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Quercetin and Resveratrol are Associated with the Downregulation of TNF-α/NF-kB/iNOS Axis-Mediated Acute Liver Injury in Rats Induced by Paracetamol Poisoning

La Quercetina y el Resveratrol están Asociados con la Regulación Decreciente de la Lesión Hepática Aguda Mediada por el Eje TNF-α/NF-kB/iNOS en Ratas Inducida por Envenenamiento con Paracetamol

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SUMMARY: Paracetamol (known as acetaminophen, or APAP) poisoning causes acute liver damage that can lead to organ failure and death. We sought to determine that APAP overdose can augment tumor necrosis factor-alpha (TNF-α)/ nuclear factor kappa B (NF-kB)/induced nitic oxide synthase (iNOS) axis-mediated hepatotoxicity in rats, and the anti-inflammatory polyphenolic compounds, quercetin (QUR) plus resveratrol (RES) can ameliorate these parameters. Therefore, we induced acute hepatotoxicity in rats using APAP overdose (2 g/kg, orally) and the protective group of rats were treated with 50 mg/kg QUR plus 30 mg/kg RES for one week before APAP ingestion. Animals were killed at day 8. APAP poisoning caused the induction of hepatic tissue levels of TNF-α, NF-kB, and iNOS, which were significantly ($p<0.05$) decreased by QUR+RES. QUR+RES, also inhibited liver injury biomarkers, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Additionally, a link between liver injury and TNF-α /NF-kB / iNOS axis mediated hepatotoxicity was observed. Thus, the presented data backing the conclusion that intoxication by paracetamol increases TNF-α / NF-kB / iNOS axis -mediated hepatotoxicity, and is protected by a combination of quercetin and resveratrol.

KEY WORDS: Acute hepatic injury; Paracetamol overdose; TNF-α /NF-kB / iNOS axis; Quercetin; Resveratrol.

INTRODUCTION

Accidental or intentional administration of toxic dose(s) of the analgesic and antipyretic drug, paracetamol causes in humans acute liver injury (McGill *et al*., 2012), which accounts in USA for approximately 50 % of admitted patients with acute liver failure (Larson *et al*., 2005). The reported cases of death in the United kingdom due to paracetamol intoxications between 1999-2001 exceeded 120 persons aged 12 and over years (Hawton *et al*., 2004). Paracetamol metabolism occurs in the liver and the hepatic antioxidant glutathione (GSH) inactivated the hepatotoxic metabolites from causing cellular damage (James *et al*., 2003). However, depletion of about 90 % of the endogenous GSH as well as the increase in the reactive oxygen species (ROS) production would cause mitochondrial damage and hepatocellular death (Henderson *et al*., 2000; Hinson *et al*., 2004). Additionally, TNF-α, NF-kB, and iNOS are reported to be associated with liver injury, fibrosis, and hepatocarcinoma (Luedde & Schwabe, 2011; Iwakiri, 2015; Robert *et al*., 2016).

Quercetin and resveratrol are natural polyphenolic compounds found in grains, fruits, and vegetables (Cudmore *et al*., 2012). They demonstrated pleiotropic effects such as inhibition of the aggravated inflammation caused by the soluble form of vascular endothelial growth factor receptor-1 released from human endothelial cells (Al-Ani, 2013), antiarrhythmic and vascular protective effects in ischemic rats due to nitric oxide release and inhibition of lactate dehydrogenase in the carotid blood (Hung *et al*., 2000), inhibit

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low density lipoprotein oxidation in humans (Frankel *et al*., 1993), and hepato-protection like the inhibition of hepatic steatosis, liver fibrosis, and hepatic sinusoidal obstruction (Faghihzadeh *et al*., 2015; Zhang *et al*., 2017). Furthermore, paracetamol intoxication was reported to be partially protected by these natural compounds in animal models via the decline in oxidative stress levels in the blood and liver tissue (Singh *et al*., 2011). However, liver protection against APAP intoxication using quercetin plus resveratrol concomitant with the inhibition of TNF-α, NF-kB, and nitrosative stress (iNOS) has not been studied before in animal models. As a result, we sought to investigate the TNF-α / NF-kB/iNOS axis-mediated liver injury induced by APAP in rats, and the protection levels provided by quercetin and resveratrol.

MATERIAL AND METHOD

Animals. The ethical committee based at King Khalid University approved the protocol used for this study. Sprague Dawley rats (170-200 g weight each) were used in this work. They were kept in a clean place at room temperature (22 °C) with a cycle of 12h light/dark as well as provided with unrestricted access to water and food.

Induction of acute liver injury and treatments. Following acclimatization for one week, one group of rats (6/group) received a single toxic dose of APAP (2 gram/kg, orally) and the protective animal group (6/group) was given 50 mg/kg QUR plus 50 mg/kg RES for one week and then received a single toxic dose of APAP (2 gram/kg, orally). Two control groups (6/group each); saline treated and drug treated without APAP were also used. Blood and liver tissue samples were harvested on day 8 under anesthesia.

Immunohistochemistry of NF-kB and iNOS. As mentioned before (Mirdad *et al*., 2022), immunohistochemical staining of deparaffinized liver sections (5 µm) were used to quantify the protein expression of NF-kB and iNOS. Following antigen retrieval, tissue sections were incubated at 40C for about 15 hours with either anti-NF-kB or anti-inducible nitric oxide synthase (iNOS) obtained from Abcam, Cambridge, UK. Liver sections were then incubated for half an hour with the secondary antibody at room temperature, followed by counterstained with Meyer's hematoxylin.

Determination of TNF-α ALT, AST, and glutathione peroxidase (GPx). ELISA kits were used as instructed by the manufacturers to assess liver tissue levels of TNF-a purchased from Abcam, Cambridge, UK, and GPx (Cayman Chemical, Ann Arbor, MI, USA) activities. ELISA kits were used to measure in the blood levels of the liver injury enzymes ALT and AST (Human Co., Germany) as instructed by the manufacturer.

Statistical analysis and morphometry. Data were handled and evaluated using the SPSS version 10.0 (SPSS, Inc., Chicago, Ill., USA). One-way ANOVA was performed succeeded by Tukey's *post hoc* test. Pearson correlation statistical analysis was performed for finding of a likely significance between two different parameters. Results were accepted to be significant if $p \leq 0.05$. Morphometry of the areas % of NF-kB and iNOS immunostaining were accomplished using "Leica Qwin 500 C" image analyzer type "Leica Qwin 500 C" from Cambridge, UK, as reported recently (Dawood *et al*., 2022).

RESULTS

Quercetin plus resveratrol inhibit TNF-α/NF-kB /iNOS axis induced by APAP in liver tissue. To determine whether APAP overdose can augment TNF-α/NF-kB/iNOS axis that is recognized to be activated in biliary cirrhosis (Li *et al*., 2016) and whether QUR+RES can protect against APAP-induced these parameters, we measured levels of liver protein expression of TNF-α, NF-kB, and iNOS in all rats' group. Ingestion of an overdose of APAP increased levels of TNF-α (Fig. 1A) and NF-kB (Figs. 1C and 1D) protein expression in liver tissue samples that were significantly ($p<0.05$) ameliorated by QUR+RES (Figs. 1A, 1E, and 1F). However, in comparison with the control group, effects of QUR+RES were still significant ($p<0.05$) in (F), which means partial inhibition of NF-kB and complete inhibition of TNF-α.

iNOS knockout mice were more protected from APAP intoxication caused liver injury than wild type mice (Bourdi *et al*., 2002). To determine the degree of iNOS level reduction by QUR+RES in our model of APAP-induced hepatotoxicity in rats, we evaluated the levels of iNOS protein in rats' groups one day post the APAP ingestion (Fig. 2). Immunohistochemistry of iNOS (Figs. 2A-E) illustrated weak positive immunostaining in the sinusoidal endothelium and negative immunostaining in cytoplasm of thehepatocytes in control groups, untreated control (Fig. 2A) and QUR+RES (Fig. 2B). Whereas, APAP group exhibited strong positive iNOS immunostaining (Fig. 2C) as compared to the control groups. Rats pretreated with QUR and RES (Fig. 2D) were significantly ($p<0.0001$) but not completely showed an inhibition of iNOS immunostaining in their liver sections. The mean area % of liver tissue iNOS immunostaining of all the groups is shown in Figure 2E. In addition, QUR+RES completely inhibited iNOS liver tissue homogenates levels that were augmented by APAP (Fig. 2F).

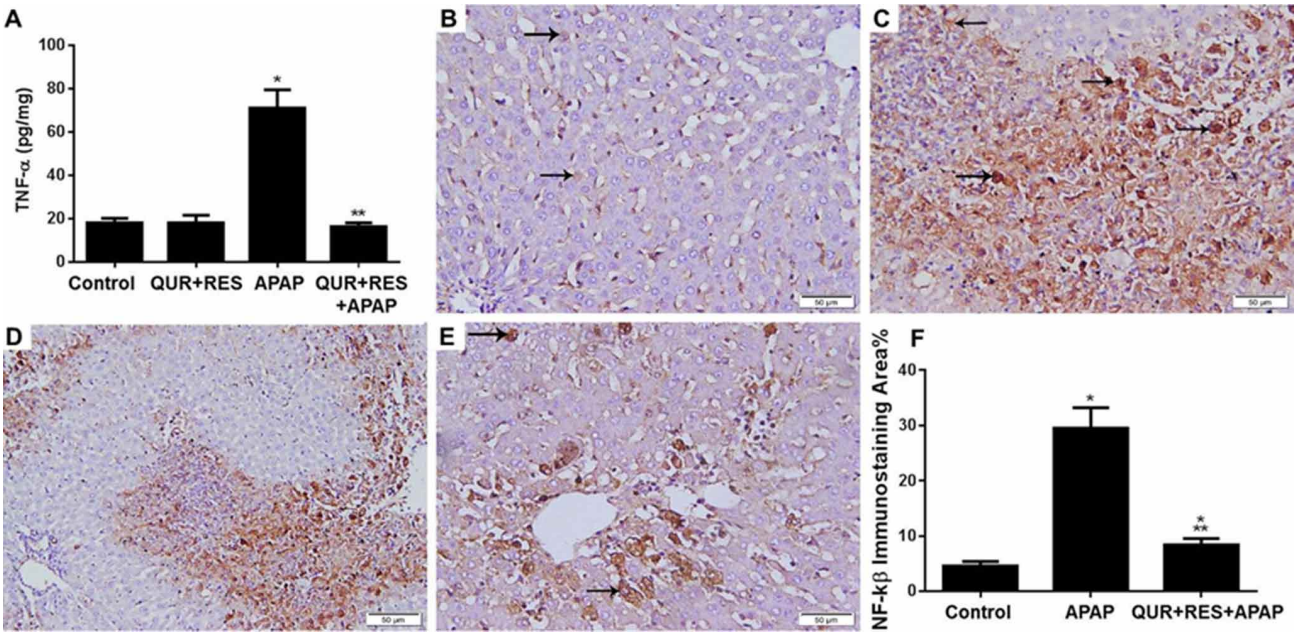

Fig. 1. Paracetamol (APAP)-induced hepatic TNF-α and NF-kB expression is inhibited by quercetin (QUR) and resveratrol (RES). Protein levels of TNF-α were estimated using western blot analysis (A). NF-kB immunohistochemistry of liver tissue samples (B, C, and E x 200; D, x100) prepared from all rats' group are shown: Control group (B); model group, APAP (C and D); and treated group, QUR+RES+APAP (E). Histograms (F) assessed the degree of NF-kB immunostaining in all rats' group. Obtainable p values are significant; *p<0.05 versus control, **p<0.05 versus APAP. TNF-α: tumor necrosis factor-alpha; NF-kB: nuclear factor kappa B.

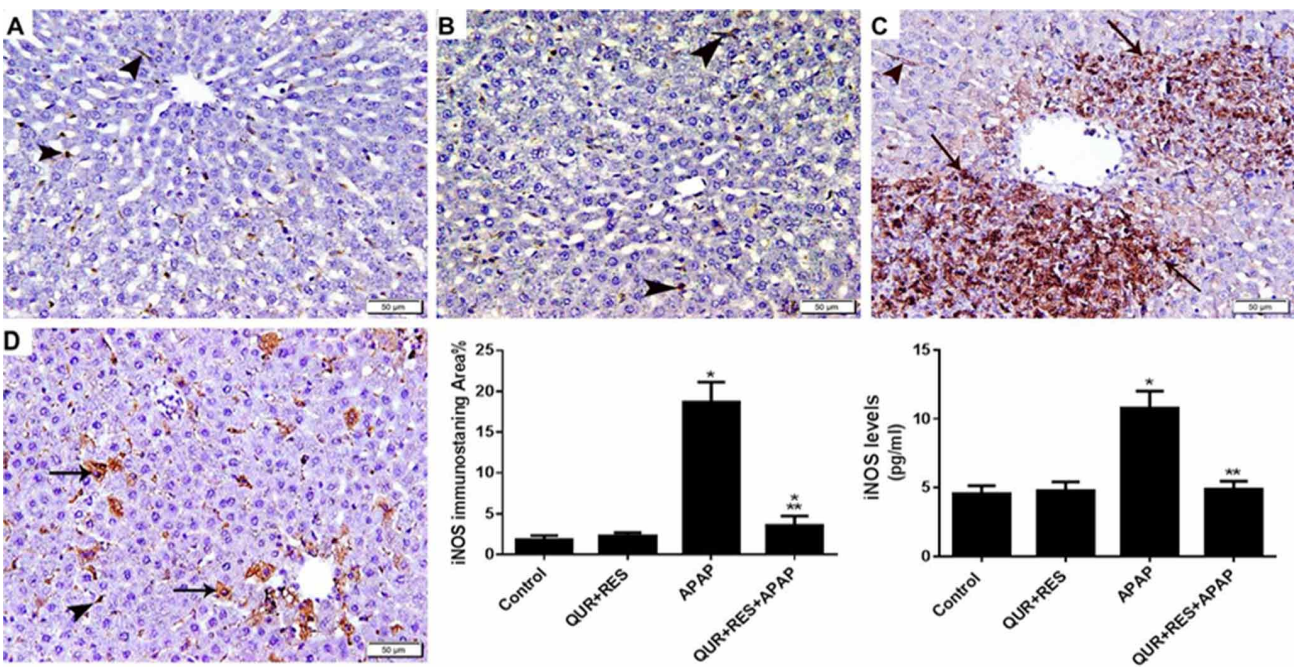

Fig. 2. Quercetin (QUR) and resveratrol (RES) are associated with the inhibition of paracetamol (APAP)-induced iNOS expression. Immunohistochemistry of iNOS of liver sections (A-D, x200) from the control (A), control QUR+RES (B), APAP (C), and QUR+RES+APAP (D) groups are illustrated. There are regions of weak positive iNOS staining (A, B, D, arrowheads) in the hepatic vascular endothelium, as well as a strong positive iNOS staining in liver tissue (C, D, arrows). (E) Degree of liver iNOS immunostaining prepared from the above groups is exemplified in histograms. (F) Liver tissue homogenates iNOS values were measured at day 8 in rats' groups. Presented p values are all significant; *p<0.0001 versus control, **p<0.0001 versus APAP. iNOS: inducible nitric oxide synthase; APAP: paracetamol.

Inhibition of glutathione and activation of liver injury enzymes upon APAP intoxication is protected by Quercetin plus resveratrol. We assessed liver tissue levels of the endogenous antioxidant glutathione peroxidase (GPx) and blood levels of hepatic injury enzymes (ALT and AST) upon ingestion of a toxic dose of APAP with and without QUR+RES. A significant ($p<0.05$) inhibition of GPx tissue levels by APAP was observed, which was significantly augmented by QUR+RES (Fig. 3A). Whereas, APAP intoxication caused a significant ($p<0.05$) increase in ALT (Fig. 2B) and AST (Fig. 2C), which were inhibited to levels compared to control group by QUR+RES.

Correlation between the score of the liver injury enzyme ALT and TNF-α/ NF-kB / iNOS axis. The link between ALT and TNF-α/ NF-kB / iNOS axis as well as GPx was evaluated. This link is significant in providing an association between these parameters because they show a role in the pathology of acute hepatic injury caused by APAP. A significant ($p<0.0001$) correlation between ALT score and TNF-α ($r = 0.985$) (Fig. 4A), NF-kB ($r = 0.944$) (Fig. 4B), and iNOS ($r = 0.939$) (Fig. 4C) was observed. Whereas, ALT *versus* GPx demonstrated a significant negative correlation ($r = - 0.928$, $p<0.0001$) (Fig. 4D).

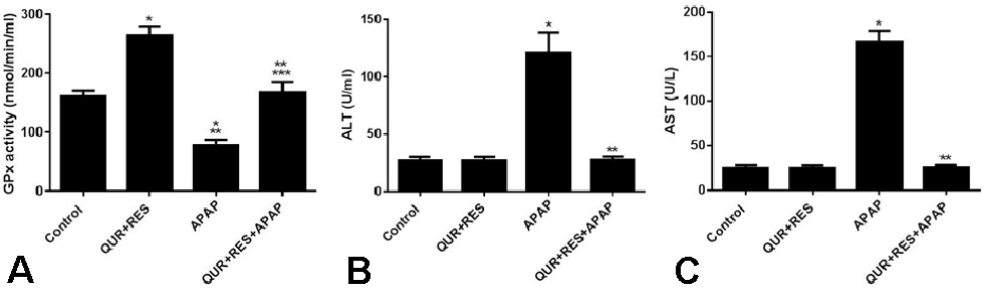

Fig. 3. Modulation of glutathione peroxidase (GPx) and liver injury enzymes (ALT and AST) by paracetamol (APAP) is protected by quercetin (QUR) and resveratrol (RES). Levels of GPx in liver tissue homogenates (A) and blood levels of ALT (B) and AST (C) were estimated in all rats' group at the end of the experiment. Obtainable p values are significant; for A: *$p<0.05$ versus control, **$p<0.05$ versus QUR+RES, ***$p<0.05$ versus APAP. For B and C: *$p<0.05$ versus control, **$p<0.05$ versus APAP. ALT: alanine aminotransferase; AST: aspartate aminotransferase.

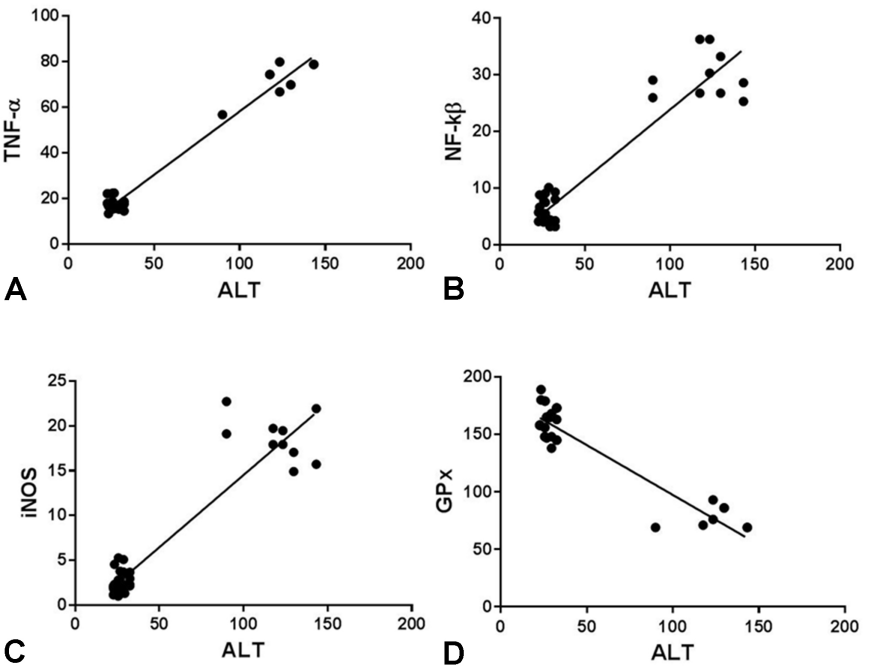

Fig. 4. Correlation between ALT score and TNF-α NF-kB / iNOS axis -mediated hepatotoxicity, and glutathione levels. The link between the liver injury enzyme ALT versus TNF-α(A), NF-kB (B), iNOS (C), and GPx (D) are shown. ALT: alanine aminotransferase; TNF-α: tumor necrosis factor-alpha; NF-kB: nuclear factor kappa B; iNOS: inducible nitric oxide synthase; GPx: glutathione peroxidase.

DISCUSSION

We used rat model of paracetamol-induced acute liver damage to examine hepatic TNF-α/NF-kB/iNOS axis as well as the level of inhibition of the endogenous antioxidant GPx with and without the incorporation of the polyphenolic compounds quercetin plus resveratrol. Our data supported the following observations; (i) APAP overdose is associated with the induction of hepatic TNF-α, NF-kB, and iNOS protein expression, and inhibition of hepatic glutathione and augmentation of liver injury enzymes; (ii) A combination of quercetin with resveratrol effectively protect against the modulation of these parameters; and (iii) a link between the specific liver injury enzyme ALT and TNF-α, NF-kB, iNOS, and GPx were observed (Fig. 5). This added more support to our report on the damaging effects of APAP overdose on the alteration of hepatocyte ultrastructure which was protected by QUR plus RES (Al Humayed *et al*., 2019).

Fig. 5. Proposed model for paracetamol (APAP)-induced acute hepatotoxicity appears protected by quercetin and resveratrol. QUR: quercetin; RES: resveratrol; TNF-a: tumor necrosis factor-alpha; NF-kB: nuclear factor kappa B; iNOS: inducible nitric oxide synthase.

The liver is a recognized target of APAP overdose that causes acute liver injury and organ failure in humans (Larson *et al*., 2005; McGill *et al*., 2012), and the data in this study demonstrated the augmentation of hepatic TNF-α/NF-kB/iNOS axis by APAP intoxication (Figs. 1 and 2). Augmentation of the above access (TNF-α/NF-kB/iNOS) was also reported (i) in a rat model of biliary cirrhosis that showed the activation of NF-kB/iNOS pathway, which was inhibited by the TNF-α inhibitor, thalidomide (Li *et al*., 2016), placing TNF-α upstream of NF-kB/iNOS pathway; and in (ii) immobilization stress for a duration of 6 hours induced in rats neurodegeneration in the brain cortex associated with the activation of TNF-α/NF-kB/iNOS axis (Madrigal *et al*., 2002). Therefore, these reports are in accordance with our data presented here. In addition, another previous report that showed administration of 1 gram per kg of APAP into rats caused hepatotoxicity associated with the upregulation of hepatic iNOS protein expression and downregulation of glutathione (GSH), which was inhibited by resveratrol (Elbe *et al*., 2018) is also in agreement with our study shown in Figure 2 (iNOS) and Figure 3 (GPx). Furthermore, effective inhibition of paracetamol induced acute liver injury by a combination of quercetin and resveratrol demonstrated by our data mirrors a recent study (Dallak *et al*., 2022) that also presented effective amelioration by QUR+RES to paracetamol induced substantial alterations to kidney ultrastructure that was linked with the downregulation of inflammation and oxidative stress.

In conclusion, this report demonstrated the upregulation of TNF-α/NF-kB/iNOS axis mediated acute hepatotoxicity caused by paracetamol overdose in rats, which was associated with the augmentation of liver injury enzyme and amelioration of glutathione. All these parameters were modulated by QUR+RES.

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RESUMEN: El envenenamiento por paracetamol (conocido como acetaminofeno o APAP) causa daño hepático agudo que puede provocar una insuficiencia orgánica y la muerte. El objetivo de este trabajo fue determinar si la sobredosis de APAP puede aumentar la hepatotoxicidad mediada por el eje del factor de necrosis tumoral alfa (TNF-α)/factor nuclear kappa B (NF-kB)/óxido nítico sintasa inducida (iNOS) en ratas, y si el polifenólico antiinflamatorio compuesto por quercetina (QUR) más resveratrol (RES) pueden mejorar estos parámetros. Por lo tanto, inducimos hepatotoxicidad aguda en ratas usando una sobredosis de APAP (2 g/kg, por vía oral). El grupo protector de ratas se trató con 50 mg/kg de QUR más 30 mg/kg de RES durante una semana antes de la ingestión de APAP. Los animales se sacrificaron el día 8. El envenenamiento con APAP en el tejido hepático provocó la inducción de niveles de TNF-α, NF-kB e iNOS, que se redujeron significativamente ($p<0,05$) con QUR+RES. QUR+RES, también inhibió los biomarcadores de daño hepático, la alanina aminotransferasa (ALT) y el aspartato aminotransferasa (AST). Además, se observó una relación entre la lesión hepática y la hepatotoxicidad mediada por el eje TNF-α /NF-kB/iNOS. Por lo tanto, los datos presentados respaldan la conclusión de que la in-

toxicación por paracetamol aumenta la hepatotoxicidad mediada por el eje TNF-α /NF-kB / iNOS, y está protegida por una combinación de quercetina y resveratrol.

PALABRAS CLAVE: Lesión hepática aguda; Sobredosis de paracetamol; Eje TNF-α/ NF-kB / iNOS; Quercetina; Resveratrol.

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