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Protective Effect of Rosmarinic Acid and Epigallocatechin Gallate Against Doxorubicin-İnduced Cytotoxicity and Genotoxicity on CHO-K1 Cells

Protective Effect of Phenolic Compounds

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ABSTRACT

Objectives: The chemotherapeutic drug Doxorubicin affects not only cancer cells but also healthy cells in an undesirable manner. The purpose of this study was to investigate the protective role of Rosmarinic acid and Epigallocatechin gallate alone and in combination against Doxorubicin-induced oxidative stress, cytotoxicity and genotoxicity in healthy cells. In addition, this study evaluated the protein expression of the mammalian target of rapamycin (mTOR) protein in Chinese Hamster Ovary cell line (CHO-K1).
Materials and Methods: The cell viability was analyzed by WST-1 cytotoxicity assay. mTOR in CHO-K1 cell line was determined by western blot analysis. DNA damage was analyzed using comet assay. Reactive oxygen species levels were determined microscopically, using dihydroethidium, staining method.
Results: It was found that Rosmarinic acid showed more effective protection against Doxorubicin-induced cytotoxicity. Epigallocatechin gallate and Rosmarinic acid did not exert a genotoxic effect but Doxorubicin increased genotoxicity in CHO-K1. Rosmarinic acid and Epigallocatechin gallate significantly reduced the genotoxic effects of Doxorubicin in the comet assay. In the group treated with doxorubicin, the expression level of the mammalian target protein of rapamycin decreased from 250 nM to 2000 nM concentrations. Epigallocatechin gallate decreased mTOR protein levels when administered alone or in combination with Doxorubicin, but Rosmarinic acid did not show this effect. Rosmarinic acid decreased the intracellular reactive oxygen species generation in CHO-K1 cells. However, EGCG did not protect against oxidative stress and damaged cells due to its pro-oxidant properties at high concentrations.
Conclusion: Epigallocatechin gallate and rosmarinic acid are promising plant-derived active components. Another important point is the evaluation of the safety of herbal products. It should be taken into account 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 chemotherapeutic agent in the anthracycline class, preferred for first-line treatment in pediatric and adult patients. It is utilized across various cancer types such as breast, stomach, prostate, soft tissues, and bone sarcomas. The side effects of DOX typically stem from its cytotoxicity and genotoxicity, as it isn't solely selective against cancer cells, causing damage to healthy cells as well. Consequently, unlike primary tumors, secondary malignant tumors may develop due to treatment during or after chemotherapy.[1]

The genotoxicity of DOX in the formation of secondary tumors is the primary factor, attributed to the damage inflicted on cells by the free radicals it generates. Phytochemicals exhibit effectiveness in protecting against oxidative damage induced by free radicals. Research has shown the potential of natural substances to protect against the adverse effects of chemical drugs without compromising their therapeutic efficacy, owing to 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 encompassing antioxidative, anti-inflammatory, antimutagenic, antiangiogenic, anti-apoptotic, and anti-fibrotic 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 and black 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, showcasing robust antioxidative, anti-inflammatory, and anticarcinogenic attributes.[5] EGCG and RA are both phenolic compounds, whereas RA is a phenolic acid, EGCG is a tannin with a flavan-3-ol structure that has been esterified with gallic acid. Compared to RA, EGCG has more phenolic -OH groups (Figure 1).

mTOR participates in several signaling pathways that are 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, a study demonstrated that combining mTOR inhibitors with DOX resulted in an increased therapeutic response in leiomyosarcoma patients compared to treatment 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 on a CHO-K1 cell line. Also, the effect of these substances on cell proliferation through the mTOR expression level was evaluated.

MATERIALS AND METHODS

Sample preparation

Doxorubicin hydrochloride, Rosmarinic acid, and (−) -Epigallocatechin gallate (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 the concentrations of 1mM, 2 mM and 400 µM in the complete Ham's F12 culture medium, respectively.

Cell line and culture conditions

Chinese hamster ovary (CHO-K1) cell line was obtained from the Institute of Pharmacology and Toxicology, Würzburg, Germany. It was cultured in Ham's medium F12 supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotics (10000 U/ml penicillin and 50 mg/ml streptomycin). Cell cultures were cultivated in a humidified incubator at 37°C with 5% CO_2. Twice a week, cells were passaged using a 0.25% trypsin solution. Reagents for cell culture have been obtained from (Gibco, Carlsbad, CA).

WST-1 Cytotoxicity Assay

The viability was measured by using the WST-1 (Roche, Germany) colorimetric assay. Cells were seeded ($5x10^3$ cells in 100 µl of culture medium) into 96-well plates, and were grown for 24 h. Then, cells were exposed to 100 µl/well of newly prepared medium containing the tested substance for 24 h, 48 h and 72 h. After the end of the incubation period, the medium was withdrawn, the cells were twice washed with PBS, then 100 µl of WST-1 was added to each well. The wells were then incubated for four hours at 37 ^{o}C. After 4 h, absorbance was measured at 450 nm in a microplate reader (Thermo Multiskan Ascent, USA) after 4 hours. (a-c)/(b-c) x 100 was used for calculating 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 IC50 (half maximal inhibitory concentration) was assessed from the dose-response curves.[9]

Alkaline Comet Assay

After being seeded in a 6-well plate, the cells were treated for four hours 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 do this, 15 µl of staining solution with fluorescein diacetate (Serva Electrophoresis GmbH, Germany) and gel red (Biotium, USA) was combined with 35 µl of 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. 45 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 (DMSO) 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 minutes 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 minutes. After electrophoresis, the slides were fixed in frigid methanol for 5 minutes and neutralized with Tris buffer for 5 minutes. Following drying, 20 µl of GelRed solution per slide was used to stain the slides and they were examined using a 200-fold magnification fluorescent microscope (Labophot-2; Nikon GmbH, Germany) using Komet 6-software (Komet version 6, ANDOR^{TM} Technology). A percentage of the 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 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, coloring the nucleus 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 grey values of 200 cells in each treatment for quantification.[12]

Western blot analysis

Western blot analysis was carried out as described previously.[13] Briefly, total protein from the CHO-K1 cells were extracted using Radioimmunoprecipitation (RIPA) lysis buffer (SantaCruz, Texas, USA) added with phenylmethylsulphonylfluoride, protease inhibitor cocktail and sodium orthovanadate. Then, each lane was filled with 20 μg of the whole lysate, which was then electrophoretically separated using a NuPAGE 4-12% Bis-Tris gel (Invitrogen^{TM}, USA) and electroblotted onto nitrocellulose transfer membrane. (Advansta, San Jose, USA). The membrane was blocked for an hour 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, then it was incubated for an hour at room temperature with the anti-mouse lgG secondary antibody (1:2000 dilution, Cell Signaling), and finally rinsed with TBS-T. mTOR protein expression was detected using chemiluminescent substrate (Thermo Scientific, USA), and immunoblot images were taken and bands measured by 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 for calculating the expression levels of each protein.

Statistical Analysis

Data were expressed as the mean ± SEM and analyzed using the GraphPad Prism 9 software (GraphPad, Boston, USA). The differences among means have been analyzed by ANOVA test followed by Dunnett's analysis. The treatment group's data and the control group's data were compared. It was considered statistically significant when $p < 0.05$.

RESULTS

WST-1 Cell Proliferation Assay

In cells treated with DOX and EGCG, the relative cell proliferation consistently decreased in a dose- and time-dependent manner. Table 1 presents the IC50 values for cells treated with DOX and EGCG at 24, 48, and 72 hours. However, treatment with RA at various concentrations (ranging from 0.0625 mM to 1 mM) for 24, 48, and 72 hours did not reduce the viability of CHO-K1 cells. Consequently, the IC50 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 on CHO-K1 cells were examined using the WST-1 assay. To evaluate the protective impact, the DOX concentration of 500 nM, as determined by its IC50 value, was chosen for the investigation. CHO-K1 cells were subjected to different dosages of EGCG, RA, and their combination for 24 hours. The results exhibited a statistically significant protective effect of both EGCG and RA against the cytotoxicity induced by DOX. Notably, the concentration of RA at 1 mM demonstrated the most pronounced protective effect against the cytotoxicity caused by DOX (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- 0.5, 1, 2, and 4 hours (Figure 3B). ROS production increased notably in cells treated with 1000 nM DOX for 0.5 hours or longer. This rise was statistically significant after a 2-hour treatment when compared with the control group. Figure 4 demonstrates 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 to the DOX-treated group. In contrast, EGCG alone and in combination with RA did not demonstrate any reduction in ROS formation compared to the DOX-treated group (Figure 5). Notably, the application of 100 µM EGCG resulted in an increase in ROS levels within CHO-K1 cells.

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 when compared to the control group (data not shown). In cells treated with DOX, there was an evident dependent on dose increase in DNA damage. (Figure 6a). This effect was statistically significant at concentrations of 1000, 2000, and 4000 nM compared to the negative control group. As illustrated in Figure 6b, the administration of RA led to a notable and dose-dependent decrease in cells exhibiting DNA damage. In contrast, EGCG alone or in combination with RA did not demonstrate a protective effect against DOX-induced genotoxicity (Figure 6c and d)

mTOR protein expression in CHO-K1 cell

The protein levels of mTOR, which plays a significant role in oxidative stress, were assessed by Western blot. mTOR protein expression levels decreased in the DOX group compared to the control group (Figure 7a). mTOR protein expression level was significantly higher in the group administered RA at the concentration of 1mM with DOX compared to the group administered DOX alone. (Figure 7b). A significant decrease in mTOR expression level was observed in EGCG treated group alone or in combination with DOX when compared to the negative control group. However, these differences were not significant compared to DOX treated group (Figure 7c).

DISCUSSION

Genotoxicity is one of the most important mechanisms of adverse effects related to DOX therapy as an anticancer drug. Various pharmacologic treatments, including hematopoietic cytokines, iron-chelating agents, and antioxidants, have been under study to mitigate the adverse effects of DOX.[14,15] In light of this, our hypothesis centered 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 diminished DOX-induced ROS formation, while EGCG did not exhibit protective activity in this context. Furthermore, when RA and EGCG were co-applied, no significant reduction in ROS formation was observed. Additionally, EGCG did not demonstrate a protective effect against DOX-induced genotoxicity in the comet assay within CHO-K1 cells, whereas RA exhibited a significant protective effect. Previous studies have revealed the dual nature of EGCG, possessing both anti-oxidant and pro-oxidant properties.[16] Catechins, including EGCG, can undergo auto-oxidation 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 pro-oxidant 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 ones. 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, concentrations of 0.5 and 1 mM of RA demonstrated a protective effect against DOX-induced genotoxicity in CHO-K1 cells. These findings align with earlier reports suggesting that concentrations of 0.28, 0.56, and 1.12 mM of RA did not induce genotoxic effects and notably decreased DOX-induced DNA damage in V79 cells over a 3-hour period.[20]

This finding suggests that the protection against DNA damage induced by DOX could be associated with the 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 offer protection against ROS production and the genotoxic effects induced by DOX. For instance, in one such study, thymoquinone was observed to mitigate 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 the cytotoxicity induced by DOX in non-cancerous cell lines. 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 DOX-induced cytotoxicity.[23] Additionally, silymarin, the 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 that DOX generated in cardiomyocytes by regulating the oxidative response and apoptotic processes that were mediated by the Bcl-2/Bax ratio.[25]

In response to a wide variety of extracellular stimuli, including growth hormones, availability of nutrients, 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 in numerous cancer cell lines.[28] Studies have reported EGCG as an inhibitor of both PI3K and mTOR pathways.[29] Interestingly, mTOR expression levels decreased with escalating doses of DOX compared to the control group. This reduction in mTOR levels was believed to be induced by 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 reactive oxygen species 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, while high-intensity stress may suppress mTOR.[31] There are different reports that mTOR is inhibited or activated by oxidative stress. This difference is thought to vary depending on the cell line or the type of oxidant.[32] In this study, the mTOR level decreased in the EGCG-administered groups. This observation may be due to the pro-oxidant property of EGCG.[33] When RA was co-administered with DOX, it increased the level of mTOR when compared to the only DOX applied cells. Lou et al. (2016) showed that RA stimulates liver regeneration through the mTOR pathway. Strong and persistent mTOR activation caused by RA treatment increased RA-mediated hepatocyte proliferation.[34] However, the interaction of the mTOR pathway and RA has not been extensively characterized in the literature.

Study limitations

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

CONCLUSION

In conclusion, this study showed that RA application protect against DOX-induced toxicity by 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 with EGCG, which exhibited both pro-oxidant and antioxidative properties. Identifying plants that protect against genotoxic agents and secondary cancers caused by treatment with chemotherapy may be used in the near future to reduce the side effects of chemotherapeutic treatment.

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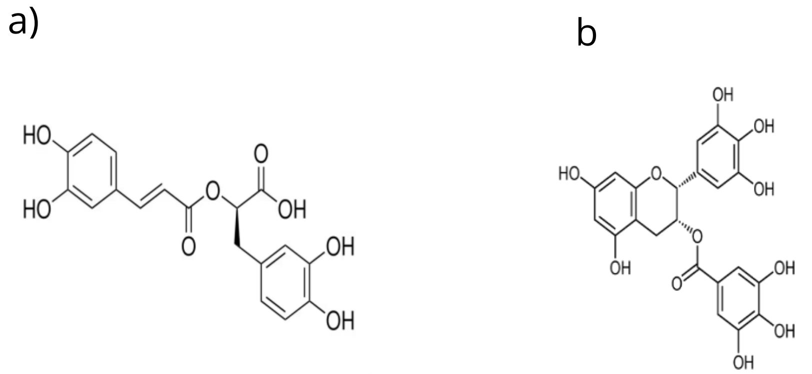

Figure 1. (a). Chemical structure of Rosmarinic acid. (b). Chemical structure of Epigallocatechin gallate

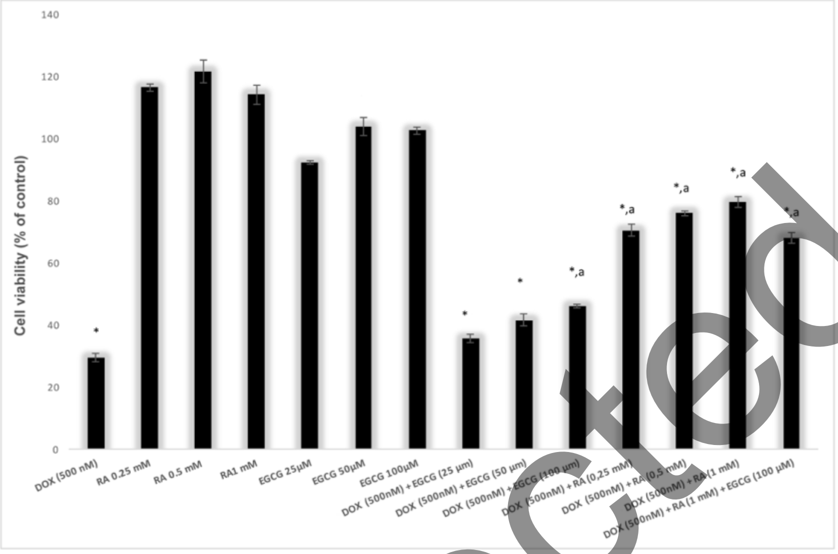

Figure 2. Cells were treated with different concentrations of DOX, RA, EGCG, and their combinations for 24h. Viability was quantitated by WST-1 assay. *p ≤ 0.05 vs. Control group and ^{a}p ≤ 0.05 vs. DOX 500 nM group

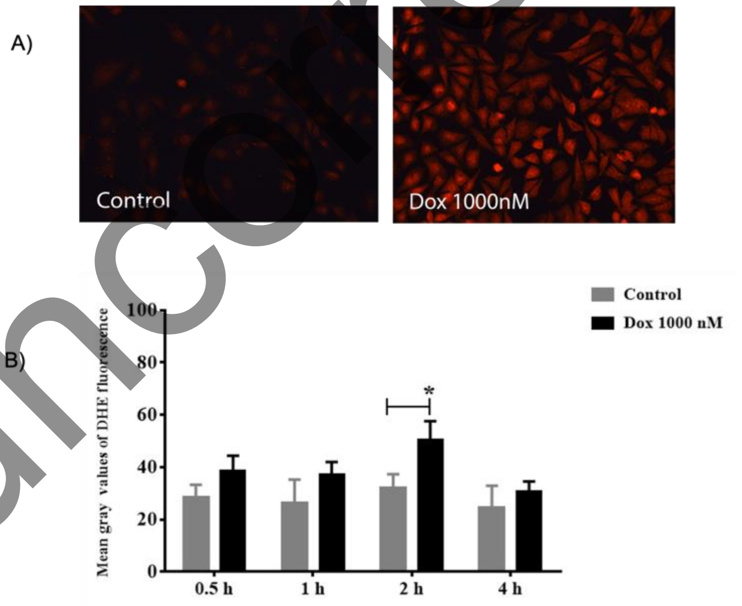

Figure 3. ROS formation in CHO-K1 cells treated with 1000 nM Dox for 0.5 to 2 hours using DHE assay. A). DHE fluorescence was quantified using image j software, which measured the mean grey value of 200 cells. B). Results are shown as mean ± SEM of three separate tests. *p ≤ 0.05 vs. Control group. Dox: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

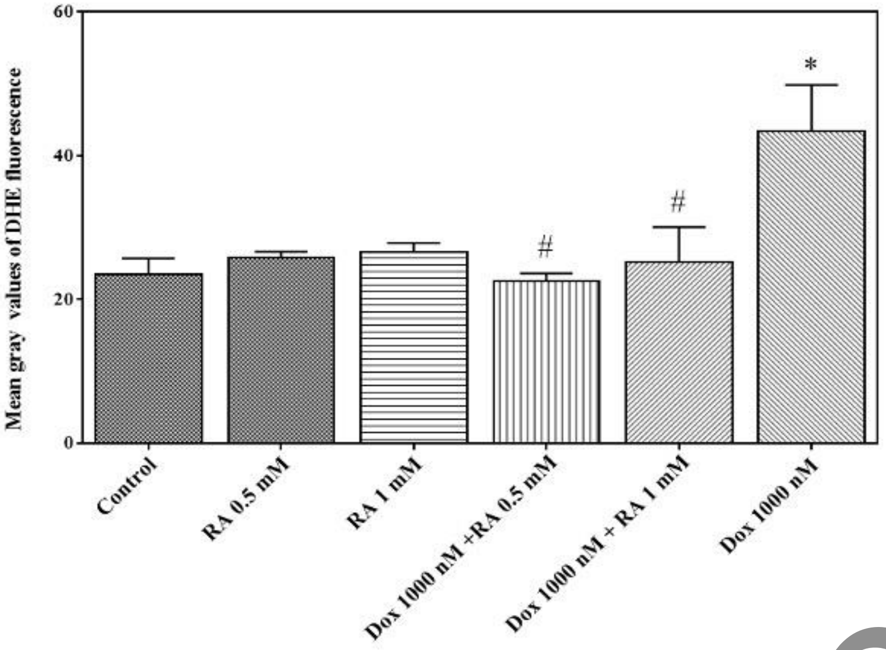

Figure 4. ROS production and its inhibition by RA in CHO-K1 cells. DHE fluorescence was quantified using image j software, which measured the mean grey value of 200 cells Results are shown as mean ± SEM of three independent tests. Kruskal Wallis test was used for analysis, *p ≤ 0.05 vs. Control and #p≤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

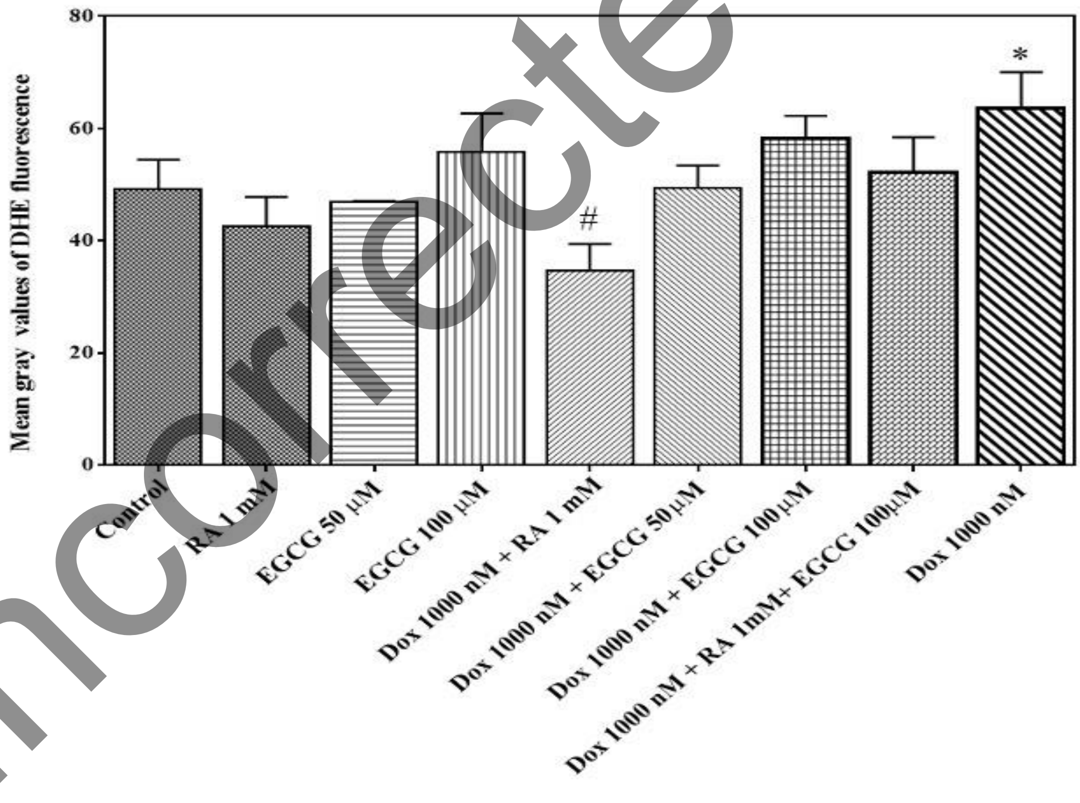

Figure 5. Intracellular ROS after treating the cells with RA 1mM and EGCG (50, 100 µM) for 2 h with and without the addition of Dox. DHE fluorescence was quantified using image j software, which measured the mean grey value of 200 cells. Results are shown as mean ± SEM of three independent tests. *p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

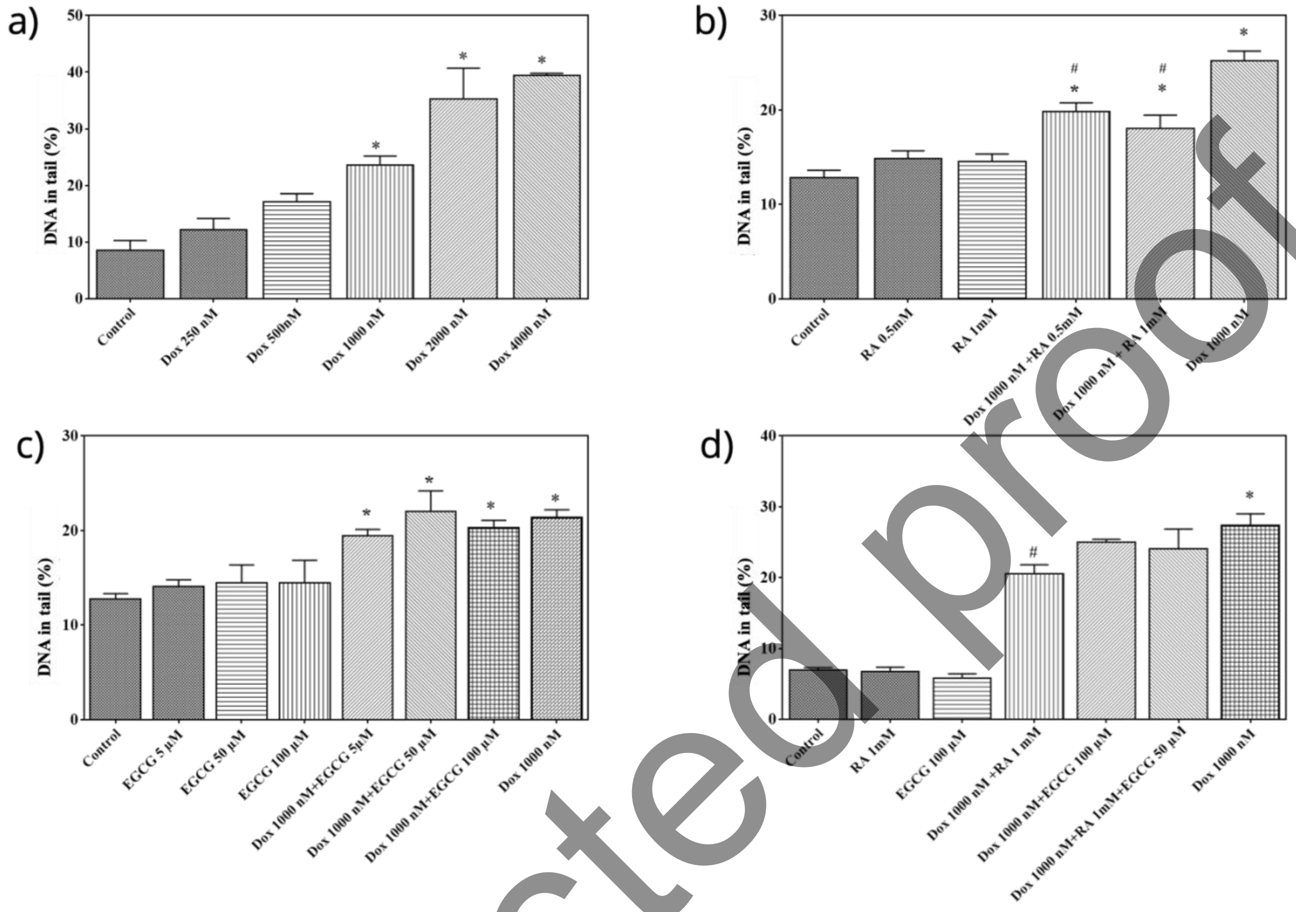

Figure 6. Alkaline comet assay results (tail intensity) obtained in CHO-K1 cells after treatment with different compounds. **a)** Concentration-dependent increase in DNA damage after 4 hours of DOX treatment in CHO-K1 cells. *$p \leq 0.05$ vs. Control group. **b)** DNA damage induction by Dox treatment 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 ± Standard Error of Mean (SEM) of 3 independent experiments. Dox: Doxorubicin, RA: Rosmarinic acid, EGCG: Epigallocatechin gallate

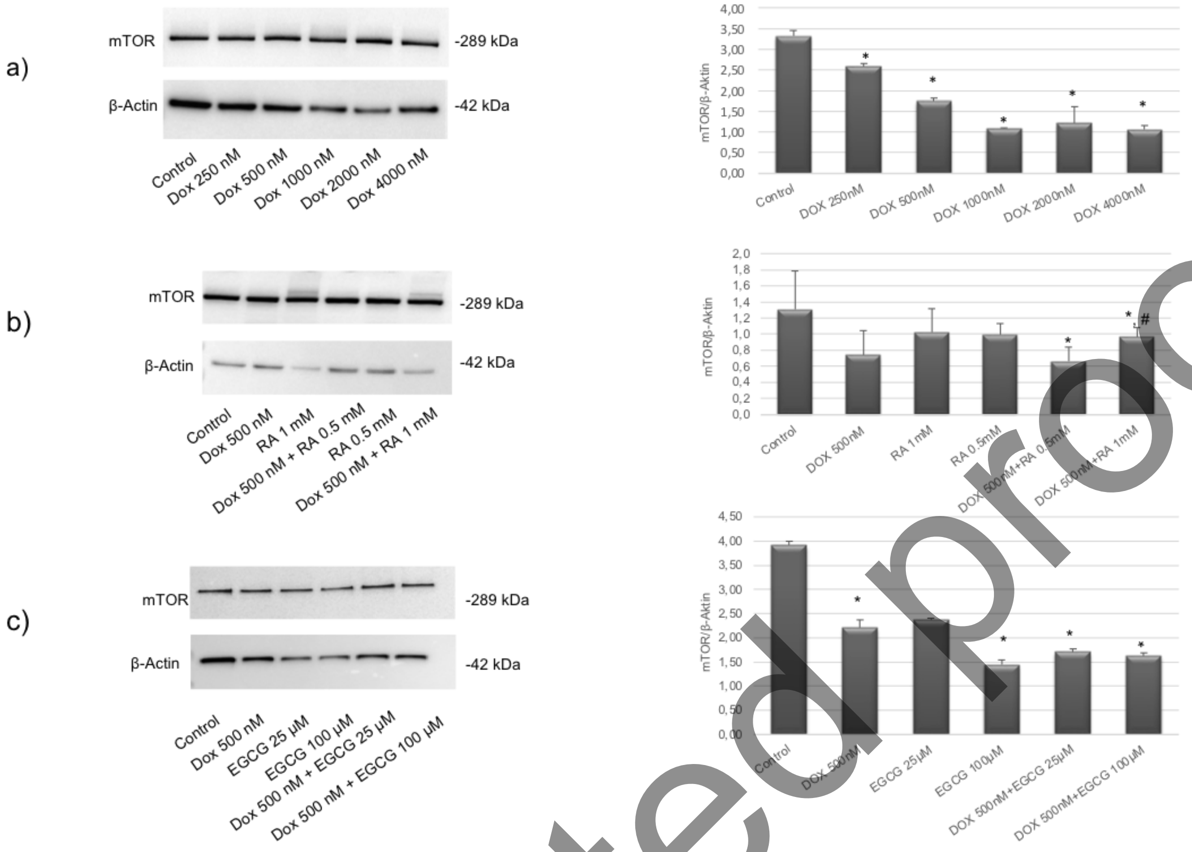

Figure 7. Western blot analysis a) mTOR protein level after 24 h of DOX treatment in CHO-K1 cells. *p ≤ 0.05 vs. Control group. b) Dox treatment with or without RA in CHO-K1 cells. *p ≤ 0.05 vs. Control and #p≤ 0.05 vs. Dox 500nM. c) Dox treatment with or without EGCG in CHO-K1 cells. *p ≤ 0.05 vs. Control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

Table 1. IC50-values of CHO-K1 cells after 24, 48 and 72 h of incubation with DOX and EGCG.

Compounds	**IC50**		
	24 h	48 h	72 h
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

Values are expressed as mean ± SD of triplicate experiments.