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ORIGINAL ARTICLE

Protective Effects of Rosmarinic Acid and Epigallocatechin Gallate Against Doxorubicin-Induced Cytotoxicity and Genotoxicity in CHO-K1 Cells

Sinem HELVACIOĞLU[1], Muhammed HAMİTOĞLU[2]*, Ecem YILDIRIM[3], Şenay VURAL KORKUT[4], Aylin YABA[3], Ahmet AYDIN[2]

[1]İstinye University Faculty of Pharmacy, Department of Pharmaceutical Toxicology, İstanbul, Türkiye
[2]Yeditepe University Faculty of Pharmacy, Department of Pharmaceutical Toxicology, İstanbul, Türkiye
[3]Yeditepe University Faculty of Medicine, Department of Histology and Embryology, İstanbul, Türkiye
[4]Yıldız Technical University Faculty of Arts and Science, Department of Molecular Biology and Genetics, İstanbul, Türkiye

ABSTRACT

Objectives: The chemotherapeutic drug doxorubicin (DOX) affects not only cancer cells but also healthy cells in an undesirable manner. The purpose of this study was to investigate the protective roles of rosmarinic acid (RA) and Epigallocatechin gallate (EGCG) alone and in combination against DOX-induced oxidative stress, cytotoxicity, and genotoxicity in healthy cells. In addition, this study evaluated the expression of the mammalian target of rapamycin (mTOR) protein in the Chinese hamster ovary cell line (CHO-K1).

Materials and Methods: Cell viability was analyzed using the WST-1 cytotoxicity assay. mTOR expression in the CHO-K1 cell line was determined by western blotting. DNA damage was analyzed using a comet assay. Reactive oxygen species (ROS) levels were determined microscopically using the dihydroethidium staining method.

Results: RA demonstrated superior protective effects against DOX-induced cytotoxicity compared to EGCG. Epigallocatechin gallate and RA did not exert genotoxic effects, but DOX increased genotoxicity in CHO-K1. Neither RA nor EGCG exhibited genotoxic effects; however, DOX significantly increased genotoxicity in CHO-K1 cells. Both RA and EGCG markedly reduced DOX-induced genotoxicity, as confirmed by the comet assay. In the DOX-treated group, the expression of mTOR protein was notably suppressed. EGCG further reduced mTOR protein levels when administered alone or in combination with DOX, whereas RA did not exhibit a similar effect. RA decreased intracellular generation of ROS in CHO-K1 cells. However, at high concentrations, Epigallocatechin gallate did not protect against oxidative stress and cell damage due to its prooxidant properties.

Conclusion: Epigallocatechin gallate and RA are promising plant-derived active components. Another important point is the evaluation of the safety of herbal products. It should be considered that herbal products may increase the toxicity of chemotherapeutic agents.

Keywords: DNA damage, comet assay, Western blot, mTOR, WST-1 assay, oxidative stress

INTRODUCTION

Doxorubicin (DOX) is a broad-spectrum anthracycline-based chemotherapeutic agent widely used as a first-line treatment for pediatric and adult patients. It is commonly employed for various cancer types, including breast stomach, and prostate cancer, as well as soft tissue and bone sarcomas. However, the cytotoxic and genotoxic effects of DOX are not limited to cancer cells, as it also damages healthy cells. Consequently, secondary malignant tumors may arise during or after chemotherapy, distinguishing them from primary tumors.[1]

The genotoxicity of DOX, primarily resulting from free radicals generated during its metabolism, plays a central role in secondary tumor development. Phytochemicals effectively protect against free radical-induced oxidative damage. Studies

*Correspondence: mohammad.saz@yeditepe.edu.tr, ORCID-ID: orcid.org/0000-0002-4545-0756
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have demonstrated the potential of natural substances to protect against the adverse effects of chemical drugs without compromising their therapeutic efficacy because of their inherent antioxidant capacity.[2] Rosmarinic acid (RA), an ester derived from caffeic acid and 3,4-dihydroxyphenyllactic acid, is found in a variety of Lamiaceae family plants.[3] It demonstrates diverse biological activities, including antioxidative, anti-inflammatory, antimutagenic, antiangiogenic, antiapoptotic, and antifibrotic properties. In particular, it is a natural antioxidant that can compete with unsaturated fatty acids for binding to lipid peroxyl groups to terminate the chain reaction of lipid peroxidation and reduce the rate of lipid peroxidation. The ability of RA to scavenge radiation-induced reactive oxygen species (ROS).[4] Additionally, epigallocatechin gallate (EGCG), another plant-based compound, serves as a phenolic compound prevalent in a wide array of plants, notably green tea. Its capacity to inhibit cellular oxidation and protect cells from free radical-induced damage renders it a subject of research as a potential cancer chemopreventive agent, exhibiting robust antioxidative, anti-inflammatory, and anticarcinogenic attributes.[5] EGCG and RA are both phenolic compounds, whereas RA is a stilbenoid, whereas EGCG is a tannin with a flavan-3-ol structure that has been esterified with gallic acid. Compared with RA, EGCG contains more phenolic -OH groups (Figure 1).

mTOR participates in several signaling pathways involved in the regulation of cell division, apoptosis, and autophagy in the body. Studies have established a connection between the mTOR signaling pathway and various disorders, including cancer. [6] Research suggests that rapamycin can potentially augment the antitumor effects of DOX by downregulating mTOR signaling.[7] For instance, demonstrated that combining mTOR inhibitors with DOX resulted in an increased therapeutic response in patients with leiomyosarcoma compared with DOX alone.[8]

Hence, in this study, the protective effects of RA and EGCG were investigated alone and in combination against DOX-induced genotoxicity and oxidative stress in Chinese hamster ovary cell line (CHO-K1) cells. The effects of these substances on cell proliferation were evaluated according to the mTOR expression level.

MATERIALS AND METHODS

Sample preparation

DOX hydrochloride, RA, and (-) -EGCG (Sigma-Aldrich, USA) were dissolved in phosphate-buffered saline (PBS) (Gibco, USA) to prepare a master stock solution and stored in -20°C before use. Then, the working solutions were prepared freshly at concentrations of 1, 2, and 400 µM in complete Ham's F12 culture medium.

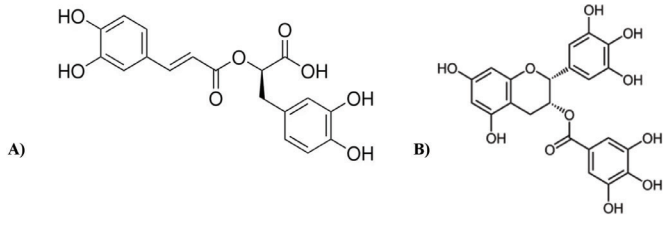

Figure 1. A) Chemical structure of RA, B) Chemical structure of EGCG
RA: Rosmarinic acid, EGCG: Epigallocatechin gallate

Cell line and culture conditions

CHO-K1 cells were obtained from the Institute of Pharmacology and Toxicology, Würzburg, Germany. The samples were cultured in Ham's medium F12 supplemented with 10% *(v/v)* fetal bovine serum and 1% *(v/v)* antibiotics (10000 U/mL penicillin and 50 mg/mL streptomycin). Cell cultures were cultured in a humidified incubator at 37 °C with 5% CO_2. Twice a week, cells were passaged through a 0.25% trypsin solution. Reagents for cell culture were obtained from (Gibco, Carlsbad, CA).

WST-1 cytotoxicity assay

Viability was measured using the WST-1 (Roche, Germany) colorimetric assay. Cells were seeded (5 x 10^3 cells in 100 µL of culture medium) were seeded into 96-well plates and grown for 24 h. The cells were then exposed to 100 µL/well of newly prepared medium containing the tested substance for 24, 48, or 72 h. After the end of the incubation period, the medium was withdrawn, and the cells were washed twice with PBS, then 100 µL of WST-1 were added to each well. The wells were then incubated for 4 h at 37 °C. After 4 h, absorbance was measured at 450 nm using a microplate reader (Thermo Multiskan Ascent, USA) after 4 h. (a-c) / (b-c) x 100 was used to calculate the percentage of cytotoxicity, where a represents the absorbance of treated cells, b represents the absorbance of control cells, and c represents the absorbance of the blank. The half maximal inhibitory concentration $(IC)_{50}$ was assessed from the dose-response curves.[9]

Alkaline comet assay

After seeding in a 6-well plate, the cells were treated for 4 h the next day with DOX, RA, EGCG, and their combinations. When the cells were harvested for the comet assay, a cell viability test was conducted. To achieve this, 15 µL of the staining solution containing fluorescein diacetate (Serva Electrophoresis GmbH, Germany) and gel red (Biotium, USA) were combined with 35 µL of the cell suspension. Cell viability was determined by counting 200 cells using an Eclipse 55i microscope fitted with a FITC filter (Nikon GmbH, Japan).[10] Twenty µL of the cell suspension and 180 µL of pre-warmed low-melting-point agarose (Carl Roth, Germany) were mixed for the comet assay. Fourty-five µL of cell agarose was loaded onto cold microscope slides previously coated with 1.5% high-melting-point agarose. Pre-cooled glass cuvettes containing the lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, and 10 mM Tris adjusted to pH 10) combined with 10% dimethyl sulfoxide and 1% Triton X-100 (Sigma Aldrich; USA) were filled with the cells on the slides. The cells were then allowed to undergo lysis at 4 °C in the dark. Following lysis, the slides were incubated for 20 min at 4 °C in electrophoresis buffer (5 M NaOH and 0.2 M Na_2EDTA, pH 13). Next, electrophoresis was run at 25 V and 0.3 A for 20 min. After electrophoresis, the slides were fixed in frigid methanol for 5 min and neutralized with Tris buffer for 5 min. Following drying, 20 µL of GelRed solution per slide was used to stain the

slides, which were examined using a 200-fold magnification fluorescence microscope (Labophot-2; Nikon GmbH, Germany) and Komet 6-software (Komet version 6, ANDOR™ Technology). The percentage of DNA in the tail region for a total of 100 cells (50 on each slide) was used to express the results.[11]

Microscopic analysis of ROS production

Dihydroethidium (DHE), (Merck Biosciences GmbH, Germany) was used to detect the superoxide anion concentration in the mitochondria of living cells. DHE is blue in the cytosol until it is oxidized, at which point it intercalates into the cell's DNA, resulting in a bright fluorescent red. After treatment, fresh medium containing 10 µM DHE was added to the cells and incubated for 20 min in the dark at room temperature. Following the incubation period, the cells were washed twice with PBS. ImageJ software was used to measure the gray values of 200 cells in each treatment for quantification.[12]

Western blot analysis

Western blotting analysis was carried out as described previously.[13] Briefly, total protein from CHO-K1 cells was extracted using radioimmunoprecipitation lysis buffer (SantaCruz, Texas, USA) with phenylmethylsulphonylfluoride, protease inhibitor cocktail, and sodium orthovanadate. Then, each lane was filled with 20 µg of the whole lysate, which was electrophoretically separated using a NuPAGE 4-12% Bis-Tris gel (Invitrogen™, USA) and electroblotted onto a nitrocellulose transfer membrane. (Advansta, San Jose, USA). The membrane was blocked for 1 h to reduce non-specific binding using 5% non-fat dry milk in TBS-T buffer (Tris-buffered saline with 0.1% tween-20). The membrane was placed with appropriate primary antibodies anti-mTOR (1:1000 dilution; Cell Signaling, Germany) overnight at 4 °C. After incubating the primary antibody, the membrane was washed three times with TBS-T for 10 min each time, incubated for 1 h at room temperature with the anti-mouse immunoglobulin G secondary antibody (1:2000 dilution, Cell Signaling), and finally rinsed with TBS-T. mTOR protein expression was detected using a chemiluminescent substrate (Thermo Scientific, USA), immunoblot images were taken, and bands were measured using Image Lab Software (BioRad, Germany). The ratio of each protein's expression level to that of β-Actin from the same samples, which served as the internal control, was used to calculate the expression of each protein.

Statistical Analysis

Data were expressed as the mean ± standard error of mean and analyzed using GraphPad Prism 9 software (GraphPad, Boston, USA). The differences among the means were analyzed using analysis of variance followed by Dunnett's analysis. The treatment and control groups were compared. It was considered statistically significant when $p < 0.05$.

RESULTS

WST-1 cell proliferation assay

In cells treated with DOX and EGCG, relative cell proliferation consistently decreased in a dose- and time-dependent manner. Table 1 presents the IC_{50} values of cells treated with DOX and EGCG at 24, 48, and 72 h. However, treatment with RA at various concentrations (ranging from 0.0625 mM to 1 mM) for 24, 48, and 72 h did not reduce the viability of CHO-K1 cells. Consequently, the IC_{50} value could not be calculated for the concentrations used in this study.

Furthermore, the possible protective effects of RA and EGCG, either alone or in combination, against DOX-induced cytotoxicity in CHO-K1 cells were examined using the WST-1 assay. To evaluate the protective effect, a DOX concentration of 500 nM, as determined by its IC_{50} value, was chosen for the investigation. CHO-K1 cells were subjected to different dosages of EGCG, RA, and their combination for 24 h. The results exhibited a statistically significant protective effect of both EGCG and RA against DOX-induced cytotoxicity. Notably, a 1 mM RA concentration demonstrated the most pronounced protective effect against DOX-induced cytotoxicity (Figure 2).

Microscopic analysis of ROS formation

The generation of ROS due to DOX administration was quantified by analyzing DHE fluorescence, with an illustrative example provided in Figure 3A. DOX was tested at a concentration of 1000 nM over different time intervals of 0.5, 1, 2, and 4 h (Figure 3B). ROS production increased notably in cells treated with 1000 nM DOX for 0.5 h. This increase was statistically significant after 2-h treatment compared with the control group.

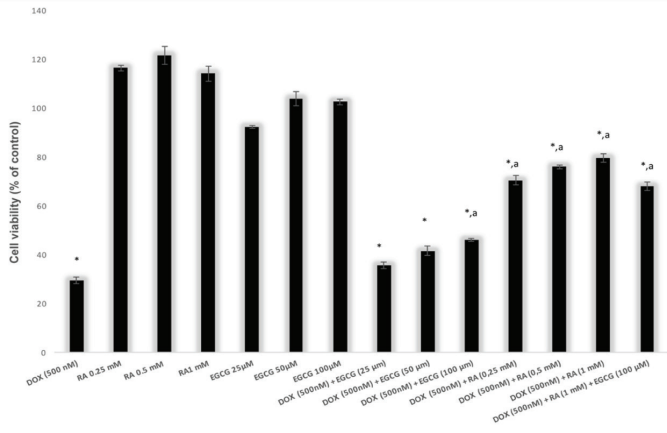

Figure 2 Cells were treated with different concentrations of DOX, RA, EGCG, and their combinations for 24 h. Viability was quantitated by WST-1 assay. *$p \leq 0.05$ vs. control group and *a*$p \leq 0.05$ vs. DOX 500 nM group.

RA: Rosmarinic acid, EGCG: Epigallocatechin gallate, DOX: Doxorubicin

Table 1. IC_{50}-values of CHO-K1 cells after 24, 48 and 72 hours of incubation with DOX and EGCG. Values are expressed as mean ± SD of triplicate experiments

Compounds	IC_{50}		
	24 hours	48 hours	72 hours
DOX (nM)	696.8 ± 1.4	467.2 ± 2.2	131 ± 2.7
EGCG (µM)	305 ± 0.4	277 ± 1.5	260.5 ± 3.3

CHO-K1: Chinese hamster ovary cell line, EGCG: Epigallocatechin gallate, DOX: Doxorubicin, SD: Standard deviation, IC_{50}: Half maximal inhibitory concentration

Figure 4 illustrates the protective effect of RA against DOX-induced oxidative stress. Across all tested concentrations, RA alone did not induce a significant increase in ROS generation; instead, it exhibited a noteworthy decrease in ROS levels compared with the DOX group. In contrast, EGCG alone or in combination with RA did not reduce ROS formation compared with the DOX-treated group (Figure 5). Notably, the application of 100 µM EGCG resulted in an increase in ROS levels in CHO-K1 cells.

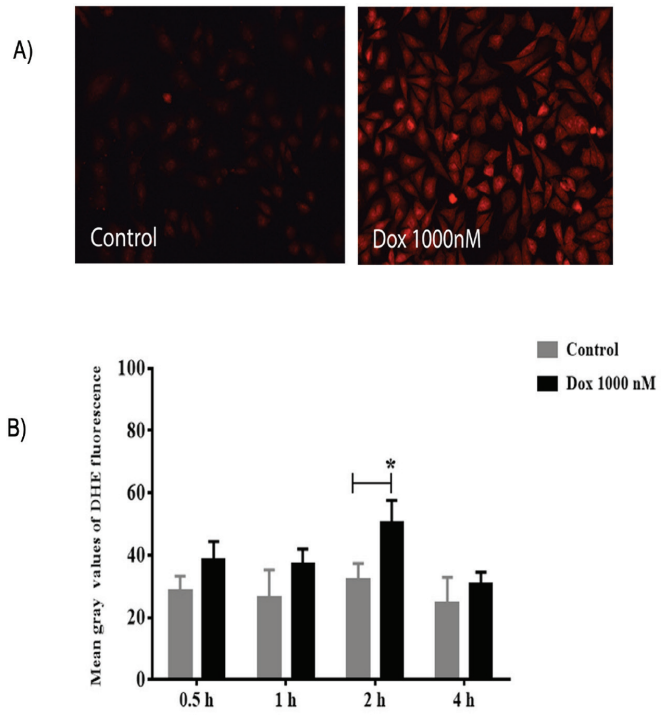

Figure 3. ROS formation in CHO-K1 cells treated with 1000 nM Dox for 0.5 to 2 hours using DHE assay. A) DHE fluorescence was quantified using image j software, which measured the mean grey value of 200 cells. B) Results are shown as mean ± SEM of three separate tests. *$p \leq 0.05$ vs. control group

RA: Rosmarinic acid, EGCG: Epigallocatechin gallate, DOX: Doxorubicin, CHO-K1: Chinese hamster ovary cell line, SEM: Standard error of mean

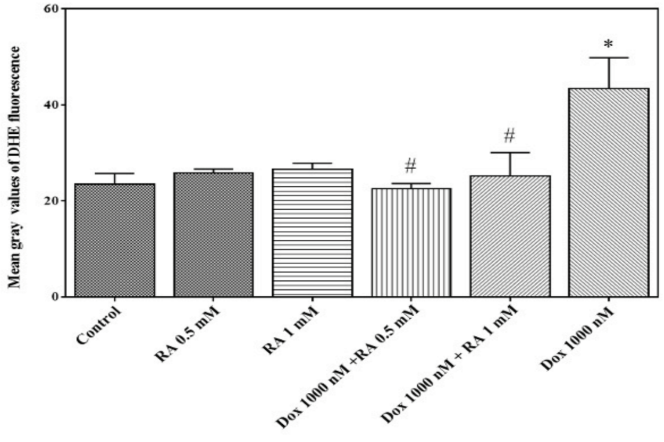

Figure 4. ROS production and its inhibition by RA in CHO-K1 cells. DHE fluorescence was quantified using image j software, which measured the mean grey value of 200 cells Results are shown as mean ± SEM of three independent tests. Kruskal-Wallis test was used for analysis, *$p \leq 0.05$ vs. control and #$p \leq 0.05$ vs. DOX 1000 nM

RA: Rosmarinic acid, DOX: Doxorubicin, CHO-K1: Chinese hamster ovary cell line, SEM: Standard error of mean, DHE: Dihydroethidium

Alkaline comet assay

According to the cell viability assay results, no significant reduction in cell viability was observed in any of the evaluated groups in the comet test compared with the control group (data not shown). In DOX-treated cells, there was an evident dependence on dose increase in DNA damage (Figure 6A). This effect was statistically significant at concentrations of 1000, 2000, and 4000 nM compared with the negative control group. As illustrated in Figure 6B, RA administration led to a notable and dose-dependent decrease in cells exhibiting DNA damage. In contrast, EGCG alone or in combination with RA did not exert a protective effect against DOX-induced genotoxicity (Figures 6C and D).

mTOR protein expression in CHO-K1 cells

The protein levels of mTOR, which plays a significant role in oxidative stress, were assessed using western blotting. mTOR protein expression levels were decreased in the DOX group compared with the control group (Figure 7A). mTOR protein expression level was significantly higher in the group administered RA at a concentration of 1 mM with DOX than in the group administered DOX alone (Figure 7B). A significant decrease in mTOR expression was observed in the EGCG-treated group, alone or in combination with DOX, compared with the negative control group. However, these differences were not significant compared with the DOX-treated group (Figure 7C).

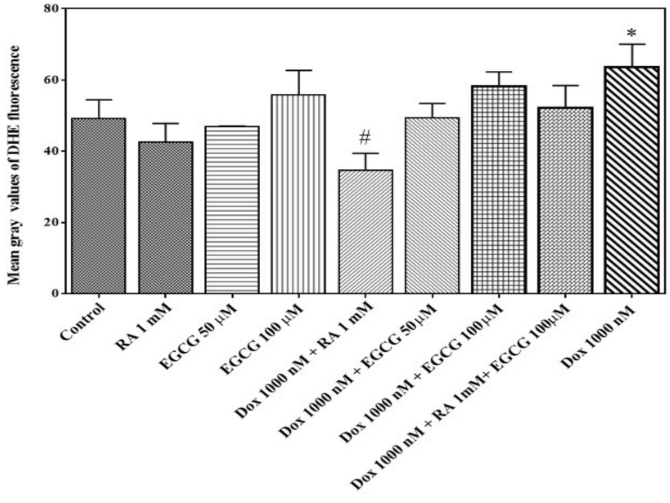

Figure 5. Intracellular ROS after treating the cells with RA 1mM and EGCG (50, 100 µM) for 2 h with and without the addition of DOX. DHE fluorescence was quantified using image j software, which measured the mean grey value of 200 cells. Results are shown as mean ± SEM of three independent tests. *$p \leq 0.05$ vs. control and #$p \leq 0.05$ vs. DOX 1000 nM

ROS: Reactive oxygen species, RA: Rosmarinic acid, EGCG: Epigallocatechin gallate, DOX: Doxorubicin, DHE: Dihydroethidium, SEM: Standard error of mean

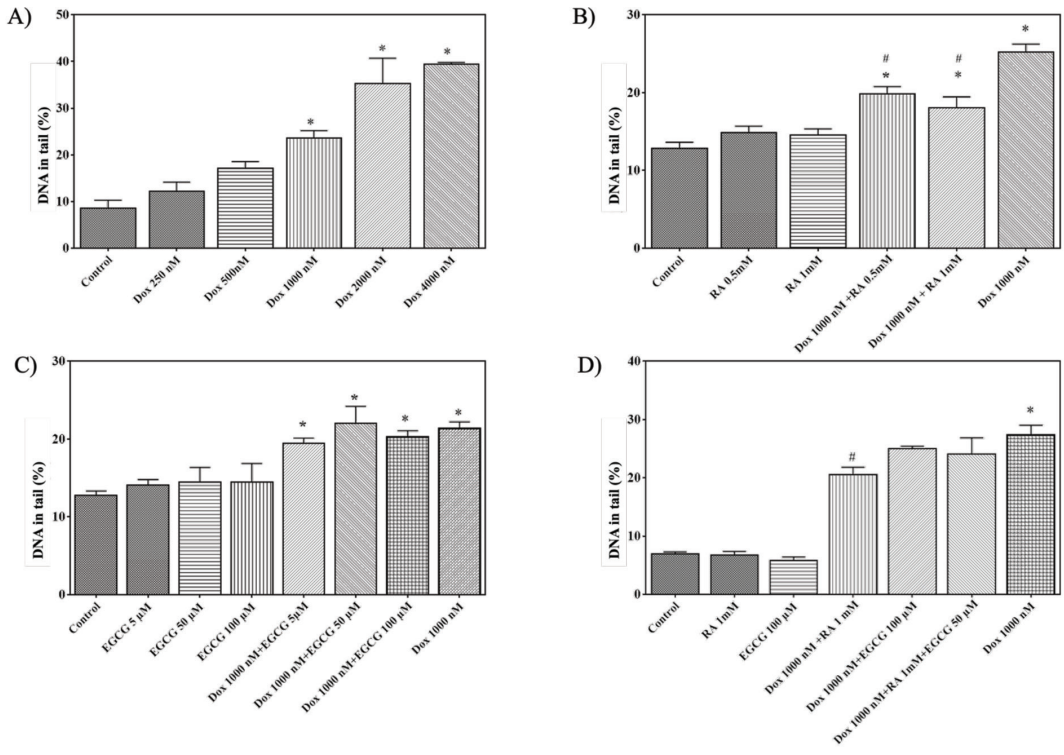

Figure 6. Alkaline comet assay results (tail intensity) obtained in CHO-K1 cells after treatment with different compounds. A) Concentration-dependent increase in DNA damage after 4 hours of DOX treatment in CHO-K1 cells. *$p \leq 0.05$ vs. control group. B) DNA damage induction by DOX treatment with or without RA inhibition in CHO-K1 cells. *$p \leq 0.05$ vs. control and #$p \leq 0.05$ vs. DOX 1000nM. C) DNA damage induction by DOX treatment with or without EGCG in CHO-K1 cells. *$p \leq 0.05$ vs. control group. D) DNA damage induction by DOX treatment with or without RA and EGCG combination in CHO-K1 cells. *$p \leq 0.05$ vs. control and #$p \leq 0.05$ vs. DOX 1000nM. In the evaluation of comet analysis results, each treatment group had 100 cells evaluated, with the findings represented as a percentage of DNA in the tail. The data are given as mean ± SEM of 3 independent experiments

RA: Rosmarinic acid, EGCG: Epigallocatechin gallate, DOX: Doxorubicin, CHO-K1: Chinese hamster ovary cell line, SEM: Standard error of mean

DISCUSSION

Genotoxicity is one of the most important mechanisms of adverse effects associated with DOX therapy as an anticancer drug. Various pharmacologic treatments, including hematopoietic cytokines, iron-chelating agents, and antioxidants, have been studied to mitigate the adverse effects of DOX.[14,15] In light of these findings, our hypothesis focused on the potential protective effects of phenolic compounds possessing antioxidant properties, such as RA and EGCG, against DOX-induced oxidative stress and DNA damage in healthy cell lines. To investigate this, a fluorescent dye-based detection method was employed to detect superoxide in CHO-K1 cells. Our findings revealed that RA significantly inhibited DOX-induced ROS formation, whereas EGCG did not exhibit protective effects in this context. Furthermore, when RA and EGCG were co-injected, no significant reduction in ROS formation was observed. Additionally, EGCG did not exert a protective effect against DOX-induced genotoxicity in the comet assay in CHO-K1 cells, whereas RA exerted a significant protective effect. Previous studies have revealed the dual nature of EGCG, which possesses both antioxidant and pro-oxidant properties.[16] Catechins, including EGCG, can undergo autooxidation and function as pro-oxidants under specific circumstances.[17] The reported anticancer activity of EGCG, including its ability to induce apoptosis in cancer cells, is attributed to these prooxidant characteristics.[18] In several studies examining EGCG's ability to prevent various cell lines from oxidative DNA damage, researchers found that at low concentrations, EGCG reduced DNA damage while acting as a pro-oxidant at higher concentrations. Specifically, it was noted that a concentration of 200 µM EGCG increased oxidative DNA damage in human lymphocyte DNA induced by H_2O_2.[19]

In the present study, 0.5 and 1 mM RA exerted protective effects against DOX-induced genotoxicity in CHO-K1 cells. These findings are consistent with earlier reports suggesting that concentrations of 0.28, 0.56, and 1.12 mM RA did not induce genotoxic effects and notably decreased DOX-induced DNA damage in V79 cells over a 3-h period.[20]

These findings suggest that protection against DNA damage induced by DOX is associated with a reduction in ROS levels. Given that the generation of free radicals constitutes one of the primary mechanisms underlying DOX's genotoxicity, a decrease in free radical formation would likely lead to a reduction in DNA damage.[21] In similar studies documented in the literature, it has been demonstrated that compounds possessing antioxidant properties can protect against ROS production and the genotoxic effects induced by DOX. For instance, in one study,

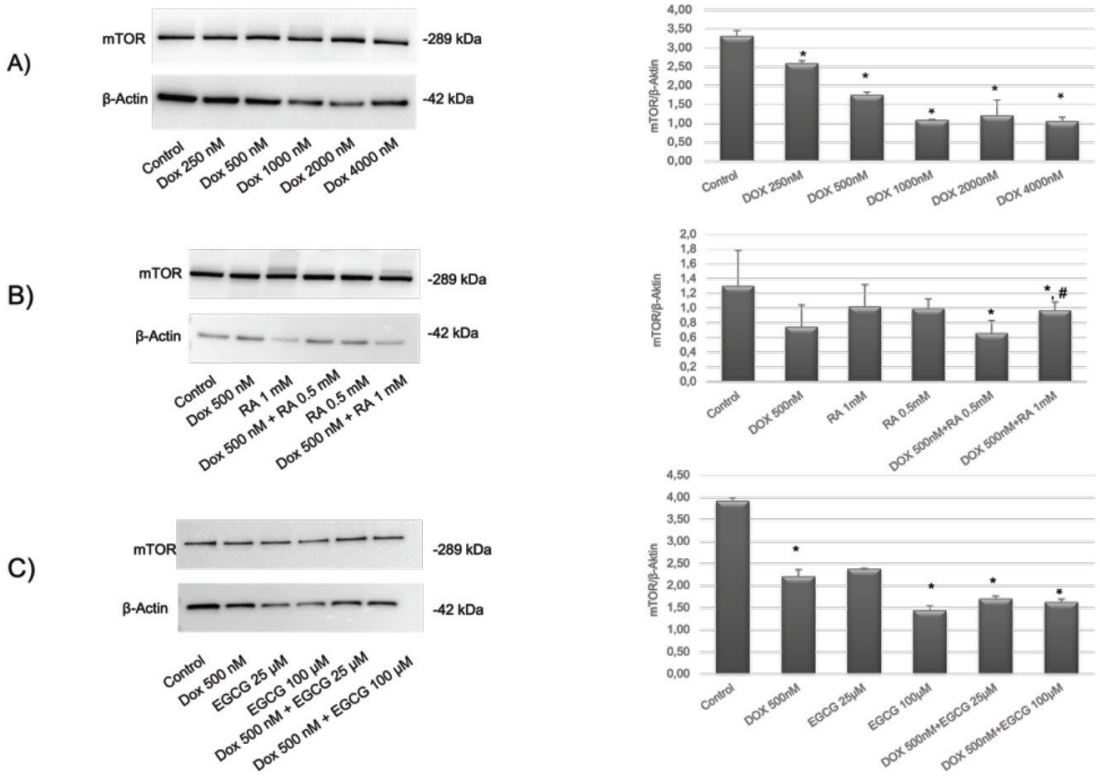

Figure 7. Western blot analysis A) mTOR protein level after 24 h of DOX treatment in CHO-K1 cells. *$p \leq 0.05$ vs. control group. B) DOX treatment with or without RA in CHO-K1 cells. *$p \leq 0.05$ vs. control and #$p \leq 0.05$ vs. DOX 500nM. C) DOX treatment with or without EGCG in CHO-K1 cells. *$p \leq 0.05$ vs. control group. Protein levels were normalized to β-actin. Data are given as a mean of (n= 3) ± SEM

mTOR: Mammalian target of rapamycin, RA: Rosmarinic acid, EGCG: Epigallocatechin gallate, DOX: Doxorubicin, CHO-K1: Chinese hamster ovary cell line, SEM: Standard error of mean

thymoquinone mitigated DNA damage and oxidative stress triggered by DOX in human leukocyte cells.[22]

Moreover, the administration of RA and EGCG, either alone or in combination, reduced DOX-induced cytotoxicity in non-cancerous cell lines. The existing literature has demonstrated the protective effects of polyphenolic compounds against DOX-induced cytotoxicity in normal cells. For example, in one study, quercetin significantly mitigated the cytotoxic effect of DOX.[23] Additionally, silymarin, a prominent flavonolignan found in *Silybum marianum* L., has been indicated to lessen DOX-induced cytotoxicity by shielding the cell membrane from damage caused by free radicals.[24] In another study, hydroxytyrosol, the primary phenolic compound found in olive oil, effectively prevented the cytotoxicity of DOX generated in cardiomyocytes by regulating the oxidative response and apoptotic processes mediated by the Bcl-2/Bax ratio.[25]

In response to a variety of extracellular stimuli, including growth hormones, nutrients availability, and stress, mTOR regulates cell proliferation and metabolism. Deregulation of the mTOR signaling system is intimately linked to aging, metabolic disorders, and malignancies.[26,27]

EGCG has exhibited inhibition of mTOR and PI3K expression in numerous cancer cell lines.[28] EGCG is an inhibitor of both the PI3K and mTOR pathways.[29] Interestingly, mTOR expression levels decreased with increasing DOX doses compared with the control group. The observed reduction in mTOR levels was attributed to oxidative stress and the formation of free radicals triggered by exposure to DOX.

In the literature, it has been stated that oxidative stress regulates mTORC1 and that ROS inhibit the mTOR signaling pathway.[30] It has been stated that moderate stress levels can trigger stress responses by inducing stress-adaptation genes and partially suppressing mTOR activity, whereas high-intensity stress may suppress mTOR.[31] There are various reports that mTOR is inhibited or activated by oxidative stress. This difference is believed to vary depending on the cell line or the type of oxidant.[32] In this study, mTOR levels were decreased in the EGCG-administered groups. This observation may be due to the pro-oxidant properties of EGCG.[33] When RA was co-administered with DOX, it increased the level of mTOR compared with DOX-treated cells. Lou et al.[34] showed that RA stimulates liver regeneration via the mTOR pathway. Strong and persistent mTOR activation caused by RA treatment increased RA-mediated hepatocyte proliferation. However, the interaction between the mTOR pathway and RA has not been extensively characterized.

Study limitations

Limited number of normal (healthy) cell lines were used in the study. Furthermore, different pathways should be studied to elucidate the protective mechanisms of phenolic compounds. *In vitro* and *in vivo* toxicity assays and clinical trials are required for the use of plant products.

CONCLUSION

In conclusion, this study showed that RA can protect against DOX-induced toxicity using different methods. However, when the two phenolic compounds were applied in combination, the protective effect against DOX-induced DNA damage was not as much as we expected. Dual behavior was observed for EGCG, which exhibited both pro-oxidant and antioxidative properties. Identification of plants that protect against genotoxic agents and secondary cancers caused by chemotherapy may be used in the near future to reduce the side effects of chemotherapy.

Ethics

Ethics Committee Approval: Not required.

Informed Consent: Not required.

Authorship Contributions

Concept: S.H., M.H., Design: S.H., M.H., A.Y., Data Collection or Processing: S.H., E.Y., Analysis or Interpretation: S.H., Ş.V.K., A.A., Literature Search: S.H., E.Y., S.V.K., A.Y., Writing: S.H., M.H.

Conflict of Interest: The authors have no conflicts of interest to declare.

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