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# Two novel anticancer compounds with minimum cardiotoxic property

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## Abstract

**Background:** Although two novel synthesized compounds with tri-aryl structures; 3-(4-chlorophenyl)-5-(4-fluorophenyl)-4-phenyl-4,5-dihydro-1,2,4-oxadiazole (A) and 3,5-bis-(4-chlorophenyl)-4-phenyl-4,5-dihydro-1,2,4-oxadiazole (B) have been previously demonstrated to possess remarkable anti-breast cancer activity, their cardiotoxicity remains a major concern due to their mechanism of action. To address this concern, we assessed the ability of these compounds to cause toxicity towards H9c2 cardiomyocytes as an in vitro model of cardiotoxicity.

**Methods:** Cytotoxic activity of both compounds was explored in vitro on H9c2 cells using MTT assay. Annexin V/PI method, intracellular ROS determination and mitochondrial membrane potential assay were applied to elucidate the mechanism of action of the cell death.

**Results:** MTT assay revealed a concentration- and time-dependent cardiotoxicity. Findings of apoptosis by double staining with annexin V and propidium iodide divulged no cell death including apoptosis and necrosis at the concentration that were effective to inhibit cancer cells proliferation (10  $\mu$ M) at 24 and 48 h. Furthermore, flow cytometric measurement of membrane potential and ROS determination using DCFH-DA verified the safe concentration of the compounds against H9c2 cells with no cardiotoxic effect. However, the higher concentration of the compounds could induce cell death through ROS-mediated mitochondrial dysfunction.

**Conclusions:** Altogether, the results represented two novel chemical molecules possessing anti-breast cancer activity with minimum cardiac side effect.

**Keywords:** Cardiotoxicity, Apoptosis, ROS, Mitochondrial potential

## Background

Cardiovascular diseases (CVDs) remains number one of the fatal diseases, which is ever increasing worldwide and hence, scientific community has concerned about it [1, 2]. Cardiotoxicity occurs as a result of the complicated working of cardiac cells and tissues or due to a chemical molecule affecting heart function and structure [3, 4]. Cardiotoxicity induced by drugs has gained attention in the past few decades. In this context, anticancer

therapy has a direct impact on cardiac function and the most common toxicities of heart are due to cancer treatment [5]. However, clinicians still use many cardiotoxic drugs due to their beneficial effects, which outweigh cardiac malformations risks [4]. As it was above-mentioned, most of the anticancer drugs exhibit a wide range of cardiovascular toxicities which results in stopping cancer treatment and affects the short- and long-term quality of life [6–8].

Inflammation and oxidative stress are mutually reliant processes, which predominantly involve in cardiovascular diseases and cancers via inducing apoptosis and necrosis [9–13]. In other words, high level of reactive oxygen species (ROS) due to oxidative stress can enfeeble cardiac cellular signaling pathways [14].

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Triaryl template-based structures found in a wide array of compounds, display various biological properties including anticancer [15, 16]. Besides it, a number of compounds with this pivotal structural feature revealed cyclooxygenase-2 (COX-2) inhibitory activity [17]. In this line, we previously reported the apoptotic activity of the two compounds; 3-(4-chlorophenyl)-5-(4-fluorophenyl)-4-phenyl-4,5-dihydro-1,2,4-oxadiazole (**A**) and 3,5-bis-(4-chlorophenyl)-4-phenyl-4,5-dihydro-1,2,4-oxadiazole (**B**) (Fig. 1) in the breast cancer cells via a COX-2 independent pathway [18], although these compounds interacted with the active site of COX-2 enzyme [19]. Notably, different hypothesis were raised by researchers for the discernible increase in cardiotoxicity due to apoptosis in the terminally differentiated cardiomyocytes [20]. Interestingly, amongst different pathophysiological consequences, cardiovascular diseases are associated with apoptosis. Indeed, a number of studies provided convincing evidence that cardiac cell death has been contributed to major heart diseases including myocardial infarction (MI), cardiomyopathies, arrhythmogenic right ventricular dysplasia, end-stage heart failure, etc. [21–23].

Given the importance of cardiac cell apoptosis and knowing that major mechanism related to the two tested compounds is triggering mitochondrial apoptosis, motivated us to evaluate the cardiotoxicity of the compounds. Since generation of ROS activates mitochondrial-mediated apoptotic signaling pathway leading to caspase 3 activation and cardiac cell apoptosis, we further focused on the understanding of molecular mechanism underlying cardiotoxicity of two compounds. To achieve it, a cell culture model created by rat cardiomyoblast cell line (H9c2) was applied.

## Materials and methods

### Cell culture

H9c2 (Rat embryonic cardiac myoblast) cell line (C585) was obtained from National Cell Bank of Pasteur

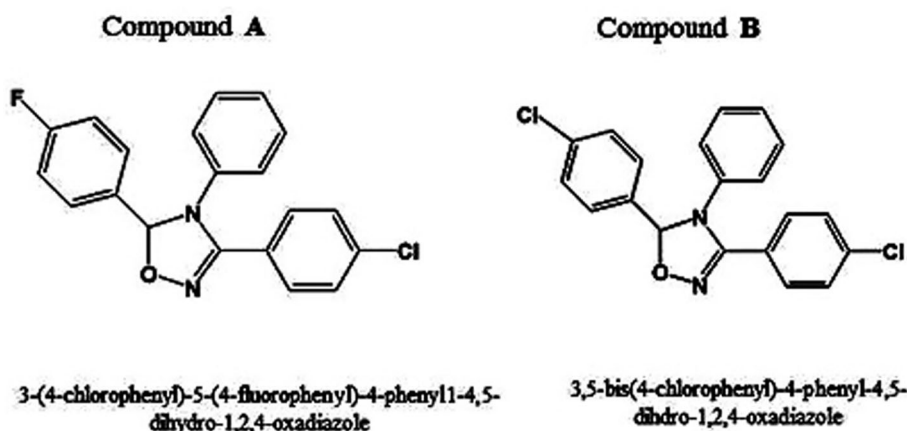
Institute of Iran. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-BRL, Rockville, IN) containing high level of glucose and 2 mM L-glutamine supplemented with penicillin-streptomycin (Gibco-BRL, Rockville, IN) and Fetal Bovine Serum (FBS) (Gibco-BRL, Rockville, IN) at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. Compounds **A** and **B** were reconstituted in Dimethyl Sulfoxide (DMSO) and the final concentration of DMSO was 0.5%. Compounds **A** and **B** were synthesized by the Medicinal Chemistry Research Laboratory at the Faculty of Pharmacy, Tehran University of Medical Sciences. Cells without treatment were considered as negative control.

### MTT assay

Cell viability was determined by MTT assay. H9c2 cells were plated in 96-well plates at 7000 cells/well for 24 h and 5000 cells/well for 48 h of treatment. Cells were exposed to different concentrations of compounds **A** and **B** to reach final concentration of 0.1–100 μM at 24 and 48 h. Afterwards, 20 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 5 mg/ml) (Sigma-Aldrich, Saint Louis, Missouri, USA) was added for an additional 4 h. The medium was removed and 200 μl of DMSO was added to each well to dissolve formazan crystals. Finally, the absorbance was measured by microplate reader (Epoch2, Biotek, USA) at 545 nm.

### Intracellular ROS determination

To determine the intracellular ROS, H9c2 cells were seeded in 6-well plates at a concentration of  $25 \times 10^4$  cells per well for 24 h and  $15 \times 10^4$  cells for 48 h of incubation. Cells were then treated with 10 and 50 μM of compounds **A** and **B** for 24 and 48 h. Following incubation of cells with 10 μM of DCFH (Sigma-Aldrich, Saint Louis, Missouri, USA) at 37 °C for 20 min, the cells were



**Fig. 1** Chemical structures of compounds **A** and **B**

washed with PBS (Phosphate Buffered Saline) and then harvested to detect ROS level by flow cytometer.

#### Mitochondrial membrane potential assay

Mitochondrial dysfunction was assessed using mitochondrial membrane potential ( $\Delta\Psi_m$ ) assay. Briefly, H9c2 cells were plated in 6-well plates as described above and then trypsinized after treating with 10 and 50  $\mu\text{M}$  of compounds **A** and **B** for 24 and 48 h. We used carbonyl 3-chlorophenylhydrazone (CCCP) as a standard control. After washing the cells with PBS, 0.5 ml of PBS buffer containing 10  $\mu\text{g}/\text{ml}$  of JC-1 (Mitoprobe JC1 assay kit, Life technologies, USA, M34152) was added to the suspend cells. Following 15–30 min of incubation at 37  $^{\circ}\text{C}$ , cells were centrifuged to discard the supernatant. Cell pellets were then re-suspended in PBS and analyzed by flow cytometry. A mitochondria-sensitive dye (JC-1) was used to monitor mitochondrial membrane potential alterations by observing the percentage of mitochondrial matrix aggregation. The aggregate JC-1 (red fluorescence) was determined at the emission wavelength of 590 nm, and the monomeric JC-1 (green fluorescence) monitored at 529 nm.

#### Apoptosis assay

The numbers of apoptotic cells were measured by AnnexinV/PI assay kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacture's instruction. To do it, H9c2 cells were washed and then harvested after treatment with 10 and 50  $\mu\text{M}$  of compounds **A** and **B**. Doxorubicin was used as a positive control. Next, cells were stained using 100  $\mu\text{l}$  of Annexin V-FLUOS labeling solution containing 2  $\mu\text{l}$  annexin V-FLUOS labeling agent, 2  $\mu\text{l}$  PI solution and 1 ml incubation buffer. Cells were then incubated for 15 min at 37  $^{\circ}\text{C}$  and subjected to flow cytometry. The percentages of viable (AnnexinV-/PI-), early apoptotic (AnnexinV+/

PI-), late apoptotic (AnnexinV+/PI+) and necrotic (AnnexinV-/PI+) cells were finally analyzed.

#### Statistical analysis

At least three biological replicates were considered for all experiments and the data were expressed as mean  $\pm$  SEM. To compare the differences between multiple parameters, one-way ANOVA followed by the Tukey's post test was applied using GraphPad Prism 6.0 Software. A  $p$  value lower than 0.05 was considered as significant.

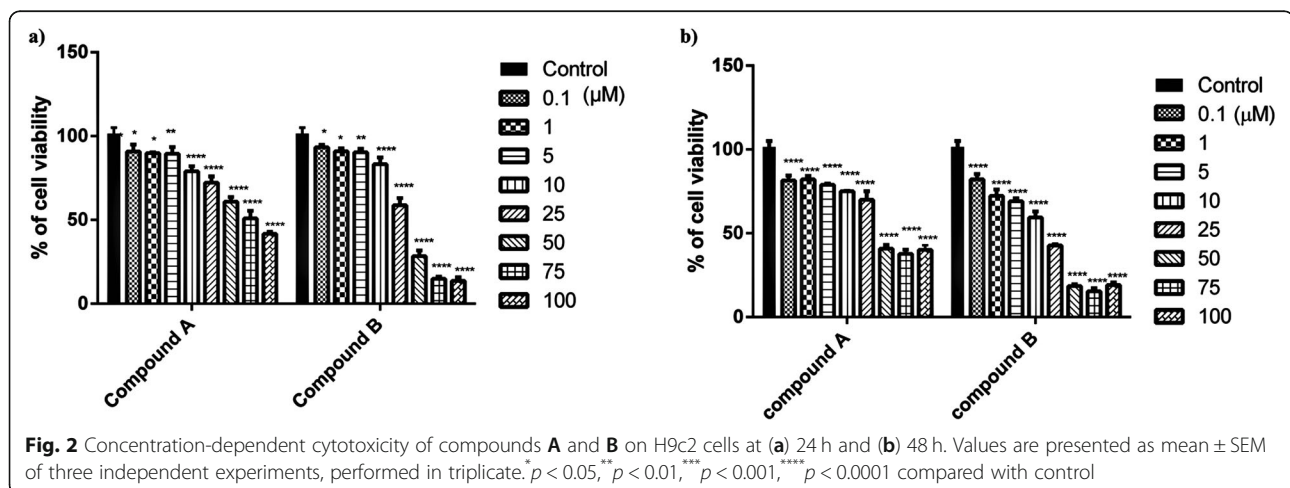
## Results

#### Compounds **A** and **B** affected viability of H9c2 cells

H9c2 cells were treated with different concentrations of compounds **A** and **B** for 24 and 48 h and cell viability was determined using MTT assay. As demonstrated in Fig. 2, both compounds reduced the cell viability in a time- and concentration-dependent manner within the concentration range of 0.1–100  $\mu\text{M}$ . 75 and 25  $\mu\text{M}$  concentrations of compound **A** reached the myocardial viability cells to 50.9 and 72.2%, respectively, after 24 h. These values for compound **B** were 14.9 and 58.7%, which were close to their  $\text{IC}_{50}$  concentrations ( $\text{IC}_{50}$  **A**:  $77.02 \pm 1.09$  and  $\text{IC}_{50}$  **B**:  $28.64 \pm 1.04$   $\mu\text{M}$ ). The vitality of the cells was also diminished more significantly after 48 h of treatment compared with that of 24 h ( $\text{IC}_{50-48\text{ h}}$  was  $45.90 \pm 1.17$   $\mu\text{M}$  for compound **A** vs.  $10.68 \pm 1.15$   $\mu\text{M}$  for compound **B**). Considering our previous findings implying the effective concentration of both compounds on breast cancer cells ( $\sim 10$   $\mu\text{M}$ ) and that this concentration is below the  $\text{IC}_{50}$  values in myocardial cells, we selected 10  $\mu\text{M}$  for use in our following experiments. Moreover, we chose a 5-fold  $\text{IC}_{50}$  concentration to verify our results.

#### Apoptosis induction by compounds **A** and **B** in H9c2 cells

Flow cytometry was applied to investigate apoptosis in the control and the compounds-treated H9c2 cells. Cell



apoptosis was determined using Annexin V-FLUOS/Propidium iodide (PI) double staining. The apoptotic rate was concentration -dependently increased in the H9c2 cells treated with both compounds at 24 h compared with the control group (Fig. 3a-e). In this context, 10  $\mu$ M of compounds A and B did not affect the cell, while a large proportion of cells were in the early apoptotic stage when the cells were subjected to both compounds at 50  $\mu$ M (Table 1). Of note, no apoptotic cells were seen upon treatment of H9c2 cells with 10  $\mu$ M of compounds A and B at 48 h (Fig. 3f-h and Table 1). Due to the highest rate of cell death following treating with 50  $\mu$ M of the compounds at 48 h, we excluded it and focused our further experiments on 10  $\mu$ M.

### Apoptosis induced by compounds A and B is through ROS generation

Previous studies have revealed that cell apoptosis can occur as a result of ROS generation through regulation of caspase [24]. To explore whether compounds A and B affect ROS generation at 10 and 50  $\mu$ M concentrations after 24 and 48 h of incubation, we used DCFH-DA to determine intracellular ROS level in H9c2 cells. DCFH-DA freely enters the cell and is oxidized to DCF by ROS inside the cell which produces green fluorescence signals. As presented in Fig. 4a, no significant shift was seen after 24 h of treatment of both compounds at 10 and 50  $\mu$ M compared with the control group. However, an observable

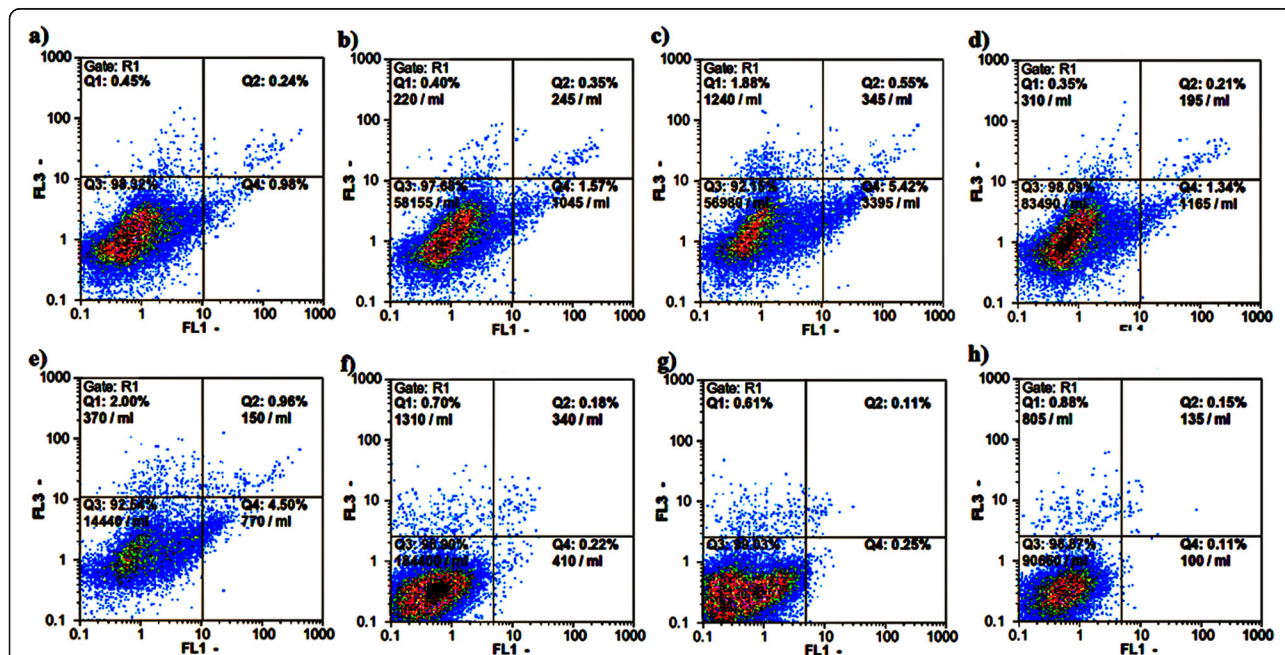
shift of fluorescence was detected in H9c2 cells treated with 10  $\mu$ M of compounds A and B at 48 h (Fig. 4b).

### Compounds A and B influenced mitochondrial potential membrane

Since mitochondrial function is highly sensitive to oxidative damages [25] and to further verify whether mitochondrial apoptosis pathway is induced by compounds A and B, the mitochondrial membrane potential (MMP) was assessed using the fluorescent dye JC-1. Red fluorescence displaying the matrix of actively respiring mitochondria is the aggregate form of JC-1 molecules, while green fluorescence is emitted from the monomeric form of the JC-1 molecules indicating mitochondrial membrane depolarization. As shown in Fig. 5a, after treating with compounds A and B at 50  $\mu$ M for 24 h, the percentage of JC-1 monomeric cells was increased compared with control, which is an indicator of the loss of MPP revealing the mitochondrial damage. No significant change was detected upon incubation of the cells with 10  $\mu$ M of compounds A and B for 24 h. Similarly, no mitochondrial dysfunction was observed following treatment of the cells with both compounds at 10  $\mu$ M for 48 h (Fig. 5b).

### Discussion

Evidences display that oxidative stress induces a variety of cardiomyocyte deaths, consisting of apoptosis,



**Fig. 3** Annexin-V/PI flow cytometric analysis to quantify apoptosis induction in H9c2 cells. Dot plot of (a) H9c2 cells alone and treated with compound A at (b) 10  $\mu$ M, (c) 50  $\mu$ M and compound B at (d) 10  $\mu$ M, (e) 50  $\mu$ M for 24 h; (f) H9c2 cells alone and treated with compounds (g) A and (h) B at 10  $\mu$ M for 48 h. The results shown are representatives of three independent experiments. Quadrant 3, living cells An<sup>-</sup>/PI<sup>-</sup>; Quadrant 4, early apoptotic cells An<sup>+</sup>/PI<sup>-</sup>; Quadrant 2, late apoptotic cells An<sup>+</sup>/PI<sup>+</sup>; Quadrant 1, necrotic cells An<sup>-</sup>/PI<sup>+</sup>

**Table 1** Percentage of H9c2 cells in each state after treatment with two concentrations of compounds **A** and **B** at 24 and 48h<sup>a</sup>

	10 $\mu$ M (24 h)			50 $\mu$ M (24 h)			10 $\mu$ M (48 h)		
	Control	Compound A	Compound B	Control	Compound A	Compound B	Control	Compound A	Compound B
Vital cells	97.7 $\pm$ 0.3	96.96 $\pm$ 0.43	97.83 $\pm$ 0.25	97.7 $\pm$ 0.3	92.29 $\pm$ 0.2 <sup>***</sup>	94.11 $\pm$ 1.5 <sup>*</sup>	98.88 $\pm$ 0.01	98.72 $\pm$ 0.19	98.98 $\pm$ 0.11
Early apoptosis	1.3 $\pm$ 0.15	1.8 $\pm$ 0.20	1.1 $\pm$ 0.25	1.3 $\pm$ 0.15	5.3 $\pm$ 0.52 <sup>****</sup>	3.0 $\pm$ 0.75 <sup>*</sup>	0.29 $\pm$ 0.04	0.29 $\pm$ 0.04	0.08 $\pm$ 0.02
Late apoptosis	0.3 $\pm$ 0.07	0.36 $\pm$ 0.01	0.32 $\pm$ 0.04	0.3 $\pm$ 0.07	0.32 $\pm$ 0.07	0.32 $\pm$ 0.04	0.16 $\pm$ 0.2	0.14 $\pm$ 0.01	0.09 $\pm$ 0.02
Necrosis	0.24 $\pm$ 0.06	0.36 $\pm$ 0.23	0.32 $\pm$ 0.04	0.24 $\pm$ 0.06	0.25 $\pm$ 0.03	0.32 $\pm$ 0.04	0.66 $\pm$ 0.02	0.64 $\pm$ 0.06	0.85 $\pm$ 0.1

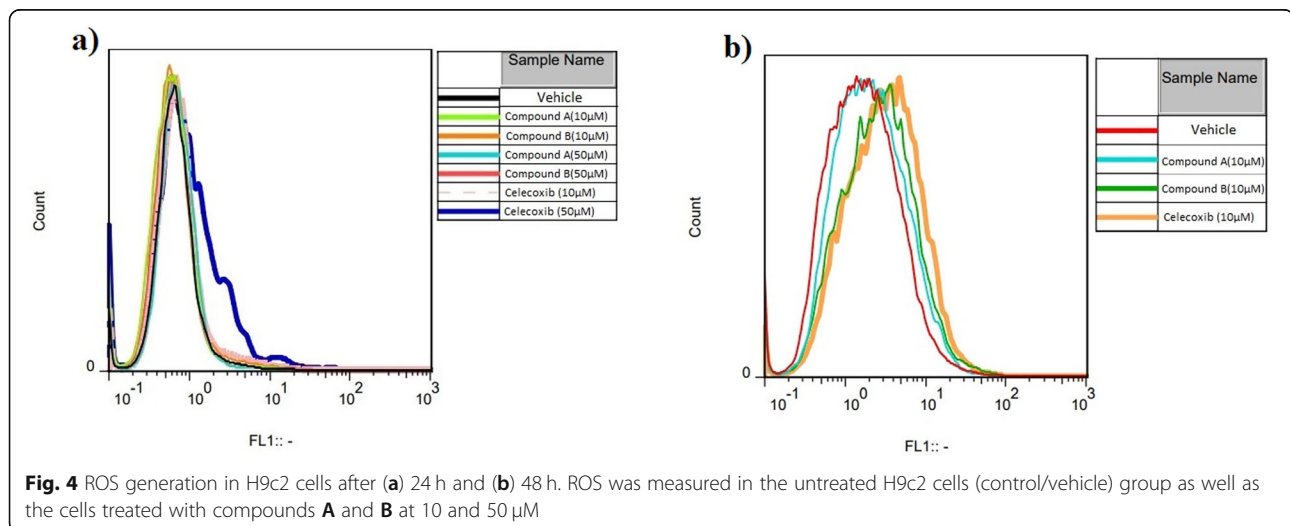
<sup>a</sup>The data presented are the mean  $\pm$  SEM of three independent experiments. <sup>\*</sup> $p < 0.05$ , <sup>\*\*</sup> $p < 0.01$ , <sup>\*\*\*</sup> $p < 0.001$ , <sup>\*\*\*\*</sup> $p < 0.0001$  compared with control

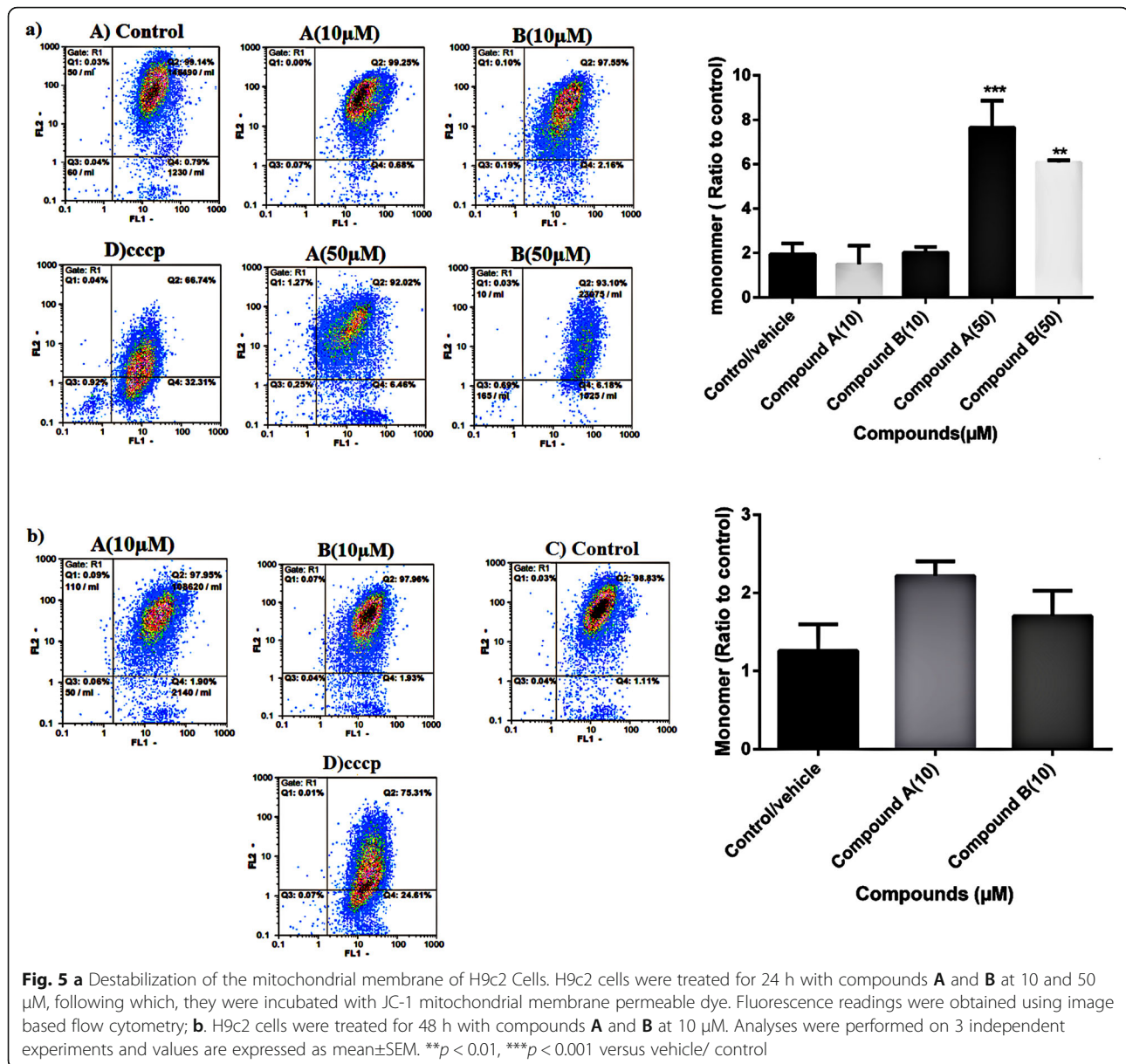
necrosis and necroptosis [26–28]. In this context, ample researches have shown that the therapeutic agents generating ROS cause cardiotoxicity [29]. Among different types of therapeutics, certain classes of chemotherapeutic agents induce more common and frequent cardiotoxic effects. On the other hand, heart dysfunction along with the promotion of cardiac hypertrophy and the loss of contractile occur due to apoptosis of cardiomyocyte, which in turn develops cardiovascular diseases [30, 31]. The anti-breast cancer activities of two novel synthesized compounds (**A** and **B**) have been previously studied [18]. Our findings revealed that these two compounds act through inducing apoptosis. Keeping in mind that apoptosis plays an important role in cardiotoxicity and in consequence heart dysfunction and knowing that these two compounds likely resemble to COX-2 inhibitors with tri-aryl structures exerting their effects via apoptosis induction [19], herein, we attempted to verify that these compounds are nontoxic to cardiac cells. Hence, we performed the in vitro experiments on H9c2 cells. This is the first study to find out the effectiveness of compounds **A** and **B** to preserve the cardiomyocyte viability.

In the current study, for an exposure time of 24 h, compounds **A** and **B** concentration-dependently

decreased H9c2 cell viability. For longer period of exposure (48 h), these compounds also caused a decrease in cell viability implying a time –dependent cell cytotoxicity. Furthermore, our findings exhibited that the higher dose of compounds **A** and **B** (50  $\mu$ M) can trigger the apoptotic process in H9c2 cells upon 24 h of treatment in which early apoptosis to a large extent was involved in the cell death. Interestingly, a concentration of 10  $\mu$ M by which a remarkable anticancer activity had been seen for the compounds against breast cancer cells [18], could activate no apoptotic process at 24 and even 48 h on cardiac cells.

A large number of studies have elucidated that intracellular ROS generation is closely associated with cell apoptosis [32]. Consistent with the apoptosis data, our results from determining the intracellular ROS level revealed no shift in the fluorescent intensity in H9c2 cells after cell exposure to 10  $\mu$ M of both compounds for 24 h. Surprisingly, when the cells were subjected to 50  $\mu$ M of compounds **A** and **B** for 24 h, the level of ROS remained unchanged in H9c2 cells which can be explained regarding the time that the ROS level was determined in the treated cells. Indeed, we assumed that following an initial increase in ROS level, it reaches the original level after 24 h. Thus, a large population of the





cells was in the early of apoptosis at 24 h, while no significant difference in ROS concentration was detected in the treated cells compared with the control. Moreover, after incubation of the cells with 10  $\mu\text{M}$  of compounds A and B for 48 h, no apoptosis process was induced, whereas a small level of ROS was produced suggesting the triggering of oxidative stress which was not strong enough to induce cell apoptosis. Our data support previous findings and indicated that ROS contributes to the regulation of apoptosis but it is generated in two steps; early and late stages in which time of ROS determination plays a key role [33].

Deregulation of the different cell death pathways causes pathological outcomes for oxidative stress-

associated diseases including ischemia-reperfusion injury that has to be considered as adverse effects of drugs [34]. The generated ROS can inaugurate and enhance important mitochondrial alterations by depolarizing MMP. In other words, ROS mainly causes depolarization and bulging of the mitochondria and then augments apoptotic mechanism through mitochondrial involvement [35, 36]. Thus, as a consequence, cytochrome *c* that is a key mediator in the mitochondrial pathway of cell death is released from mitochondria [37]. At last, upon activation of caspase-3, cell apoptosis is induced [38]. Our results demonstrated that 50  $\mu\text{M}$  of compounds A and B disturbed mitochondrial membrane potential  $\psi\text{m}$  of H9c2 at 24 h, whereas no mitochondrial

function impairment was observed following treatment of H9c2 with 10  $\mu$ M of both compounds for 24 h confirming our apoptosis results. Interestingly, it was shown that excessive ROS can damage mitochondria as well as open its permeability transition pore (PTP), and thereby induces mitochondrial permeability transition. The mitochondrial depolarization and outer membrane rupture due to these alterations result in cell apoptosis or death [39, 40]. These findings corroborate our obtained results and indicate that low dose of both compounds can induce ROS production after 48 h of treatment; however, this level of ROS was unable to induce apoptosis. Interestingly, high dose of these compounds after 24 h damaged mitochondrial potential and induced apoptosis, although it did not affected ROS generation suggesting an undetectable level of ROS due to the inappropriate time of measurement in the cells exposed to a high concentration of the compounds.

## Conclusions

Our study represented two novel chemical molecules possessing anti-breast cancer activity with minimum influence on the oxidative stress-mediated apoptosis induction on cardiomyocytes implying therapeutic potential for heart disease. However, these compounds at the higher dose can attenuate mitochondrial dysfunction and trigger apoptosis via oxidative stress. But, the detailed mechanism of action needing to be further investigated.

## Abbreviations

CVDs: Cardiovascular diseases; ROS: Reactive oxygen species; COX-2: Cyclooxygenase-2; MI: Myocardial infarction; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; DMSO: Dimethyl sulfoxide; MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DCFH: 2',7'-Dichlorofluorescein; PBS: Phosphate buffered saline; CCCP: Carbonyl 3-chlorophenylhydrazine; PI: Propidium iodide; SEM: Standard error of the mean; ANOVA: Analysis of variance; IC<sub>50</sub>: The half maximal inhibitory concentration; DCFH-DA: 2',7'-Dichlorofluorescein diacetate; MMP: Mitochondrial membrane potential; PTP: Permeability transition pore

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## Authors' contributions

TA and RT performed experiments. MA synthesized the chemical compounds. BD helped in designing the experiments. MS was the principle investigator who conceived the idea and helped in the preparation of the manuscript. All authors read and approved the final manuscript.

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## Availability of data and materials

The datasets used during the present study are available from the corresponding author on reasonable request.

## Ethics approval and consent to participate

There is no animal testing of the compound made in this study, and therefore, no animal ethical clearance is required for this study.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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