; ATO, arsenic trioxide.

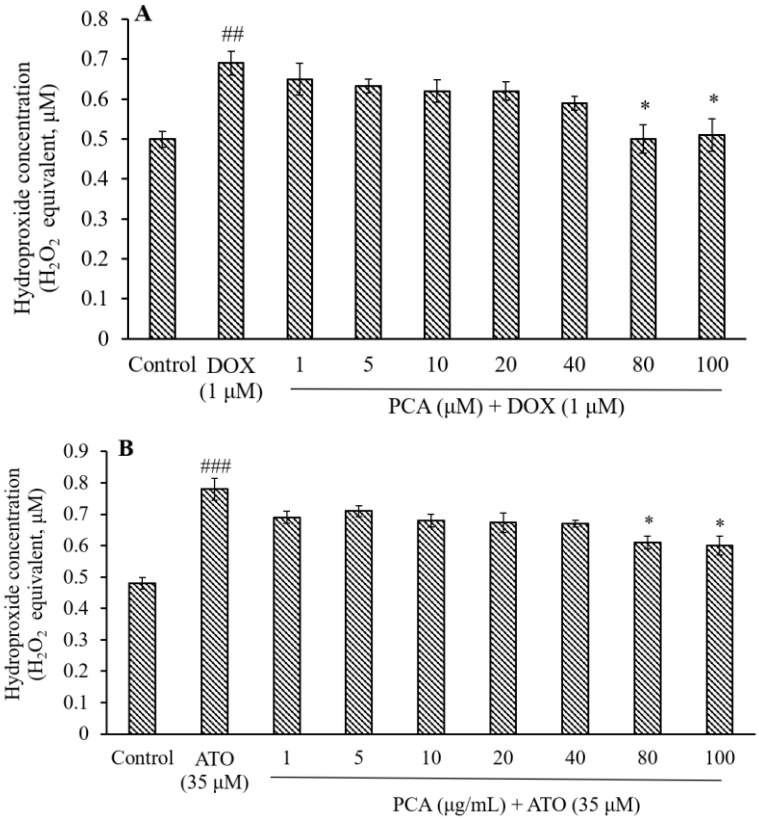

Fig. 5. Effect of PCA on hydroperoxides concentration in (A) DOX- and (B) ATO-induced toxicity in H9C2 cells determined by FOX-1 method. Values are means ± SEM. $^{##}P < 0.01$ Indicates significant differences in comparison with the control group (untreated cells); $^{*}P < 0.05$ versus DOX or ATO. PCA; Protocatechuic acid; DOX, doxorubicin; ATO, arsenic trioxide; FOX-1 ferrous ion oxidation by xylenol orange.

Effect of PCA on total antioxidant capacity

As shown in Fig. 6, exposure of H9C2 cells to DOX and ATO resulted in a significant decline in FRAP value in comparison with the control group as a measure of total antioxidant capacity. Incubation of cells with PCA markedly elevated FRAP level at the range of 10-100 µM concentration compared to the DOX or ATO groups.

Effect of PCA on TLR4 gene expression

Meaningfully higher rates of TLR4 expression were observed in H9C2 cells, after 24-h exposure to DOX and ATO, compared to the control group. Pretreatment of cardiomyocytes with PCA significantly decreased TLR4 expression at the concentrations of 80 and 100 µM in DOX- and ATO-induced cytotoxicity (Fig. 7).

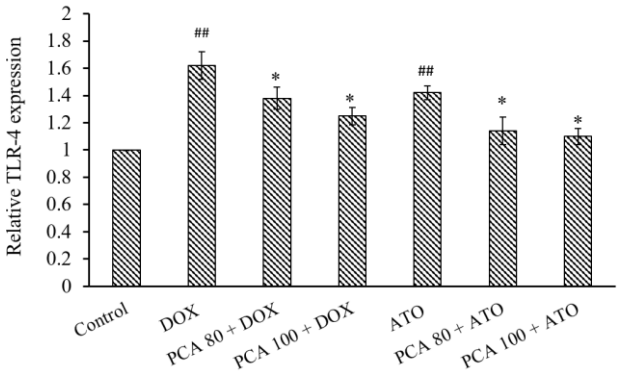

Fig. 7. Effect of PCA on TLR4 gene expression in DOX- and ATO-induced toxicity in H9C2 cells determined by quantitative real-time-polymerase chain reaction. Values are means ± SEM. $^{\#\#}P < 0.01$ Indicates significant differences in comparison with the control (untreated cells); $^{*}P < 0.05$ versus DOX or ATO. PCA; Protocatechuic acid; TLR, triggers toll-like receptors; DOX, doxorubicin; ATO, arsenic trioxide.

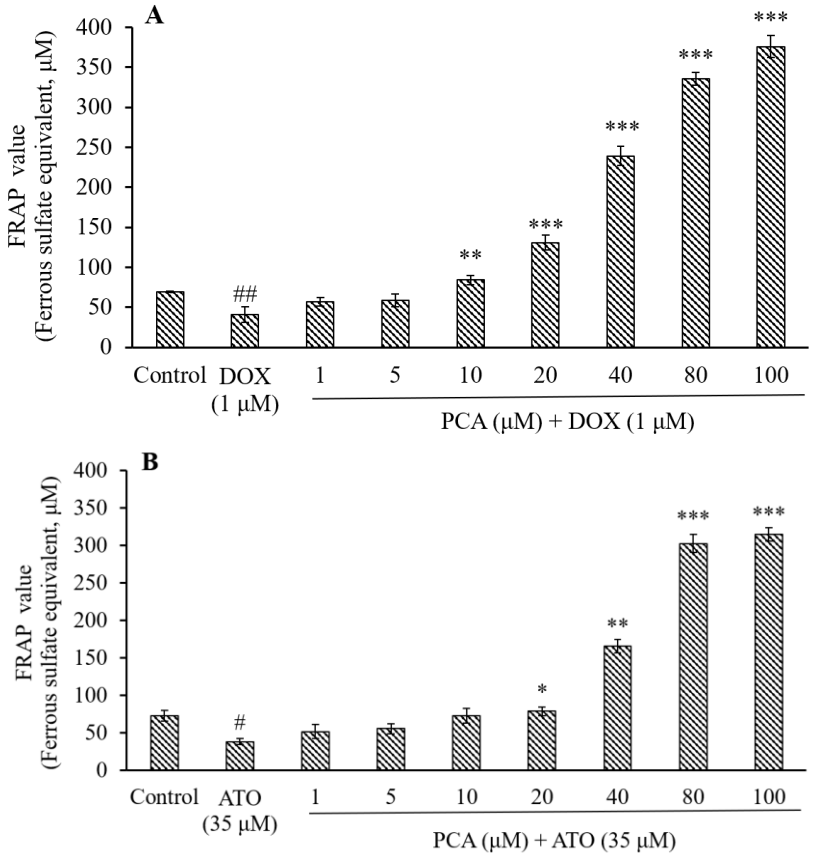

Fig. 6. Effect of PCA on FRAP value in (A) DOX- and (B) ATO-induced toxicity in H9C2 cells determined as ferrous sulfate equivalents. Values are means ± SEM. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ Indicates significant differences in comparison with the control (untreated cells); $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P <0.001$ versus DOX or ATO. PCA; Protocatechuic acid; FRAP, ferric-reducing antioxidant power; DOX, doxorubicin; ATO, arsenic trioxide.

DISCUSSION

In the current investigation, *in vitro* assessment of PCA exhibited protective and antioxidant activities through elevation in cellular viability and FRAP value, and reduction in cytotoxicity, hydroperoxides level, and TLR4 expression in H9C2 cells under toxicities caused by DOX and ATO.

PCA as a natural phenolic acid owns various nutritional uses and medicinal effects, particularly against cardiovascular disorders (23). Recent evidence has shown the cardioprotective activities of phenolic acids including hypolipidemic, antihypertrophic, antifibrotic, and blood pressure-lowering effects (24).

In this study, not only did PCA (1-100 µM) not have an inhibitory effect on H9C2 cell viability but also showed a proliferative effect at 100 µM. It noticeably prevented the cytotoxicity caused by DOX and ATO at 40-100 µM concentrations. MTT assay is a suitable method for the evaluation of cell viability not for proliferation however the absorbance of the PCA-pretreated group at the concentration of 100 µM was more than the control group. So according to the formula, a positive effect on cell proliferation was experimentally observed. This proliferative effect has been confirmed in other studies with reliable methods. In the study conducted by Guan *et al*. 4 days of incubation with PCA at 30-120 µM could enhance the proliferation of neural stem cells in cell counting kit-8 assay and bromodeoxyuridine labeling test. It also diminished the basal apoptosis of these cells by reducing the caspase-3 activity (25). In another *in vitro* investigation, PCA at the concentrations of 60 and 120 µM displayed a proliferative effect in pheochromocytoma PC12 cells which is a well-known cell line for neurosecretion and neuronal differentiation studies. Moreover, it prohibited the cytotoxicity from 1-methyl-4-phenylpyridinium ion, hydrogen peroxide, or sodium nitroprusside in PC12 cells (26). Deng and co-workers described the anti-apoptotic activity of PCA through suppressing caspases, Bax, Bid, and Fas pathways and stimulating the pro-survival signals including Bcl-2, Bcl-xL, insulin-like growth factor 1, Akt, and phosphoinositide 3-kinase in hypertensive rat heart (12). The protective and anti-apoptotic properties of PCA have also been reported in rat cardiomyocytes in an ischemic heart disease model (13).

It is noteworthy that PCA has mixed effects on normal and cancerous cells. Although PCA has displayed chemo-preventive activity against the development of some neoplasms in various tissues typically through antioxidant effects and *via* its impact on the action and metabolism of some carcinogens (27). It has shown anticancer and antimetastatic properties by inducing apoptosis and cell cycle arrest in cancer cells *in vitro* (28). Additionally, the combination of PCA with some anti-neoplastic agents such as 5-fluorouracil has shown a synergistic effect as not only was useful for decreasing the dose requirements of the anticancer drug but also has been able to stimulate its cytotoxic activity (29).

Moreover, our findings exhibited a reduction in hydroperoxides content and an increase in FRAP value in H9C2 cells after incubation with PCA confirming its antioxidative capacities similar to other studies. The potent antioxidant activities have been established for PCA through scavenging of various free radicals like superoxide anion, hydroxyl, and DPPH radicals, reducing power and the chelating ability for Fe^{2+} and Cu^{2+}, enhancing the activities of superoxide dismutase and catalase, and inhibiting NADPH oxidase (30,31).

Our results also indicated that PCA ameliorates the expression of the TLR4 gene during DOX- and ATO-induced cardiotoxicity. The role of DOX in the up-regulation of TLR2 and TLR4 expression in cardiomyocytes has been recognized in several studies (5,32,33). Improvement of cardiac function and diminution of oxidative, inflammatory, and apoptotic processes have been reported after Dox administration in mice with TLR4 deficiency (32,33). However, there are only a few reports about the effects of ATO on TLR expression in cardiotoxicity. Recently, Zheng *et al*. showed the involvement of the TLR4/NF-κB pathway and inflammatory responses in the heart damage caused by ATO in a mice model

(34). On the other hand, Nam and co-workers indicated that PCA exerts an inhibitory action on TLR4 and subsequently prevents motivation of mammalian target of rapamycin, protein kinase Akt, and NF-κB, c-Jun N-terminal kinase, and p38mitogen-activated protein kinases pathways during oxidative stress and inflammation caused by lipopolysaccharide in keratinocytes (18).

CONCLUSION

Our findings suggest that PCA can protect cardiomyocytes from DOX- and ATO-induced cytotoxicity by promoting cellular viability and total antioxidant power and lessening the total oxidant capacity and TLR4 expression. However, further *in vivo* investigations are recommended to define the clinical efficacy of PCA in the management of cardiotoxicity.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

L. Safaeian contributed to the conceptualization of the study, supervision, project administration, and editing of the manuscript; F. Shafiee contributed to the supervision and editing of the manuscript; F. Gorbani contributed to the investigation and writing the original draft of the manuscript. The finalized manuscript was approved by all authors.

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