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Original Article

Cinnamaldehyde potentiates cytotoxic and apoptogenic effects of doxorubicin in prostate cancer cell line

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Abstract

Background and purpose: Nowadays, herbal medicine has been utilized to treat various diseases such as cancer, which showed successful therapeutic efficacy in previous studies. This study for the first time evaluated the cytotoxic potential of cinnamaldehyde (CIN) alone and in combination with doxorubicin (DOX), a well-known potent anti-tumor agent, on the proliferation of prostatic cancer cell line (PC3).
Experimental approach: The cytotoxicity and apoptotic activities of CIN and DOX, either separately or together, were determined on PC3 cells by the MTT test and Annexin V/PI assay, respectively. To further investigate which apoptotic pathway participated in cell death a collection of prominent markers of apoptosis induction including caspase-3/7 activations, mitochondrial membrane potential (MMP), and phosphatidyl serine translocation were detected.
Findings/Results: The different concentrations of CIN and DOX significantly inhibited the proliferation of PC3 cells in a concentration-dependent way within a 24-h treatment. In addition, the induction of apoptosis by CIN was accompanied by an increase in the activation of caspase-3/7 in PC3 cells with IC_{50} concentrations of 12.5 and 10 µg/mL for CIN and DOX, respectively. Moreover, the morphological observations obtained from flow cytometry MMP and caspase-3/7 activity assays, altogether, revealed the potential effect of CIN on apoptosis induced in PC3 cells by DOX.
Conclusions and implications: Taken together, the current study concluded that the combination of CIN and DOX could lead to the production of a potential therapeutic agent for prostate cancer. However, further *in vivo* and clinical studies are still needed to validate this combination in prostate cancer therapy.

Keywords: Apoptosis; Cinnamaldehyde; Cytotoxicity; Doxorubicin; Prostate cancer.

INTRODUCTION

The high rate of mortality can be seen among men with prostate cancer, as one of the most prevalent types of cancer in these groups (1) because of the lack of available curative options for the advanced stage of metastatic disease (2). The most important strategy in cancer therapy is to monitor the viability and death of cancerous cells. There are two types of human prostate cancer cell lines including LNCaP and PC3, used widely by researchers to represent the overall aspects of prostate cancer (3). The literature has documented that LNCaP cells express androgen and prostate-specific antigen (PSA), indicating these cells depend on androgen and exhibit androgen-sensitive growth. While PC3 cells do not express androgen and PSA, indicating the cells are androgen-independent and exhibit an atypical pattern of cancer progression (3,4). Prostatectomy at the early stages of the disease would be an efficient tool in prostate cancer therapy, the diagnosis of its metastasis at later stages makes chemotherapy the essential way of treatment (5).

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Expensive chemotherapy drugs along with the side effects on normal cells have provided the opportunity for herbal products in new treatment ways for various cancers, especially in developing countries like Iran (6). Recent study has also shown that various factors such as dietary supplements influence the development and disease progression (7). Cinnamaldehyde (CIN), an isolated bioactive compound from the stem bark of *Cinnamomum cassia*, has displayed several biological activities, including immunomodulatory, antibacterial, and anti-angiogenic activities (8,9). In addition, cinnamon has exhibited cytotoxic and anti-cancer effects (10-13).

Doxorubicin (DOX) as a successful chemotherapeutical agent possesses wide uses in cancer therapy. However, ignoring its severe side effects on heart and kidney toxicity is inevitable. Furthermore, DOX resistance in the treatment of prostate cancer is a growing challenge. Hence, it is necessary to introduce a new treatment manner that not only decreases DOX cytotoxicity in normal cells but also reinforces its antitumor potential (12-14).

Combination chemotherapy, common chemotherapy, is a key, safe, and beneficial way to delay the resistance of cancer cells to DOX and reduce its side effects in patients. Several experiments have investigated the antitumor activity and cytotoxic effect of herbal extracts and natural compounds on DOX-induced cytotoxicity in normal cells and cancer cell lines (15-21). Moreover, the cytotoxic and anticancer activities of natural compounds have provided promising results in preventing the prevalence and progression of various cancers (22). Previous studies have shown that CIN results in the improvement of DOX efficacy in U87MG cells (12). Accordingly, the present study was planned to evaluate the effect of CIN on the efficacy of DOX in PC3 cancerous cells and investigate the possible mechanisms involved in the potentiating cytotoxic effect of DOX.

MATERIALS AND METHODS

Cell lines and chemicals

CIN, Triton® X-100, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rhodamine 123 fluorescent dye, Bradford reagent, DOX and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Company (USA). Dulbecco's modified eagle's medium (DMEM-F12), fetal bovine serum (FBS), and penicillin/streptomycin were supplied from Gibco Company (USA). Trypsin-EDTA was prepared by Bon Yakhteh Company (Iran). Caspase-3/7 activities were purchased from Kia Zist Company (Iran). The human PC3 cell line was obtained from the Pasteur Institute (Iran).

Cell culture

PC3 cells were propagated in DMEM-F12 supplemented by 1% penicillin (100 U/mL), 10% FBS, and streptomycin (100 µg/mL) under the condition of 5% CO_2 and a temperature of 37 °C. To achieve 70-80% confluency in cell proliferation, it was attempted to replace the medium at specified time intervals. Then, cells were washed with PBS, detached by adding 1 mL of 0.25% trypsin-EDTA solution and seeded in a 96-well plate.

Cell viability

The volume of 200 µL containing a density of 5×10^3 cells was seeded into 96-well culture plates. The different concentrations of CIN and DOX were prepared in a serial dilution manner in DMSO as a suitable solvent. After reaching an appropriate confluence, the cells were treated by DOX at the concentrations of 2.5, 5, 10, and 20 µg/mL either alone or in combination with CIN at the concentrations of 12.5, 25, and 50 µg/mL. Untreated cells received DMSO (0 µg/mL concentration of DOX or CIN) and were considered the control group. After 24 h, the supernatant was replaced by 20 µL of MTT (0.5 mg/mL) and incubated for 2-4 h at 37 °C. Finally, to dissolve MTT-produced formazan crystals DMSO (100 µL) was added to each well, and the optical density was measured at a wavelength of 490 nm by an ELIZA microplate reader (BioTek Instruments, USA). For further accuracy, triplicate was considered in all the MTT assays.

Annexin V-fluorescein isothiocyanate/ propidium iodide double staining assay

Cells were seeded into 6-well plates. After obtaining appropriate confluence, cells were treated with DOX alone and in combination with CIN and then incubated for 24 h. Next, the treated and control cells were collected and washed with PBS. After that, the cells were stained according to the manufacturer's instruction of the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit (CoWin Biosciences, China). Finally, flow cytometry (BD Biosciences, USA) was used to analyze the samples.

Caspase-3/7 activities

Caspase-3/7 was evaluated according to the Kit user's guide. Briefly, the number of 5×10^4 cells/mL was cultured in a 6-well plate. After being incubated overnight, cells were treated with DOX alone and in combination with CIN, and incubated for 24 h. In the following, the treated and untreated cells were gathered and washed with PBS and resuspended in a 1× assay buffer BA, mixed with the Caspase-3/7 reagent, and incubated for 20 min. Finally, each tube was mixed thoroughly with Caspase-3/7-AAD working solution for 5 min at room temperature in the dark. Finally, the absorbance was measured at 405 nm by the plate reader (BioTek, H1M, USA).

Assessment of mitochondrial membrane potential

Rhodamine 123 fluorescent dye was used for the mitochondrial membrane potential (MMP) assay, as a marker of the intrinsic pathway of apoptosis (23). PC3 cells (5×10^4) were seeded into a 6-well plate and incubated with DOX (10 µg/mL) either alone or in combination with CIN (12.5, 25, and 50 µg/mL) for 24 h. Then, to stain the cells 10 µL of rhodamine 123 (20 µM) was added to each well and incubated for 30 min at 37 °C. Thereafter, Triton X-100 was utilized to lyse cells. Finally, the changes were detected by fluorescence microscope at the excitation and emission wavelengths of 488 nm and 510 nm, respectively.

Intracellular reactive oxygen species assay

Dichlorofluorescein diacetate (DCFDA) (Sigma-Aldrich, Germany) was used for the examination of intracellular reactive oxygen species (ROS) levels. DCFDA is a non-fluorescent molecule and is converted to fluorochrome DCF due to crossing the plasma membrane and followed by other interactions. Briefly, cells cultured in 12-well plates were treated with DOX alone and in combination with CIN. After being incubated overnight, each well was exposed to 10 µL of DCFDA for 30 min. Then, Triton X-100 was used to achieve lysed cells. Finally, the excitation wavelength (488 nm) and the emission wavelength (528 nm) of cells were measured by a fluorescence microplate reader (BioTek, H1M, USA).

Statistical analysis

In the present study, all the experiments were conducted in triplicate, and all data were represented as the mean ± SEM. The one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was performed to compare the results. *P*-values < 0.05 were considered the statistical significance.

RESULTS

Effect of CIN on the DOX-induced cytotoxicity

The viability of PC3 cells in the presence of CIN in comparison with the control group was determined by the MTT assay. As shown in Fig. 1A, PC3 cells exposed to the different concentrations of CIN (0, 12.5, 25, 50, 100, and 200 µg/mL) exhibited concentration-dependently cytotoxicity with the approximate IC_{50} of 73 µg/mL. Exposure to CIN in the concentration of 100 µg/mL caused a drop in the viability of PC3 cells such that the viability of cells decreased by 69.26% (Fig. 1A). Therefore, CIN at the concentrations of 12.5, 25, and 50 µg/mL with high viability on PC3 cells was selected. Furthermore, the cytotoxic effect of DOX alone at the concentrations of 2.5, 5, 10, and 20 µg/mL on the PC3 cell line was assessed. Unsurprisingly, DOX as a chemotropic agent with the IC_{50} value of 11.45 µg/mL had a strong cytotoxic effect on PC3 cells (Fig. 1B). In addition to investigating the potencies of CIN and DOX separately to induce cell death, the cytotoxicity exerted by the various concentrations of DOX in combination with the selected concentrations of CIN (12.5, 25, and 50 µg/mL) was evaluated on PC3 cells, and the findings were compared with the only

DOX-treated cells. The presence of CIN potentiated the anti-tumor effect of DOX. As far as the 12.5 µg/mL concentration of CIN was able to decrease the DOX IC_{50} value to 3.2 µg/mL. However, 50 µg/mL concentration of CIN could exhibit the strongest effect on toxicity exerted by DOX towards PC3 cells with an IC_{50} value of 2.8 µg/mL which was lower than that in DOX alone, expressing far higher efficacy than DOX alone. Based on this finding in association with CIN, not only does it have a crucial role in the prevention of PC3 cell proliferation, but also possesses a potential ability to increase DOX (as a chemotherapeutic) anti-tumor effect. Finally, 10 µg/mL concentration of DOX was determined to combine with the selected concentrations of CIN for treating cells in the later sections of the study.

Figure 2 reveals typical apoptosis in PC3 cells treated by DOX alone in comparison with the control group. Also, the morphological abnormalities associated with the combination of DOX (10 µg/mL) and CIN at different concentrations were more prominent than the DOX group. Treatment with the combination of DOX and CIN within 24 h caused an increase in the percentage of apoptotic cells. The apoptotic cells were characterized by features including shrinkage in size, roundness in shape, rounding up, and detaching from the plate (Fig. 2).

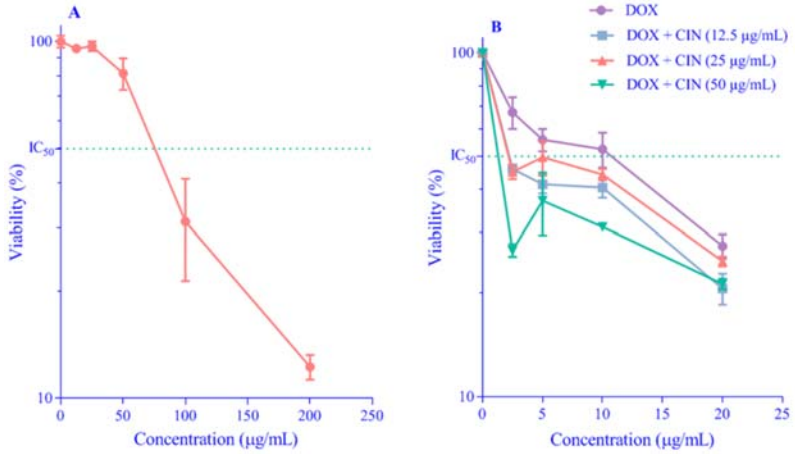

Fig. 1. Cell viability (logarithmic scale) in the PC3 cells treated with (A) CIN at the concentrations of 0, 12.5, 25, 50, 100, and 200 µg/mL and (B) DOX at the concentrations of 0, 2.5, 5, 10, and 20 µg/mL combined with the selected concentrations of CIN (12.5, 25, and 50 µg/mL). Data were expressed as the mean ± SEM, n = 3. DOX, doxorubicin; CIN, cinnamaldehyde.

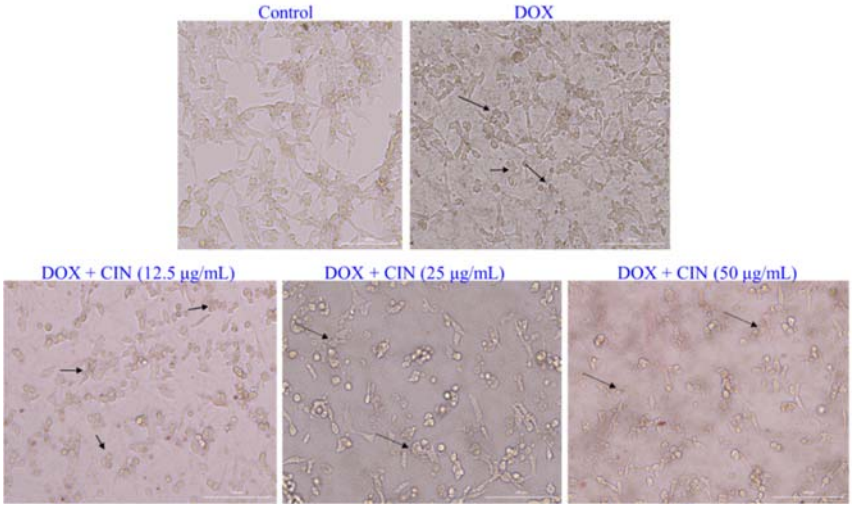

Fig. 2. Morphological changes in PC3 cells under an inverted microscope (Magnification: 10×). Untreated cells as the control group received DMSO. DOX was used at the concentration of 10 µg/mL. Black arrows indicate the distinct morphological characteristics of apoptosis. DOX, doxorubicin; CIN, cinnamaldehyde; DMSO, dimethyl sulfoxide.

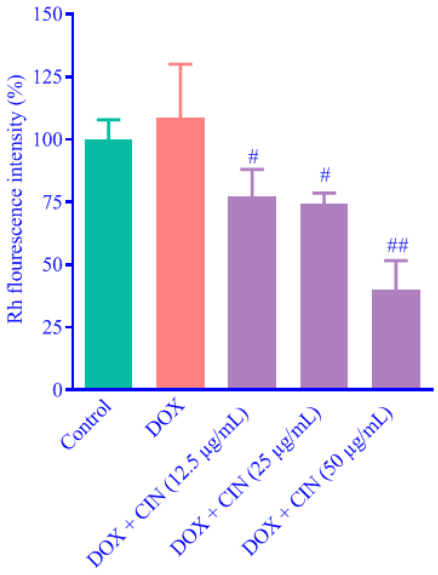

Fig. 3. Effect of different concentrations of CIN in combination with DOX on MMP collapses detected by rhodamine 123 fluorescence. Rhodamine 123 fluorescence intensity (%) expressed MMP in experimental groups. Untreated cells as the control group received DMSO. DOX was used at the concentration of 10 µg/mL. Data were presented as the mean ± SEM, n = 3. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ indicate significant differences from the DOX group. Rh, rhodamine 123; DOX, doxorubicin; CIN, cinnamaldehyde; MMP, mitochondrial membrane potential.

Effect of DOX in combination with CIN on MMP

The different key markers of apoptosis were explored to confirm mechanisms involved in cell death induced by CIN in PC3 cells. The findings showed that CIN at concentrations of 12.5, 25, and 50 µg/mL significantly induced depolarization in the MMP when compared with the DOX group. The concentration of 50 µg/mL had the most remarkable reduction (approximately 40%) in rhodamine 123 fluorescence intensity (Fig. 3). The results revealed the potential ability of CIN in combination with DOX to induce apoptosis through the mitochondrial pathway in PC3 cells.

Effect of CIN on the DOX-induced apoptosis

To further investigate mechanisms involved in cytotoxicity induced by DOX + CIN in PC3 cells, apoptosis induction was confirmed by Annexin V/PI double staining flow cytometric assay. Figure 4A indicates dot plots of the double-stained PC3 cell line. Furthermore, the percentages of viable cells (PI/FITC -/-), necrotic cells (PI/FITC +/-), late apoptotic cells (PI/FITC +/+), and early apoptotic cells (PI/FITC -/+) have been displayed. The results of flow cytometry revealed that the percentage of late apoptotic cells increased from 0.069% in control cells to 3.54% after treatment by DOX. With increasing CIN concentration, the population of late apoptotic cells also increases. So in the highest concentration of CIN, this population reaches 59.9% (Fig. 4B).

Effect of CIN on the DOX-induced ROS generation

To detect whether CIN-enhanced DOX potency resulted from increasing ROS generation, the fluorescent probe of DCF was utilized. DOX alone increased ROS levels in PC3 cells in comparison to the control group, significantly (Fig. 5). Interestingly, the treatment of cells with DOX in combination with CIN different concentrations reduced the intracellular ROS levels. However, these changes were not significant and concentration-dependent.

Effect of CIN on the DOX-induced caspase activation

To clarify the apoptotic pathway involved in DOX-induced the death of PC3 cells in combination with CIN, the activation of caspase-3/7, which is crucial in initiating apoptosis cascades, was analyzed. As shown in Fig. 6, treatment with DOX alone increased the levels of caspase-3/7 in the PC3 cells compared to control cells. Moreover, adding CIN at the concentrations of 25 and 50 µg/mL to DOX could significantly potentiate the DOX anti-tumor effect on the activation of caspase-3/7 (Fig. 6).

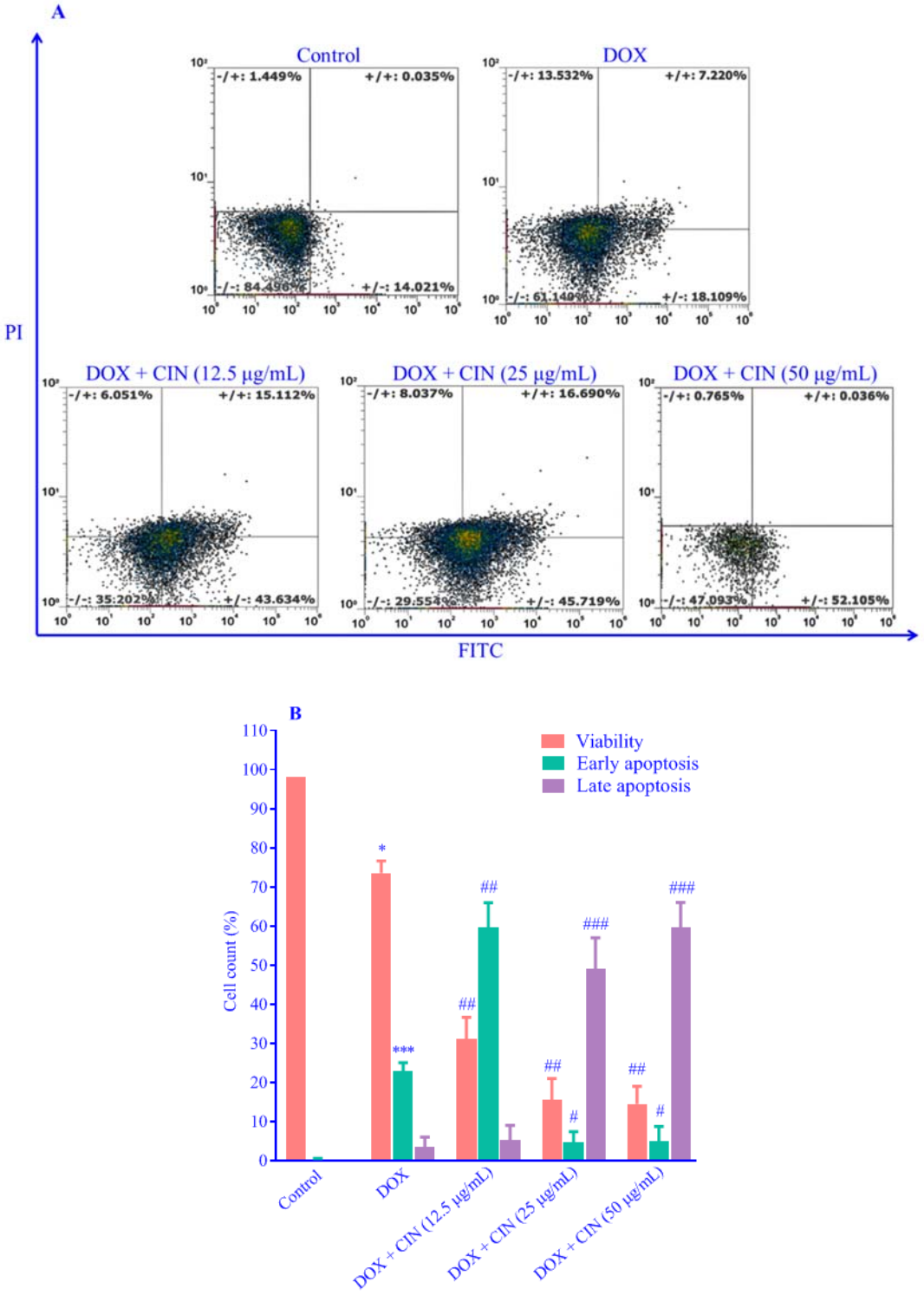

Fig. 4. Detection of apoptosis by flow cytometry assay. (A) Flow cytometry dot plot represents Annexin-V-FITC staining in the X-axis and PI in the Y-axis and (B) summary of percentages of the Annexin V-FITC apoptosis assay. Untreated cells as the control group received DMSO. DOX was used at the concentration of 10 µg/mL. Data were presented as the mean ± SEM, n = 3. $^{*}P < 0.05$ and $^{***}P < 0.001$ demonstrate significant differences in comparison with the respective control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus respective DOX group. DOX, doxorubicin; CIN, cinnamaldehyde; PI, propidium iodide; FITC, fluorescein isothiocyanate.

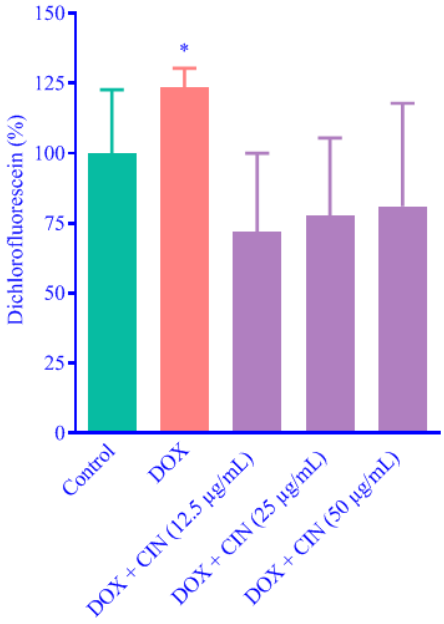

Fig. 5. Effect of different concentrations of CIN on DOX-induced ROS generation detected by DCF. Untreated cells as the control group received DMSO. DOX was used at the concentration of 10 μg/mL. Data were expressed as the mean ± SEM, n = 3. $^{*}P < 0.05$ indicates a significant difference from the control group. DOX, doxorubicin; CIN, cinnamaldehyde; DCF, dichlorofluorescein; ROS, reactive oxygen species.

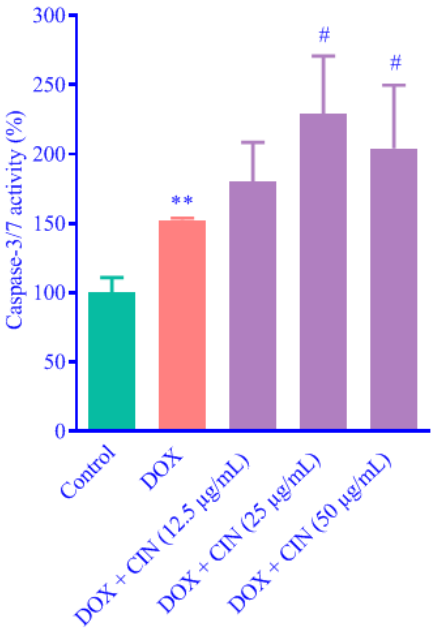

Fig. 6. Caspase activity in PC3 human prostat carcinoma cells following treatment with various concentrations of CIN in combination with DOX for 24 h. Untreated cells as the control group received DMSO. DOX was used at the concentration of 10 μg/mL. Data were presented as the mean ± SEM, n = 3. $^{**}P < 0.01$ indicates significant difference with control group; $^{\#}P < 0.05$ versus DOX group. CIN, cinnamaldehyde; DOX, doxorubicin.

DISCUSSION

Cancer chemotherapy needs to maximize the efficacy of drugs that are currently used while minimizing their side effects. One possible way is to add natural compounds to single-agent therapy providing a more effective treatment (24). The combination of natural compounds and chemotherapeutic agents has been extensively studied for potential effectiveness and reducing the need for chemotherapy (6) One study revealed a significant reduction in drug resistance in the presence of several compounds, which can cause cancer cells to become sensitive to chemotherapy drugs (25). CIN has been identified to be an anti-proliferative substance as well as an effective agent in inducing apoptotic cell death against several types of human cancer cells (10,11,26-30). A previous study examined that CIN induced the apoptosis of U87MG cells along with an increase in the toxic effects of DOX and its anti-cancer effects (12).

This study showed that CIN can sensitize PC3 cells to respond more to the cytotoxic effect of DOX. It further was indicated that CIN in combination with DOX affected mitochondrial potential, caspase-3/7 activation and apoptosis in the prostate cancer cells. There was no study supporting the anticancer effect of CIN in prostate cancer cells while numerous studies have revealed the effects of CIN on other types of human cancer cells. Lee *et al*. reported the cytotoxic effect of trans-CIN against some human cancer cell lines, and showed various results according to the type of cancer cell lines. Trans-CIN exhibited potent cytotoxic activity against HeLa, SK-MEL-2, XF498, and HCT-15 tumor cell lines, which were comparable with cisplatin (31). Another study assessed the antihepatoma activity of CIN on human hepatoma PLC/PRF/5 (CD95-negative) cells and showed that cells exposed with CIN (1 μM) for 12 and 24 h exhibited 47% and 64.30% cell death, respectively (32). Moreover, Yu *et al*. examined the effect of CIN

on cytotoxicity induced by fluorouracil (5-FU) and oxaliplatin on HT-29 and LoVo colorectal carcinoma cell lines and revealed the synergistic effect of CIN on the cytotoxicity induced by the chemotherapeutic agents in these cell lines (33).

As a chemotherapy drug needs to be selected in a way that has a minimal cytotoxic effect on normal cells, some studies evaluated the cytotoxicity of CIN on non-cancerous cell lines. Ranjitkar *et al*. directly compared the cytotoxicity of trans-CIN on both HeLa cancerous and non-cancerous fibroblast cell lines and demonstrated that trans-CIN had a significant cytotoxic effect at the concentration higher than 200 µg/mL on normal cell type (34). Zhang *et al*. evaluated the effect of CIN treatment on the proliferative responses in normal cells such as FHs 74 Int intestinal epithelial cells and HIEC as well as the various types of colorectal cancer cells. The findings of the clonogenic assay showed that CIN was less toxic to non-cancerous intestinal epithelial cells (35). Another study investigated and compared the cytotoxic effects of an aqueous cinnamon extract from the bark of *Cinnamomum zeylanicum* L. with CIN on primary and cancer cell lines. The observations demonstrated that CIN had 15-25% cytotoxicity on mouse primary fibroblasts and exhibited 65-80% cell survival at a far more toxic concentration than the cancerous cells (36).

The induction of apoptosis is one of the most important mechanisms for cancer treatment (37,38). When apoptosis begins, it cannot be stopped, so apoptosis is a highly regulated process. Apoptosis begins in two ways. In the intrinsic pathway, changes are made within the cell, while in the extrinsic pathway, cells kill themselves because of external signals. It is noteworthy that the apoptotic internal pathway can be activated by weak external signals (39-41). In the current study, dead cell staining was conducted by Annexin V/PI, caspase-3/7 activity, and MMP assay to confirm the apoptosis mechanism. Caspase-3 is activated in the cell through external (death ligand) and internal (mitochondria) pathways. Caspase-3 exists in the form of zymogen because if its activity is not regulated, cells are indiscriminately killed (42,43). Caspase-7 acts as an initiating caspase by cleaving, thus activating downstream executioner caspases, and initiating apoptosis (44). The precursor of caspase-7 is cleaved by caspase-3, caspase-10, and caspase-9. It is activated upon cell death stimuli and induces apoptosis (45). The present results showed that DOX increased caspase-3/7 activity. Moreover, the combination of CIN and DOX enhanced caspase-3/7 activity in cells. The results of flow cytometry clearly revealed the increment of apoptosis in the cells. The high number of cells in the Annexin V+/PI- (as an indicator of apoptotic status) was shown to be associated with increased cell death in CIN + DOX-treated cells compared with DOX-treated cells. The morphological study of DOX-treated cells in the presence or absence of DOX showed an increase in cell death and cell abnormalities such as contraction and vacuolation. As previously mentioned, the loss of MMP is one of the main factors in the intrinsic apoptosis pathway (23). DOX alone was not able to destroy MMP, but adding CIN led to the collapse of MMP. This can improve the involvement of mitochondria in the apoptosis induced by DOX in the presence of CIN. On the other hand, ROS are known as one of the most important inducers of apoptosis. Generally, the level of ROS in cancer cells is higher than normal ones, which leads to more sensitivity of cancer cells. Furthermore, there are strives to apply agents producing ROS as one of the cancer therapeutic strategies (46). It is well known that oxidative stress has an important role in apoptosis induced by DOX in cancer cells (18). Some studies showed that CIN could increase the ROS formation in different cancer cells. Han *et al*. demonstrated that CIN causes growth inhibition and the induction of apoptosis in prostate cancer-associated fibroblasts. Also, CIN can suppress oxidative stress (44). Therefore, the current study assessed ROS production in PC3 cells exposed to DOX in the presence of CIN. As anticipated, DOX increased ROS generation. On the other hand, CIN not only did not potentiate DOX-induced ROS generation but also inhibited it, non-significantly. The result identified that the potential effect of CIN against apoptosis induced by DOX was not mediated through the enhancement of ROS.

CONCLUSION

The present study showed that CIN as an available natural compound may have anticancer potential activity due to its cytotoxicity against the PC3 cancer cell line. CIN also potentiates the apoptotic effect of DOX in PC3 cells by reducing MMP and enhancing caspase-3/7 activation. Overall, CIN could be introduced as a promising and effective natural-derived anticancer and therapeutics agent alone or in combination with DOX. However, there is still an urgency to investigate the activities and side effects of DOX.

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Conflict of interest statement

The authors declare no conflict of interest for this study.

Authors' contributions

A. Abbassi designed and performed experiments, analyzed data and wrote the manuscript; L. Hosseinzadeh and P. Yaghmaei supervised the work, designed the study, corrected the manuscript, and provided the facilities for the study. The final version of the manuscript was approved by all authors.

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