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Galangin Protects AML-12 Cells Against Dactinomycin Induced Hepatotoxicity

Galangin AML-12 Hücrelerini Daktinomisine Bağlı Gelişen Hepatotoksisiteye Karşı Korur

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ABSTRACT

Aim: The purpose of this study was to evaluate the effects of galangin (Gal) on dactinomycin induced hepatotoxicity *in vitro*.

Materials and Methods: AML-12 cell line was divided into 4 groups as the control, Gal, dactinomycin, and Gal+dactinomycin groups. IC50 dose was determined by the thiazolyl blue tetrazolium bromide test. Gene expressions of glutathione (GSH), superoxide dismutase (SOD), catalase, caspase 3 (Cas-3), Cas-9, apoptotic protease activating factor-1 (Apaf-1), B cell CLL/lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax), tumor protein p53 (p53), second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein (smac/DIABLO), topoisomerase (Top) I, and Top II were determined with quantitative real-time polymerase chain reaction analysis.

Results: Dactinomycin elevated the expression of SOD, catalase, and GSH in response to oxidative effects. In the Gal+dactinomycin group, Gal administration reduced Apaf-1 expression and increased Bcl-2 expression with antiapoptotic effects. In the dactinomycin group, p53 levels increased due to the defense mechanism against DNA damage. Gal increased smac/DIABLO expression to remove damaged structures. Bcl-2 and smac/DIABLO expression levels in the groups were inversely proportional. In the Gal+dactinomycin group, Top II expression level was lower than in the dactinomycin group. This result indicated that double strand of DNA damage was diminished by Gal.

Conclusion: Gal protected against the hepatotoxicity due to dactinomycin with antioxidant and antiapoptotic effects. Further experimental studies are needed to establish the use of Gal in liver damage.

Keywords: Dactinomycin, galangin, AML-12 cell line, hepatotoxicity, oxidative stress, apoptosis

ÖZ

Amaç: Bu çalışmanın amacı galangin (Gal) daktinomisin kaynaklı hepatotoksisite üzerindeki etkilerini *in vitro* olarak incelemektir.

Gereç ve Yöntem: AML-12 hücre hattı, kontrol, Gal, daktinomisin ve Gal+daktinomisin olmak üzere 4 gruba ayrıldı. IC50 dozu tiazolil mavi tetrazolyum bromid yöntemi ile belirlendi. Glutatyon (GSH), süperoksid dismutaz (SOD), katalaz, kaspaz-3 (Cas-3), Cas-9, apoptotik proteaz aktive edici faktör 1 (Apaf-1), Bcl-2, Bax, p53, apoptoz bağlayıcı protein inhibitörü (smac/DIABLO), topoizomeraz (Top) I ve Top II gen ekspresyonları Kantitatif gerçek zamanlı polimeraz zincir reaksiyonu analizi ile incelendi.

Bulgular: Daktinomisin, oksidatif etkilere yanıt olarak SOD, katalaz ve GSH ekspresyonunu artırdı. Gal+daktinomisin grubunda Gal uygulaması, antiapoptotik etkilerle Apaf-1 ekspresyonunu azaltırken, Bcl-2 ekspresyonunu artırdı. Daktinomisin grubunda DNA hasarına karşı savunma mekanizması nedeniyle p53 seviyeleri arttı. Gal, hasarlı yapıları kaldırmak amacıyla smac/DIABLO ekspresyonunu artırdı. Gruplardaki Bcl-2 ve smac/DIABLO ekspresyon seviyeleri ters orantılı idi. Gal+daktinomisin grubunda Top II ekspresyon düzeyi, daktinomisin grubuna göre daha düşük bulundu. Bu durum, çift sarmal DNA hasarının Gal tarafından azaltıldığını göstermektedir.

Anahtar Kelimeler: Daktinomisin, galangin, AML-12 hücre hattı, hepatotoksisite, oksidatif stres, apoptoz

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INTRODUCTION

Dactinomycin is a cytotoxic drug with a polypeptide structure that has anti-cancer activity derived from *Streptomyces parvulus*. Dactinomycin is used to treat Wilms tumor in children[1]. Dactinomysin's common side effects include myelosuppression, mucositis, and hepatotoxicity[1,2]. The risk of hepatotoxicity increases when dactinomycin is combined with radiation therapy[3]. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were found to be elevated in the serum of individuals receiving dactinomycin treatment. The risk of hepatotoxicity is higher with dactinomycin therapy, especially in young children[4,5]. Obliteration of hepatic veins, severe hepatic obstruction, portal hypertension, and hepatocyte destruction are among the detrimental effects of dactinomycin that lead to liver injury[6].

In order to avoid the many adverse effects of anticancer medications on the liver and to cure hepatotoxicity if it has already occurred, phytotherapy is thought to be a useful strategy. Plants ameliorate hepatotoxicity through a variety of mechanisms. Hepatoprotective effects could be found in several phytochemicals, including flavonoids, monoterpenes, and phenols. A bioactive flavonoid called galangin (Gal) is present in honey and *Alpinia officinarum*. Gal has been demonstrated to be well tolerated and safe in rodents without adverse effects[7]. Gal has been found to have antioxidant and anti-inflammatory properties in many experimental animal studies. In addition, Gal protected against carbon tetrachloride (CCl_4)-induced hepatotoxicity and fibrosis by reducing oxidative stress and inhibiting the activation and proliferation of hepatic stellate cells. Gal was also found to ameliorate apoptosis via modulating antioxidant defense mechanisms in rats with ischemia-reperfusion (I/R)-induced liver damage. Gal has been shown to reduce oxidative stress and sustain mitochondrial activity in diabetic rats, thereby alleviating liver damage[8].

Although some pharmacological effects of Gal have been investigated, there are no studies on its ability to prevent dactinomycin-induced liver injury in the AML-12 cell line. Therefore, in our study, we investigated the potential of Gal to prevent the hepatotoxicity of dactinomycin.

MATERIALS AND METHODS

Groups

Our study was planned into 4 groups: control, Gal, dactinomycin, and Gal+dactinomycin.

Chemicals

The Gal and dactinomycin solutions were prepared in an aqueous solution containing 0.01% DMSO, and the Gal+dactinomycin mixture was prepared in a 1:1 ratio.

Cell Culture

AML-12 cells (ATCC®, CRL-2254 TM) were grown in Eagle's Minimum Essential Medium, Dulbecco's Modified Eagle's Medium, Ham's F-12 growth medium supplemented with 5% fetal bovine serum, 100 IU/mL penicillin, 10 mg/mL streptomycin and 1% L-glutamine. They were placed in the incubator that contained 95% moisture and 5% CO_2 at 37 °C. Our study started in the 5th passage and ended in the 12th passage.

Determination of Substance Concentrations to Be Administered to Cell Lines by Thiazolyl Blue Tetrazolium Bromide Method

180 µL cell culture medium was inoculated in 96 well plates with $1x10^5$ cells in each well to calculate IC_{50} values for all groups to be used in the research. After the incubation of 24 hours, substances were administered to the related groups except for the control group and doses are shown at Figure 1 (in a volume of 20 µL). Then, all groups were left in the incubator (37 °C, 5% CO_2) for 24 hours. The control group was administered an aqueous solution containing 0.01% DMSO. MTT solution (20 µL, 5 mg/mL) was added to each well. After 3 hours, DMSO (200 µL) was added to dissolve formazan crystals. The absorption value was measured using a microplate scanner at 492 nm (Thermo Scientific Multiskan Go). The control group was regarded 100% alive and the IC_{50} dose was calculated by probit analysis. MTT test was run in four replicates in all groups.

RNA Isolation and cDNA Synthesis

AML-12 cells were inoculated 3 times in culture plates to have $3x10^6$ cells in each well. After 24 hours, chemicals were administered at the dose of AML-12 cell IC_{50}. RNA was isolated (PureLink RNA Mini Kit) from the obtained cells according to the manufacturer's instructions. Concentrations and purity values of the obtained RNA samples were determined with nanodrop (NaNoQ OPTIZEN). cDNA synthesis was carried out from RNA samples (high capacity cDNA reverse transcription kit) according to the manufacturer's instructions.

Quantitative Real-Time Polymerase Chain Reaction Analysis

Quant Studio 6 Flex device of SYBR Select Master Mix was used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis of gene expressions of the cells associating with SOD, CAT, GSH and gene expressions of the cells associating with caspase (Cas-3), Cas-9, Apaf-1, Bcl-2 associated X protein (Bax), B cell CLL/lymphoma-2 (Bcl-2), tumor protein p53 (p53), smac/DIABLO, Top I, Top II. PCR conditions were determined as: 1 cycle was 2 minutes at 50 °C, 10 minutes at 95 °C, afterwards 50 cycles for denaturation were 15 seconds

at 95 °C, and 1 second at 60 °C for annealing and extension. Comparative cycle threshold (2-ΔΔCt) method (User Bulletin 2, Applied Biosystems) was performed for the analysis of mRNA expression levels. To obtain a copy of the GSH gene sequences was selected "Nucleotide" from the National Center for Biotechnology Information. After that, relevant organism/gene name was entered in the search box, and FASTA was determined and the relevant genes were designed. Relative fold-changes in gene expression were calculated by comparing the experimental groups to the control group and were normalized to the expression of β-actin mRNA (Table 1).

Statistical Analysis

IC_{50} value was calculated by applying probit analysis to percent viability data obtained by MTT test. One-way ANOVA test and post hoc Tukey were administered to the relative fold-change values of gene expressions. Values at $p<0.05$ were accepted to be significant. Probit analysis and ANOVA test were done with Statistical Package for the Social Sciences 20 software (IBM).

Table 1. Primer sequences of analyzed genes for qRT-PCR analysis

Gene	Primer sequences (forward/reverse)
SOD	F: AGCTGCACCACAGCAAGCAC[8] R: TCCACCACCCTTAGGGCTCA
CAT	F: TCCGGGATCTTTTTAACGCCATTG[9] R: TCGAGCACGGTAGGGACAGTTCAC
GSH	F: ACTTGGCACTCCTCTCCTGA R: AGGCACTAGAACCTGCTGGA
Cas-3	F: GGTATTGAGACAGACAGTGG[10] R: CATGGGATCTGTTTCTTTGC
Cas-9	F: GAGTCAGGCTCTTCCTTTG[10] R: CCTCAAACTCTCAAGAGCAC
Apaf-1	F: GATATGGAATGTCTCAGATGGCC[11] R: GGTCTGTGAGGACTCCCCA
Bax	F: TTCATCCAGGATCGAGCAGA[10] R: GCAAAGTAGAAGGCAACG
Bcl-2	F: ATGTGTGTGGAGAGCGTCAA[10] R: ACAGTTCCACAAAGGCATCC
p53	F: CACGAGCGCTGCTCAGATAGC[10] R: ACAGGCACAAACACGCACAAA
Smac/DIABLO	F: CTCTGTGGCTGAGGGTTGAT[12] R: TTGTAGATGATGCCCACAGG
Top I	F: TCATACTGAACCCCAGCTCC[10] R: GTCCTGCAAGTGCTTGTTCA
Top II	F: CTTCTCTGATATGGACAAACATAAGATTCC[10] R: GGACTGTGGGACAACAGGACAATAC

SOD: Superoxide dismutase, GSH: Glutathione, Cas: Caspase, qRT-PCR: Quantitative real-time polymerase chain reaction

RESULTS

In order to investigate the effects of Gal, dactinomycin and Gal+dactinomycin on the viability of AML-12 cell lines, MTT assays were performed for 24 hours. MTT assay results detected that Gal, dactinomycin and Gal+dactinomycin reduced the cell viability on the AML-12 cell line that was dependent on the dose (Figure 1). IC_{50} doses were identified as 30.354 μM in Gal, 2.853 in dactinomycin and 3.262 μM in Gal+dactinomycin.

SOD mRNA expression levels increased in the dactinomycin (281,43-folds) and Gal+dactinomycin (15.04-folds) groups compared to the control group ($p<0.05$). SOD mRNA expression levels were elevated in the dactinomycin group (33.62-folds) compared to the Gal group ($p<0.05$) (Figure 2A).

Dactinomycin group showed an increase in CAT mRNA expression in response to the control (58.86-folds) and Gal groups (5.98-folds) ($p<0.05$) (Figure 2B).

The Gal (20,88 folds), dactinomycin (75-folds), and Gal+dactinomycin (76.92-folds) groups all showed an increase in GSH mRNA expression compared to controls ($p<0.05$). Compared to the Gal group, GSH mRNA expression levels in the dactinomycin (3.61-folds) and Gal+dactinomycin (3.7-folds) groups were increased ($p<0.05$) (Figure 2C).

When compared to the control, Cas-3 mRNA expression increased in the Gal (6.89-folds) and Gal+dactinomycin (5.68-folds) groups ($p<0.05$). Compared to the Gal group, dactinomycin group (4.82-folds) showed a decrease in Cas-3 mRNA expression ($p<0.05$) (Figure 2D).

In the Gal (12.12-folds) and Gal+dactinomycin (4.25-folds) groups, Cas-9 mRNA expression increased in comparison to control ($p<0.05$). In the dactinomycin (8.78-folds) and Gal+dactinomycin (2.85-folds) groups, Cas-9 mRNA expression decreased in comparison to the Gal group ($p<0.05$) (Figure 2E).

In comparison to the control and Gal groups, Apaf-1 mRNA expression was higher in the dactinomycin (30.34-folds and 14.81-folds) and Gal+dactinomycin (62.61-folds and 10.26-folds) groups ($p<0.05$) (Figure 2F).

When compared to the control, Bax mRNA expression increased in the Gal (11.90-folds), dactinomycin (12.19-folds), and Dactinomycin+Gal (14.04-folds) groups ($p<0.05$) (Figure 3A).

In comparison to the control group, Bcl-2 mRNA expression elevated in the Gal+dactinomycin group (2.43-folds) whereas it dropped in the Gal group (0.45-folds) ($p<0.05$). When compared to the Gal group, Bcl-2 mRNA expression was higher in the dactinomycin (2.11-folds) and Gal+dactinomycin (5.4-folds) groups (Figure 3B).

p53 mRNA expression increased in all groups when compared to the control group (54.55-folds for Gal group, 201.59-folds for dactinomycin group, 11.99-folds for Gal+dactinomycin group) ($p<0.05$). p53 mRNA expression increased in the dactinomycin group (3.64-folds), and decreased in the Gal+dactinomycin group (4.72-folds) compared to the Gal group ($p<0.05$) (Figure 3C).

In comparison to the control group, Smac/DIABLO mRNA expression increased in all groups (105.12-folds for Gal group, 59.75-folds for dactinomycin group, 56.55-folds for Gal+dactinomycin group) ($p<0.05$). In comparison to the Gal group, Smac/DIABLO mRNA expression was reduced in the dactinomycin (1.76-folds) and Gal+dactinomycin (2.26-folds) groups ($p<0.05$) (Figure 3D).

When compared to the control group and the Gal group, the expression of the top 1 mRNA was higher in the dactinomycin (3.13-folds and 3.52-folds) and Gal+dactinomycin groups (6.83-folds and 7.67-folds) ($p<0.05$) (Figure 3E).

Top II mRNA expression was higher in the dactinomycin group (1.58-folds) than in the control group ($p<0.05$). Top II mRNA expression was increased in the dactinomycin group (1.98-folds) compared to the Gal group ($p<0.05$) (Figure 3F).

DISCUSSION

Dactinomycin has serious toxic effects that could lead to hepatic injury. Depending on the dosage, combination therapy and conditions related to patients, dactinomycin elevates serum AST and ALT levels. This clinical situation could progress to liver damage[13]. One of the most significant and natural flavonoids is the polyphenolic molecule Gal[14]. In addition to Gal's beneficial effects in the treatment of cancer, it has been shown to be antigenotoxic against chemotherapy and radiotherapy[14,15]. Our study has demonstrated that Gal provides hepatoprotective effects against dactinomycin-induced injury *in vitro*.

Antioxidant enzymes present in cells include SOD and CAT. SOD converts superoxide anion to H_2O_2, which then is reduced to H_2O by CAT. Moreover, the antioxidant compound GSH

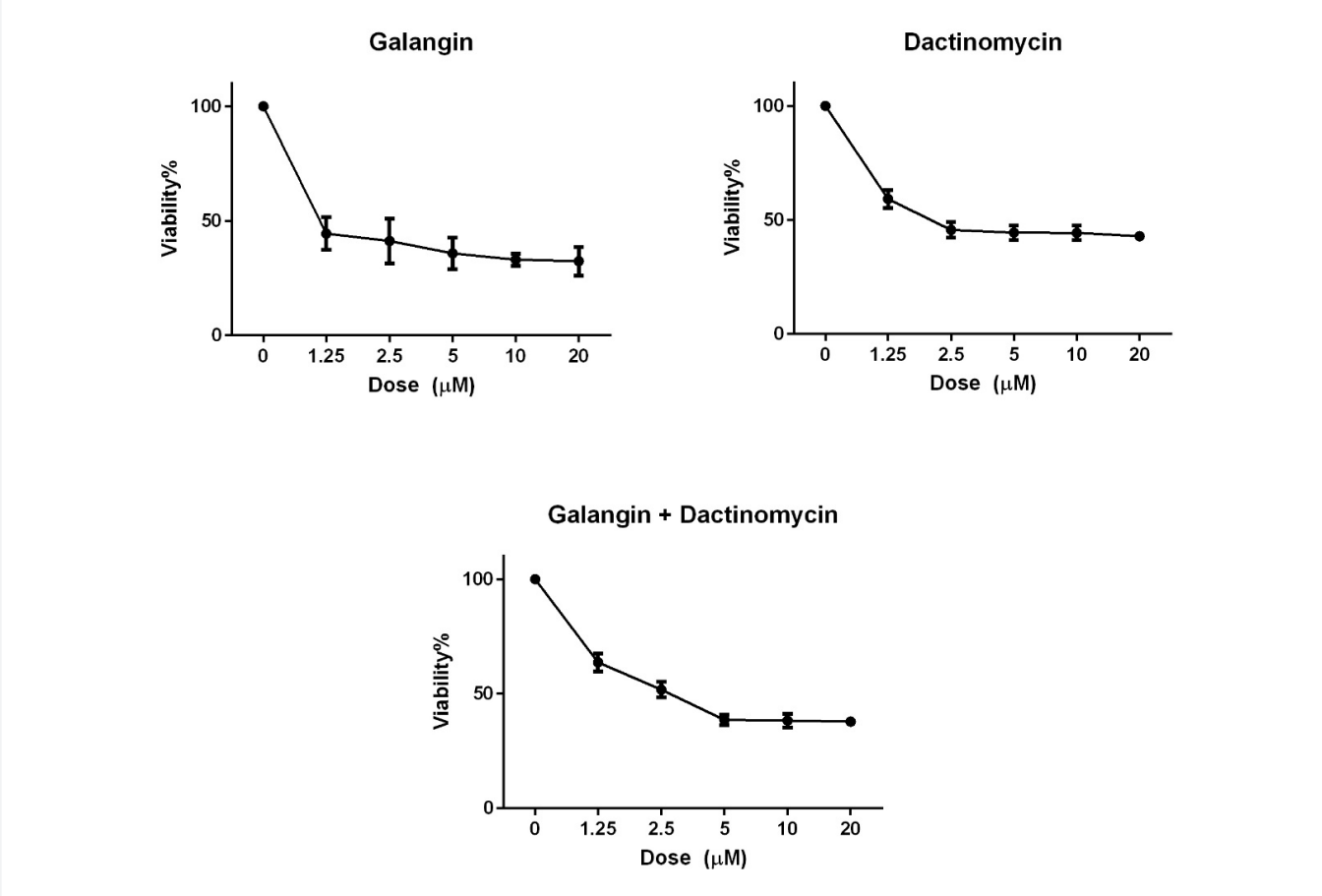

Figure 1. MTT assay results of each treatment group. Vertical bars represent standard deviation (n=4, mean±standard deviation) (viability %=Sample absorbance average / control absorbance average × 100)

reacts with free radicals, and it generates oxidized glutathione. According to the previous studies, Gal has antioxidant activity[16]. Supportively, in our study, Gal administration has elevated antioxidant enzymes SOD and CAT mRNA expression. Furthermore, it has increased GSH mRNA expression. According to our results, dactinomycin has also elevated SOD, CAT, and GSH mRNA expressions. This situation could be dependent on the response to oxidant effects of dactinomycin, since the cell

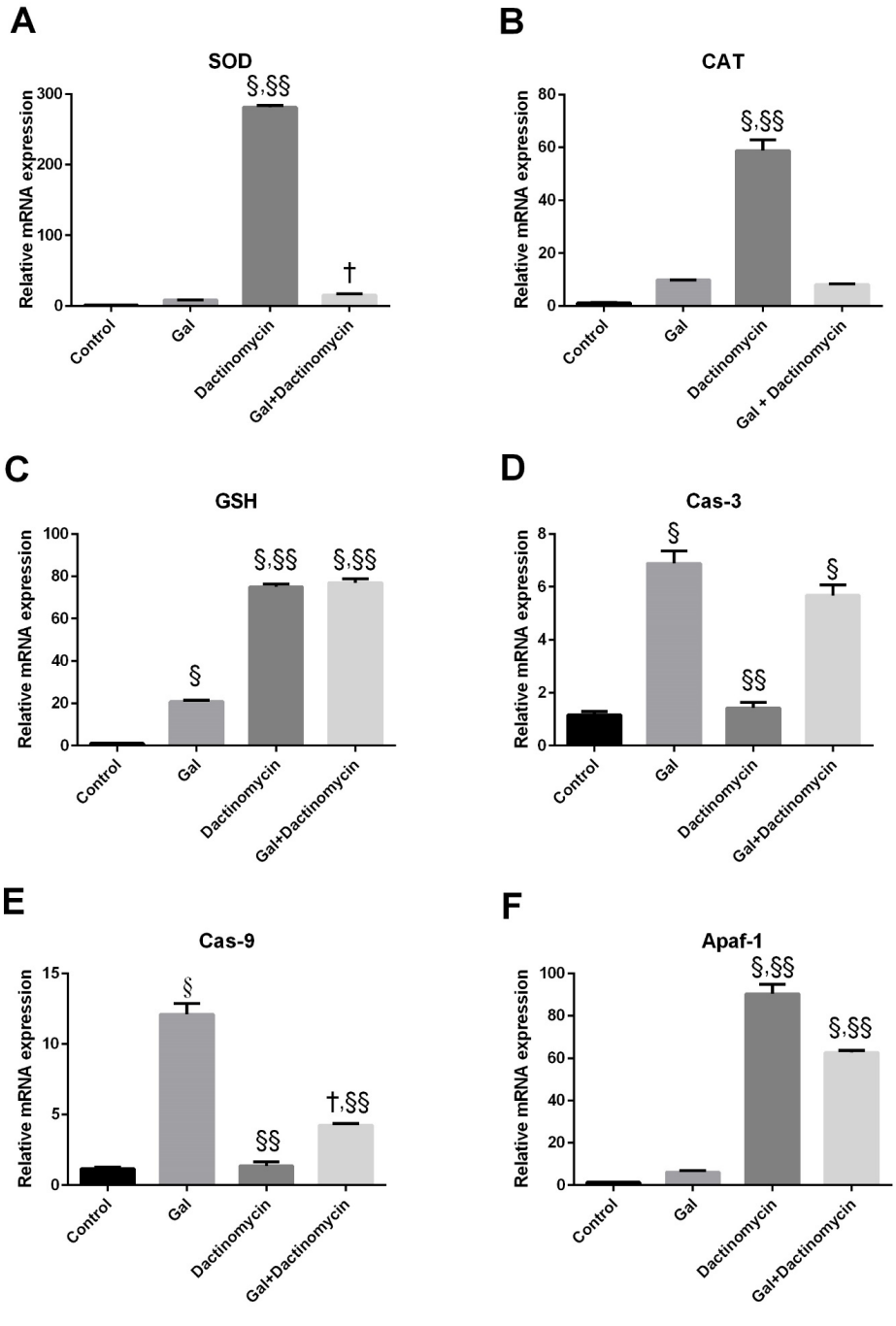

Figure 2. SOD (A), CAT (B), GSH (C), Cas-3 (D), Cas-9 (E), and Apaf-1 (F) relative mRNA expression

*p<0.05, †p<0.01, ‡p<0.001, §p<0.0001 compared to the control group; **p<0.05, ††p<0.01, ‡‡p<0.001, §§p<0.0001 compared to the Gal group

SOD: Superoxide dismutase, GSH: Glutathione, Cas: Caspase

was able to raise the antioxidant activity. Due to the protective effects of Gal, in the Gal+dactinomycin group, SOD and CAT mRNA expressions wer lower than in the dactinomycin group.

Apoptosis is the term for the controlled cell death that maintains equilibrium in living organisms. Apoptosis plays an important role in various diseases' progression. Dactinomycin triggers apoptosis in many tissues, which has positive effects in chemotherapy. However, apoptosis could also play a role in adverse effects, such as hepatic injury. During the apoptosis in cells, mitochondria releases cytochrome-c which generates a complex with Apaf-1, and ATP. Thereby in the cytosol, an

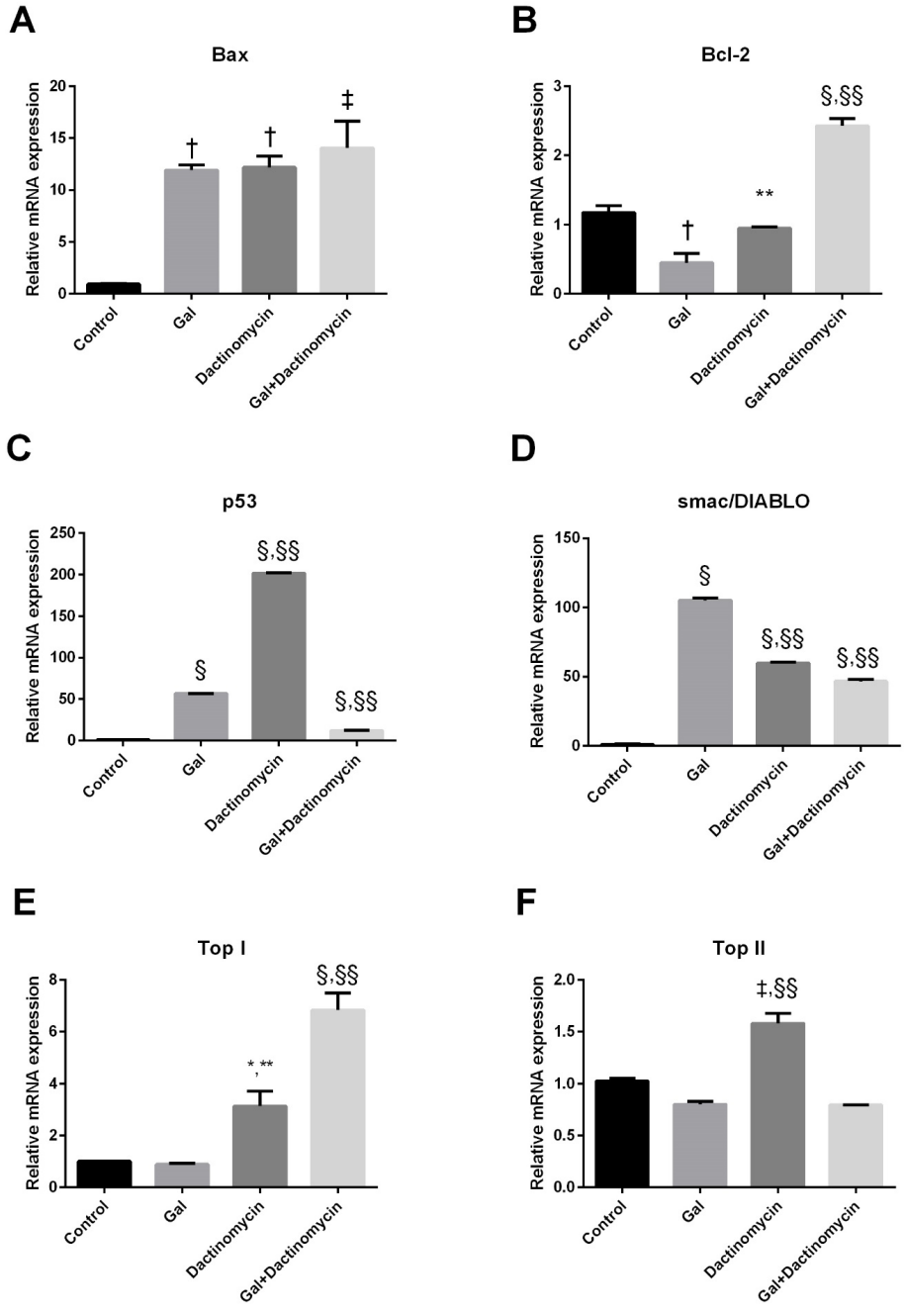

Figure 3. Bax (A), Bcl-2 (B), p53 (C), smac/DIABLO (D), Top I (E), and Top II (F) relative mRNA expression

*p<0.05, †p<0.01, ‡p<0.001, §p<0.0001 compared to the control group; **p<0.05, ††p<0.01, ‡‡p<0.001, §§p<0.0001 compared to the Gal group

Gal: Galangin

apoptosome complex is produced, which activates inhibitor Cas, and Cas-9. Moreover, Cas-9 activates Cas-3 which is an effector Cas. According to our results, in the dactinomycin group, Apaf-1 mRNA expression levels increased significantly; however, expression levels of Cas-3 and Cas-9 were not elevated. This condition indicates that liver damage-related apoptosis pathway has not progressed up to Cas-9 and Cas-3[17].

Bax is a protein that provides a proapoptotic effect. In our study, Gal and dactinomycin increased Bax gene expression with respect to the control group and induced apoptosis. Supportively, Zhang et al.[18] demonstrated that Gal triggerred apoptosis through elevation in Bax levels. Bcl-2 is one of the antiapoptotic proteins. According to our results, in the Gal+dactinomycin group, Bcl-2 expression levels were higher than in the dactinomycin group, which indicates that Gal has exerted an antiapoptotic effect. P53 is a protein that provides DNA repair. In the dactinomycin group, p53 levels increased due to the defense mechanism against DNA damage. Furthermore, it has been known that p53 could sensitize the cells to apoptosis by increasing Apaf-1 levels. Supportively, according to our results, in the dactinomycin group, both of the p53 and Apaf-1 levels were increased[19]. Moreover, Gal elevated p53 levels. According to literature, Gal increases p53 levels that prompts cell autophagy and alleviates cellular metabolic stress[20].

During the apoptosis, mitochondria releases smac/DIABLO that exacerbates apoptosis depending on decreasing IAPs inhibition on caspases[21]. Supportively, in our study, elevated smac/DIABLO expression with Gal administration provides removing damaged structures. smac/DIABLO release is attenuated by Bcl-2[22]. In this regard, results of our study have exhibited that Bcl-2 and smac/DIABLO transcript levels in the groups are inversely proportional. In a recent study, similar results have been obtained with ellagic acid, which is a phenolic compound. It has been shown that ellagic acid elevates smac/DIABLO expression in a dose-dependent manner that provides anti-cancer activity[23].

DNA-topoisomerases are essential enzymes for regulating DNA structure and metabolism, including DNA replication, transcription, and chromosomal segregation. Top I enzymes are typically monomers that break the single strand of the DNA double helix. Top II enzymes with two or more subunits are capable of breaking both strands of DNA's double helix[24]. Various situations as many diseases, oxidative stress and inflammation could evelate the topoisomerase levels[25]. According to our results, when Gal was administered with dactinomycin, Top II level reduced with respect to the dactinomycin group. This result has indicated that double strand of DNA damage is diminished by Gal. However, the amount of Gal administered along with dactinomycin was inadequate to repair the single strand of DNA damage, since Top I level in Gal+dactinomycin group was detected to be increased.

CONCLUSION

Our study has demonstrated that Gal could protect against hepatotoxicity due to dactinomycin through alleviating the oxidative stress, abnormalities in apoptotic pathways, and double strand of DNA damage in the liver tissue. Further experimental studies are needed in order to use the Gal in hepatotoxicity treatment.

Ethics

Ethics Committee Approval and Informed Consent: Since our study is a cell culture study, Ethics Committee Form and Informed Consent Form are not required.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: M.A., Ç.O., E.B., Design: M.A., Ç.O., E.B., Data Collection or Processing: M.A., Ç.O., E.B., Analysis or Interpretation: M.A., Ç.O., E.B., Z.A.Ç.Y., Literature Search: M.A., E.B., Z.A.Ç.Y., Writing: M.A., Z.A.Ç.Y.

Conflict of Interest: No conflict of interest was declared by the authors.

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