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School of Pharmacy & Pharmaceutical Sciences
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Original Article

Evaluation of the cytotoxic and apoptogenic effects of cinnamaldehyde on U87MG cells alone and in combination with doxorubicin

Abbas Abbasi[1], Marziyeh Hajialyani[2], Leila Hosseinzadeh[2,*], Fereshteh Jalilian[3], Parichehr Yaghmaei[1], Sahar Jamshidi Navid[4], and Hajar Motamed[2]

[1]Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, I.R. Iran.
[2]Pharmaceutical Sciences Research Center, Health Institute, Kermanshah University of Medical Sciences, Kermanshah, I.R. Iran.
[3]Medical Biology Research Center, Health Technology Institute, Kermanshah University of Medical Sciences, Kermanshah, I.R. Iran.
[4]Pharmacology and Toxicology Department, School of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, I.R. Iran.

Abstract

Background and purpose: In the present study, we tried for the first time to examine whether cinnamaldehyde (CA), with herbal nature, can be co-administrated with doxorubicin (DOX, as an anticancer drug) toward U87MG glioblastoma cells to potentiate its cytotoxic effect and overcome or reduce its side effects.
Experimental approach: The cytotoxic effect of DOX and CA, either individually or in combination, were evaluated on U87MG cells using the MTT method. The mechanism of action was studied by investigating the mode of cell death using caspase-3 and 9 activations, mitochondrial membrane potential (MMP) as well as sub G1 analysis. The expression of apoptosis- related genes (Bcl-2 and Bax) was also examined.
Findings / Results: Cellular toxicity assay revealed that CA and DOX can potentially reduce the viability of U87MG cells with IC_{50} at 11.6 and 5 µg/mL, respectively. Exposure with the combination of CA and DOX significantly increased cytotoxic effect of DOX on U87MG cells. The results of SUBG1, MMP, and also caspase-3 and -9 activity assays, in association with the results corresponding to the Bax and Bcl-2 gene expressions, altogether revealed that CA can induce apoptosis on U87MG cells. Moreover, apoptogenic effects of DOX were found to be potentiated by CA.
Conclusion and implications: The results of this study revealed the promising cytotoxic and apoptogenic role of CA on U87MG cells. Additionally, our findings demonstrated that CA is able to enhance the apoptosis induced by DOX on human glioblastoma cells. Collectively, these data suggested that co-exposure of CA and DOX could be effective for treatment of glioblastoma, but further *in vivo* and clinical studies are still needed to prove these results.

Keywords: Apoptosis; Cinnamaldehyde; Cytotoxicity; Doxorubicin; U87MG**.**

INTRODUCTION

Glioblastoma is among the most aggressive cerebral tumors, with high incidence and mortality rates (1,2). Chemotherapy, surgery, and radiation are the most common management strategies for treatment of glioma (3). Although the common medications may cause a slight increase in the median survival, they could be just palliative care for most of the patients and their tumors cannot be fully cured (4). On the other hand, achieving an efficient chemotherapy, which can capably pass blood-brain barrier and also selectively eradicate tumor cells, without destructing normal cells, is still a prominent issue (5). Over the years, providing an adjuvant to chemotherapy has been the subject of various studies for enhancing response rate to chemotherapy, reducing side effects and minimizing treatment resistance.

*Corresponding author: L. Hosseinzadeh
Tel: +98-8338250271, Fax: +98-8334276493
Email: Lhoseinzadeh@kums.ac.ir

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Plants, as rich sources of bioactive compounds, can be promising alternatives or adjuvant for cancer chemotherapy. Various medicinal plants demonstrated potential anticancer effects without causing troublesome side effects, making these safe compounds preferable for cancer treatment (6). Cinnamaldehyde (CA) has long been used either as a spice or as an herbal-based active compound in traditional herbal medicine, which possesses antitumor, antifungal, mutagenic, and chemo-preventive properties (7). The antiproliferative and pro-apoptotic effects of CA against various cancer cell lines, including breast, leukemia, ovarian, and lung tumor cells, have been corroborated in previous studies (8-12). Its anticarcinogenic mechanism has been attributed to the mitochondrial depolarization (13), activation of the pro-apoptotic Bcl-2 proteins and caspase-3, and inhibition of glutathione S-transferase, and ATPases (9,14).

Doxorubicin is an anticancer drug used for treatment of a wide range of cancers (15). The most dangerous side effect of this drug is dilated cardiomyopathy, which leads to progressive heart failure. Combination of chemotherapy (co-chemotherapy) is a strategy to make cancer therapy more effective, safe, and suppress side effect of DOX (16-19).

In this study, we investigated the effects of CA on apoptosis and cytotoxicity induced by DOX towards U87MG cells. To the best of our knowledge, the role of CA therapy in combination with chemotherapeutic agents, such as DOX, has not been clearly addressed thus far. Furthermore, we aimed to elucidate the mechanisms possibly involved in the apoptogenic activity of CA by examining its effect, solely and in combination with DOX, on apoptosis markers and apoptotic gene expressions.

MATERIALS AND METHODS

Materials

Cinnamaldehyde, Triton® X-100, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rhodamine 123 fluorescent dye, Bradford reagent, doxorubicin and dimethyl solphoxide (DMSO) were purchased from Sigma Aldrich (St Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM), fetal bovin serum (FBS), and penicillin/streptomycin were supplied from Gibo (USA). Trypsin-EDTA was prepared from Bon Yakhteh (I.R. Iran).

Cell culture

Human U87MG cells were purchased from pasture institute and were propagated in DMEM supplemented with 10% (v/v) heat inactivated FBS and 1% penicillin/streptomycin (100/100 U/mL) at 37 °C in 95% CO_2 humidified incubator. Medium was replenished at specified time intervals until the cell proliferation reached appropriate population density of 70-80% confluence.

Cell viability assay

Cellular toxicity of DOX and CA were examined in U87MG cells using MTT method. Cells were seeded into 96-well culture plates at a same density of 2.0×10^4 cells/well and in a volume of 200 µL. Stock solutions of CA and DOX were prepared separately in DMSO and different concentrations of each compound were prepared after serial dilution of stock solutions. The final concentration of DMSO in the medium was always 0.5%. After 24 h, 2 µL from each of DOX and CA solutions at different concentrations was added to each well. For this assay, a group of cells was kept untreated (control), and other groups were treated with different concentrations of CA and DOX, either individually or in combination. For individual examinations the concentrations of CA were 8, 16, 32, 64, and 124 µg/mL and concentrations of DOX were 5.43, 10.86, and 16.29 µg/mL. The cotreatment experiments were performed using 8, 16, 32 µg/mL of CA and 1.80 and 8.25 µg/mL of DOX. At appropriate time intervals, the medium was replenished with 20 µL of 0.5 mg/mL of MTT in growth medium and plates were further incubated for 3 h at 37 °C. After the incubation period, MTT-formazan products were solubilized by 100 µL DMSO. The optical density at wavelength of 570 nm was detected on an Eliza micro plate reader (BioTek Instruments, USA), while the reference wavelength was

630 nm. Growth inhibition was calculated and IC_{50} concentration was obtained, which was corresponding to the concentration required for killing 50% of cells. All the MTT assays were conducted in triplicate (20).

Sub G1 phase analysis

U87MG cells (10^4 cells) were treated with CA (8 µg/mL) individually or in combination with DOX (5 µg/mL), for 24 h. Cells were harvested and labeled with propidium iodide (PI), and then analyzed by flow cytometer (Partec TM, Germany). It must be noted that DNA content histogram obtained by this method, can be estimated for sub G1phase in cells cycle and not for proportions of cells in all cell cycle phases.

Caspase-3 and -9 activities

The activity of caspase-3 and -9 was determined by the Sigma colorimetric caspase-3 and -9 kit according to the manufacturer's instructions. For this purpose, 1×10^6 cells were seeded and incubated for 24 h with CA (8, 16, and 32 µg/mL) and DOX (5 µg/mL), either individually or in combination. Subsequently, cells were trypsinized and centrifuged at 1200 rpm and lysed with 50 µL lysis buffer and incubated on ice for 10 min. The extraction of the protein content of cells was achieved by centrifugation of lysates at 16000-20000 rpm and 4 °C for 5 min. The substrate reaction buffers containing caspase-3 and caspase-9 (1 mM) were added separately to the supernatant and incubated for 1 h at 37 °C. The absorbance was then measured at 405 nm using a plate reader (BioTek, H1M, USA). The protein content was determined by Bradford method using bovine serum albumin as a standard.

Measurement of mitochondrial membrane potential

In this study mitochondrial membrane potential (MMP) was measured using rhodamine 123 fluorescent dye. U87MG cells were plated in a 6-well culture plate and incubated with DOX at 5 µg/mL and CA at 8, 16, and 32 µg/mL either individually or in combination, for 24 h. After incubation period, the treated and control cells were further incubated with 15 µL rhodamine 123 (20 µM) for 30 min at 37 °C. Thereafter, cells were lysed with one mL Triton® X100 and the amount of their fluorescence was measured at a wavelength of 488-510 nm using fluorescence microplate reader.

Real time polymerase chain reaction analysis (RT-PCR)

In order to evaluate the expressions of Bax and Bcl2 genes, U87MG cells (10^4 cells) were treated with CA (8 µg/mL) individually or in combination with DOX (5 µg/mL). After 24 h treatment, total RNA of cells (7×10^5 cells) was extracted using high pure isolation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Total RNA was assessed qualitatively and quantitatively using a spectrophotometer (NanoDrop™ 2000, USA) and samples stored at -80 ºC for further investigations. A one-step quantitative test was performed on RNA expression using CYBR Green kit. The primer sequences were completely similar to those performed in our previous study (20). The cDNA synthesis was carried out at thermal cycler conditions corresponding to 15 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C to denature DNA and 45 s at 60 °C to anneal and extend the template (20). The melting curve was analyzed at temperatures in range of 65-95 °C with a temperature transient rate of 0.1 °C/s. All reactions were performed in triplicate in a Stratagene MX 3000P system (USA). The values obtained for the target gene expression were normalized to β-actin and analyzed by the relative gene expression $2^{-\Delta\Delta CT}$ method (20) using the following equation:

$-\Delta\Delta CT$ = (CT target - CT β-actin) Unknown - (CT target - CT β-actin) calibrator

where, CT is threshold cycle.

Statistical analysis

In the present study, all the experiments were conducted in triplicate and reported values were represented as the mean value ± SEM. The One-way analysis of variance (ANOVA) following Tukey's post-hoc test was performed to compare the results. The statistical significance of variations could be confirmed at $P < 0.05$.

RESULTS

Effect of cinnamaldehyde on cytotoxicity induced by doxorubicin

In order to examine the effect of CA on the proliferation of U87MG cells, cells were treated with different concentrations of CA (8, 16, 32, 64, and 128 µg/mL), and viability percentage of cells in the presence (treatment groups) or absence of CA (control group) were compared. According to the results, CA dose-dependently affected the viability of U87MG cells and significantly inhibited proliferation of cells. The IC_{50} concentration of CA was 11.6 µg/mL. The cytotoxic effect of DOX (5.43, 10.86, and 16.29 µg/mL) was also examined. As it was expected, DOX significantly exerted cytotoxic effect on U87MG cells. The IC_{50} of DOX was found to be 5 µg/mL (Table 1).

In order to examine combinatorial effects of DOX and CA toward U87MG cells, three different combinations were assessed. The effects were tested at 8, 16, and 32 µg/mL of CA with DOX. The results were compared with viability percentage of cells treated only with DOX (Table 1). The presence of CA in culture medium (even at low concentrations) potentiated the cytotoxic effect of DOX. The cells survival significantly decreased and IC_{50} of the mixture of DOX and CA (8, 16, and 32 µg/mL) was 1.75, 1.83, and 1.79 µg/mL, respectively, which was smaller than IC_{50} of DOX alone (5 µg/mL). Such reduction in cell survival was more pronounced when cells were treated with CA at 8 µg/mL. It can be concluded that CA at 8 µg/mL possessed the most potentiating effect on toxicity induced by DOX toward U87MG cells. According to these results, beside the anti-proliferative effect of CA toward U87MG cells, it also possesses the ability of combining with DOX (as a chemotherapeutic) to enhance its effect on the tumor cells.

Effects of cinnamaldehyde and doxorubicin on morphological changes

To examine whether CA (in combination with DOX) can increase cell death by inducing apoptosis, the morphology of cells before and after treatment was observed. Figure 1 illustrates the morphological changes before and after treatment with DOX (5 µg/mL), CA (8, 16, 32 µg/mL), and their combinations.

It could be observed that CA significantly increased the percentage of apoptotic cells in a concentration-dependent manner (Fig. 1). The morphological abnormalities (shrinkage, and vacuolization), growth inhibition, and detachment of cells were evident in the CA-treated cells, especially at the highest dose (32 µg/mL), while no significant abnormality and cellular death was observed after exposure to 8 µg/mL of CA. Apoptosis and morphological abnormalities were more prominent when U87MG cells were treated with a mixture of DOX (5 µg/mL) and CA at different levels. It was found that CA potentiates the apoptotic effect of DOX on the U87MG cells. A large number of cells died after exposure of DOX and CA at 32 µg/mL.

Sub G1 analysis results

The apoptotic properties of CA and its effect on DOX-induced apoptosis was examined by propidium iodide staining (PI) staining and flow cytometry to evaluate the sub-G1 peak resulting from DNA fragmentation. Flow cytometry histograms of cells treated with CA and co-treated with CA and DOX demonstrated an increase in the percentage of cells in SubG1 phase after exposure of CA and more significantly the combination of CA and DOX (Fig. 2).

Effect of cinnamaldehyde on doxorubicin-induced mitochondrial membrane potential collapse

In order to examine the effect of CA on MMP, MMP test was performed using a cell permeable cationic fluorescent dye. Depolarization of MMP caused by DOX (5 µg/mL) induced damage of the outer membrane (about 40% decrease in rhodamine 123 fluorescence compare to control cells).

Table 1. Comparison of IC_{50} in different groups of treatments. Data represent mean ± SEM, n = 3.

Treatments	IC_{50} (µg/mL)
DOX	5 ± 0.16
CA	11.6 ± 0.65
DOX + CA (8 µg/mL)	1.75 ± 0.08
DOX + CA (16 µg/mL)	1.83 ± 0.32
DOX + CA (32 µg/mL)	1.79 ± 0.29

DOX, Doxorubicin; CA, cinnamaldehyde.

This damage was found to be potentiated following exposure of CA (8, 16, and 32 µg/mL). The most significant reduction in rhodamine 123 fluorescence occurred after exposure of CA at 8 µg/mL. These results showed that CA, in combination with DOX, is able to induce apoptosis dose-dependently in U87MG cells, and this alteration was more pronounced at the lowest dose (8 µg/mL) (Fig. 3).

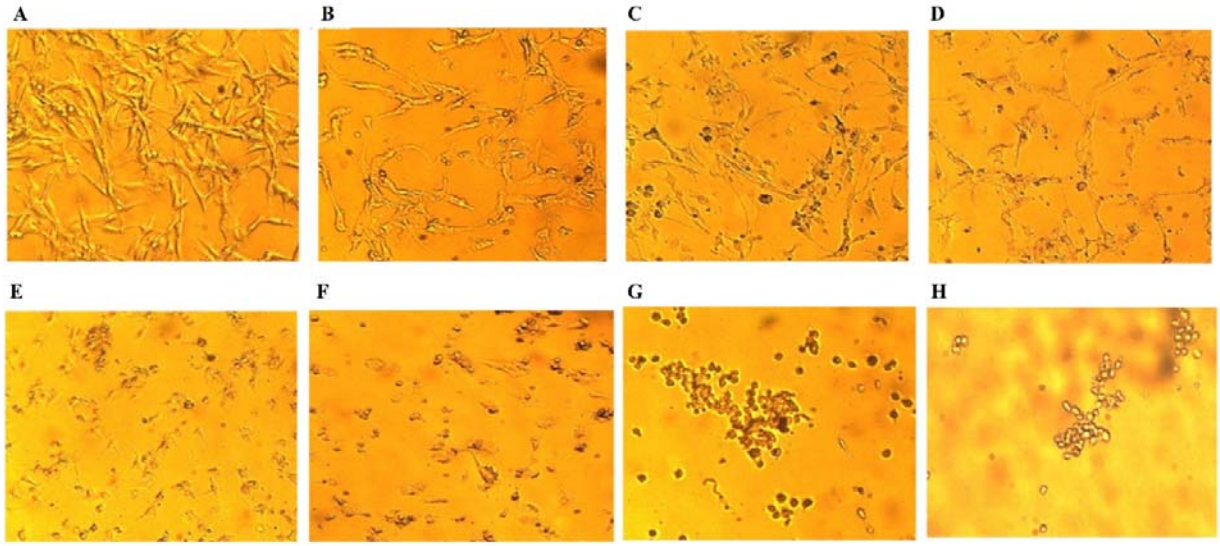

Fig. 1. U87MG cells morphological changes treated with DOX (5 µg/mL) and different concentrations of CA. (A) Control, (B) DOX (5 µg/mL), (C) CA (8 µg/mL), (D) DOX + CA (8 µg/mL), (E) CA (16 µg/mL), (F) DOX + CA (16 µg/mL), (G) CA (32 µg/mL), and (H) DOX + CA (32 µg/mL). CA, cinnamaldehyde; DOX, doxorubicin.

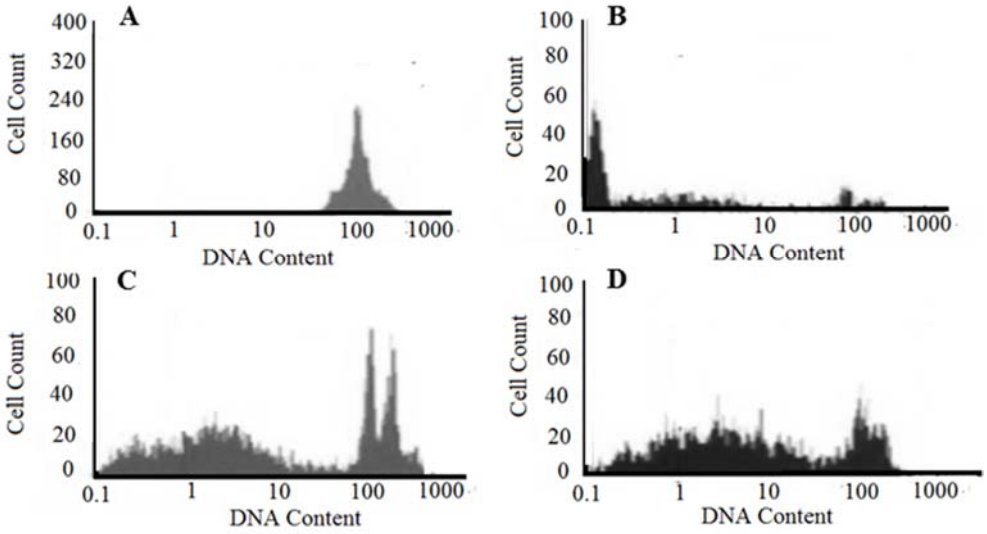

Fig. 2. Sub G1 cell cycle arrest induced by DOX and CA on U87MG cell line. (A) Control, (B) DOX (5 µg/mL), (C) CA (8 µg/mL), and (D) CA (8 µg/mL) + DOX (5 µg/mL). U87MG cells (10^4 cells) were treated for 24 h. Cells were harvested and labeled with propidium iodide (PI), and then analyzed by flow cytometer. CA, cinnamaldehyde; DOX, doxorubicin;

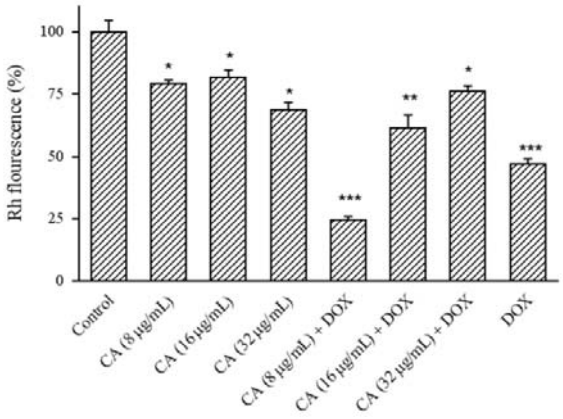

Fig. 3. Effect of different concentrations of CA (8, 16, and 32 µg/mL) on DOX (5 µg/mL)-induced mitochondrial membrane potential (MMP) collapse. U87MG cells were exposed to test compounds 24 h before analysis. Results are expressed as mean ± SEM of three separate experiments. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 indicate significant differences in comparison with control. CA, Cinnamaldehyde; DOX, doxorubicin.

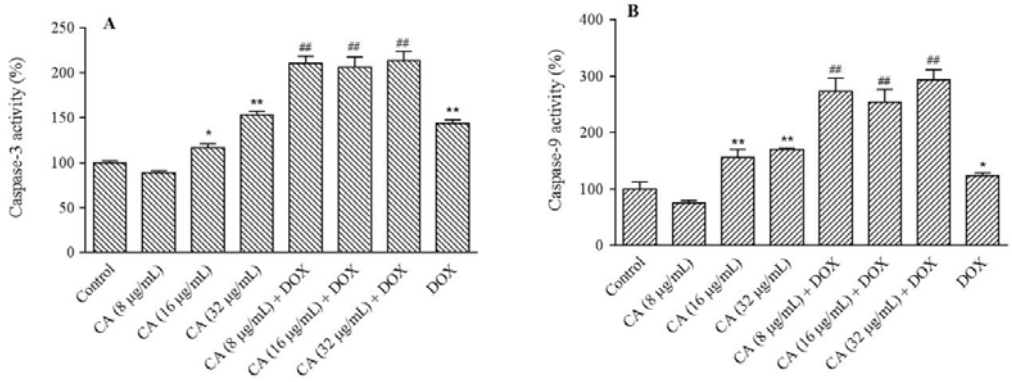

Fig. 4. Effect of different concentrations of CA (8, 16, and 32 µg/mL) and DOX (5 µg/mL) on caspase-3 and -9 activities. Firstly cells pretreated with CA for 24 h before exposure to DOX (5 µg/mL). Caspase-3 and -9 activities measured by colorimetric detection of p-nitroaniline and expressed as percent of control. Results are expressed as mean ± SEM, n = 3. **P* < 0.05 and ***P* < 0.01 indicate significant differences in comparison with control; and $^{\#\#}P < 0.01$ shows significant differences relative to DOX group. CA, Cinnamaldehyde; DOX, doxorubicin.

Effects cinnamaldehyde and doxorubicinon caspase-3 and -9 activities

Caspase-3 and -9 activities possess prominent role in the executioner caspase-activated pathways and mitochondrial apoptotic pathway, respectively (21,22). We speculated that CA could potentiate apoptosis induced by DOX in U87MG cells. In order to elucidate which apoptosis pathways are involved in the death of U87MG cells, the effect of CA and DOX on caspase-3 and -9 activities was examined (Fig. 4). Our findings showed that the level of caspase-3 and -9 increased upon the application of DOX in comparison with control group. CA with antiproliferative effect on U87MG cells elevated caspase-3 and -9 activities and significantly enhanced the effect of DOX on the activity level of caspase-3.

Effects of cinnamaldehyde and doxorubicin on the expression of Bax and Bcl-2 genes

In order to examine the effect of CA at 8 µg/mL on the expression of Bcl2 and Bax apoptotic genes, which participate in the mitochondrial apoptotic pathway, RT-PCR method was performed on U87MG cells (Fig. 5). The results imply that CA, as well as DOX can successfully up-regulate the pro-apoptotic genes expression compared to control cells. Although the tested concentrations of CA and DOX increased the levels of both pro-apoptotic and anti-apoptotic gen expressions (Bax and Bcl-2)

simultaneously, the increase of Bax was more pronounced than Bcl-2 level. This caused increasing the level of Bax/Bcl-2 ratio, after treatment of U87MG cells with DOX and CA, individually. It was also observed that exposure of cells to the combination of CA (8 µg/mL) and DOX (5 µg/mL) caused a significant increase in the level of Bax and a decrease in the level of Bcl-2 compared to DOX. The increase in the ratio of Bax/Bcl-2 was more pronounced in co-treatment of CA with DOX, rather than CA and DOX alone.

DISCUSSION

Cancer is taken into account as one of the death-leading diseases for human beings, and is responsible for 13% of deaths in the world. Various medicaments have been considered for disease treatment, while the toxicity of chemotherapeutic agents, their limited effectiveness, and resistance to these drugs are still remained as major issues. Therefore, several attempts have been devoted to the develop of medicaments, which are capable of inducing apoptosis in cancerous cells without causing major adverse effects (23-25). Herbal-derived compounds and their phytochemicals have been extensively investigated for their possible effectiveness and chemo-preventive properties, solely or in combination with other chemotherapeutic agents. Some of these compounds could potentially attenuate the pathways causing treatment resistance and consequently sensitize different tumor cells to chemotherapeutic drugs (26). This study aimed to investigate whether CA is capable of sensitizing U87MG cells to the chemotherapeutic agents like DOX. For this purpose, cytotoxic effect of CA, alone and in combination with DOX was studied on U87MG cells. This study showed that CA significantly induced toxicity in U87MG cells, and interestingly potentiated the toxicity of DOX toward U87MG cells. This indicated the synergistic interaction between DOX and CA, corroborating the successful role of CA in sensitizing glioblastoma cells to DOX as the promising chemotherapeutic agent for these tumor cells. The synergistic interaction of CA and other chemotherapeutic drugs has been demonstrated in different studies (10,27).

The morphological study on cells before and after treatment with DOX and CA, alone or in combination, revealed the increase of cellular death and cell abnormalities such as shrinkage and vacuolization, upon treatment with CA (concentrations higher than 16 µg/mL). These effects were more prominent for the cells co-treated with DOX and CA. The fraction of cells in SubG1 phase increased and depolarization of mitochondrial membrane potential (caused by the DOX) potentiated in the presence of CA.

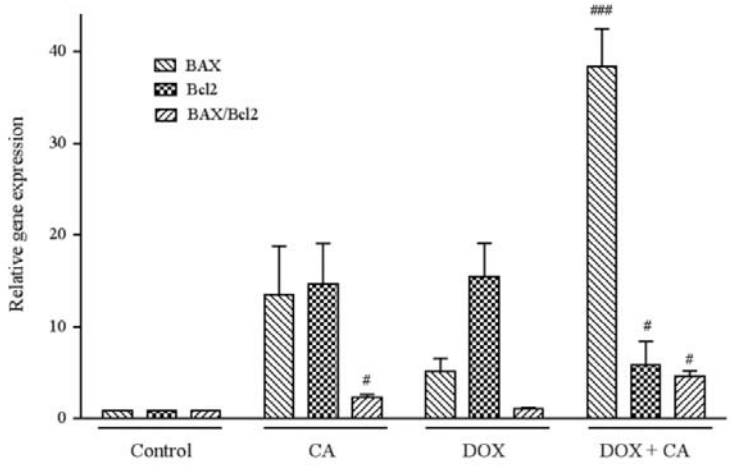

Fig. 5. Effects of CA at 8 µg/mL alone and in combination with DOX (5 µg/mL) on expressions of Bax and Bcl-2 genes in U87MG cells. RNA was isolated, reverse transcribed to cDNA, and then amplified by a real-time PCR detection system to measure mRNA levels of Bax and Bcl-2. Target genes were normalized to β.actin. The data are presented as the mean ± SEM from three independent experiments. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ *vs* corresponding DOX group. CA, Cinnamaldehyde; DOX, doxorubicin.

In order to elucidate the mechanism through which CA induced the apoptosis in U87MG cells, the caspase-3 and -9 activities were also determined and compared. It is believed that the apoptosis is mediated through intrinsic (characterized by mitochondrial dysfunction and activation of caspase-9) and extrinsic (characterized by activation and cleavage of caspase-8 and caspase-3) pathways (26,28,29). Based on the ability of CA in increasing both caspase-3 and -9, it can be concluded that apoptotic mechanism of CA is probably through intrinsic pathway. These results were in agreement with the results of other studies (9,30).

Furthermore, we have determined the antitumor effect of CA and DOX on the levels of Bax and Bcl2 gene expressions, as prognostic markers of apoptosis. The results indicated that CA increased the expression of both genes, but the increase in the Bax expression was more prominent. Bcl-2 possesses anti-apoptotic properties and stabilizes the mitochondria membrane by preventing the release of cytochrome-c (31). Bax is a pro-apoptotic protein that accelerates cell death (31). Results obtained from RT-PCR in the U87MG cells demonstrated that CA increased the Bax/Bcl2 ratio compared to control cells. The high ratio of Bax/Bcl2 in the presence of CA, in association with its effects on MMP, caspase-3 and -9 activation, suggest a remarkable link between CA and cell death in U87MG cells, and it could prove that CA potentiated the anticancer effects of DOX. These results were in line with the results obtained from the flow-cytometry, suggesting that cells treated with both DOX and CA had more levels of apoptosis. High percentage of cells in Sub-G1 phase (as an indicative of apoptotic cells) was demonstrated to be associated with increase of cellular death in cells co-treated with CA and DOX, rather than control cells and DOX treated ones.

Finally, according to the results of this study, it can be concluded that CA, as the main ingredient in cinnamon oil, has the potential for inducing apoptosis in glioblastoma cells and even sensitizing them to DOX. It exerted apoptotic activities toward several types of cancer and tumor cells. The apoptotic effects of CA against colon cancer, breast (32), blood (30,33), lung, skin, kidney and prostate (34) cancers have been confirmed in the other studies. A study showed that CA is mainly responsible for the anticancer activity of cinnamon (35).

CONCLUSION

The results of the present study, for the first time indicated that CA can be co-administrated with DOX to potentiate its cytotoxicity toward U87MG cells for treatment of glioblastoma. CA decreased the level of MMP of U87MG cells and induced apoptosis through intrinsic pathway. CA showed synergistic interactions with DOX, sensitizing U87MG cells to this chemotherapeutic drug. Collectively, these data suggest that CA (as a compound with different biological activities) could be introduced as a supplement effective for enhancing the efficacy of DOX.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest for this study.

AUTHORS' CONTRIBUTION

A. Abbasi designed and performed experiments, analyzed data, and co-wrote the paper. M. Hajialyani, wrote the manuscript. L. Hosseinzadeh supervised the work, corrected the manuscript, and provided the facilities for the study. P. Yaghmaei designed the study. F. Jalilian and S. Jamshidi performed experiments. H. Motamed performed flow cytometry experiment and analyses.

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