ORIGINAL ARTICLE

DOI: 10.4274/nkmj.galenos.2023.27147
Nam Kem Med J 2023;11(3):193-201

Puerarin Protects from Methotrexate Induced Hepatotoxicity in AML-12 Cells

Puerarin AML-12 Hücrelerinde Metotreksatın Neden Olduğu Hepatoksisiteden Korur

Melek AKINCI[1], Çağatay OLTULU[2], Elvan BAKAR[3], Zatiye Ayça ÇEVİKELLİ YAKUT[4]

[1]*Trakya University Faculty of Pharmacy, Department of Pharmacology, Edirne, Turkey*
[2]*Trakya University Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Edirne, Turkey*
[3]*Trakya University Faculty of Pharmacy, Department of Basic Pharmacy, Edirne, Turkey*
[4]*Trakya University Faculty of Pharmacy, Department of Pharmacognosy, Edirne; Marmara University Faculty of Pharmacy, Department of Pharmacology, İstanbul, Turkey*

ABSTRACT

Aim: The purpose of this study was to look into the effects of puerarin (PR) on methotrexate (MTX)-induced hepatotoxicity *in vitro*.

Materials and Methods: We designed our research with four groups in the AML-12 cell line: control, PR, MTX, and PR+MTX groups. Administered concentration levels to the cell lines were determined with the MTT test. To investigate oxidative stress, the expression levels of glutathione, superoxide dismutase, and catalase were determined with quantitative real-time-polymerase chain reaction (qRT-PCR) analysis. To evaluate the role of apoptosis pathways in MTX induced hepatotoxicity and the hepatoprotective effects of PR, gene expressions of caspase 3 (Cas-3), Cas-9, apoptotic protease activating factor-1, Bcl-2, Bax, p53, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein (smac/DIABLO), topoisomerase (Top) I, and Top II were investigated with qRT-PCR.

Results: MTX impaired the antioxidant defense through SOD but elevated the expression of catalase and glutathione due to an increase in free radicals. In the PR+MTX group, SOD expression increased and catalase and glutathione expression decreased compared to the MTX group. Cas-9, Apaf-1, and Top I gene expression levels were reduced in group PR. In the group of PR+MTX, PR application increased the expression of Bax, p53, and smac/DIABLO while decreasing the expression of Bcl-2, which resulted in the elimination of damaged structures by apoptosis.

Conclusion: PR alleviated the hepatotoxicity caused by MTX with its antioxidant effects and positive effects on apoptosis pathways. However, different dose studies are needed because PR could not prevent double-strand damage in DNA due to MTX and there is an increase in Top I expression in the PR group.

Keywords: Methotrexate, puerarin, hepatoprotective, oxidative stress, apoptosis

ÖZ

Amaç: Puerarinin (PR) metotreksat (MTX) nedenli hepatotoksisite üzerindeki etkilerinin incelenmesi hedeflenmiştir.

Gereç ve Yöntem: Çalışmamız AML-12 hücre hattında kontrol, PR, MTX, PR+MTX olacak şekilde dört grup olarak planlandı. Hücre hatlarına uygulanacak madde konsantrasyonları MTT yöntemi ile belirlendi. Oksidatif stresi irdelemek amacıyla süperoksid dismutaz (SOD), katalaz ve glutatyon ekspresyon düzeyleri kantitatif gerçek zamanlı-polimeraz zincir reaksiyonu (qRT-PZR) analizi ile ölçüldü. MTX'in neden olduğu hepatotoksisitede ve PR'nin hepatoprotektif etkilerinde apoptoz yolaklarının rolünü değerlendirmek amacıyla qRT-PZR analizi ile kaspaz-3 (Cas-3), Cas-9, apoptotik proteaz aktive edici faktör 1, Bax, Bcl-2, p53, ikinci mitokondri türevli kaspaz aktivatörü/doğrudan apoptoz bağlayıcı protein inhibitörü (smac/DIABLO), topoizomeraz (Top) I, Top II gen ekspresyonları incelendi.

Bulgular: MTX SOD üzerinden antioksidan savunmayı zayıflattı, fakat serbest radikal artışı nedeni ile katalaz ve glutatyon ekspresyonunu artırdı. PR+MTX grubunda, MTX grubuna göre, SOD ekspresyonu arttı, katalaz ve glutatyon ekspresyonları azaldı. PR grubunda, Cas-9, Apaf-1 ve Top I gen ekspresyon düzeyleri azaldı. PR+MTX grubunda PR uygulaması Bax, p53 ve smac/DIABLO ekspresyonunu artırarak ve Bcl2 ekspresyonunu azaltarak hasarlı yapıların apoptozla ortadan kaldırılmasını sağladı.

Address for Correspondence: Melek AKINCI MD, Trakya University Faculty of Pharmacy, Department of Pharmacology, Edirne, Turkey
Phone: +90 505 896 45 58 **E-mail:** melektamer@trakya.edu.tr **ORCID ID:** orcid.org/0000-0003-3879-4232
Received: 17.03.2023 **Accepted:** 11.05.2023

Sonuç: PR, MTX'in neden olduğu hepatotoksisiteyi antioksidan etkileri ve apoptoz yolaklarındaki olumlu etkileri ile hafifletmiştir. Fakat PR MTX'e bağlı gelişen DNA'daki çift iplik hasarını önleyemediği ve PR grubunda Top I ekspresyonunda artış geliştiği için, farklı doz çalışmalarına ihtiyaç duyulmaktadır.

Anahtar Kelimeler: Metotreksat, puerarin, hepatoprotektif, oksidatif stres, apoptoz

INTRODUCTION

Cancer, which is the second-leading cause of death in the world, is a heterogeneous disease characterized by irreversible disruption of cellular homeostasis and function. Cancer treatment choices include surgery, chemotherapy, radiation, and palliative care[1]. Chemotherapy is the use of chemotherapy medications to cure cancer. Methotrexate (MTX) is an antineoplastic and immunosuppressive agent used for the treatment of different malignancies and autoimmune disorders such as psoriatic dermatomyositis and rheumatoid arthritis[2]. MTX, a folate antagonist, affects nucleic acid synthesis by competitively inhibiting dihydrofolate reductase[3].

Chemotherapeutic medicines, which are effective and commonly used cancer treatments, lead to cytotoxic side effects. Serious adverse effects such as hepatotoxicity, nephrotoxicity, testicular dysfunction, and cardiotoxicity have been documented in individuals receiving low-dose MTX[2]. Alcohol intake, abnormal liver enzyme levels, a history of liver disease, and the presence of chronic disorders such as obesity, diabetes, and hyperlipidemia all raise the risk of MTX-induced hepatotoxicity[4]. MTX is known to raise blood aminotransferase levels, and long term MTX treatment has been related to the onset of fibrosis, fatty liver disease, and cirrhosis[5]. Although the mechanism of hepatotoxicity induced by MTX has not been fully understood, several recent studies have proposed that oxidative stress and inflammation are key factors involved. Studies have indicated that an excess of reactive oxygen species and nitric oxide, coupled with insufficient antioxidant defenses, play a crucial role in the development of MTX-induced liver damage[2]. Hence, it has been suggested that natural products possessing antioxidant and anti-inflammatory properties could potentially mitigate the hepatotoxicity caused by MTX[2]. Puerarin (PR), an isoflavone glycoside, is the primary antioxidant present in *Pueraria lobata*, which is commonly used for treating liver illnesses in traditional Chinese medicine. Positive effects of PR on the liver have been found in various investigations. For example, PR demonstrated hepatoprotective effects in an experimental liver injury model induced with carbon tetrachloride in rats[6]. In another experimental study conducted on rats by Chen et al.[7] (2013), the PR was reported to have repaired liver damage triggered with chronic alcohol consumption. Based on this knowledge, we also investigated the effects of PR on liver damage in the AML-12 cell line with MTX in our research, taking into consideration oxidative stress, apoptosis, and DNA damage.

The protective impact of PR on the hepatotoxic effects of MTX was examined in our research. Glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) enzymes, and genes encoding Caspase-3 (Cas-3), Caspase-9 (Cas-9), apoptotic protease activating factor-1 (Apaf-1), Bcl-2, Bax, p53, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (smac/DIABLO), Topoisomerase I and II (Top I, Top II) proteins, were investigated. Thus, the interactions between the pathways implicated in apoptosis were investigated, as well as their impacts on nuclear gene expression.

METARIALS AND METHODS

Groups

Our study consisted of 4 groups as the control group, PR group, MTX group, and PR+MTX group.

Chemicals

Eagle's Minimum Essential Medium (EMEM) (320-026-CL), HAMS F 12 (318-010-CL), and trypsin/EDTA (325-542-EL) were purchased from Multicell (Wisent Bioproducts, St-Bruno, QC, Canada). Dulbecco's modified Eagle's medium (DMEM) (320-026-CL), penicillin-streptomycin (Gibco 15070063), fetal bovine serum (FBS) (Gibco 26140079), and L-glutamine (Gibco 25030081) were supplied from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Thiazolyl Blue Tetrazolium Bromide (MTT) was taken from Biocompare (New York, USA). Dimethyl sulfoxide (DMSO) (Merck 67-68-5) and Phosphate buffered saline (PBS) (Merck 524650) were obtained from Merck-Millipore (Darmstadt, Germany). The PureLink RNA Mini Kit (121-830-18A) was taken from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). SYBR Select Master Mix and high capacity cDNA reverse transcription kit (8368814) were supplied from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA, USA).

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

Cell Culture

AML-12 (alpha mouse liver 12) cells are hepatocytes isolated from the normal liver of a 3-month-old mouse. AML-12 (ATCC®, CRL-2254™), 5% heat-inactivated FBS; nutrient medium contains 100 IU/mL penicillin, 10 mg/mL streptomycin and 1%

L-glutamine, 1:1 ratio of EMEM, DMEM, HAMS F12 are seeded in flasks and they are placed in the incubator that contains 95% moisture and 5% CO_2 at 37 °C. Our study started in the 5th passage and ended in the 12th passage.

Dose Determination with MTT Method

180 µL AML-12 cells were seeded in 96 well plates to have $1x10^6$ cells in each well to determine IC_{50} values of all groups to be used in the study. They were left to incubate for 24 hours to enable the cells to adhere onto the plate wells. All substances were administered to all 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. An aqueous solution containing 0.01% DMSO was applied to the control group. Aqueous solutions of PR and MTX containing 0.01% DMSO were prepared. In a 1:1 (v/v) ratio, PR and MTX were combined. Each well received 20 µL of MTT (5 mg/mL) solution. To dissolve formazan crystals 200 µL of 0.01% DMSO were added after 3 hours. The absorption value was calculated 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 seeded 3 times in culture plates to have $3x10^6$ cells in each well. After 24 hours, AML-12 cells were administered the chemical applications of the experimental groups at the dose of IC_{50} for 24 hours. RNA was isolated (PureLink RNA Mini Kit) from the obtained cells. 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).

qRT-PCR Analysis

Quant Studio 6 Flex device of SYBR Select Master Mix was used for quantitative real-time-polymerase chain reaction (qRT-PCR) analysis of enzyme expressions of the cells associating with SOD, CAT, GSH and gene expressions of the cells associating with Cas-3, Cas-9, Apaf-1, Bax, Bcl-2, p53, smac/DIABLO, Top I, Top II. PCR conditions were determined as follows: 1 cycle was 2 minutes at 50 °C and 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. mRNA expression levels were analyzed by comparative cycle threshold (2-ΔΔCt) method (User Bulletin 2, Applied Biosystems). To obtain a

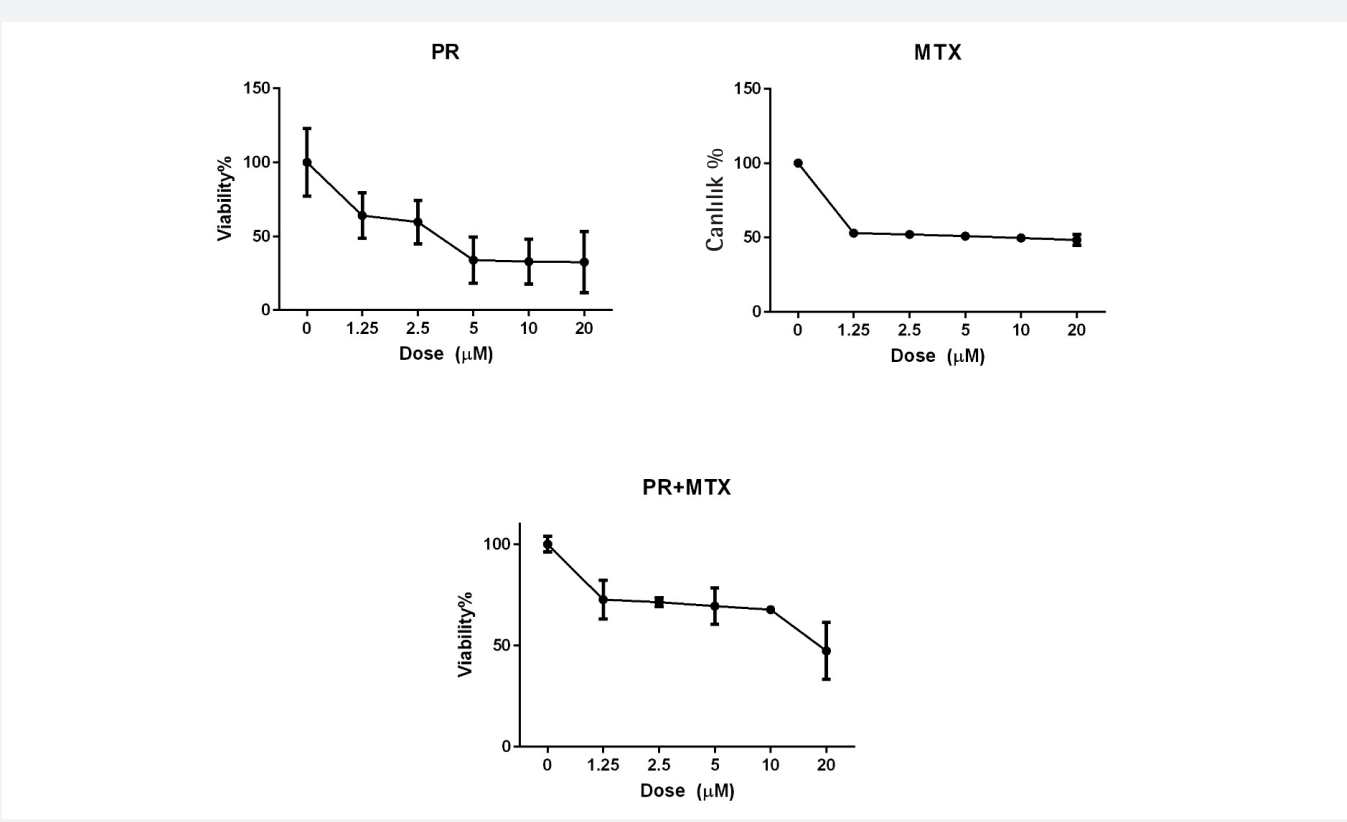

Figure 1. MTT assay results of each treatment group. Vertical bars represent standard deviation (n=4, mean±standard deviation) (viability %=Sample absorbance average/control absorbance average × 100)

PR: Puerarin group, MTX: Methotrexate group, PR+MTX: Puerarin+methotrexate combination group

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. After the application of the AML-12 cell line at IC_{50} doses for 24 hours to the AML-12 cells, 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).

RESULTS

To evaluate the effect of PR, MTX, and the PR+MTX combination on the viability of AML-12 cell lines, we conducted MTT assays for a duration of 24 hours. MTT assay results indicated that exposure of the AML-12 cell line to different concentrations of PR, MTX, and the PR+MTX combination for 24 hours caused a reduction in cell viability that depended on the dose, as shown in Figure 1. IC_{50} doses were determined as 3.28 µM in PR, 7.97 in MTX and 18.98 µM in PR+MTX.

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

qRT-PCR: Quantitative real-time-polymerase chain reaction, Cas: Caspase

PR administration significantly elevated SOD expression levels in comparison to the control group ($p<0.0001$). Although MTX administration led to a significant increase in SOD expression compared to the control group ($p<0.01$), it was significantly lower than in the PR group ($p<0.0001$). When compared to the PR group, the PR+MTX group had significantly lower SOD expression levels ($p<0.0001$). While there was no significant difference between the PR+MTX and MTX groups, SOD expression was higher in PR+MTX, as shown in Figure 2A.

Comparisons of CAT expression levels among the control, MTX, and PR groups revealed a statistically significant increase in CAT expression in the MTX group ($p<0.0001$). Compared to the control and PR groups, the PR+MTX group exhibited significantly higher levels of CAT expression ($p<0.0001$ and $p<0.001$, respectively), as shown in Figure 2B.

Statistical analysis of GSH expression levels revealed an increase in the MTX-administered groups compared to both the control and PR groups ($p<0.0001$). With respect to the control group, PR administration increased GSH expression levels ($p<0.01$), as shown in Figure 2C.

Cas-3 gene expression was higher in the PR ($p<0.0001$), MTX ($p<0.05$), and PR+MTX ($p<0.0001$) groups compared to the control group. When compared to the PR group, MTX administration significantly reduced Cas-3 gene expression ($p<0.0001$). Cas-3 gene expression was determined higher in the PR+MTX group than in the PR group ($p<0.0001$) (Figure 2D).

With MTX administration, Cas-9 gene expression increased significantly compared to the control group and PR group ($p<0.0001$). Although Cas-9 gene expression decreased in the PR+MTX group compared to the MTX group, that difference was not significant. Cas-9 gene expression was higher in the PR+MTX group than in the PR group ($p<0.0001$). When PR was administered alone, Cas-9 gene expression increased significantly compared to the control group ($p<0.001$) (Figure 2E).

In comparison to the control and PR groups, MTX administration significantly raised the Apaf-1 gene expression ($p<0.0001$). Although expression of Apaf-1 gene reduced in the PR+MTX group compared to the MTX group, that difference was not significant. Compared to the control and PR groups, Apaf-1 gene expression was higher in the PR+MTX group ($p<0.0001$) (Figure 2F).

Bax gene expression increased significantly with PR administration compared to the control group (p<0.0001). MTX administration decreased Bax gene expression compared to the PR group (p<0.0001). Bax gene expression in the PR+MTX group was higher compared to the control group (p<0.05), but significantly lower compared to the PR group (p<0.0001) (Figure 3A).

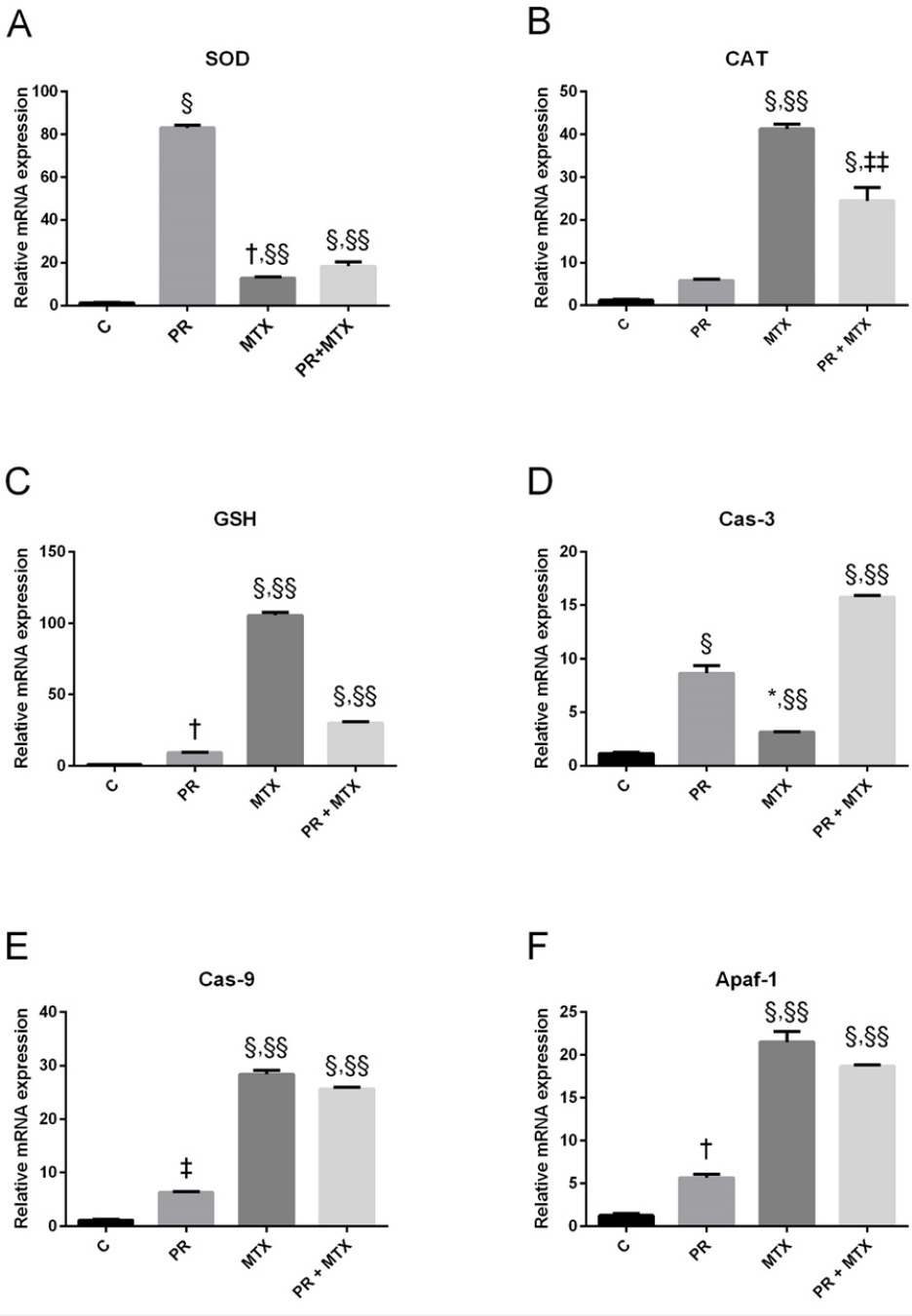

Figure 2. SOD (A), CAT (B), GSH (C), Cas-3 (D), Cas-9 (E), and Apaf-1 (F) relative mRNA expression

*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 PR group

C: Control group, PR: Puerarin group, MTX: Methotrexate group, PR+MTX: Puerarin+methotrexate combination group

MTX administration significantly increased Bcl-2 expression compared to the PR group ($p<0.001$). Bcl-2 expression was significantly lower in the PR group compared to the control group ($p<0.05$) (Figure 3B).

PR and MTX administrations elevated p53 gene expression significantly compared to the control group. Statistical significance of that increase was determined as $p<0.001$ in the PR group and MTX group, and $p<0.0001$ in the PR+MTX group. Gene expression on p53 was found to be higher in the PR+MTX group than in the PR group and control group ($p<0.0001$) (Figure 3C).

PR administration increased smac/DIABLO gene expression level compared to the control group ($p<0.0001$). Expression of smac/DIABLO gene level in the PR+MTX group was significantly higher than in the control group ($p<0.001$). The smac/DIABLO gene expression level in the PR+MTX group was lower than in the PR group ($p<0.0001$). When PR was administered with MTX, the smac/DIABLO gene expression level was found to be higher than in the MTX group, although it was not statistically significant (Figure 3D).

Top I gene expression increased significantly with MTX administration compared to the control group and PR group ($p<0.001$ and $p<0.05$). Decrease in the expression of Top I was observed in the PR+MTX group compared to the MTX group, which was not found statistically significant. In the PR group, Top I gene expression was elevated when compared to the control group ($p<0.01$) (Figure 3E).

PR and MTX administrations increased Top II gene expression compared to the control group. Statistical significance of this increase was determined as $p<0.01$ in the PR group, $p<0.001$ in the MTX group, and $p<0.0001$ in the PR+MTX group. Top II expression was higher in the PR+MTX group than in the PR group ($p<0.001$) (Figure 3F).

DISCUSSION

According to our results, the PR administration has a protective effect against MTX-induced liver damage *in vitro*. Our study is the first to investigate the effects of PR on the development of MTX-related liver damage. Furthermore, our research retains its originality by casting light on the mechanisms underpinning MTX-caused liver damage.

SOD and CAT are antioxidant enzymes found in cells. SOD removes the superoxide anion by converting it to H_2O_2, which is reduced to H_2O by CAT. Previous research has revealed that MTX causes oxidative stress by reducing SOD levels[3]. In our study, while PR administration raised SOD expression by demonstrating an antioxidant effect, MTX administration significantly reduced SOD levels compared to the PR group. Supportively, according to literature, PR provides antioxidant and antiinflammatory activities in many diseases such as nervous system diseases, respiratory diseases, osteoporosis, and liver diseases[13]. In addition, PR alleviated renal damage in MTX induced experimental study[14].

According to literature, when the amount of superoxide anion in the cell surpasses the detoxification capacity, CAT activity rises to enhance antioxidant defense and CAT contributes to GSH formation by raising GSH reductase activity[15]. It is also known that exposing cells to hydrogen peroxide enhances CAT function[16]. In our study, MTX administration increased the cell's exposure to free radicals and increased CAT expression. PR administration with MTX attenuated this increase in CAT levels.

GSH is a tripeptide with a low molecular weight that acts as an antioxidant. When GSH combines with free radicals, it produces oxidized glutathione (GSSG) and other disulfides[3]. GSH levels could rise in response to elevated amounts of free radicals in order to avoid oxidative stress from causing disease[17-19]. In the study, GSH levels were found to be significantly higher in the MTX group. This situation developed in response to the free radicals whose levels increased with MTX administration, and the cell was able to increase GSH levels, which had a protective effect against oxidative stress. High CAT expression in this group also contributed to increasing GSH levels[15]. In the PR+MTX group, depending on the protective effects of PR against oxidative stress, GSH levels were found to be lower than in the group administered MTX alone. When cells are continuously exposed to free radicals, their amounts may drop as a consequence of the conversion of reduced GSH to disulfides. In experimental studies on animals, it has been shown that MTX administration reduces the level of GSH in liver tissue. It is believed that GSH deficiency contributes to MTX-induced liver tissue injury[3].

Apoptosis refers to the programmed cell death ensuring homeostasis in living organisms. In addition to genes, RNA, protein synthesis and energy also play a key role in regulating the apoptosis. It is known that abnormalities in the apoptotic pathway occur in many diseases, such as diabetes, infection, and tumor, which are affected by oxidative stress and inflammation. Both the internal (mitochondrial) and external pathways contribute to the development of apoptosis. DNA damage and hypoxia are intracellular signals that trigger apoptosis. Chemotherapeutic drugs, external factors such as radiation, and the activation of death receptors could be shown as the examples of extracellular signals[20]. MTX induces apoptosis in various of tissues[3]. The apoptosis induced by MTX has a beneficial impact in cancer treatment by enabling cancer cells to be eliminated. However, abnormalities in apoptosis pathways could have negative impacts in a variety of organs, including the liver. For instance, the administration of MTX

to rats has resulted in liver tissue damage through apoptosis mechanisms in a number of experimental investigations[3,21].

Caspases that activate each other in a proteolytic way are known as cysteine proteases. While Cas-9 is one of the initiator caspases, Cas-3 could be given as an example of effector caspase. They inactivate enzymes involved in DNA repair and replication[20]. When a cell undergoes apoptosis, cytochrome-c release from the mitochondria rises. Cytochrome-c, Apaf-1, and ATP create a complex known as "apoptosome" in the cytosol.

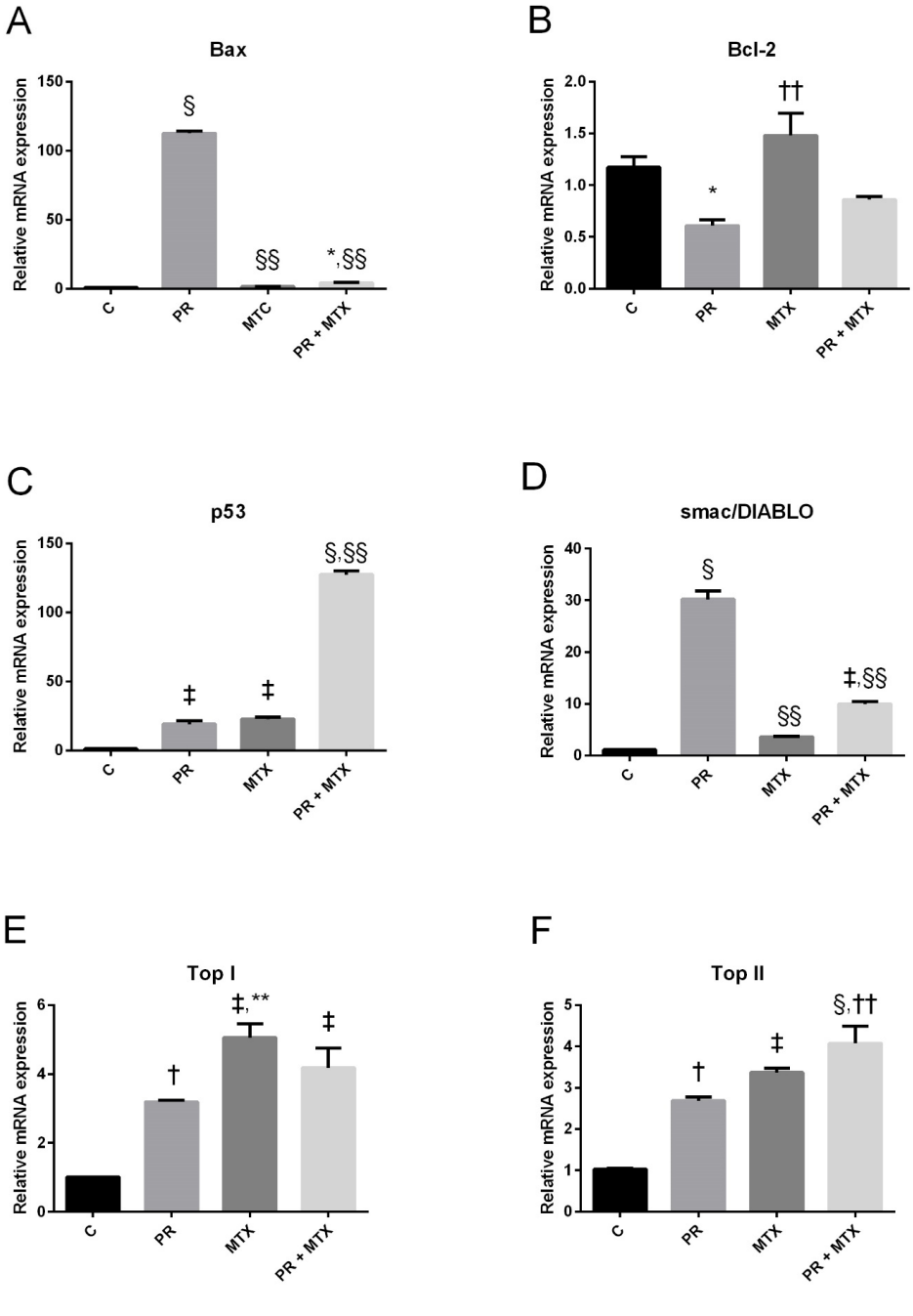

Figure 3. Bax (A), Bcl-2 (B), p53 (C), smac/DIABLO (D), Top I (E), and Top II (F) relative mRNA expression

*p<0.05, †: p<0.01, ǂ: p<0.001, §: p<0.0001 compared to the control group.

**p<0.05, ††: p<0.01, ǂǂ: p<0.001, §§: p<0.0001 compared to the PR group.

C: Control group, PR: Puerarin group, MTX: Methotrexate group, PR+MTX: Puerarin+methotrexate combination group

Cas-9 is activated by the apoptosome and Cas-3 is activated by Cas-9. Supportively, Apaf-1 and Cas-9 expression levels were detected to be elevated in the MTX group in our research. The fact that the expression level of Cas-3 was not high in this group indicates that the pathway has not progressed up to Cas-3. An experimental liver damage model generated with MTX administration in rats has demonstrated that MTX causes apoptosis in liver tissue by increasing Cas-9 and Cas-3 activity[22]. In our study, when PR was administered along with MTX, the decrease in both Apaf-1 and Cas-9 gene expression compared to the MTX group indicated the protective effect of PR on hepatotoxicity caused by MTX.

Proteins such as the Bcl-2 family, caspases, p53, and cytotome-c regulate apoptosis. The Bcl-2 family includes both proapoptotic and antiapoptotic proteins. One of the parameters we investigated in our study is that Bax has a proapoptotic effect, while Bcl-2 is one of the antiapoptotic proteins[20]. In our study, PR administration along with MTX increased Bax gene expression compared to the control group and induced apoptosis of damaged structures. According to Chen et al.[23] (2012), PR treatment in ischemic pulmonary artery smooth muscle cells raised Bax levels, which in turn increased apoptosis. Recent research has demonstrated that isoflavones and flavone glycosides promote the production of the protein Bax, which contributes to the destruction of cancer cells[24,25].

In our study, as an indicator of cell damage, Bcl-2 gene expression levels elevated in the MTX group to prevent cell death. This result is supported by the fact reported in previous studies that in individuals with chronic hepatitis, Bcl-2 expression rises in paralel with blood transaminase levels[26]. Furthermore, it has been determined that the Bcl-2 expression level is correlated with inflammation in the liver of patients with autoimmune hepatitis[27]. Additionally, the increase in the Bcl-2 levels is involved in the hepatocarcinogenesis development[28]. In a clinical research, it was found that Bcl-2 expression dropped with antiviral therapy in hepatitis patients[26]. In our study, Bcl-2 gene expression was observed to be lower in the MTX+PR group than in the MTX group. P53 is a transcription element that gives cells the opportunity to fix damaged DNA. When the harm is irreparable, it induces apoptosis by raising the production of Bax and Apaf-1 while suppressing Bcl-2[20]. In our study, p53 levels increased as a result of a defense mechanism against DNA damage in the PR+MTX group. The experimental hepatotoxicity model caused by acetaminophen in animals has shown that p53 has a protective impact against liver injury, which is consistent with our results[29,30].

Smac/DIABLO, a recently identified apoptosis protein inhibitor (IAP) binding protein, is released from mitochondria during apoptosis and potentiates apoptosis by attenuating IAPs inhibition on caspases[31]. In our research, we hypothesize that enhanced smac/DIABLO expression with PR administration has a protective impact on removing damaged structures. Bcl-2 reduces smac/DIABLO release[32]. The findings of our research show that Bcl-2 and smac/DIABLO transcript levels in the groups are inversely proportional.

DNA-topoisomerases are crucial enzymes in metabolism and regulation of DNA structure, including DNA replication, transcription, and chromosome segregation. Top I enzymes usually consist of a monomer and break the single strand of the DNA double helix. Top II enzymes with two or more subunits could break both of the double helix of DNA[33]. The level of topoisomerases rises as a cellular response to various conditions that result in cellular damage, such as infection, inflammation, or oxidative stress[34]. In our study, when PR was administered with MTX, the decrease in Top I level compared to the MTX group indicates that single strand damage in DNA is diminished. The increase in the Top I level in PR group indicates that the dose of PR applied is high for this cell. In our research, the amount of PR that was administered along with MTX was inadequate to repair the double strand of DNA damage. As a result, Top II level in the PR+MTX group was determined to be elevated.

CONCLUSION

Our research has clarified that PR could be effective in hepatotixicity of MTX, which is a widely used treatment option in various cancer diseases. PR mitigated the oxidative stress, abnormalities in apoptotic pathways, and DNA single strand damage in the liver tissue due to MTX administration. Our study would guide the experiments in animals and clinical studies. Since PR could not prevent double-strand DNA damage caused by MTX and there was an increase in Top I expression in the PR group, different dose studies are needed.

Ethics

Ethics Committee Approval and Informed Consent: Since our study is a cell culture study, ethics committee approval and informed consent are not required.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: M.A., Ç.O., E.B., 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.

Financial Disclosure: This study was funded by Trakya University Scientific Research Projects Committee with the grant number 2018/274.

REFERENCES

1. McCahill LE, Krouse RS, Chu DZ, Juarez G, Uman GC, Ferrell BR, et al. Decision making in palliative surgery. J Am Coll Surg. 2002;195:411-22.
2. Alfwuaires MA. Galangin mitigates oxidative stress, inflammation, and apoptosis in a rat model of methotrexate hepatotoxicity. Environ Sci Pollut Res Int. 2022;29:20279-88.
3. Ali N, Rashid S, Nafees S, Hasan SK, Sultana S. Beneficial effects of Chrysin against Methotrexate-induced hepatotoxicity via attenuation of oxidative stress and apoptosis. Mol Cell Biochem. 2014;385:215-23.
4. Bath RK, Brar NK, Forouhar FA, Wu GY. A review of methotrexate-associated hepatotoxicity. J Dig Dis. 2014;15:517-24.
5. Methotrexate. LiverTox: Clinical and Research Information on Drug-Induced Liver Injury [Internet]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK548219/
6. Xia DZ, Zhang PH, Fu Y, Yu WF, Ju MT. Hepatoprotective activity of puerarin against carbon tetrachloride-induced injuries in rats: a randomized controlled trial. Food Chem Toxicol. 2013;59:90-5.
7. Chen X, Li R, Liang T, Zhang K, Gao Y, Xu L. Puerarin improves metabolic function leading to hepatoprotective effects in chronic alcohol-induced liver injury in rats. Phytomedicine. 2013;20:849-52.
8. Tam NN, Gao Y, Leung YK, Ho SM. Androgenic regulation of oxidative stress in the rat prostate: involvement of NAD(P)H oxidases and antioxidant defense machinery during prostatic involution and regrowth. Am J Pathol. 2003;163:2513-22.
9. Dkhil MA, Moneim AE, Al-Quraishy S. Indigofera oblongifolia Ameliorates Lead Acetate-Induced Testicular Oxidative Damage and Apoptosis in a Rat Model. Biol Trace Elem Res. 2016;173:354-61.
10. Oltulu C, Akıncı M, Elvan B. Antitumor Activity of Etoposide, Puerarin, Galangin and Their Combinations in Neuroblastoma Cells, International Journal of Life Sciences and Biotechnology. 2022;5:407-23.
11. Yakovlev AG, Ota K, Wang G, Movsesyan V, Bao WL, Yoshihara K, et al. Differential expression of apoptotic protease-activating factor-1 and caspase-3 genes and susceptibility to apoptosis during brain development and after traumatic brain injury. J Neurosci. 2001;21:7439-46.
12. Tokatlı C, Doğanlar O, Doğalar ZB. Meriç Delta Balıklarında Çevre Kirliliğinin Genotoksik Etkileri: Antioksidan Savunma, Isı Şok Protein Sinyali ve DNA Hasar-Onarım Mekanizmaları. Journal of Limnology and Freshwater Fisheries Research. 2020;6:14-24.
13. Liu Q, Liu Z, Huo X, Wang C, Meng Q, Sun H, et al. Puerarin improves methotrexate-induced renal damage by up-regulating renal expression of Oat1 and Oat3 in vivo and in vitro. Biomed Pharmacother. 2018;103:915-22.
14. Wang D, Bu T, Li Y, He Y, Yang F, Zou L. Pharmacological Activity, Pharmacokinetics, and Clinical Research Progress of Puerarin. Antioxidants (Basel). 2022;11:2121.
15. Aguiló A, Tauler P, Fuentespina E, Tur JA, Córdova A, Pons A. Antioxidant response to oxidative stress induced by exhaustive exercise. Physiol Behav. 2005;84:1-7.
16. Kenney MC, Chwa M, Atilano SR, Tran A, Carballo M, Saghizadeh M, et al. Increased levels of catalase and cathepsin V/L2 but decreased TIMP-1 in keratoconus corneas: evidence that oxidative stress plays a role in this disorder. Invest Ophthalmol Vis Sci. 2005;46:823-32.
17. Chakravarthi S, Jessop CE, Bulleid NJ. The role of glutathione in disulphide bond formation and endoplasmic-reticulum-generated oxidative stress. EMBO Rep. 2006;7:271-5.
18. Sthijns MM, Weseler AR, Bast A, Haenen GR. Time in Redox Adaptation Processes: From Evolution to Hormesis. Int J Mol Sci. 2016;17:1649.
19. Chowdhury FA, Mahboob S, Saha A, Jahan A, Islam MN. Effect of oxidative stress on glutathione reductase activity of Escherichia coli clinical isolates from patients with urinary tract infection. J Immunol Clin Microbiol. 2017:2.
20. Coşkun G, Özgür H. Apoptoz ve Nekrozun Moleküler Mekanizması. Arşiv. 2011;20:145-58.
21. Khalaf MM, Hassanein EHM, Shalkami AS, Hemeida RAM, Mohamed WR. Diallyl Disulfide Attenuates Methotrexate-Induced Hepatic Oxidative Injury, Inflammation and Apoptosis and Enhances its Anti-Tumor Activity. Curr Mol Pharmacol. 2022;15:213-26.
22. Ali N, Rashid S, Nafees S, Hasan SK, Shahid A, Majed F, et al. Protective effect of Chlorogenic acid against methotrexate induced oxidative stress, inflammation and apoptosis in rat liver: An experimental approach. Chem Biol Interact. 2017;272:80-91.
23. Chen C, Chen C, Wang Z, Wang L, Yang L, Ding M, et al. Puerarin induces mitochondria-dependent apoptosis in hypoxic human pulmonary arterial smooth muscle cells. PLoS One. 2012;7:e34181.
24. Li Y, Kong D, Bao B, Ahmad A, Sarkar FH. Induction of cancer cell death by isoflavone: the role of multiple signaling pathways. Nutrients. 2011;3:877-96.
25. Abusaliya A, Ha SE, Bhosale PB, Kim HH, Park MY, Vetrivel P, et al. Glycosidic favonoids and their potential applications in cancer research: a review. Molecular & Cellular Toxicology. 2022;18:9–16.
26. Anatol P, Danuta P, Janusz D, Bozena P. Expression of bcl-2 protein in chronic hepatitis C: effect of interferon alpha 2b with ribavirin therapy. World J Gastroenterol. 2005;11:2949-52.
27. Yachida M, Kurokohchi K, Arima K, Nishioka M. Increased bcl-2 expression in lymphocytes and its association with hepatocellular damage in patients with autoimmune hepatitis. Clin Exp Immunol. 1999;116:140-5.
28. MaS, Chen GG: Lai PBS: Bcl-2 Family Members in Hepatocellular Carcinoma (HCC) – Mechanisms and Therapeutic Potentials. In: Chen GG, Lai PBS (eds), Apoptosis in Carcinogenesis and Chemotherapy. Springer. 2009;219-35.
29. Huo Y, Yin S, Yan M, Win S, Aung Than T, Aghajan M, et al. Protective role of p53 in acetaminophen hepatotoxicity. Free Radic Biol Med. 2017;106:111-7.
30. Sun J, Wen Y, Zhou Y, Jiang Y, Chen Y, Zhang H, et al. p53 attenuates acetaminophen-induced hepatotoxicity by regulating drug-metabolizing enzymes and transporter expression. Cell Death Dis. 2018;9:536.
31. Roberts DL, Merrison W, MacFarlane M, Cohen GM. The inhibitor of apoptosis protein-binding domain of Smac is not essential for its proapoptotic activity. J Cell Biol. 2001;153:221-8.
32. Adrain C, Creagh EM, Martin SJ. Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. EMBO J. 2001;20:6627-36.
33. Mastrangelo S, Attina G, Triarico S, Romano A, Maurizi P, Ruggiero A. The DNA-topoisomerase Inhibitors in Cancer Therapy. Biomedical and Pharmacology Journal. 2022;15:553-62.
34. Rialdi A, Campisi L, Zhao N, Lagda AC, Pietzsch C, Ho JSY, et al. Topoisomerase 1 inhibition suppresses inflammatory genes and protects from death by inflammation. Science. 2016;352:aad7993.