FULL LENGTH	Iranian Biomedical Journal 30 (5): 310-318 September 2026

Ellagic Acid May Potentiate Doxorubicin Efficacy in Osteosarcoma Cells through Apoptotic Activation and P-glycoprotein Suppression

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OPEN ACCESS

Article type: Research Article
Received: June 27, 2026
Revised: August 12, 2026
Accepted: August 25, 2026
Published online: August 26, 2026

How to cite:
Mohammadzadeh Boukani L, Valizadeh A, Golchin A, Molavand M, Vaghari-Tabari M, Yousefi B. Ellagic Acid May Potentiate Doxorubicin Efficacy in Osteosarcoma Cells through Apoptotic Activation and P-glycoprotein Suppression. *Iran. Biomed. J.* 2026; 30 (5): 310-318.

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ABSTRACT

Background: Osteosarcoma (OSA) frequently develops resistance to doxorubicin (DOX), attenuating the effectiveness of treatment. Ellagic acid (EA), a natural polyphenol, has shown a chemosensitizing effect in various cancers, but its impact on OSA cells has been minimally studied. The present study examined whether EA could increase the sensitivity of Saos-2 OSA cells to DOX by affecting apoptosis and the mechanism of multidrug resistance.

Methods: The effects of EA and DOX, alone and in combination, were evaluated in Saos-2 cells. To evaluate the responses of these cells to treatments, we analyzed their viability using the MTT assay, apoptotic activity through cell death detection ELISA, and gene expression via quantitative real-time PCR. Data were analyzed via one-way ANOVA, and all the experiments were carried out in triplicate.

Results: DOX exhibited concentration-dependent cytotoxicity in Saos-2 cells. EA synergistically enhanced the efficacy of DOX, with a decrease in the effective concentration of the drug. The combination treatment elevated apoptosis, enhanced the expression of the pro-apoptotic gene (*Bax*), and increased the *Bax/Bcl-2* ratio. In addition, EA attenuated the expression of *MDR1* and reduced the levels of p-glycoprotein, proposing dual chemosensitizing mechanisms.

Conclusion: EA enhances the effectiveness of DOX in Saos-2 OSA cells in a synergistic manner, possibly by targeting both apoptosis and *MDR1*/P glycoproteins pathways. Our findings thus justify further research into EA as a potential chemosensitizer aimed at enhancing DOX-based chemotherapy for OSA. ***DOI: 10.22034/IBJ.30.5.310***

Keywords: Apoptosis, Doxorubicin, Ellagic acid, Osteosarcoma

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1. INTRODUCTION

Osteosarcoma (OSA) is defined as the most prevalent form of primary malignant tumors affecting the bone, primarily seen in adolescents and young adults. Owing to its dismal prognosis, lack of appropriate treatment modalities, and early occurrence of metastases, OSA has caused a significant number of deaths[1,2]. Despite the introduction of different modes of treatment like neoadjuvant chemotherapy, radiotherapy, surgical resection, and adjuvant therapy, only a small percentage of patients suffering from OSA have shown increased five-year survival rates, which fluctuate between 20% and 30% in the last several years[3,4]. Therefore, this situation emphasizes the need for developing novel strategies to combat chemoresistance and improve clinical outcomes.

Among numerous chemotherapeutic drugs employed for the management of OSA, doxorubicin (DOX), an anthracycline antibiotic, continues to be an integral part of standard chemotherapy protocols[5,6]. However, its usage is significantly limited by both dose-dependent and irreversible cardiac toxicities, leading to cardiomyopathy, along with the emergence of multidrug resistance (MDR) mediated by the ATP-binding cassette (ABC) transporter family, particularly P-glycoproteins (P-gp)[7,8]. In response to these issues, extensive studies have focused on developing chemosensitizers, capable of augmenting therapeutic efficacy while concomitantly decreasing drug dosage. Recently, much attention has turned toward naturally occurring compounds owing to their advantageous features, including multi-targeted pharmacological effects, lower incidence of side effects, and probable economic benefits[9-11].

Ellagic acid (EA), a natural polyphenol derived from pomegranate, berries, and nuts[12,13] belongs to a group of promising chemosensitizers demonstrating diverse bioactivities against various tumor cells. This effect is achieved through the modulation of inflammatory and oxidative stress pathways, along with the regulation of different signal transduction molecules[14,15]. Studies have clarified the ability of EA to make cancer cells more responsive to conventional chemotherapeutic agents[16-18]. It has been observed that in breast cancer cells, treatment with EA significantly decreases MDR1 expression and enhances the anti-tumoral effects of cisplatin[19]. Similarly, in colon cancer, EA potentiated the anti-cancerous activities of 5-fluorouracil, probably via the elevation of the Bax/Bcl-2 ratio and caspase-3 activity[20]. These results suggest that EA may act through pathways relevant to OSA treatment, although its specific effects in this type of cancer are not yet well characterized.

The molecular mechanisms responsible for the ability of EA to sensitize cells are multifaceted. The anti-cancer mechanism of action of EA occurs due to its ability to halt cell cycle progression at the G1 phase by altering the activity of cyclin-dependent kinases. Apoptotic changes are induced by alterations in the mitochondrial membrane potential, which lead to modifications in the ratio of Bcl-2/Bax and activation of caspase signaling pathways. It also inhibits the function and expression of P-gp in drug-resistant cells[20-22]. While it has been shown that EA can increase the effectiveness of conventional chemo-therapeutic agents by inducing apoptosis and inhibiting MDR1/p-gp in a number of cancer types, such as breast and colon cancer, its effects in OSA have still been largely uninvestigated.

OSA is different from most other solid tumors due to its special bone microenvironment, a high rate of both intrinsic and acquired drug resistance, and frequent TP53 mutations (including a complete loss of p53 function)[23-25]. As a result, findings from other types of malignancy cannot be directly applied to OSA. Specifically, no prior investigations have been performed on the impact of EA in enhancing the cytotoxicity of DOX in a p53-null OSA model, via p53-independent mechanisms. In light of these gaps in knowledge regarding this disease, our objective was to study the impact of the combined effects of EA and DOX on Saos-2 cell lines, focusing especially on two parameters, namely (i) modulating bax/bcl-2 apoptosis MDR/p-gp.

2. MATERIALS AND METHODS

2.1. Cell culture

The Saos-2 cell line was acquired from the Pasteur Institute of Iran, Tehran, Iran. The cells were cultured in RPMI-1640 medium supplemented with 10% Fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin solution (Sigma-Aldrich, St. Louis, MO, USA). They were maintained under standard culture conditions in a humidified atmosphere containing 5% CO_2 at 37°C. When the cells reached 70–80% confluency, subculturing was performed using 0.25% trypsin-EDTA (Sigma-Aldrich).

2.2. Drug preparation and treatment

EA (≥95% pure; Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) to prepare a 10 mM stock solution. An appropriate dilution of the stock was then used to prepare the desired working concentrations in the culture medium. For DOX hydrochloride treatment, a 1 mM stock solution of DOX (Pfizer, New York, NY, USA) was prepared using sterile phosphate-buffered saline (PBS) and allowed to dissolve on a shaker overnight. It should be noted that the final concentration of DMSO in the samples never exceeded 0.1%. Preliminary data confirmed that this concentration of DMSO does not influence the viability of cancerous cell types.

2.3. Experimental design

The cells were seeded onto 96-well plates at a density of 5 × 10^3 cells per well and permitted to adhere for a period of 24 hours before being treated. The experiment consisted of the following groups: (1) control, (2) DOX-treated, (3) EA-treated, and (4) combination treatment (EA + DOX) groups. The concentrations of EA and DOX varied depending on the experimental assay. After being treated for 24 hours, the cells were subjected to various experiments described below. Each experiment was carried out in triplicate and repeated at least three times independently.

2.4. Cell viability assessment

The cell viability test was performed utilizing an established protocol based on the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, 10 µL of a 5 mg/mL MTT reagent in PBS was introduced to each well and incubated at 37°C for four hours. Following this process, the formazan crystals generated during the reaction were solubilized via the addition of 100 µL of DMSO, and the absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA). Percentages representing the proportion of live cells relative to untreated controls were subsequently calculated from the absorbance values.

2.5. Detection of apoptosis using ELISA

The extent of apoptosis was evaluated using the Cell Death Detection ELISA PLUS kit (Roche Diagnostics, Mannheim, Germany), which measures the levels of cytoplasmic DNA fragments associated with histone proteins (mono- and oligonucleosomes). Saos-2 cells were seeded (1 × 10^4 cells per well) onto 96-well plates and incubated either with EA, DOX, or their combinations for periods ranging from 24 to 72 h. After treatment, the cells were lysed, and the supernatant was transferred to a microplate coated with streptavidin. Afterwards, the lysate was mixed with a solution containing biotin-labeled anti-histone antibody and peroxidase-conjugated anti-DNA antibody and incubated at room temperature for 2 hours. Then, after thorough washing, we used ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] substrate to quantify the immunocomplex content of the samples. Next, we read absorbance values at 405 nm using a microplate reader (BioTek Instruments). Finally, we compared apoptosis rates among different experimental groups.

2.6. Western blot analysis

To examine the protein expression of p-gp, we performed Western blot analysis. Cells were first lysed in RIPA buffer (Thermo Fisher Scientific) supplemented with protease and phosphatase inhibitor cocktails (Roche Diagnostics). The concentration of each sample was measured via the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA), with bovine serum albumin as the standard. Each sample, containing 50 µg of total protein, was separated via SDS-PAGE using 10–12% gels and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking the membranes with a 5% non-fat milk solution (in TBST) at room temperature for one hour, the membranes were allowed to incubate at 4°C overnight with the primary antibodies anti-P-gp (1:500; Abcam, #ab170904, USA) and anti-β-actin (1:5000; Sigma-Aldrich, #A5441), used as a loading control. The membranes were then left at room temperature for two hours to react with horseradish peroxidase-conjugated secondary antibody at a dilution of 1:5000 (Santa Cruz Biotechnology, USA). The protein bands were made visible with the use of an enhanced chemiluminescence substrate (Pierce, Thermo Fisher Scientific), and quantification was carried out via ImageJ Software (NIH, Bethesda, MD, USA).

2.7. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA extraction was performed using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), based on the manufacturer's instructions. RNA concentration and purity were determined with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Complementary DNA synthesis was done using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), with 1 µg of total RNA as the template. The qRT-PCR analysis was conducted on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Green Master Mix (Applied Biosystems). Table 1 lists the primer sequences used in this study. Gene expression levels were normalized to GAPDH and then calculated by applying the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primer sequences used for qRT-PCR analysis

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Product size (bp)
Bax	CCCGAGAGGTCTTTTTCCGAG	CCAGCCCATGATGGTTCTGAT	145
Bcl-2	GGTGGGGTCATGTGTGTGG	CGGTTCAGGTACTCAGTCATCC	174
MDR	TTGCTGCTTACATTCAGGTTTCA	AGCCTATCTCCTGTCGCATTA	135
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG	238

2.8. Statistical analysis

The results were expressed as means ± SD of at least three independent experiments. Statistical analyses were performed using GraphPad Prism version 7.02 (GraphPad Software, San Diego, CA, USA), utilizing one-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons.

3. RESULTS

3.1. Cytotoxicity of DOX on Saos-2 cells

To test the effectiveness of DOX as an inhibitor of Saos-2 OSA cell proliferation, we performed an MTT assay on these cells using a range of concentrations of DOX from 0.1 to 5 μM. At submicromolar concentrations (≤0.5 μm), DOX displayed minimal apparent cytotoxic activity, since there was no significant decrease in cell viability beyond 85% after 24 h (Fig. 1A). However, increasing the concentration of DOX had an inhibitory impact on cell viability. The reduction in viability after treatment with 1 μM of DOX alone was significant (62.4 ± 4.1%; n = 6) when compared to the control condition ($p < 0.01$ vs. control). Similar observations were made using higher concentrations; viability was further decreased by treatment with 2 μM (41.3 ± 3.8%; n = 6) or 5 μM (28.5 ± 2.9%; n = 6) of DOX ($p < 0.001$ vs. control). The half-maximal inhibitory concentration (IC_{50}) of DOX for Saos-2 cells was calculated as 1.2 ± 0.07 μM, showing only moderate sensitivity of the cells to DOX. This conclusion is corroborated by the drastic decrease seen on the dose-response curve between 0.5 μM and 2 μM, suggesting that this particular compound has a very small therapeutic window in terms of cytotoxic activity. Additionally, repeated experiments showed consistent results (inter-assay CVs <10% at all concentrations except near the IC_{50}, where values approached 12-15%). A slightly greater variability was noted near the IC_{50}, likely due to the stochastic differences in the cellular response at the critical thresholds of drug-induced stress. The ability of EA to modulate the cytotoxic properties of DOX was examined by systematic application of different combinations. It was determined that the use of EA alone (at concentrations ranging from 0 to 100 μM) resulted in a mild reduction in cell proliferation (not greater than 20% even at 50 μM). However, when used in combination with subtoxic levels of DOX (0.1–2 μM), EA elevated the effect of DOX in a concentration-dependent manner (Fig. 1B). The strongest synergistic effects were observed at EA concentrations of 20-50 μM. The addition of EA decreased the DOX IC_{50} from 1.2 ± 0.07 μM to 0.75 ± 0.06 μM. This significant decrement indicates a 1.6-fold improvement. Furthermore, isobolograms demonstrated that this interaction was not merely an additive effect but a true synergistic effect (combination index at ED_{50} = 0.72 ± 0.05). Of significant clinical importance is the fact that this synergistic interaction occurred within the physiologically relevant and acceptable DOX dosage range (0.5–1 μM); the addition of EA within this range achieved a further substantial increase in the cytotoxic effect, reducing cell viability to 35–45% ($p < 0.01$; Fig. 1C). The reproducibility of these results was verified by three independent biological replicates, with variations between experiments remaining below 15% for all combination indices. Thus, EA was suggested as a powerful chemosensitizer capable of significantly improving the therapeutic index of DOX in OSA cells.

3.2. Modulation of apoptotic pathways

To explore molecular mechanisms underlying the synergistic cytotoxic effects of EA and DOX, key regulatory proteins involved in apoptosis were examined via qRT-PCR. In this study, we observed significant modulation of the genes implicated in mitochondrial-mediated apoptosis. We observed that combination treatment with EA at 27 μM and DOX at 1 μM increased the transcription of the pro-apoptotic gene *Bax* (B-cell lymphoma-2 homologue 2), whose levels were significantly elevated (~2.5-fold; $p < 0.001$) relative to the untreated control (Fig. 2). This significant upregulation of *BAX* was even more pronounced than that produced by only DOX (~1.3-fold; $p < 0.05$) or even EA on its own, which showed no significant difference from controls. Meanwhile, the expression level of the anti-apoptotic *BCL-2* gene dropped by ~40%, resulting in a substantial ~4.8-fold elevation in the *BAX/BCL-2* ratio (Fig. 2B), an essential determinant for triggering cell death via apoptosis. To analyze the capacity of DOX, EA, and their combination to trigger cell death processes in Saos-2 cells, we used the Cell Death Detection ELISA PLUS kit (Roche Diagnostics, Mannheim, Germany). Both EA (27 μm) and DOX (1 μm) resulted in a significant augmentation of apoptosis versus the control group ($p < 0.05$ and $p < 0.01$, respectively; Fig. 3). However, the application of the two compounds together showed even higher rates of apoptosis, which reached statistical significance ($p < 0.001$). Since the Saos-2 cells lack p53, these effects are mediated by p53-independent mechanisms. These results offer a mechanism explaining how natural compounds such as EA might enhance conventional chemotherapy by acting on basic cell death pathways.

3.3. Suppression of MDR mechanisms

The ability of EA to overcome chemoresistance was examined by assessing its effects on key MDR markers in Saos-2 cells. The qRT-PCR analysis showed that co-treatment with EA (27 μM) and DOX significantly

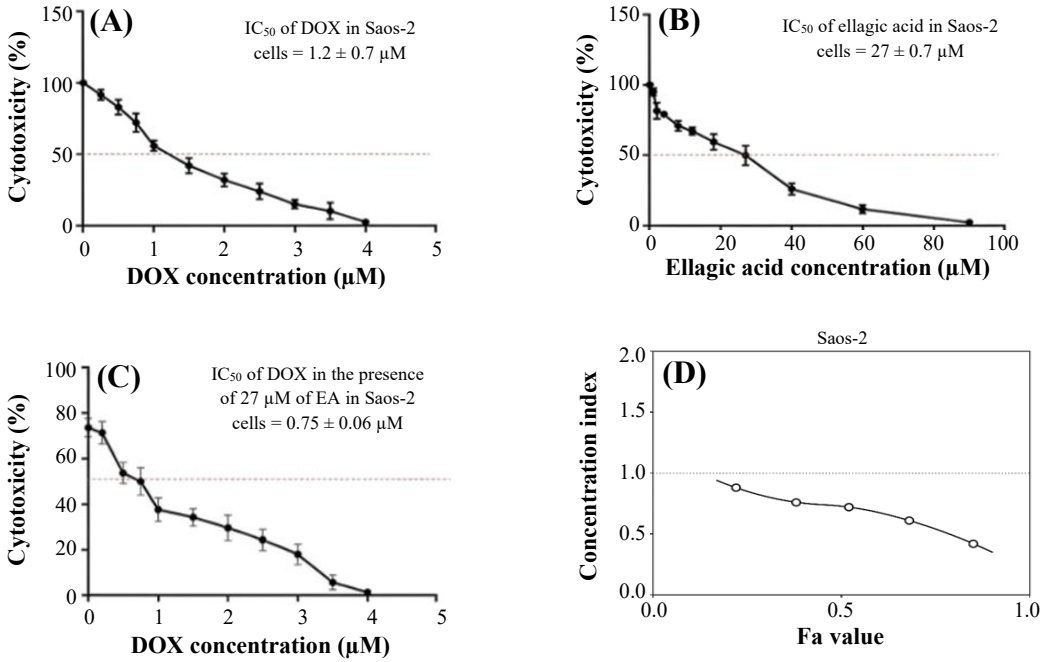

Fig. 1. (A) Effect of DOX on the cytotoxicity of Saos-2 cells. The cells were treated with increasing concentrations of DOX (0.1–5 µM) for 24 h; (B) effect of EA on the cytotoxicity of Saos-2 cells. The cells were treated with EA (20–50 µM) for 24 h; (C) synergistic cytotoxicity of EA and DOX in Saos-2 cells. The cells were treated with DOX in the presence of EA (20–50 µM) for 24 h ($p < 0.01$ vs. DOX monotherapy). Cell viability was determined by MTT assay. Data are shown as mean ± SD of three independent experiments (n = 3). Statistical significance was assessed by one-way ANOVA, followed by Tukey's post hoc test.

reduced the expression of the *MDR1* gene, leading to a 60.3 ± 5.2% decrease when compared with the untreated controls ($p < 0.001$). This level of suppression was much greater than that seen in the cells treated with either compound individually; EA alone caused a 15-20% reduction, and DOX alone led to a 25-30% reduction. At the protein level, Western blotting analysis indicated a similar decrease in P-gp expression. In the combination therapy, there was a 52.7 ± 4.8% reduction in P-gp levels compared to treatment with either EA or DOX alone, which showed a reduction of only 18.3 ± 3.1% for EA and 22.6 ± 3.9% for DOX, respectively ($p < 0.001$). The very high degree of correlation observed between changes in MDR1 mRNA (r = 0.89) and P-gp protein expression indicates that this form of regulation through gene transcription is likely to be the principal mode by which P-gp is suppressed as a result of our treatments (Fig. 4). However, we were unable to carry out a rhodamine-123 accumulation assay to investigate P-gp activity. The extent of this MDR

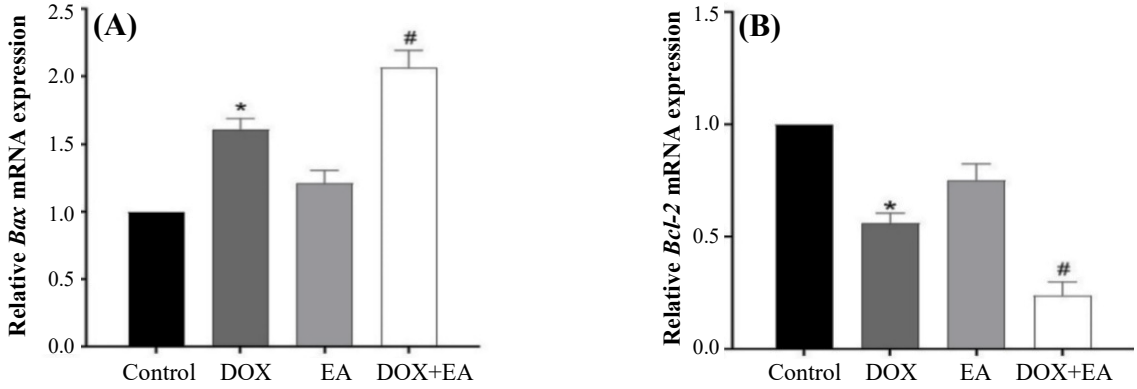

Fig. 2. Relative mRNA expression of apoptotic regulators in Saos-2 cells. The cells were treated with EA (27 µM), DOX (1 µM), or their combination for 24 h. The expression levels of (A) *Bax* and (B) *Bcl-2* were quantified by qRT-PCR and normalized to *GAPDH* using the $2^{-\Delta\Delta Ct}$ method. Data are presented as the mean ± SD of three independent experiments (n = 3). Statistical analysis was performed by one-way ANOVA, followed by Tukey's post hoc test ($^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. control for combination treatments.

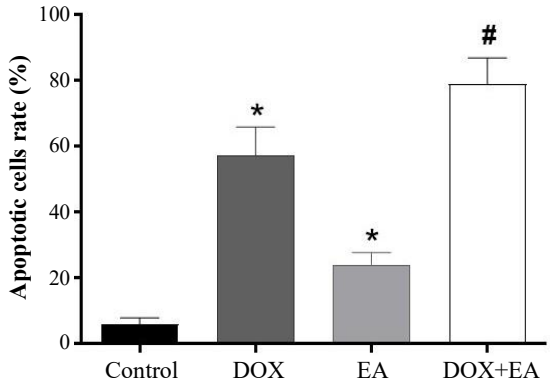

Fig. 3. Quantification of apoptosis in Saos-2 cells. The cells were treated with EA (27 μM), DOX (1 μM), or their combination for 24 h. Apoptosis was measured using the Cell Death Detection ELISA PLUS kit. Data are presented as the mean ± SD of three independent experiments (n = 3). Statistical analysis was performed using one-way ANOVA, followed by Tukey's post hoc test ($^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. control for combination treatments).

modulation, especially in combination with DOX, offers a solid mechanistic explanation for the chemosensitization effects observed in our experimental model.

4. DISCUSSION

The ability of EA to increase the cytotoxic effect of DOX in Saos-2 OSA cells seems to be due to several molecular mechanisms, which could address both the efficacy and resistance issues in the treatment of OSA. Firstly, the combination treatment of EA and DOX was found to cause a significant rise in the Bax/Bcl-2 mRNA ratio (4.8-fold), indicating that the intrinsic apoptotic pathways were activated. The strong apoptotic response observed with the combination treatment has shown that there is a synergistic interaction between EA and DOX in Saos-2 OSA cells. The second mechanism, which involves the suppression of MDR1 and downregulation of P-gp, provides strong evidence for the role of EA in overcoming chemoresistance, supporting the earlier results reported by Saltoglu et al. in breast cancer cells[19]. This finding becomes especially important in light of the pharmacokinetics study by Gu et al., who have shown that EA nanoparticles greatly improve the bioavailability of this compound[26], offering a promising future perspective for achieving biologically relevant systemic exposure in vivo.

The difference that has found between the results of this study with compared to those of Lin et al.[27]. Regarding the cytotoxicity of monotherapy is probably due to both the concentration of EA used and different metabolic profiles specific to various cell types, as pointed out by Boehning et al.[28]. Our study fills several gaps in the current literature. While Kao et al. have found that EA causes changes in the Bax/Bcl-2 ratio in colon cancer[29], we have shown that this effect is enhanced in OSA when EA is used in combination with DOX. These findings provide a basis for further investigation of the potential of EA to enhance DOX efficacy in osteosarcoma. First, the pharmacological attainability of the subtoxic EA concentration (27 μM), which exhibited synergistic activity with DOX, may potentially be improved through the development of advanced EA delivery systems, particularly nanoformulation-based approaches. Second, by simultaneously targeting both apoptosis and drug resistance pathways, we address two major clinical problems in OSA treatment. Third, the cell type-specific effects of EA on mitochondrial function and cellular respiration, which were noted by Boehning et al.[28], raise the possibility that metabolic characteristics could serve as potential biomarkers for predicting treatment responses. The importance of modifying the Bax/Bcl-2 axis to increase DOX sensitivity in OSA has been recently highlighted. As demonstrated by Cao et al., simultaneous targeting of Bcl-2 and Bcl-xL can overcome DOX resistance in OSA models[30]. These results align with our findings, which demonstrated a 4.8-fold increase in the Bax/Bcl-2 ratio following

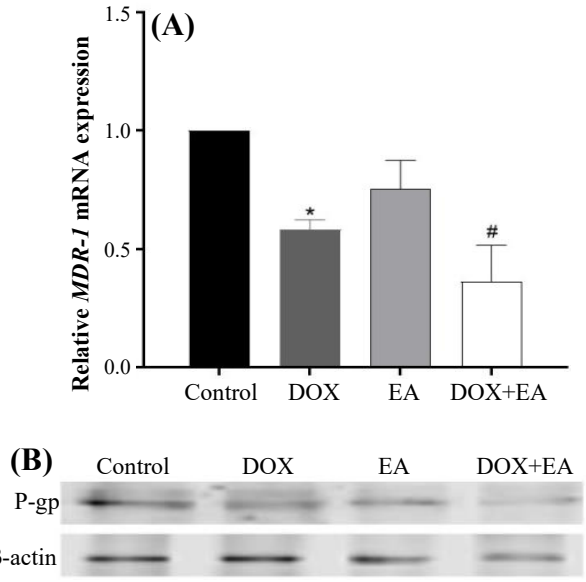

Fig. 4. Effect of EA and DOX on *MDR1* mRNA and P-gp protein expression in Saos-2 cells. (A) Relative *MDR1* mRNA levels determined by qRT-PCR after 24 h of treatment with EA (27 μM), DOX (1 μM), or their combination (normalized to GAPDH); (B) P-gp protein levels assessed by Western blot under the same treatment conditions; β-actin served as a loading control. Data are presented as mean ± SD of three independent experiments (n = 3). Statistical analysis was performed by one-way ANOVA, followed by Tukey's post hoc test ($^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. control for combination treatment).

combination treatment with EA and DOX further supports the view that natural compounds can be effective in activating the mitochondrial apoptotic pathway in OSA cells. Besides, another study has demonstrated that the combination of EA acid and etoposide can synergistically induce apoptosis in canine OSA cells via the upregulation of Bax and downregulation of Bcl-2, together with caspase activation[31]. Although that recent study was conducted in a different species and used a different drug, the results are similar to ours and suggest that the pro-apoptotic activity of EA may be maintained across different OSA models.

Regarding multidrug resistance, the overexpression of P-gp/ABCB1 is still a major cause of DOX failure in OSA. Recent studies have kept on emphasizing the role of ABC transporters in drug efflux as a key resistance mechanism. In this context, a number of strategies, such as the use of natural compounds and nanocarriers, have been investigated to reduce both MDR1 mRNA and P-gp protein expression[32-35]. Our results indicated that EA markedly reduced both MDR1 mRNA and P-gp protein levels, thus providing further evidence that EA can interfere with this resistance pathway. A study published in 2024 by Lu et al. also indicated that a copper nanoplatform coordinated with EA and loaded with DOX could suppress P-gp activity via depleting ATP and thus overcome drug resistance, which supports the potential of an EA-based nanoplatform to improve intracellular DOX retention[32]. Our findings suggest that the sensitizing effects of EA may be independent of p53. Very recent studies have also reported that natural compounds may enhance DOX sensitivity in OSA cells in both p53-wild-type and p53-deficient cells, which supports the potential for such agents to be used more broadly[36-38].

Taken together, our findings provide further support for the potential role of natural polyphenols as multi-targeted chemosensitizing agents in OSA, through their ability to promote apoptosis and reduce P-gp protein expression that natural polyphenols can function as multi-targeted chemosensitizers in OSA by both promoting apoptosis and reducing P-gp protein levels. Such approaches would overcome the present limitations of two-dimensional culture systems while also providing the necessary preclinical data for moving into clinical applications. Our study indicated that EA has the potential to be a promising chemosensitizer for the treatment of OSA, owing to its unique ability to enhance apoptosis and modulate MDR1 expression. Future work should focus on proteomic validation of the apoptosis markers (cleaved caspases 3/9) using Western blot analysis, and in vivo validation in orthotopic OSA models while monitoring for cardiac toxicity, and also investigation of epigenetic modifications via ChIP-seq, extending upon the framework established by Abbott et al.[39]. The mechanistic insights offered in this study provide a starting point for the development of targeted combination therapies. Nevertheless, our study has some limitations. For instance, the study was carried out using a single cell line; it did not include an in vivo component, and it did not assess organ toxicity. Furthermore, Western blot analysis of the apoptosis markers was not done, and our evaluation was limited to gene expression. On the other hand, a rhodamine-123 accumulation assay was not performed to examine P-gp activity. Thus, our findings should be regarded as a preliminary study as an indication of directions for future research, and no definite conclusions can be drawn from these results.

5. CONCLUSION

This study offers preliminary evidence that EA may increase the therapeutic effect of DOX in Saos-2 OSA cells by means of a multi-targeted mechanism. The combination treatment showed synergistic effects, possibly as a result of upregulating Bax and downregulating Bcl-2, which in turn caused more apoptosis through a p53-independent pathway. This phenomenon involved the downregulation of MDR1/P-gp expression, reducing the amount of DOX needed for cytotoxicity. Our findings provide further evidence supporting the potential application of natural compounds as chemosensitizing agents in cancer therapy. The synergistic effect observed between EA and DOX suggests a promising approach for enhancing treatment efficacy in OSA while possibly reducing the side effects of traditional chemotherapy. Further preclinical/clinical studies are needed to completely evaluate the therapeutic potential of such a combination strategy.

DECLARATION

Acknowledgments

This paper is the outcome of the Medicine Degree thesis (thesis no. 74373) approved by Tabriz University of Medical Sciences, Tabriz, Iran. The authors would also like to thank the Shohada Clinical Research Development Unit, Shohada Hospital, Tabriz University of Medical Sciences, Tabriz, Iran.

Generative AI and AI-assisted technologies

During the preparation of this work, the authors used ChatGPT (OpenAI) for English editing. After using this tool, the authors carefully reviewed and edited the content and took full responsibility for the final version of the manuscript.

Ethical approval

This study was conducted in accordance with

institutional guidelines and approved by the Research Ethics Committee of Tabriz University of Medical Sciences, Tabriz, Iran (ethical code IR.TBZMED.VCR.REC.1404.030). As the study utilized commercially available cell lines, additional ethical approval was not required. All experiments were performed in compliance with relevant biosafety regulations.

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

LMB: writing–original draft, methodology, investigation, data curation; AV: methodology, investigation, data curation; AG: review and editing; MM: visualization, investigation; MVT: conceptualization, writing–review & editing; BY: conceptualization, validation, writing–review & editing, supervision, investigation.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Supplementary information

The online version does not contain supplementary material.

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