ORIGINAL ARTICLE

Rosmarinic acid alleviate hepatotoxicity induced by cyclophosphamide in rats

El ácido rosmarínico alivia la hepatotoxicidad inducida por ciclofosfamida en ratas

Cemre Uçar-Ekin[1], *Fırat Aşır*[2]*, *Fırat Şahin*[2], *and Şehmus Kaplan*[2]

[1]*Department of Physiology;* [2]*Department of Histology and Embryology. Faculty of Medicine, Dicle University, Diyarbakır, Turkey*

Abstract

Objective: *This study aimed to investigate the hepatoprotective effects of Rosmarinic acid (RA) against cyclophosphamide (CP)-induced liver injury in rats.* **Methods:** *Twenty-one male Wistar Albino rats were divided into three groups: Control, CP, and CP + RA. Hepatotoxicity was induced by administering CP (20 mg/kg/day) intraperitoneally for 14 days. RA (20 mg/kg/day) was administered for 14 days after CP induction. Serum biochemical parameters including malondialdehyde (MDA), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured. Liver tissues underwent histological and immunohistochemical analysis for B-cell lymphoma 2 (Bcl-2), apoptotic protease activating factor 1 (Apaf-1), nuclear factor erythroid 2-related factor 2 (Nrf-2), and tumor necrosis factor (TNF)-α. In addition, in silico analysis was performed to explore potential molecular targets of RA and their biological pathways.* **Results:** *CP significantly increased liver weight, MDA content, ALT and AST enzyme activities, indicating hepatic oxidative stress and injury. Histologically, CP caused severe hepatocellular damage characterized by hepatocyte degeneration, hemorrhage, and disrupted hepatic architecture. Immunohistochemically, CP exposure upregulated pro-apoptotic (Apaf-1), oxidative stress (Nrf-2), and inflammatory (TNF-α) markers, while downregulating anti-apoptotic (Bcl-2) proteins. RA administration significantly reversed these biochemical and histopathological changes. In silico analysis revealed RA interacts with multiple inflammatory and oxidative stress pathways, reinforcing its hepatoprotective role.* **Conclusion:** *RA demonstrates significant hepatoprotective activity against CP-induced liver toxicity by attenuating oxidative stress, inflammation, and apoptosis pathways. RA represents a promising therapeutic agent to manage drug-induced hepatotoxicity.*

Keywords: *Apoptosis. Cyclophosphamide. Hepatotoxicity. Inflammation. Oxidative stress.*

Resumen

Objetivo: *Investigar los efectos hepatoprotectores del ácido rosmarínico (AR) frente a la lesión hepática inducida por ciclofosfamida (CP) en ratas.* **Métodos:** *Se dividieron 21 ratas macho Wistar Albino en tres grupos: control, CP y CP + AR. La hepatotoxicidad fue inducida administrando CP (20 mg/kg/día) por vía intraperitoneal durante 14 días. Posteriormente se administró AR (20 mg/kg/día) durante 14 días. Se midieron parámetros bioquímicos séricos incluyendo malondialdehído (MDA), alanina aminotransferasa (ALT) y aspartato aminotransferasa (AST). Se realizaron análisis histológicos e inmunohistoquímicos en tejidos hepáticos para Bcl-2, Apaf-1, Nrf-2 y TNF-α. Además, se realizó un análisis in silico para explorar posibles dianas moleculares del AR y sus vías biológicas relacionadas.* **Resultados:** *El CP incrementó significativamente el peso hepático, el contenido de MDA y las actividades de ALT y AST, indicando estrés oxidativo y lesión hepática. Histológicamente, el CP causó daño hepatocelular grave caracterizado por degeneración hepatocitaria, hemorragia y arquitectura hepática alterada. Inmuno-*

Correspondence:
Fırat Aşır
E-mail: firatasir@gmail.com

Date of reception: 09-01-2025
Date of acceptance: 08-06-2025
DOI: 10.24875/CIRU.25000020

Cir Cir. 2025;93(5):546-555
Contents available at PubMed
www.cirugiaycirujanos.com

histoquímicamente, la exposición al CP aumentó los marcadores proapoptóticos (Apaf-1), de estrés oxidativo (Nrf-2) e inflamatorios (TNF-α), y disminuyó las proteínas antiapoptóticas (Bcl-2). La administración de AR revirtió de manera significativa estos cambios bioquímicos e histopatológicos. El análisis in silico reveló que el AR interactúa con múltiples vías inflamatorias y de estrés oxidativo, fortaleciendo su rol hepatoprotector. Conclusione: El AR mostró actividad hepatoprotectora significativa contra la toxicidad hepática inducida por CP, atenuando las vías de estrés oxidativo, inflamación y apoptosis. El AR representa un agente terapéutico prometedor para manejar la hepatotoxicidad inducida por fármacos.

Palabras clave: *Apoptosis. Ciclofosfamida. Hepatotoxicidad. Inflamación. Estrés oxidativo.*

Introduction

Cyclophosphamide (CP) is a medication and has been mainly used in the treatment of malignant and non-malignant disease for many years. CP exerts its effects by alkylating deoxyribonucleic acid (DNA), disrupting DNA replication and introducing DNA breaks[1]. Cytotoxic and mutagenic effects of CP are mostly observed in proliferating cells. Studies showed that CP is associated with significant toxicity across multiple organs such as the heart, testes, urinary bladder, gonads, and limits the use of this drug in cancer treatment[2]. Since CP is primarily metabolized by hepatic enzymes through hepatic oxidation system, hepatotoxicity is a major side effect of CP. Metabolization of CP generates active metabolites which cause immunosuppression and cytotoxicity[3]. These metabolites induce apoptosis and inflammatory response, leading to tissue damage[4,5].

Rosmarinic acid (RA) is a constituent of *Rosmarinus officinalis* and various other plants[6]. RA exhibits a wide range of beneficial properties including antioxidant[7], anti-inflammatory[8], antiapoptotic[9], antimicrobial, antimutagenic, anti-cancer, antidepressant, antiangiogenic, antiallergenic activities and additionally hepatoprotective, cardioprotective nephroprotective effects[10-12]. Many studies have investigated the role of RA in liver diseases and showed possible action of mechanisms of RA on the liver. Osakabe et al.[13] found that RA protected the liver injury against lipopolysaccharide by conferring anti-tumor necrosis factor (TNF)-α and superoxide dismutase (SOD) activity. Another study demonstrated that RA can mitigate acute oxidative liver injury by reducing serum markers of hepatotoxicity, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and lipid peroxidation, following exposure to a pro-oxidant agent[14]. Touiss et al. found that RA alleviated hepatotoxicity caused by administration of carbon tetrachloride (CCL4) by lowering serum ALT, AST, LDH levels. The authors also found that animals restored weight loss after RA treatment[15].

An experimental study showed that RA treatment significantly reduced TNF-α expression release ameliorating collagen-induced arthritis under *in vivo* conditions, suggesting a potential novel therapy for bone disorders such as rheumatoid arthritis and osteoporosis[16]. Liang et al.[17] revealed that RA treatment against asthma mouse model prevented inflammation, favored the antioxidant enzymes in lung tissues. A study investigated the *in vivo* and *in vitro* effects of RA on samples from humans and revealed that RA increased serum SOD and decreased the serum malondialdehyde (MDA)[18]. Taking all these into consideration, RA has been found to exert different biological activities in *in vitro* and *in vivo* studies.

Given that CP has cytotoxic effects on many organs including liver, we conducted biochemical, histochemical, and bioinformatic analysis to show differential roles (anti-inflammatory, anti-oxidant, anti-apoptotic) of RA on CP-induced hepatic injury.

Methods

Study design

All animal experimentations were approved by the Animal Experiments Local Ethics Committee of Dicle University (date: April 24, 2024 and approval number: 694769). This study was conducted in accordance with the ARRIVE guidelines (Animal Research: Reporting of *in vivo* Experiments) to ensure transparent and reproducible reporting of animal research[19]. Twenty-one male Wistar Albino rats (15-16 weeks old, weighing 200-250 g) were assigned to 3 groups (7 rats/group). The animals were housed in their cages at 12/12-h day/night period at 23 ± 1°C. The animals were fed with water and animal pellets *ad libitum*. CP (Endoxan®, Baxter Oncology GmbH, Halle, Germany) and RA (catalog no: sc-202796, Santa Cruz Biotechnology Inc, Heidelberg, Germany) were commercially purchased. RA was dissolved in 1% ethanol. The mixture was daily prepared and freshly injected intraperitoneally (i.p.) to rats at the

same time of day each day. The sample size per group (n = 7) was determined based on previous studies with similar designs that evaluated hepatotoxicity and protective agents using CP in rodents. The selected number ensured adequate statistical comparison while minimizing animal usage (Gpower v3.1 software, analysis, power: 80%, effect size: 0.6, α = 0.05). The dose of RA (20 mg/kg/day) was chosen in accordance with previous studies which demonstrated that this dose significantly ameliorated liver and kidney injury induced by toxic substances in animal models[20,21].

- Control group (n = 7): Animals were given 1 mL of physiological saline solution daily for 14 days. No further treatment was done.
- CP group (n = 7): Animals were administered 20 mg/kg CP daily for 14 days. No further treatment was done.
- CP + RA group (n = 7): Animals were administered 20 mg/kg CP and then 20 mg/kg RA daily for 14 days. No further treatment was done.

At the end of the 14th day, animals were euthanized with an intramuscular injection of 90 mg/kg ketamine (Ketasol; Richter Pharma AG, Feldgasse 19, Wels, Austria) and 8 mg/kg xylazine (Rompun; Bayer, Leverkusen, Germany). Blood samples of animals were collected for biochemical analysis. Liver organs were excised and weighted for each animal per group, and tissue samples were dissected for routine paraffin wax tissue embedding protocol. Final weight of liver organs was measured and an average value was calculated per group (n = 7). Average weight of livers per group was statistically compared with other groups.

Determination of MDA levels and hepatic enzyme activity

Intracardiac blood samples were collected for colorimetric analysis of blood parameters. MDA (#MAK085, Merck, Germany), alanine transaminase (ALT, #MAK052, Merck, Germany), and AST (AST, #MAK055, Merck, Germany) kits were commercially purchased. Blood samples from each rat were centrifuged at 2000 rpm for 10 min, and the supernatant was collected. Serum plasma analyzed for MDA content (oxidative stress marker), ALT (specific hepatic injury marker), and AST (non-specific hepatic injury marker) enzymatic activities according to the manufacturer's instructions. MDA content was measured in nmol/L. Enzymatic activities of ALT and AST were measured in U/L.

Immunohistochemical protocol

Hepatic tissue samples were fixed in 10% formaldehyde solution and passed through ascending ethanol series. Samples were treated with xylene solution and incubated in paraffin wax. Paraffin blocks of tissue samples were cut in 5 μm thick. Sections were deparaffinized, passed through a descending ethanol series, and treated with antigen retrieval solution. Samples were washed in phosphate-buffered saline (PBS) and incubated with 3% hydrogen peroxide for 20 min before blocking solution for 8 min. Samples were incubated with primary antibody B-cell lymphoma-2 (Bcl-2), apoptotic protease activating factor 1 (Apaf-1) and TNF-α, (catalog no: sc-65891, sc-7382, sc-52746, Santa Cruz Biotechnology Inc, Heidelberg, Germany, dilution ratio:1/100) and Nuclear factor erythroid 2-related factor 2 (Nrf-2) (catalog no: AF0639, Affinity Biosciences, US, dilution ratio:1/100). Samples were washed in PBS, biotinylated with a secondary antibody and treated with streptavidin peroxidase for a complex of avidin-biotin peroxidase for 15 min. Diaminobenzidine chromogen was used to visualize expression. Counterstaining was performed with Harris hematoxylin, and the preparations were mounted. Sections were imaged with Zeiss light microscope[22].

Semi-quantitative histological score

The staining intensity (expression) of primary antibodies was measured by Image J software (version 1.53, http://imagej. nih.gov/ij). Signal intensity was measured by the method of Crowe et al.[23]. Quantification was recorded by analyzing 5 fields from each specimen per group according to the method described by Aşır et al.[24]. In specimens, the brown color represented a positive expression of the antibody of interest, while the blue color represented a negative expression of the antibody of interest. Signal intensity from the analyzed field was calculated by dividing the intensity of the antibody of interest by the whole area of the specimen. A value for staining area/whole area was calculated for each specimen from five fields. An average value was measured for each group and recorded as semi-quantitative immunohistochemistry scoring. All images were processed and quantified using ImageJ software.

In silico analysis

To elucidate the pathways potentially implicated in regulating the mitigating effects of RA on CP toxicity,

Table 1. Hepatic weight, serum MDA, ALT, and AST levels in experimental groups after 14 days of treatment

Parameters	Control (n = 7)	CP (n = 7)	CP + RA (n = 7)	Group comparisons
Hepatic weight, g	2.4 (1.7-2.6)	3.8 (3.3-4.2)	2.5 (1.8-2.7)	Control versus CP < 0.01 CP versus CP + RA < 0.01
MDA (nmol/L)	9.2 (7.9-9.7)	32 (25-39)	13 (12-17)	Control versus CP < 0.0001 CP versus CP + RA < 0.05
ALT (U/L)	50 (46-53)	149 (143-153)	118 (110-123)	Control versus CP < 0.0001 CP versus CP + RA < 0.05
AST (U/L)	54 (52-57)	179 (168-185)	203 (98-106)	Control versus CP < 0.0001 CP versus CP + RA < 0.05

*Data was presented as median (Q1-Q3). Test: Kruskal Wallis (*post hoc* Dunn's test); CP: cyclophosphamide; RA: Rosmarinic acid; AST: aspartate; MDA: malondialdehyde; ALT: alanine transaminase; AST: aspartate aminotransferase.

an analysis was conducted on the RA targets associated with Apaf-1, Bcl-2, Nrf-2, and TNF-α, along with their functional annotations. Potential protein targets of RA were identified by exploring various databases, including SwissTargetPrediction (https://www.swisstargetprediction.ch, accessed on: 20 February 2024), STITCH (http://stitch.embl.de/accessed on: February 20, 2024) and the ChEMBL database (https://www.ebi.ac.uk/chembl/accessed on: February 20, 2024). For the STITCH database and interactors of the 4 genes, STITCH: protein (maximum interactors: 50) and Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, https://string-db.org): protein modules (maximum interactors: 100) of Cytoscape v3.10.1 (San Diego, CA, USA) were utilized, respectively. The confidence cut-off values were maintained at the default level of 0.40, representing a moderate level of confidence[25]. Parameters for SwissTargetPrediction and ChEMBL were also set to default, except for specifying the species as Homo sapiens. To detect the shared targets of RA and 4 proteins of interest, Venn diagrams were constructed using the jvenn tool[26]. Afterward, kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis, which refers to a computational approach that identifies biological pathways significantly associated with a list of genes or proteins of interest, was conducted using these shared proteins through ShinyGO 0.80 (http://bioinformatics.sdstate.edu/go/, accessed on: February 25, 2024)[27]. Pathway enrichment analysis refers to a computational approach that identifies biological pathways significantly associated with a list of genes or proteins of interest. Bar plots were generated for the top 10 ranked pathways with a false discovery rate below 0.05, encompassing KEGG and gene ontology biological process.

Statistical analysis

All statistical analyses were performed by GraphPad Prism Software (Version 9.2.0; Graph Pad Software, Inc., San Diego, CA). Data distribution was analyzed by the Shapiro-Wilk test. The non-normal distributed data was recorded as median (interquartile range, Q1-Q3). Multiple comparisons were performed by Kruskal-Wallis and followed by *post hoc* Dunn's test. Significance level was shown as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

Results

RA reduced hepatic weight, MDA content, and ALT-AST activities after CP induction

Table 1 demonstrates the protective effects of RA against CP-induced hepatic toxicity in rats. CP administration significantly increased hepatic weight, serum MDA levels, and liver enzyme activities (ALT and AST) compared to the control group, indicating oxidative stress, liver inflammation, and hepatocellular damage. Specifically, hepatic weight rose from a median of 2.4 g in the control group to 3.8 g in the CP group ($p < 0.01$), while MDA levels markedly increased from 9.2 nmol/L to 32 nmol/L ($p < 0.0001$), reflecting heightened lipid peroxidation. Serum ALT and AST levels were also significantly elevated in the CP group ($p < 0.0001$), confirming hepatocellular injury. Treatment with RA significantly reduced all these parameters, including hepatic weight and MDA levels ($p < 0.01$, $p < 0.05$, respectively), as well as ALT and AST activities ($p < 0.05$), suggesting that RA alleviates CP-induced

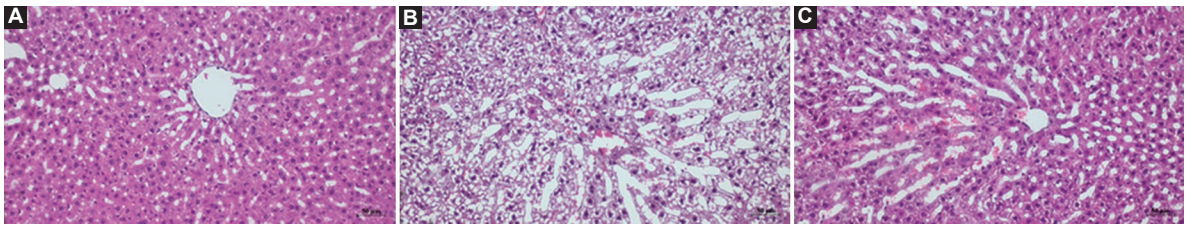

Figure 1. *Microscopic sections of hepatic tissue from control* **(A)**, *cyclophosphamide (CP)* **(B)** *and CP + rosmarinic acid* **(C)** *groups. Hematoxylin eosin staining, scale bar: 50 μm, magnification: ×20.*

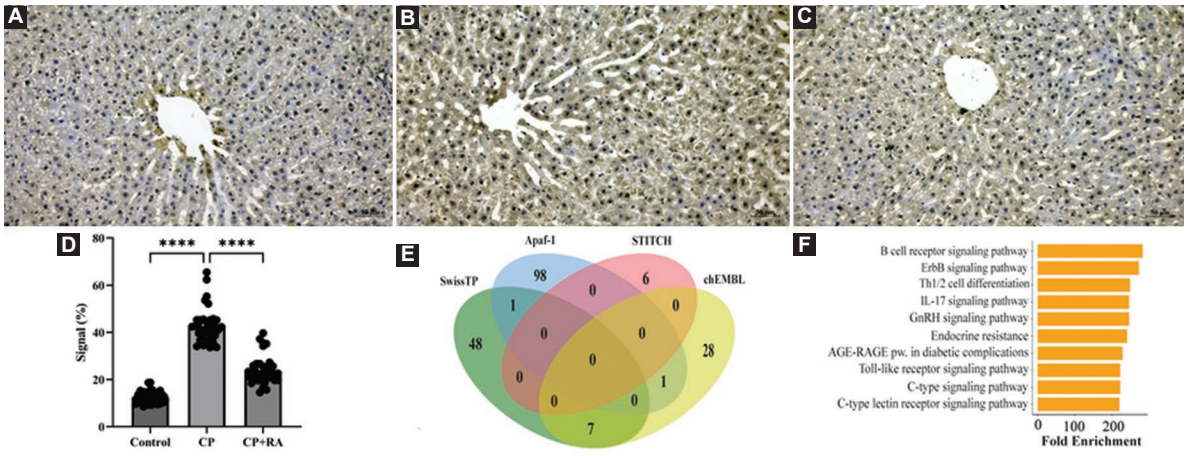

Figure 2. *Immunoexpression of pro-apoptotic marker of apoptotic protease activating factor 1 (Apaf-1) in hepatic tissues of control* **(A)**, *cyclophosphamide (CP)* **(B)** *and CP + rosmarinic acid (RA)* **(C)** *groups, scale bar: 50 μm, magnification: ×20;* **(D)** *Immunostaining scores in groups;* **(E)** *Intersection of RA protein targets with Apaf-1.* **(F)** *Kyoto encyclopedia of genes and genomes analysis of Apaf-1-associated RA targets. Significance level was shown as *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.*

hepatotoxicity through antioxidant and hepatoprotective mechanisms.

RA alleviated the histopathological alterations in hepatic tissue

Normal histology was observed in hepatic tissue with radial organization of hepatocytes, regular central vein, and hepatic sinusoids (Fig. 1A). CP toxicity caused pathology in hepatic tissue (Fig. 1B). Hepatocyte degeneration with pyknotic nuclei, dilated sinusoids with hemorrhage, glycogen depletion and disruption in radial organizations were observed in CP group. RA treatment improved the histology of liver tissue in CP + RA group, suggesting hepatoprotective effects of RA on liver tissue (Fig. 1C). Hepatic cords with hepatocytes, radial organization, and sinusoids were restored to normal histology, however, hemorrhage was continued. These findings suggest that RA has hepatoprotective effects against adverse histological changes of CP in hepatic tissue.

Impacts of RA on apoptotic pathways were analyzed by Apaf-1 expression (Fig. 2). Apaf1 expression in hepatocytes was mainly negative in control group (Fig. 2A). In CP group, overexpression of Apaf1 was observed in hepatocytes due to toxic effects of CP in liver cells (Fig. 2B). RA treatment downregulated Apaf-1 expression in CP + RA group, indicating anti-apoptotic properties of RA in hepatic tissue (Fig. 2C). Semi-quantitative analysis also showed that Apaf-1 expression was significantly reduced in CP + RA group, compared to CP group (Fig. 2D). The intersection between the targets of RA and the Apaf-1 protein network revealed 2 common target proteins (Fig. 2E). The KEGG analysis of these shared targets highlighted pathways such as the B cell receptor signaling pathway, Erythroblastic oncogene B signaling pathway, Th1 and Th2 cell differentiation (Fig. 2F). In the enrichment bar graphs, the term “fold enrichment” indicates the ratio between the observed and expected number of genes involved in a specific pathway, highlighting how prominently a particular pathway is

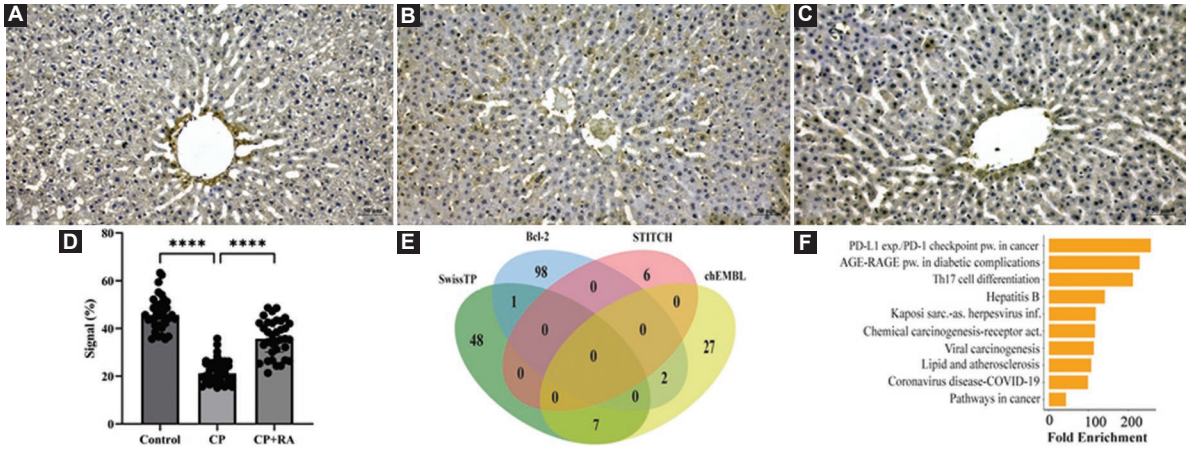

Figure 3. *Immunoexpression of anti-apoptotic marker of B-cell lymphoma 2 (Bcl-2) in hepatic tissues of control* **(A)**, *cyclophosphamide (CP)* **(B)** *and CP + rosmarinic acid (RA)* **(C)** *groups, Scale bar: 50 μm, magnification: x20;* **(D)** *Immunostaining scores in groups.* **(E)** *Intersection of RA protein targets with Bcl-2.* **(F)** *Kyoto encyclopedia of genes and genomes analysis of Bcl-2-associated RA targets. Significance level was shown as *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.*

represented among the shared targets. Here, "targets" refer to the individual proteins identified as common interactors between RA and the CP-related key molecules (Apaf-1, Bcl-2, Nrf-2, and TNF-α), whereas "pathways" represent the broader biological processes in which these targets are significantly involved. These findings showed that RA exerts its anti-apoptotic effect on Apaf-1 associated pathways to influence immune response and cellular signaling cascades.

Anti-apoptotic effect of RA was shown by evaluating immune stained sections of hepatic tissue with Bcl-2 (Fig. 3). Bcl-2 expression was moderate in hepatocytes in control group (Fig. 3A). CP toxicity induced apoptotic pathway, leading to downregulation of Bcl-2 expression in hepatic sections of CP group compared to control group (Fig. 3B). Upregulation of Bcl-2 was observed in hepatocytes after RA treatment in CP+RA group (Fig. 3C). Immune score for Bcl-2 was also increased after RA treatment in CP + RA group compared to CP group (Fig. 3D). The intersection of the RA targets with the Bcl-2 protein network identified 3 common target proteins (Fig. 3E). The enriched pathways of these shared targets encompassed the programmed death-ligand 1 (PD-L1)/programmed death-1 (PD-1) pathways in cancer, advanced glycation end products (AGE)- receptors of advanced glycation end products (RAGE) signaling pathway in diabetic complications, Th17 cell differentiation (Fig. 3F). Our results indicated that RA interacts with Bcl-2 with many pathways such as immune modulation and inflammation, in addition to apoptotic pathways.

RA showed antioxidant activity in CP induced hepatotoxicity

To show antioxidant effects of RA, hepatic sections were immunostained with Nrf-2 (Fig. 4). Nrf-2 expression was moderate in control sections (Fig. 4A). Compared to control group, CP toxicity increased reactive oxygen species in hepatocytes, causing oxidative stress and elevation of Nrf-2 expression in CP group (Fig. 4B). With antioxidant effects of RA on hepatocytes, Nrf-2 expression was downregulated in hepatocytes after RA treatment in CP+RA group (Fig. 4C). Semi-quantitative analysis showed Nrf-2 expression was significantly decreased after RA treatment in CP + RA group compared to CP group (Fig. 4D). The intersection of RA targets with the Nrf-2 protein network identified 5 common target proteins ((Fig. 4E). The enriched pathways for the shared targets comprised leishmaniasis, PD-L1/PD-1 pathways in cancer, and interleukin (IL)-17 signaling pathway (Fig. 4F). Our results highlighted that RA interacted with oxidative stress and additionally with immune responses and inflammatory processes through Nrf-2 mediated mechanisms.

RA showed anti-inflammatory activity in CP-induced hepatotoxicity

Role of RA in the inflammatory pathway was shown though expression of TNF-α (Fig. 5). TNF-α expression was mainly negative in control group (Fig. 5A). CP

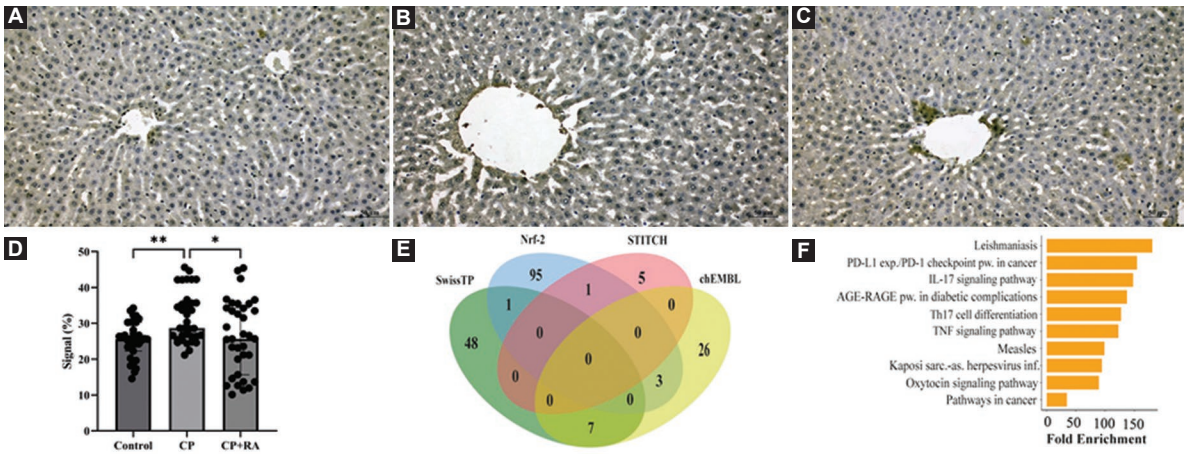

Figure 4. *Immunoexpression of oxidative stress marker of Nuclear factor erythroid 2-related factor 2 (Nrf-2) in hepatic tissues of control* **(A)**, *cyclophosphamide (CP)* **(B)** *and CP + rosmarinic acid (RA)* **(C)** *groups, scale bar: 50 μm, magnification: x20;* **(D)** *Immunostaining scores in groups;* **(E)** *Intersection of RA protein targets with Nrf-2.* **(F)** *kyoto encyclopedia of genes and genomes analysis of Nrf-2-associated RA targets. Significance level was shown as *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.*

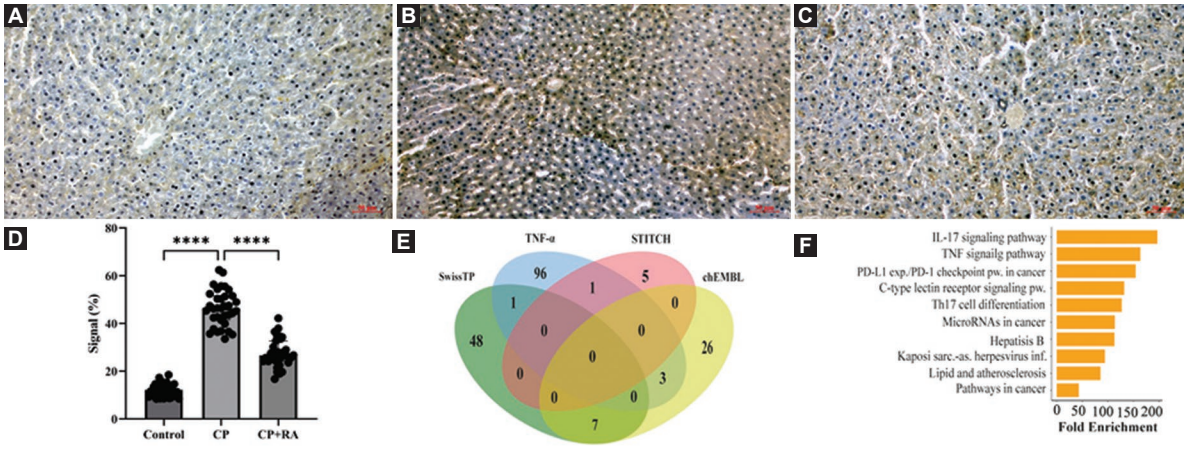

Figure 5. *Immunoexpression of inflammation marker of tumor necrosis factor (TNF)-α in hepatic tissues of control* **(A)**, *cyclophosphamide (CP)* **(B)** *and CP + rosmarinic acid (RA)* **(C)** *Groups, scale bar: 50 μm, magnification: x20;* **(D)** *Immunostaining scores in groups;* **(E)** *Intersection of RA protein targets with TNF-α.* **(F)** *Kyoto encyclopedia of genes and genomes analysis of TNF-α-associated RA targets. Significance level was shown as *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.*

injection caused hepatotoxicity and promoted inflammatory response in hepatic tissue, leading to upregulation of TNF-α expression in hepatocytes compared to control group (Fig. 5B). RA administration showed anti-inflammatory effects in hepatocytes, downregulating the TNF-α expression in hepatic tissues in CP + RA group (Fig. 5C). Immune scores also reflected similar results of histological staining, indicating TNF-α expression was significantly decreased after RA treatment in CP + RA group compared to CP group (Fig. 5D). 5 common target proteins were identified in intersection of the RA targets with TNF-α interactors (Fig. 5E). The enriched pathways of these common targets included the IL-17 signaling pathway, TNF signaling pathway, PD-L1 expression and PD-1 checkpoint pathway in cancer (Fig. 5F). These data signify that RA's role in inflammatory process and cellular differentiation through TNF-α mediated mechanisms.

Summary of our study is shown in table 2. The table presented the key protein targets affected by CP toxicity, the reversing effects of RA treatment, and their associated cellular signaling pathways identified through *in silico* analysis.

Discussion

The present study suggests pharmacological perspective targeting an oxidative stress, apoptosis, and inflammation disease module as a potential target of CP induced hepatotoxicity. *In silico* analysis can also

Table 2. Summary of immunohistological and *in silico* findings

Target protein	Role in CP toxicity	Effect of RA treatment	Pathways involved
Apaf-1	Apoptosis ↑	Suppresses apoptosis	BCR signaling, ErbB signaling
Bcl-2	Anti-apoptotic defense ↓	Enhances anti-apoptosis	PD-L1 pathway, AGE-RAGE pathway
Nrf-2	Oxidative stress response ↑	Reduces oxidative damage	Nrf2 pathway, immune regulation
TNF-α	Inflammation ↑	Inhibits inflammatory response	IL-17 signaling, TNF signaling

↑: upregulation of molecular expression or activity.

↓: downregulation of molecular expression or activity.

CP: cyclophosphamide; RA: rosmarinic acid; Bcl-2: B-cell lymphoma 2; Apaf-1: apoptotic protease activating factor 1; Nrf-2: nuclear factor erythroid 2-related factor 2; TNF-α: tumor necrosis factor; PD-L1: programmed death-ligand 1; AGE-RAGE: advanced glycation end products-receptors of advanced glycation end products; IL: interleukin; TNF: tumor necrosis factor.

reveal the association of these mechanisms and pathways related to RA. Administration of RA after CP injection ameliorated MDA content, ALT-AST activities and toxicity in hepatic tissue of rats.

In certain instances, the administration of chemotherapeutic agents may lead to the occurrence of adverse effects, thereby posing potential challenges to patient health and treatment outcomes[28]. CP, a commonly used chemotherapeutic agent, has been associated with hepatotoxicity, indicating its potential to induce liver damage. This hepatotoxic effect has been documented in various studies, suggesting a need for close monitoring of liver function in patients undergoing CP treatment[29,30].

Jia et al.[31] found that RA showed hepatoprotective effects on ovalbumin-induced hepatic injury by lowering serum ALT, AST activities and preventing expression of proinflammatory cytokines (TNF-α, IL-4 and IL-6) in liver. Another study on cholestatic liver showed that RA treatment improved hepatic histopathology, serum biochemical parameters, oxidative stress and fibrosis. The inflammatory response was also suppressed by RA treatment through modulation of NF-κB activity[32]. In our study, RA treatment effectively mitigated CP-induced hepatotoxicity, as evidenced by reduced hepatic weights, improved liver histology, and decreased levels of MDA, ALT, and AST, demonstrating its antioxidant and hepatoprotective properties.

Studies showed that RA can inhibit pro-apoptotic pathway Apaf-1 expression or promote anti-apoptotic pathway through Bcl-2 expression. Yıldızhan et al.[33] found that RA treatment protected kidney tissue against deltamethrin toxicity, by suppressing apoptosis through inhibition of Apaf-1 expression. In an *in vitro* study of pancreatic β cell culture, RA was found to protect β-cells against streptozotocin toxicity by upregulating increased expression of Bcl-2 and maintaining normal morphology of β-cells[34]. RA administration suppressed CP-induced apoptosis by increasing Bcl-2 and reducing Apaf-1 expression in hepatic tissue. Supporting this, *in silico* analysis identified key enriched pathways associated with these targets, including the B cell receptor and ErbB signaling pathways for Apaf-1, and PD-L1/PD-1 and AGE-RAGE signaling pathways for Bcl-2. These pathways are closely linked to inflammatory regulation[35], oxidative stress and apoptotic control[36], suggesting that RA exerts its protective effects through modulation of multiple signaling networks involved in CP-induced liver injury. *In silico* pathway analysis is a valuable tool for predicting potential biological pathways and interactions based on gene expression data. In the context of our study with RA's hepatoprotective effects, bioinformatic analysis can suggest involvement in pathways related to apoptosis and immune signaling[37,38]. However, such predictions are based on existing databases and known interactions, which may not account for novel or context-specific mechanisms. Therefore, while our bioinformatical analysis provides hypotheses, it does not offer definitive proof of pathway involvement.

Oxidative stress is an indicator of cell death and Nrf-2 is a key transcription factor that regulates genes involved in antioxidant mechanisms[39]. Yu et al.[40] studied the role of RA in neuroinflammation and found that RA administration significantly modulated Nrf-2 expression in histologically damaged brains. In a CCl_4-induced hepatotoxicity, RA was found to show hepatoprotective effects by improving antioxidant mechanism, inhibiting inflammation, and hepatocyte apoptosis through Nrf-2 signaling pathway[41]. Recent studies have further elucidated the molecular pathways underlying the hepatoprotective effects of RA. Lu et al.[38] reported that RA attenuated CCL4-induced liver injury by activating the Nrf2 signaling pathway, thereby enhancing antioxidant defense mechanisms and reducing oxidative stress and inflammation. Luo et al.[42] demonstrated that RA mitigates toosendanin-induced

hepatic damage by restoring autophagic flux and lysosomal functions through activation of the JAK2/STAT3/CTSC signaling axis. Moreover, Karaca et al.[43] found that RA protects against tramadol-induced hepatorenal toxicity by modulating multiple pathways, including oxidative stress, inflammation, endoplasmic reticulum stress, and apoptosis. Collectively, these findings are consistent with our results, emphasizing RA's multifaceted role in reducing drug-induced hepatic injury thorugh diverse molecular mechanisms.

RA can influence the activity and response of the immune system, potentially aiding in immune function regulation and defense against pathogens or harmful substances[44]. RA has been shown to prevent inflammatory diseases via preventing activity of TNF-α through its anti-inflammatory activity. In an experimental osteoarthritis rat model, Hu et al.[45] showed that RA inhibited extracellular matrix formation and production of IL-6 and prevented activation of IL-1β induced genes. Immunostaining revealed that CP-induced liver injury was associated with elevated Nrf-2 and TNF-α expression, reflecting increased oxidative stress and inflammation. RA treatment significantly reduced the expression of both markers, supporting its antioxidant and anti-inflammatory effects. KEGG pathway analysis identified shared targets between RA and Nrf-2 or TNF-α, highlighting enrichment in immune and stress-related pathways, including PD-1/PD-L1, IL-17, and AGE-RAGE signaling. Notably, the association of RA-Nrf-2 targets with pathways involved in leishmaniasis[46] suggests broader immunomodulatory roles for RA. The strong link between TNF-α and IL-17 signaling further implies that RA may exert protective effects by modulating cytokine networks implicated in hepatic and renal toxicity[47]. Together, these findings support the role of RA in mitigating CP-induced liver damage through coordinated regulation of oxidative and inflammatory pathways.

Conclusion

This experimental study sheds light on the potential protective effects of RA against CP induced hepatotoxicity. Our findings demonstrate that RA ameliorated CP-induced liver damage as evidenced by reduced hepatic weight, serum liver enzymes, decreased histopathological alterations, and enhanced antioxidant defenses. The mechanistic insights revealed RA's ability to attenuate oxidative stress, inflammation, and apoptosis pathways, which are pivotal in CP-induced liver injury. For the future, RA may be a potential therapeutic candidate for clinical use. In addition, interaction between RA and CP metabolism may promise as an adjuvant therapy for CP -based chemotherapy regimens.

Limitations and future perspectives

The limitations of this study include a small sample size and potential biases due to animal model use, which may affect the generalizability of the findings. Methodological constraints and resource limitations may have also influenced the study outcomes and their applicability to broader contexts. In future studies, mechanisms through which RA may be effective in reducing CP toxicity could be elucidated at the experimental level.

Funding

The authors declare that they have not received funding.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical considerations

Protection of humans and animals. The authors declare that the procedures followed complied with the ethical standards of the responsible human experimentation committee and adhered to the World Medical Association and the Declaration of Helsinki. The procedures were approved by the Institutional Ethics Committee.

Confidentiality, informed consent, and ethical approval. The authors have followed their institution's confidentiality protocols, obtained informed consent from patients, and received approval from the Ethics Committee. The SAGER guidelines were followed according to the nature of the study.

Declaration on the use of artificial intelligence. The authors declare that no generative artificial intelligence was used in the writing of this manuscript.

References

1. Cuce G, Cetinkaya S, Koc T, Esen HH, Limandal C, Balci T, et al. Chemoprotective effect of vitamin E in cyclophosphamide-induced hepatotoxicity in rats. Chem Biol Interact. 2015;232:7-11.
2. Bhattacharjee A, Basu A, Biswas J, Bhattacharya S. Nano-Se attenuates cyclophosphamide-induced pulmonary injury through modulation of oxidative stress and DNA damage in Swiss albino mice. Mol Cell Biochem. 2015;405:243-56.

3. Oyagbemi AA, Omobowale OT, Asenuga ER, Akinleye AS, Ogunsanwo RO, Saba AB. Cyclophosphamide-induced hepatotoxicity in wistar rats: the modulatory role of gallic acid as a hepatoprotective and chemopreventive phytochemical. Int J Prev Med. 2016;7:51.
4. Mazur L, Czyzewska A, Bochenek M. Flow cytometric detection of apoptotic bone marrow cells with fractional DNA content after application of WR-2721, cyclophosphamide, cisplatin, and exposure of mice to gamma rays. Hum Exp Toxicol. 2002;21:335-41.
5. Shokrzadeh M, Ahmadi A, Naghshvar F, Chabra A, Jafarinejhad M. Prophylactic efficacy of melatonin on cyclophosphamide-induced liver toxicity in mice. Biomed Res Int. 2014;2014:470425.
6. Dewanjee S, Gangopadhyay M, Das U, Sahu R, Samanta A, Banerjee P. Signal transducer and oxidative stress mediated modulation of phenylpropanoid pathway to enhance rosmarinic acid biosynthesis in fungi elicited whole plant culture of *Solenostemon scutellarioides*. Enzyme Microb Technol. 2014;66:1-9.
7. Zhang Y, Li X, Wang Z. Antioxidant activities of leaf extract of *Salvia miltiorrhiza* Bunge and related phenolic constituents. Food Chem Toxicol. 2010;48:2656-62.
8. Chu X, Ci X, He J, Jiang L, Wei M, Cao Q, et al. Effects of a natural prolyl oligopeptidase inhibitor, rosmarinic acid, on lipopolysaccharide-induced acute lung injury in mice. Molecules. 2012;17:3586-98.
9. Lee HJ, Cho HS, Park E, Kim S, Lee SY, Kim CS, et al. Rosmarinic acid protects human dopaminergic neuronal cells against hydrogen peroxide-induced apoptosis. Toxicology. 2008;250:109-15.
10. Huang SS, Zheng RL. Rosmarinic acid inhibits angiogenesis and its mechanism of action *in vitro*. Cancer Lett. 2006;239:271-80.
11. Karthik D, Viswanathan P, Anuradha CV. Administration of rosmarinic acid reduces cardiopathology and blood pressure through inhibition of p22phox NADPH oxidase in fructose-fed hypertensive rats. J Cardiovasc Pharmacol. 2011;58:514-21.
12. Moon DO, Kim MO, Lee JD, Choi YH, Kim GY. Rosmarinic acid sensitizes cell death through suppression of TNF-alpha-induced NF-kappaB activation and ROS generation in human leukemia U937 cells. Cancer Lett. 2010;288:183-91.
13. Osakabe N, Yasuda A, Natsume M, Sanbongi C, Kato Y, Osawa T, et al. Rosmarinic acid, a major polyphenolic component of *Perilla frutescens*, reduces lipopolysaccharide (LPS)-induced liver injury in d-galactosamine (d-GalN)-sensitized mice. Free Radic Biol Med. 2002;33:798-806.
14. Yang SY, Hong CO, Lee GP, Kim CT, Lee KW. The hepatoprotection of caffeic acid and rosmarinic acid, major compounds of *Perilla frutescens*, against t-BHP-induced oxidative liver damage. Food Chem Toxicol. 2013;55:92-9.
15. Touiss I, Ouahhoud S, Harnafi M, Khatib S, Bekkouch O, Amrani S, et al. Toxicological evaluation and hepatoprotective efficacy of rosmarinic acid-rich extract from *Ocimum basilicum* L. Evid Based Complement Alternat Med. 2021;2021:6676998.
16. Hsu YC, Cheng CP, Chang DM. Plectranthus amboinicus attenuates inflammatory bone erosion in mice with collagen-induced arthritis by downregulation of RANKL-induced NFATc1 expression. J Rheumatol. 2011;38:1844-57.
17. Liang Z, Wu L, Deng X, Liang Q, Xu Y, Deng R, et al. The antioxidant rosmarinic acid ameliorates oxidative lung damage in experimental allergic asthma via modulation of NADPH oxidases and antioxidant enzymes. Inflammation. 2020;43:1902-12.
18. Bilto YY, Alabdallat NG. *In vitro* and *in vivo* antioxidant related effects of rosemary (*Rosmarinus officinalis* L.) extracts in humans. Am J Clin Exp Med. 2015;3:213-21.
19. Du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. PLoS Biol. 2020;18:e3000410.
20. Domitrović R, Skoda M, Vasiljev Marchesi V, Cvijanović O, Pernjak Pugel E, Stefan MB. Rosmarinic acid ameliorates acute liver damage and fibrogenesis in carbon tetrachloride-intoxicated mice. Food Chem Toxicol. 2013;51:370-8.
21. Akhter J, Khan J, Baghel M, Beg MM, Goswami P, Afjal MA, et al. NLRP3 inflammasome in rosmarinic acid-afforded attenuation of acute kidney injury in mice. Sci Rep. 2022;12:1313.
22. Ermiş IS, Deveci E, Aşır F. Effects of quince gel and hesperidin mixture on experimental endometriosis. Molecules. 2023;28:5945.
23. Crowe AR, Yue W. Semi-quantitative determination of protein expression using immunohistochemistry staining and analysis: an integrated protocol. Bio Protoc. 2019;9:e3465.
24. Aşır F, Oğlak SC, Ağaçayak E, Alabalık U. Homeobox A Cluster 7 (HOXA7) protein expression increased in the placentas of patients with preterm delivery. Perinat J. 2023;31:213-8.
25. Keşim DA, Aşır F, Ayaz H, Korak T. The effects of ellagic acid on experimental corrosive esophageal burn injury. Curr Issues Mol Biol. 2024;46:1579-92.
26. Bardou P, Mariette J, Escudié F, Djemiel C, Klopp C. jvenn: an interactive Venn diagram viewer. BMC Bioinformatics. 2014;15:293.
27. Ge SX, Jung D, Yao R. ShinyGO: a graphical gene-set enrichment tool for animals and plants. Bioinformatics. 2020;36:2628-9.
28. Weijl NI, Cleton FJ, Osanto S. Free radicals and antioxidants in chemotherapy-induced toxicity. Cancer Treat Rev. 1997;23:209-40.
29. Zarei M, Shivanandappa T. Amelioration of cyclophosphamide-induced hepatotoxicity by the root extract of *Decalepis hamiltonii* in mice. Food Chem Toxicol. 2013;57:179-84.
30. Gunes S, Ayhanci A, Sahinturk V, Altay DU, Uyar R. Carvacrol attenuates cyclophosphamide-induced oxidative stress in rat kidney. Can J Physiol Pharmacol. 2017;95:844-9.
31. Jia B, Shang J, Zeng H, Wang X, Fang M, Xu L, et al. Hepatoprotective effects of rosmarinic acid on ovalbumin-induced intestinal food allergy mouse model. Molecules. 2023;28:788.
32. Lin SY, Wang YY, Chen WY, Liao SL, Chou ST, Yang CP, et al. Hepatoprotective activities of rosmarinic acid against extrahepatic cholestasis in rats. Food Chem Toxicol. 2017;108:214-23.
33. Yildizhan E, Ülger BV, Akkuş M, Katanalp ÖF, Gökalp Özkorkmaz E, Yıldızhan İ. Rosmarinic acid ameliorates deltamethrin induced hepatotoxicity and nephrotoxicity. Med Rec. 2023;5:400-5.
34. El-Huneidi W, Anjum S, Mohammed AK, Bin Eshaq S, Abdrabh S, Bustanji Y, et al. Rosemarinic acid protects β-cell from STZ-induced cell damage via modulating NF-κβ pathway. Heliyon. 2023;9:e19234.
35. Qin W, Hu L, Zhang X, Jiang S, Li J, Zhang Z, et al. The diverse function of PD-1/PD-L pathway beyond cancer. Front Immunol. 2019;10:2298.
36. Zgutka K, Tkacz M, Tomasiak P, Tarnowski M. A role for advanced glycation end products in molecular ageing. Int J Mol Sci. 2023; 24:9881.
37. Yao Y, Li R, Liu D, Long L, He N. Rosmarinic acid alleviates acetaminophen-induced hepatotoxicity by targeting Nrf2 and NEK7-NLRP3 signaling pathway. Ecotoxicol Environ Saf. 2022;241:113773.
38. Lu YH, Hong Y, Zhang TY, Chen YX, Wei ZJ, Gao CY. Rosmarinic acid exerts anti-inflammatory effect and relieves oxidative stress via Nrf2 activation in carbon tetrachloride-induced liver damage. Food Nutr Res. 2022;66:8359-72.
39. Ngo V, Duennwald ML. Nrf2 and oxidative stress: a general overview of mechanisms and implications in human disease. Antioxidants (Basel). 2022;11:2345.
40. Yu Y, Li Y, Qi K, Xu W, Wei Y. Rosmarinic acid relieves LPS-induced sickness and depressive-like behaviors in mice by activating the BDNF/Nrf2 signaling and autophagy pathway. Behav Brain Res. 2022; 433:114006.
41. Dahchour A. Anxiolytic and antidepressive potentials of rosmarinic acid: a review with a focus on antioxidant and anti-inflammatory effects. Pharmacol Res. 2022;184:106421.
42. Luo L, Lin J, Chen S, Ni J, Peng H, Shen F, et al. Rosmarinic acid alleviates toosendanin-induced liver injury through restoration of autophagic flux and lysosomal function by activating JAK2/STAT3/CTSC pathway. J Ethnopharmacol. 2024;330:118196.
43. Karaca O, Akaras N, Şimşek H, Gür C, İleritürk M, Küçükler S, et al. Therapeutic potential of rosmarinic acid in tramadol-induced hepatorenal toxicity: modulation of oxidative stress, inflammation, RAGE/NLRP3, ER stress, apoptosis, and tissue functions parameters. Food Chem Toxicol. 2025;197:115275.
44. Zhao J, Xu L, Jin D, Xin Y, Tian L, Wang T, et al. Rosmarinic acid and related dietary supplements: potential applications in the prevention and treatment of cancer. Biomolecules. 2022;12:1410.
45. Hu ZN, Huang LJ, Chen WP. The inhibitory effects of rosmarinic acid on catabolism induced by IL-1β in rat chondrocyte. Acta Biochim Pol. 2018;65:535-8.
46. Clementi A, Battaglia G, Floris M, Castellino P, Ronco C, Cruz DN. Renal involvement in leishmaniasis: a review of the literature. NDT Plus. 2011;4:147-52.
47. Sheng Y, Chen K, Jiang W, Wu Z, Zhang W, Jing H, et al. PD-1 restrains IL-17A production from γδ T cells to modulate acute radiation-induced lung injury. Transl Lung Cancer Res. 2021;10:685-98.