Hepatoprotective effect of raspberry ketone against carbon tetrachloride-induced rat hepatic injury

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ABSTRACT. Raspberry ketone (RK) is a natural phenolic compound. The aim of this study was to evaluate the therapeutic detoxification of RK against carbon tetrachloride (CCl_4)-induced acute liver injury *in vivo* and to explore the underlying mechanism, including whether RK regulates inflammation and apoptosis. In this study, seven groups of rats were used: I, Control; II, received 200 mg/kg RK for 5 days (PO); III, received a single dose of CCl_4 diluted with olive oil (1:1 v/v; 1 mL/kg body weight) intraperitoneally on the fifth day; IV, V, VI, and VII received 25 mg/kg, 50 mg/kg, 100, and 200 mg/kg RK (PO) daily for 5 days, respectively, with a CCl_4-intraperitoneal injection on the fifth day. Histopathology, ultra-microstructural examination via transmission electron microscopy, immunohistochemical detection of NF-κB and cytochrome c, DNA fragmentation, and the levels of malondialdehyde, glutathione, tumor necrosis factor-α, and caspase-9 were detected in the liver. Serum liver transaminases were also measured. CCl_4 induced a significant elevation of serum liver transaminases, as well as increased hepatic malondialdehyde, tumor necrosis factor-α, and caspase-9. In

addition, CCl_4 increased NF-κB activation, cytochrome c expression, and DNA fragmentation. However, CCl_4 decreased hepatic glutathione content. RK pre-treatment significantly ameliorated CCl_4 hepatotoxicity, with the highest dose nearly normalizing all measured parameters. In conclusion, RK is a promising protective agent against CCl_4 hepatotoxicity, possibly through antioxidant, anti-inflammatory, and anti-apoptotic activities.

Key words: Carbon tetrachloride; Raspberry ketone; Anti-inflammatory; Anti-apoptotic; Hepatotoxicity

Abbreviations: ALT: Alanine Transaminase; AST: Aspartate Transaminase; GSH: Reduced Glutathione; NF-κB: Nuclear Factor-κB; MDA: Malondialdehyde; RK: Raspberry, Ketone; ROS: Reactive Oxygen Species; TBARS: Thiobarbituric Acid Reactive Substance; TNF-α: Tumor Necrosis Factor-α

INTRODUCTION

Liver disease is one of the major causes of morbidity and mortality across the world. According to WHO estimates, approximately 500 million people are living with chronic hepatitis infections, resulting in the death of more than one million people annually (Al-Asmari et al., 2014). There is no commonly accepted, effective, conventional drug therapy that prevents or reverses liver damage (Luper, 1998). Conventional drug therapies for many common liver disorders, including non-alcoholic fatty liver disease and viral hepatitis, have limited efficacy and potentially life-threatening side effects. Therefore, it is necessary to search for complementary and alternative medicine (CAM), for the treatment of liver disease (Mahmud et al. 2012). Medicinal plants serve as a vital source of potentially useful new compounds. Several studies have reported the therapeutic efficacy of phytomedicines, such as silymarin and curcumin, in the management of liver dysfunction (Luper, 1998; Miliman et al., 2000).

Much attention has been directed toward the potential health-promoting properties of phenolic phytochemicals (Block, 1992). Plant-derived phenolic compounds are well known for their antioxidant properties. Recent evidence indicates that these compounds may confer anti-inflammatory and/or inflammatory response-stabilizing activities, which would have important implications in health maintenance and disease risk reduction (Joseph et al., 2016). Plants containing phenolic compounds have been proven to possess many pharmacological effects, such as cardioprotective, anticancer, and hepatoprotective properties (Croft, 1998).

Rheosmin, or Raspberry ketone (RK), is a natural phenolic compound. RK,4- (4-hydroxyphenyl) butan-2-one, occurs in various fruits, including raspberries, cranberries, and blackberries. It is biosynthesized from coumaroyl-CoA. RK was discovered in blackberries by Japanese researchers. The Flavour & Extract Manufacturers Association (FEMA) placed rheosmin on ‘‘GRAS” (generally regarded as safe) status (Opdyke, 1978). RK is well absorbed orally; approximately 90% of the dose is excreted as metabolites via the urine within 24 h in rats, guinea pigs, and rabbits. However, the biological activities of RK are not fully elucidated. Although products containing this compound are marketed for weight loss, this effect has not been studied in humans. Morimoto *et al*. (2005) reported that a dose of 1% RK was sufficient to prevent high-fat diet-induced increases in body and tissue weights in mice. This effect was reported to stem from increased norepinephrine-induced lipolysis, altering lipid metabolism.

However, the study by Zhou *et al.* (2008) illustrated that RK can also treat nonalcoholic steatohepatitis (NASH) by minimizing oxidative stress and lipid peroxidation. These effects maintained the balance between oxidation and antioxidation, preventing damage in liver cells, improving leptin resistance, and modulating inflammatory responses. It was believed that RK had a dual effect of liver protection and fat reduction. There have been no reports on the effect of RK on acute hepatic injury and whether it possesses anti-inflammatory or anti-apoptotic activities in the liver.

The transcription factor, nuclear factor κ-B (NF-κB), regulates the expression of various genes, including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2), which play critical roles in apoptosis, tumorigenesis, immune responses, and acute-phase inflammatory responses (Li and Verma, 2002).

After stimulation and degradation of its inhibitor, free NF-κB translocate to the nucleus, where it binds to κB-binding sites in the promoter regions of target genes, and induces the transcription of various inflammatory

genes. Due to its ubiquitous role, NF-κB is a current target for treating various diseases (Baeuerle and Baltimore, 1996).

CCl_4-induced liver injury is one of the most widely used experimental models for exogenous toxin-induced hepatotoxicity, and is a commonly used model for the screening of anti-hepatotoxic and/or hepatoprotective activities of drugs. Several investigative groups reported that CCl_4 could lead to lipid peroxidation of unsaturated fatty acids in plasma and organelle membranes after its metabolic activation by hepatic microsomal cytochrome P450, leading to serious hepatocyte damage (Lee et al., 2003). Ratziu and Zelber-Sagi, 2009; Chen et al., 2012; Weber et al. 2003). CCl_4 causes a wide array of dysfunctions in the liver, including triglyceride accumulation, centrilobular necrosis, polyribosomal desegregation, and depression of protein synthesis (Mahran et al., 1996).

The aims of the present study were to assess whether RK possesses *in vivo* protective effects against CCl_4-induced liver injury in rats and to explore the probable mechanisms of action involved.

MATERIALS AND METHODS

Chemicals required

CCl_4 was obtained from Sigma-Aldrich (St. Louis, MO, USA). Raspberry ketone was obtained from Extrasynthese (Genay, France). Ether, n-butanol, 1,1,3,3-tetramethoxypropane, potassium dihydrogen phosphate, reduced glutathione (GSH), Ellman's Reagent (5,5'-dithio-bis- (2-nitrobenzoic acid), trichloroacetic acid (TCA), and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (St. Louis, MO). Alanine transaminase (ALT) and aspartate transaminase (AST) kits were obtained from Sigma-Aldrich (St. Louis, MO). Rat TNF-α and caspase-9 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Ray biotech, Inc. (Norcross, USA) and Cloud-Clone Corp. (Houston, USA), respectively. Immunohistochemistry (IHC) antibodies for NF-κB and cytochrome C were obtained from Thermo Fisher Scientific (USA). The DNA extraction kit was obtained from Qiagen (Hilden, Germany). Other chemicals were of high analytical reagent grade.

Experimental design

Adult healthy male Wistar rats (150–200 g) were obtained from the animal farm at King Saud University, Saudi Arabia, Riyadh. Animals were housed at 4 rats per standard polypropylene cage. The rats were acclimatized for 1week before beginning the experiments. The animals were provided water and standard chow *ad libitum* and were maintained under controlled conditions of temperature, humidity, and light (12:12 h light: dark cycle). Handling of animals followed the guidelines for the care and use of animals for scientific purposes, with ethical approval in 19-2-16 for animal protocol, according to King Saud University instructions.

Fifty-six adult rats were randomly divided into seven groups, eight rats per group. The experiment was performed for 6 days. Group I (Control), serving as an untreated control, received vehicle daily via gavage. Group II (RK) received 200 mg/kg of RK dissolved in distilled water daily for 5 days via gavage. Group III (CCl_4) rats received vehicle daily via gavage, and a single CCl_4 dose was injected intra-peritoneally (1mL/kg of 1:1 v/v CCl_4: corn oil) on the fifth day (Sinduh et al., 2015). Group IV, V, VI, and VII (RK+CCl_4) received an oral administration of 25 mg/kg, 50 mg/kg, 100 mg/kg, and 200 mg/kg RK daily for 5 days via oral tube, respectively, and were injected intraperitoneally with a single dose of CCl_4 (1mL/kg of 1:1 v/v CCl_4: corn oil) on the fifth day of the experiment, 1 h after the RK dose.

The animals of all groups were euthanized by fast decapitation on the sixth day. Animals were anesthetized with ether, and euthanized 24 h after CCl_4 administration. Blood samples were collected, allowed to stand for 30 min, centrifuged at 3000 rpm for 15 min at 4°C to separate serum, and then stored at −80°C for the different biochemical assays. The liver was immediately removed, dried, weighed, and divided into four parts. The first part was transferred immediately into 10% phosphate-buffered formaldehyde for histological and immuno-histochemical studies. The second part was transferred immediately into 2.5% glutaraldehyde for electron microscopic examination. The third part was weighed and homogenized immediately in ice-cold 50 mM Tris-HCl (pH 7.4) to yield a 50% (w/v) homogenate to be used for various biochemical determinations. The fourth part was stored at −20°C until use.

Liver function tests

To assess liver cell damage, serum levels of ALT and AST were assayed using diagnostic spectrophotometric kits, according to the manufacturer's instructions.

Determination of oxidative stress markers

Twenty percent liver homogenate was used for determination of lipid peroxidation and reduced glutathione (GSH) as markers of oxidative stress. Lipid peroxides (LPO) were determined spectrophotometrically as thiobarbituric acid-reactive substances (TBARs), according to the method of Mihara and Uchiyama (1978). The colorimetric determination of TBARs was based on the reaction of malondialdehyde (MDA) with thiobarbituric acid at low pH and high temperature. The resulting pink product was extracted with n-butanol, and the absorbance was determined spectrophotometrically at 535 nm.

The estimation of GSH was determined using a spectrophotometer, according to Ellman's method (1959). An aliquot of 0.5 mL of tissue homogenate was used. Proteins were precipitated using TCA, and samples were centrifuged at 3000 rpm for 10 min. The resulting supernatant was used for determination of GSH using Ellman's reagent. The absorbance was measured at 412 nm.

Detection of TNF-α and caspase 9 using ELISA kits

TNF-α and caspase-9 (Raybiotech and Cloud-Clone) were detected in hepatic tissue homogenates using standard ELISA kits, according to the manufacturer's instruction.

Total DNA extraction and fragmentation analysis

Thirty milligrams of each tissue sample were homogenized in RTL lysis buffer (Qiagen) containing 1% 2-mercaptoethanol. Total DNA was extracted using an All Prep DNA/RNA Mini kit (Qiagen, Cat# 80204), following the manufacturer's manual, and the DNA was eluted with 50 µL of the elution buffer provided. The extracted DNA was quantified using a NanoDrop-8000, and DNA fragmentation was assessed via agarose gel (1.5%) electrophoresis. Gels were trans-illuminated with a 300 nm UV light, and a photographic record was made (Sellins and Cohen, 1995).

Histopathological examination

The liver samples were fixed with 10% phosphate-buffered formaldehyde for 24h and then washed with tap water. Serial dilutions of alcohol (methyl, ethyl, and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56°C in a hot air oven for 24 h. Paraffin beeswax tissue blocks were prepared, and 5–6-µm-thick sections were cut using a sledge microtome. The tissue sections were mounted on glass slides, deparaffinized, and stained with hematoxylin and eosin for histopathological examinations with a light microscope (Domitrovic et al., 2012).

Observation of changes of liver tissue ultra-microstructure by transmission electron microscopy

After sampling, 2.5% of glutaraldehyde (prepared with dipotassium sodium arsenate) was used to fix the samples for 2 h. Samples were washed with phosphate-buffered saline three times. After fixing with 1% osmic acid (CsO_4), samples were washed three times with phosphate-buffered saline. Samples were then sequentially dehydrated with 50 and 70% alcohol, followed by 80, 90, and 100% acetone. After immersion overnight, samples were polymerized at 35, 45, and 60°C for 24 h (total of 72 h). Samples were sectioned (50–70 nm) with an ultramicrotome, treated with uranyl acetate–lead citrate dye, and then observed with a transmission electron microscope (model H-7650) (Wang et al., 2012).

Immunohistochemical detection of NF-κB and Cytochrome c

Formalin-fixed tissues were embedded in paraffin and sectioned (5-µm thickness). The liver sections were mounted overnight, deparaffinized in xylene, rehydrated with a graded series of reducing ethanol concentrations, and boiled in antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA) for 5 min. Slides were immersed in peroxidase-blocking reagent (Dako, Botany Bay, NSW, Australia) for 10 min and incubated in a humidified chamber with blocking goat serum (Dako) for 30 min. Sections were incubated with anti-NF-κB antibody (rabbit polyclonal, 1:500) in blocking solution for 12 h at 4 °C for NF-κB detection, or cytochrome c antibody (mouse monoclonal, Clone 7H8.2C1) for cytochrome C detection. They were then re-equilibrated at 25°C and washed with PBS, incubated with horseradish peroxidase (HRP) antibody conjugates (1:2500) in blocking solution without Tween® 20 for 2 h at 25°C.

Specimens were washed with PBS and incubated at room temperature with 0.2% 3,3′-diaminobenzidine (DAB) until the desired stain intensity developed, followed by washing in distilled water. Sections were counterstained with hematoxylin, dehydrated in a graded series of increasing ethanol concentrations, and mounted with di-n-butylphthalate-polystyrene-xylene (DPX) (Liu et al., 2013).

Immunoreactivity was assessed in a blinded manner by three independent observers, and the total number of positively stained cells was quantified.

Statistical analysis

Results are reported as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA). If the overall *p*-value was found statistically significant ($p \leq 0.05$), further comparisons among groups were made according to post-hoc Tukey's test. All statistical analyses were performed using GraphPad InStat 3 (GraphPad Software, Inc. La Jolla, CA, USA) software. Graphs were drawn using GraphPad Prism version 4 software (GraphPad Software).

RESULTS

Liver function tests: ALT and AST

The levels of serum ALT and AST were significantly higher in the CCl_4-treated animals compared with those of the control group. Treatment with RK (200 mg/kg) alone did not reveal any changes in the levels of the tested parameters. Pre-treatment with RK significantly and dose-dependently decreased the levels of ALT and AST in the CCl_4 model (Figures 1A and 1B).

Figure 1: A

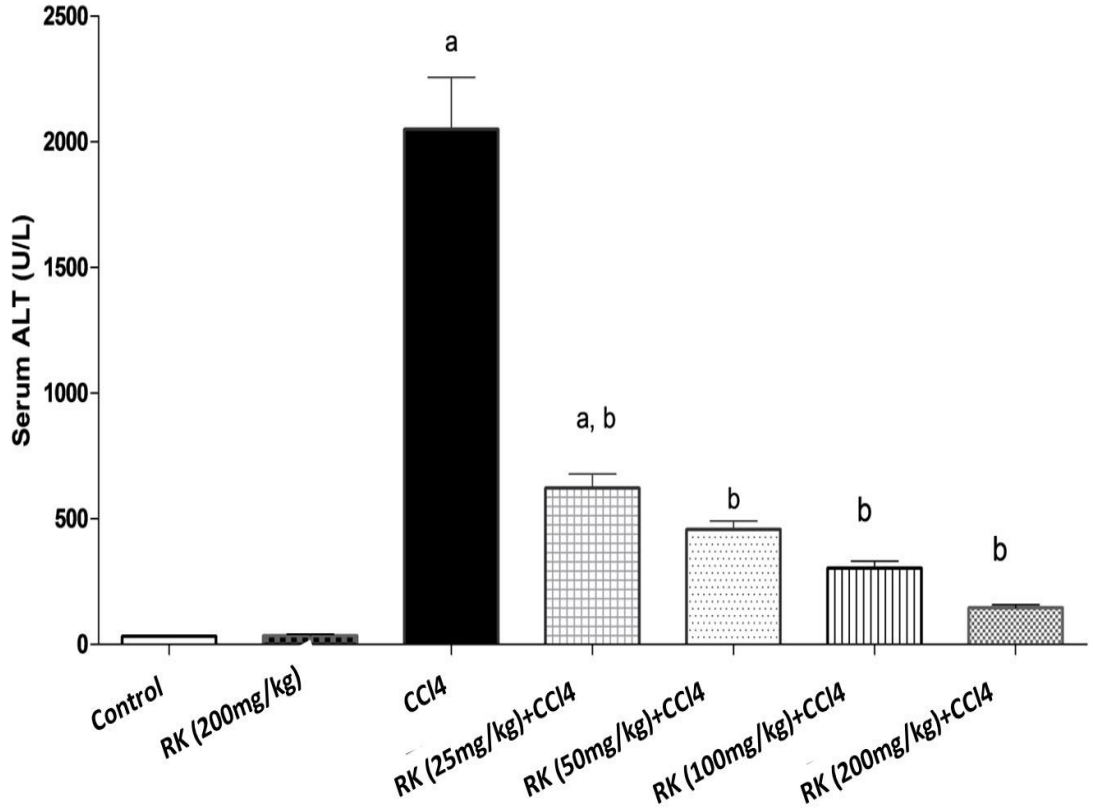


Figure.1: B

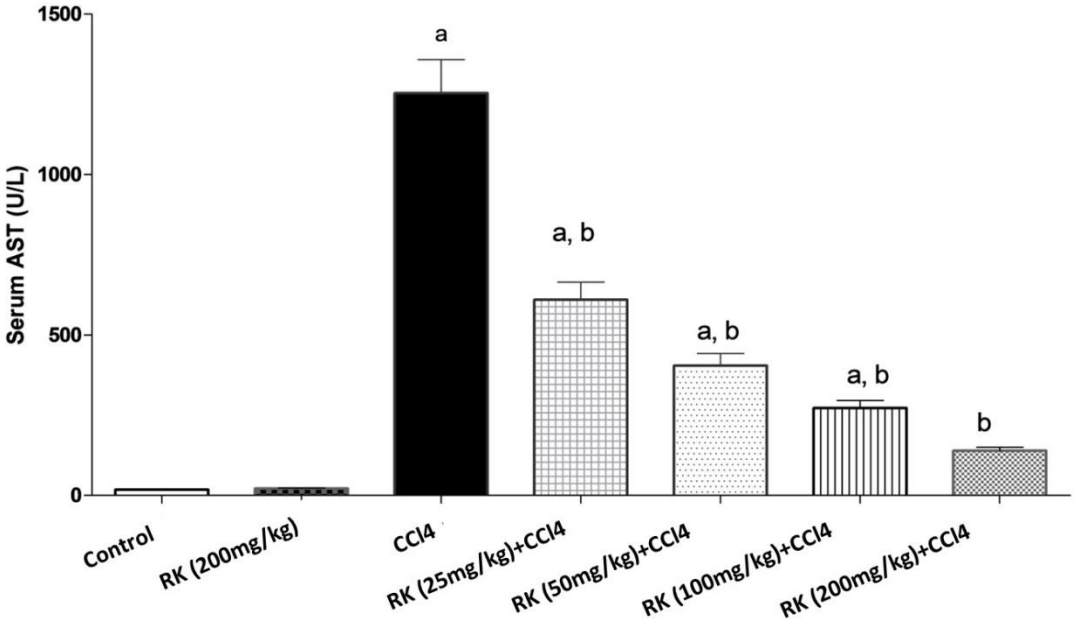
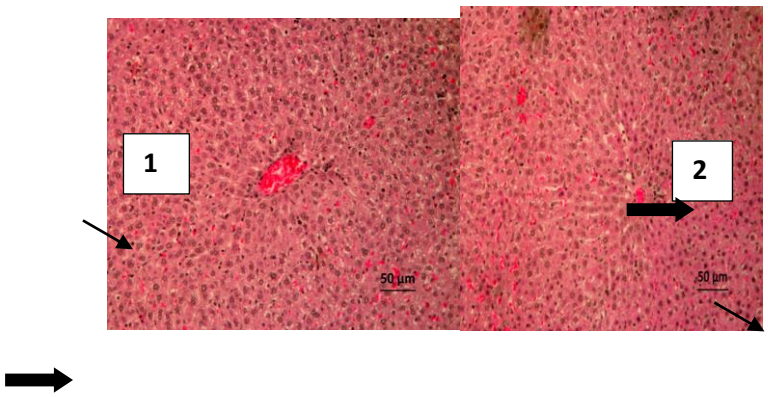


Figure 1. Serum ALT (U/L, part A) and AST (U/L, part B) in different animal groups. Each bar represents the mean of 8 rats +SEM. [a]$p \leq 0.05$with respect to the Control group; [b]$p \leq 0.05$ with respect to the CCl_4 group. ALT, alanine transaminase; AST, aspartate transaminase; RK, raspberry ketone.

Liver histopathology: Histological examination

Rats in the Control group and those treated with RK alone (Figure 2A, images 1 and 2) showed no morphological changes in the liver, and there were no significant differences found between the two groups. CCl_4 administration resulted in histopathologic changes of the liver, including vacuolation, lymphocytic infiltration, congestion of the central veins, obliteration of the blood sinusoids, pyknotic nuclei, and massive necrosis in the centrilobular area (Figure 2A, image 3).



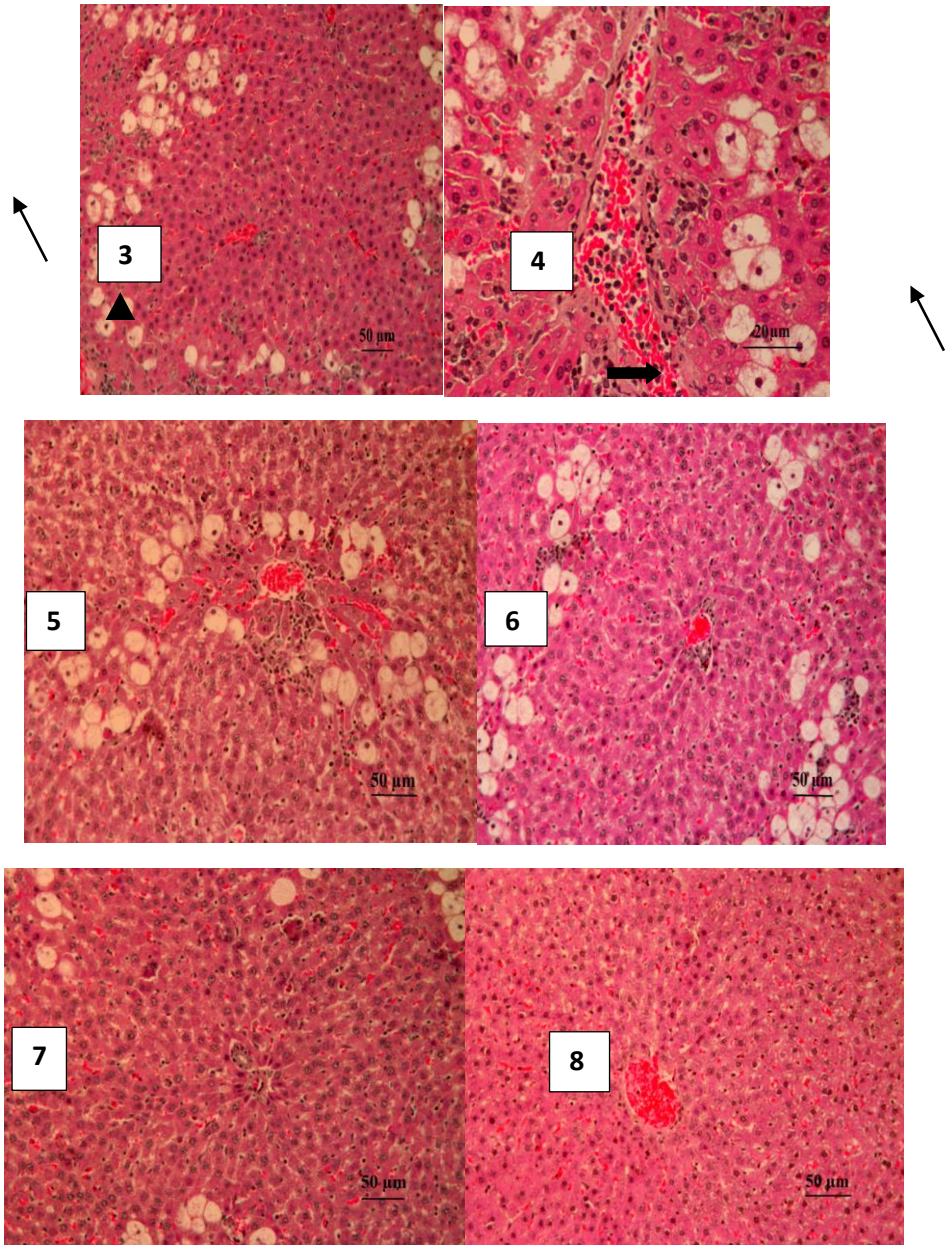


Figure 2A: Histological examinations of liver tissue sections stained with hematoxylin and eosin dye demonstrating the effect of RK on CCl_4-induced hepatic damage in rats. Light micrographs showing groups treated with vehicle (image 1) and 200 mg/kg RK (image 2), showing the normal hepatic architecture of the central vein (↑) and hepatic strands (→), CCl_4 (images 3 and 4, 20 and 40× magnification, respectively) showing vacuolation (↑), lymphocytic infiltration (→), congestion of the central veins (▲), obliteration of the blood sinusoids, pyknotic nuclei, and massive necrosis in centrilobular area, 25, 50, 100, and 200 mg/kg RK+ CCl_4 (images 5-8, respectively),showing hepatic damage decreasing in a dose-dependent manner. Rats receiving the highest dose of RK had normal hepatocytes that resembled the control group. Each image is representative of those from 8 similarly treated rats. All images are 20× magnification, except image 4 (40× magnification). RK, raspberry ketone.

Treatment with RK dose-dependently reduced the pathological changes in the liver (Figure 2A, images 5-8). These results show that RK administration protected the liver from hepatotoxicity induced by CCl_4.

Ultra-microstructure by transmission electron microscopy

In the control and RK-treated groups (Figure 2B, images 1 and 2), the ultra-microstructure of rat liver cells was normal. The liver cell nuclei were round or nearly round. The rough endoplasmic reticulum and the cytochondriome were abundant and clear. In the CCl_4 group (Figure 2B, image 3), the hepatocytes were obviously abnormal. The liver cell matrix was filled with large numbers of vacuoles, and the nuclei were no longer round. In the RK+ CCl_4 group (Figure 2B, image 4), the nuclei of the liver cells were normal. The

cytochondriome and the rough endoplasmic reticulum were abundant, and only small vacuoles were found in the matrix.

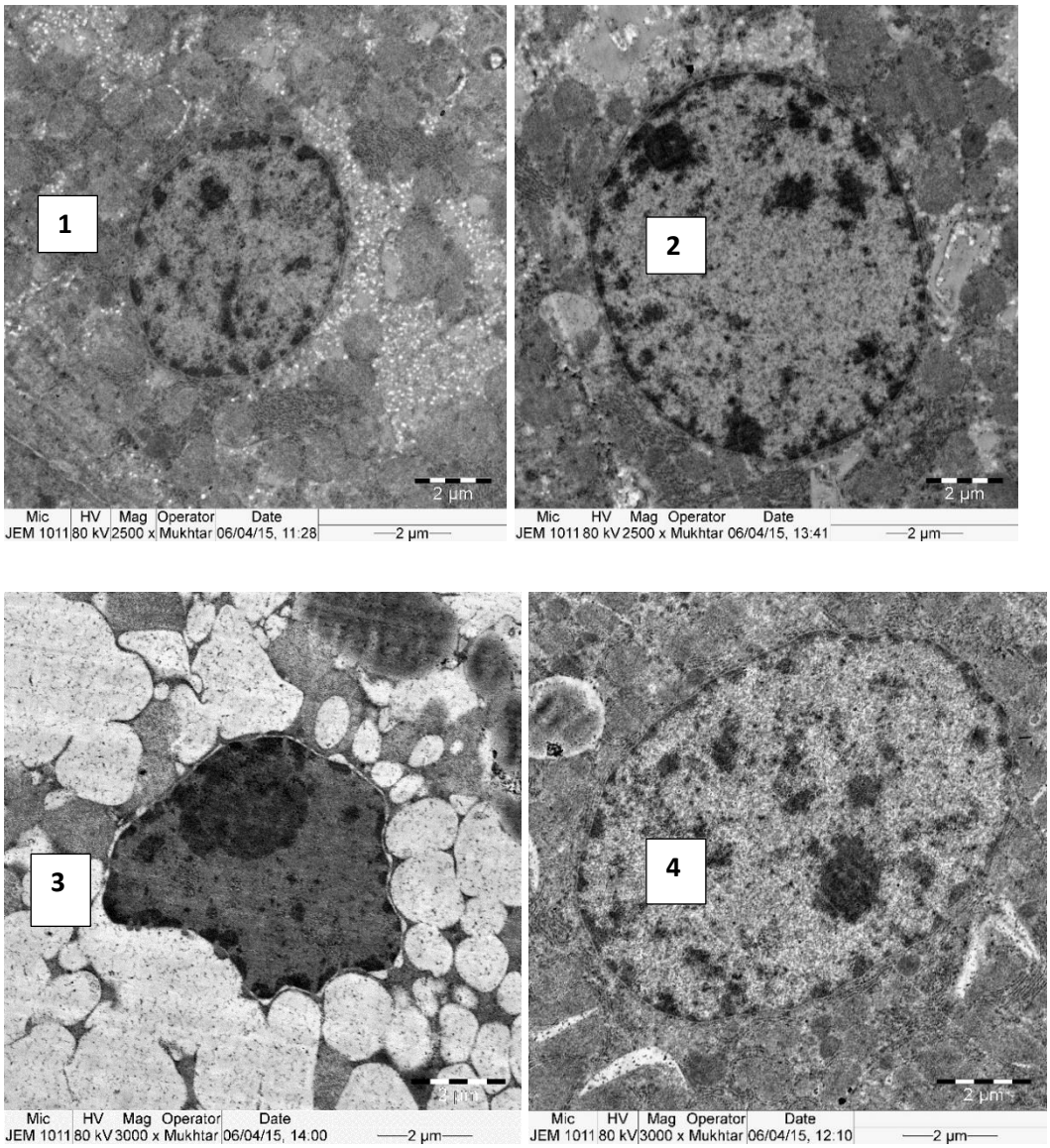


Figure 2B. The Appearance of Liver Cells by Transmission Electron Microscopy: (1) Control Group, (2) Rk (200 mg/kg) Group, (3) Ccl_4 Group, (4) Rk (200 mg/kg) + Ccl_4 Group. Rk, Raspberry Ketone.

Oxidative Stress Markers

Effect of CCl_4 and RK treatment on LPO levels

CCl_4 treatment significantly enhanced ($p \leq 0.01$) the LPO in liver tissues compared to that of the control group, as evidenced by increased TBARS. Pre-treatment with different concentrations of RK ameliorated LPO increases that would have been caused by CCl_4-induced hepatic injury. RK (200 mg/kg) alone did not cause any changes in the levels of LPO; Its LPO values were like those of the control group (Figure 3A).

Effect of CCl_4 and RK treatment on GSH levels

CCl_4 treatment significantly ($p \leq 0.01$) reduced the level of GSH compared to that of the control group (Figure 3B). RK pre-treatment at doses of 25 mg/kg, 50 mg/kg, 100 mg/kg, and 200 mg/kg significantly and dose-

dependently increased GSH levels compared to those of CCl_4 treatment alone ($p \leq 0.05$, 0.05, 0.01, and 0.001 respectively). RK (200 mg/kg) alone did not alter the levels of GSH compared to those of the control group.

Figure 3A

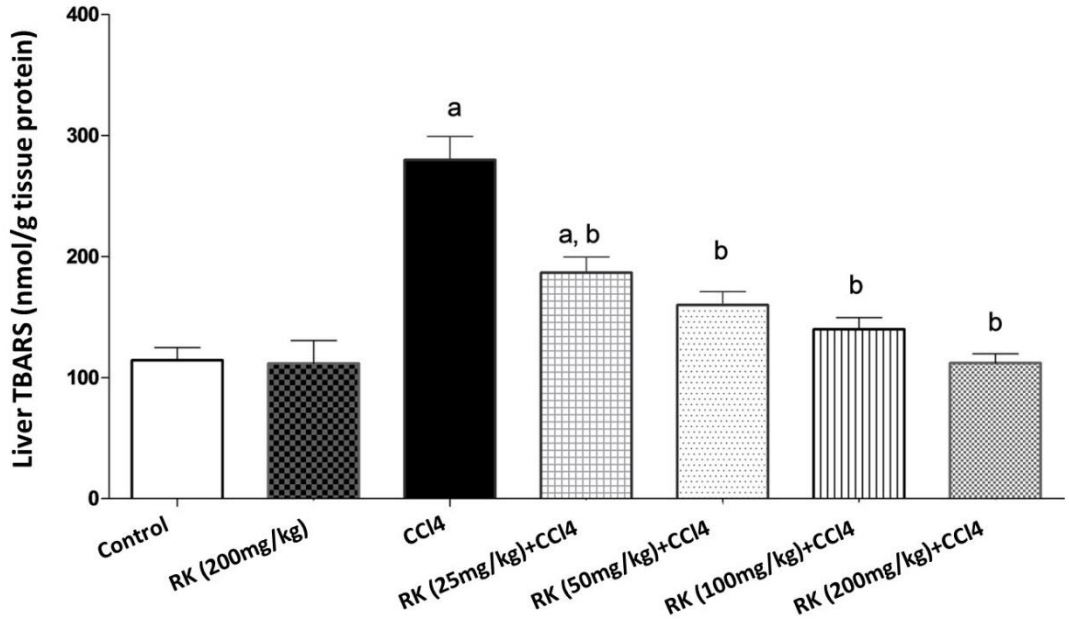


Figure 3B

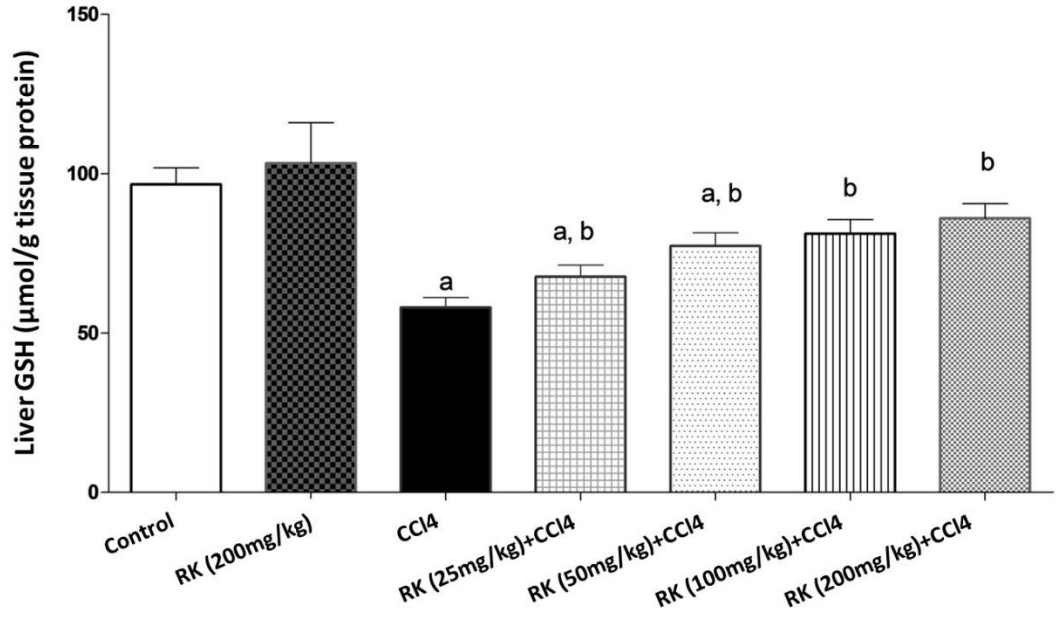


Figure 3: A: Liver TBARS (nmol/g tissue protein), and B: liver GSH (µmole/g tissue protein) in different animal groups. Each value represents the mean of 8 rats + SEM. [a]$p \leq 0.05$ with respect to the Control group; [b]$p \leq 0.05$ with respect to the CCl_4 group. TBARS: Thiobarbituric Acid-Reactive Substances; GSH: Reduced Glutathione; RK: Raspberry Ketone.

Inflammatory response markers

Nuclear factor-κB

Immunohistochemical analysis of NF-κB expression in the liver of the control group (Figure 4A) and rats treated with RK (200 mg/kg) (Figure 4B) showed low NF-κB immunoreactivity. In contrast, strong NF-κB immunoreactivity was found in hepatocytes of CCl_4-treated rats, with numerous positive nuclei (Figure 4C). Pre-treatment of CCl_4-injured rats with RK (200 mg/kg) produced a similar result as the control group, lacking nuclear immunoreactivity (Figure 4D).

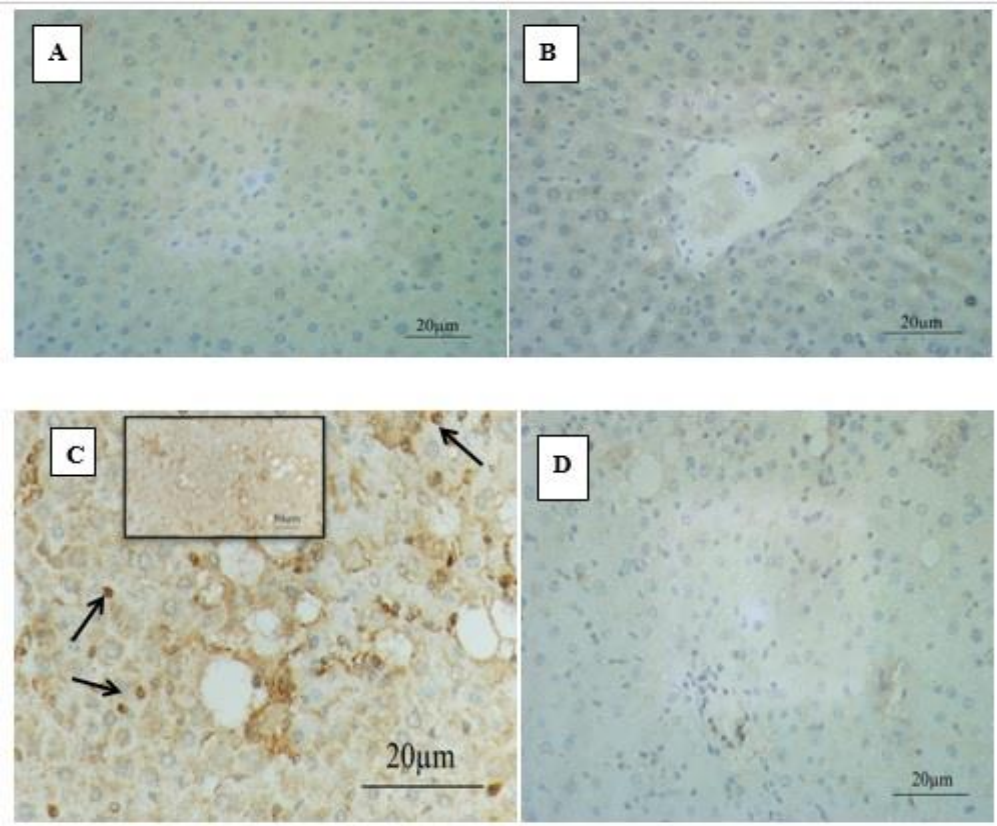


Figure 4: Immunohistochemistry was performed for NF-κB expression in rat livers in different animal groups. Rats treated with vehicle (A), RK (200 mg/kg) (B), CCl_4 (C), RK (200 mg/kg) + CCl_4 (D). Arrows show NF-κB positive nuclei. Representative results from 8 similarly treated rats are shown. Immunohistochemistry staining, original magnifications 40×.RK, raspberry ketone.

Tumor necrosis factor-α

As shown in Figure 5, the level of TNF-α quantified via ELISA markedly increased in the livers of rats treated with CCl_4. Rats treated with RK (200 mg/kg) prior to CCl_4 exhibited a significant ($p \leq 0.01$) suppression in the level of TNF-α compared to that from rats treated with CCl_4 alone. The values of the RK and RK + CCl_4 groups were comparable to those of the control group (Figure 5).

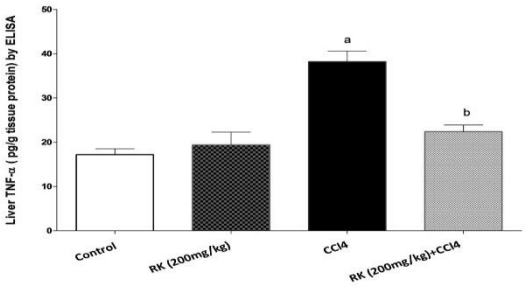


Figure 5. Liver TNF-α by ELISA (pg/g tissue protein) in different animal groups. Each value represents the mean of 8 rats +S.D. $^{a}p \leq 0.05$ compared to Control group; $^{b}p \leq 0.01$ compared to CCl_4 group. TNF-α: Tumor Necrosis Factor-α; ELISA: Enzyme-Linked Immunosorbent Assay; RK: Raspberry Ketone.

Kidney genomic DNA

Figure 6 shows the qualitative changes in the integrity of the liver genomic DNA, which is indicative of apoptosis. Agarose gel electrophoresis shows that CCl_4 treatment resulted in the fragmentation of DNA into oligonucleosome-length fragments (lane 4). However, DNA isolated from control rats (lane 2) and RK (200 mg/kg) treated rats (lane 3) showed no DNA fragmentation. DNA of rats treated with RK (200 mg/kg) prior to CCl_4 (lane 5) showed marked improvement in the integrity of the liver genomic DNA.

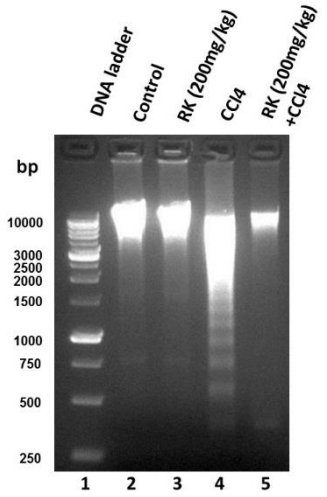


Figure 6. Liver DNA fragmentation in control and experimental rats. Lane 1: DNA ladder, 2: control group, 3: group treated with RK (200 mg/kg),4: group treated with CCl_4,5: group pre-treated with RK (200 mg/kg) and then CCl_4.

Caspase-9 activity

The apoptosis that was induced in the CCl_4 group correlated with elevatedcaspase-9 activity, which is also known as an index of apoptosis. However, the group treated with RK prior to CCl_4 had significantly reduced caspase-9 activity (Figure 7).

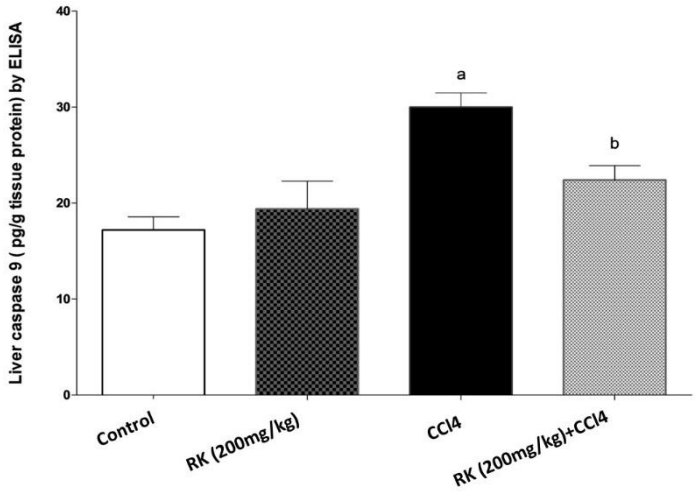


Figure 7. Caspase-9 activity by ELISA (pg/g tissue protein) in different animal groups. Each value represents the mean of 8 rats +S.D. $^{a}p \leq 0.05$ compared to Control group; $^{b}p \leq 0.05$ compared to CCl_4 group. ELISA, enzyme-linked immunosorbent assay; RK, raspberry ketone.

Cytochrome c protein expression

Cytochrome c protein expression was assessed by immunohistochemistry in the livers of the control group (Figure 8A) and rats treated with RK (200 mg/kg) (Figure 8B); both groups showed low cytochrome c immuno-reactivity. In contrast, strong cytochrome c expression was found in hepatocytes of CCl_4-treated rats (Figure

8C). Pre-treatment of CCl_4-injured rats with RK (200 mg/kg) reduced cytochrome c protein expression (Figure 8D).

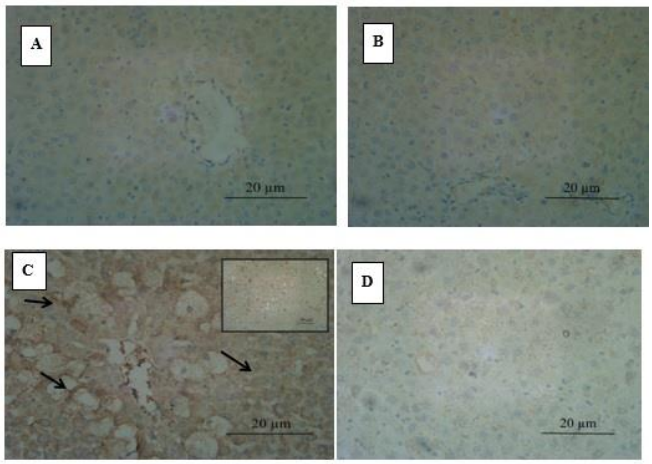


Figure 8. Immunohistochemistry was performed for expression of cytochrome c in rat livers. Rats treated with vehicle (A), RK (200 mg/kg) (B), CCl_4 (C), RK (200 mg/kg) and then CCl_4 (D). Arrows show cytochrome c positive expression. Representative results from 8 similarly treated rats are shown. Original magnification 40×.RK, raspberry ketone.

DISCUSSION

This study focused on the protective effect of RK against CCl_4-induced hepatotoxicity in male rats. RK is a major aromatic compound of raspberries and used in cosmetics and as a food-flavouring agent (Beekwilder et al., 2007).

Raspberry fruits contain various components, such as vitamins, organic acids, flavonoids, ellagic acid, and anthocyanins. In recent years many researchers have examined the effect of plant extracts as hepatoprotectives agents. In the present study, we used four doses of RK (25, 50, 100, 200 mg/kg animal body weight) and our data showed that RK administered protected rats from CCl_4-induced liver injury. The therapeutic benefits were more powerful for the higher dose (200 mg/kg bw) than for the lower doses as indicated by reducing the level of liver enzymes in serum (ALT and AST) and other biomarkers. This result coincides with result obtained by silymarin treatment in rats with the same dose (Clichici et al., 2016). A similar result was obtained using curcumin at the same dose and the extract *Curcuma longa* L (Lee et al., 2017).

The liver is the largest organ of the human body. In addition to its crucial role in the metabolism of nutrients, the liver is also responsible for the biotransformation of drugs and chemicals to protect the body against toxic foreign materials (Aghel et al., 2011). In this process, the liver is exposed to high concentrations of toxic chemicals and their metabolites, which may cause liver injury. There are more than 100 well-known liver diseases with diversified etiopathologies. The most frequent causes of hepatic disease include infectious agents (especially hepatitis A, B, and C), obesity-related fatty liver disease, xenobiotics-induced liver injury (e.g., alcohol, drugs, and chemicals), inherited and genetic liver diseases, autoimmune hepatitis, liver cirrhosis, and primary or secondary liver cancer (Al-Asmari, 2014).

CCl_4-induced acute liver injury in mice and rats is widely used as an experimental animal model for screening hepatoprotective drugs (Corcoran and Ray, 1992). Morio et al., 2001; Yuan et al., 2008; Lee et al., 2011). Generally, metabolites such as CCl_3 radicals, oxidative stress liver injuries, and the induction of apoptosis are the main reasons for CCl_4-induced hepatotoxicity. GSH is the key antioxidant in the liver, and its homeostasis is associated with various toxin-induced liver injuries (Zhang et al., 2014).

The detection of liver injury involves analyses that include assaying liver function and oxidative stress markers such as AST, ALT, lipid peroxidation, and GSH. CCl_4 causes the depletion of GSH content that plays an important role in scavenging free radicals and other reactive oxygen species. GSH protects cellular molecules from oxidative damage, including membrane lipid peroxidation (Yadav et al., 1997). Liu et al., 2002). Gumieniczek,2005).

Increased free radical generation in injured hepatic tissue could result in hepatic necrosis (Jaeschke et al., 2002). TBARS is a major reactive aldehyde, generated during the peroxidation of polyunsaturated fatty acids; it is a useful indicator of oxidative damage (Cheeseman, 1993).

Our present results also show that exposure of rats to CCl_4 causes significant increases in the release of ALT and AST, and increased lipid peroxidation, which indicates that the CCl_4 causes severe liver cell damage due to free radical-induced hepatic injuries (Alkreathy et al., 2014). After treatment with RK, GSH content and LPO levels markedly improved. In addition, the activities of serum ALT and AST decreased significantly.

Furthermore, the histopathological observations support the results obtained from the enzyme assays. The histology of the control and RK groups revealed a normal hepatic architecture (Figure 2, images 1 and 2). In the CCl_4-induced group, severe hepatotoxicity (i.e., massive fatty changes, necrosis, ballooning degeneration, and broad infiltration of lymphocytes and Kupffer cells around the central vein) was observed (Figure 2, images 3 and 4). The histological architecture of liver sections of rats treated with different doses of RK showed a relatively normal lobular pattern, with a mild degree of fatty change, necrosis, and lymphocyte infiltration compared to control group histology (Figure 2, images 5-8). The combination of ultrastructural examination of liver and histopathological observation of hematoxylin and eosin staining under the light and electron microscopes revealed remarkable histological regeneration.

The results of hepatoprotective effects of different RK doses on CCl_4-treated rats are shown in Figure 1. These effects are exemplified by a decrease in the activities of serum ALT, AST, and reduced lipid peroxidation in the RK + CCl_4 group versus the control group. Oxidative stress and inflammation play a central role in acute liver damage induced by toxic compounds (Khan et al., 2011). Reyes-Gordillo et al., 2007).

We showed that RK also reduces oxidative liver damage of lipid to protect the stability of liver cell membranes, reduce secretion of the inflammatory factor, TNF-α, and lessen inflammation of liver cells in CCl_4-treated rats, perhaps through enhancement of peroxisome proliferator-activated receptor-γ (PPAR-γ) expression (Wang et al., 2012). Several toxins have been described that can damage DNA by oxidation or alkylation, and ultimately lead to apoptotic cell death. CCl_4-induced toxicity in isolated hepatocytes is mediated by a direct solvent injury to cell membranes (Berger et al., 1986).

However, CCl_4 induces the release of cytokines, which may contribute to pathophysiologic processes, culminating in hepatocyte apoptosis after toxic injury to the liver (Czaja et al., 1995). Leist et al., 1997).

Our study found that the serum level of the inflammatory cytokine, TNF-α, and NF-κB expression increased significantly in the CCl_4 group. TNF-α can affect the cytochrome P450 enzyme system to cause cytochondriome damage, further inflammatory reactions, and hepatocellular damage. After treatment with RK, serum TNF-αcontent decreased significantly, which suggests that RK can reduce the release of TNF-αby inhibiting NF-κB activation and decreasing intracellular ROS production (Luedde and Schwabe, 2011).

In addition, RK has been shown to inhibit NF-κB activation by suppressing IκB-α phosphorylation and subsequent degradation, which could regulate inflammation (Jeong and Jeong, 2010). In the present study, CCl_4 could have degraded the DNA of rat liver tissue by generating free radicals. In addition, the increase in the activity of caspase-9 and cytochrome c expression indicated the induction of apoptosis. Apoptosis can be caused by drugs and toxins (Corcoran et al., 1994) and is responsible for various degenerative diseases (Manierea et al., 2005). Sahreen et al., 2014). The free radical products of lipid peroxidation can react with DNA, causing oxidative damage through the formation of M_1d Gadducts and the mutagenic pirimedopurinone adduct of deoxyguanosine (Marnett, 2000). RK could cause a decrease in DNA fragmentation and apoptosis through a reduction in oxidative stress.

These findings show, for the first time, the hepatoprotective activity of RK. RK ameliorates hepatic oxidative stress and suppresses inflammation and apoptosis in CCl_4-injured liver. In addition, it appears to play an important role in the prevention of acute liver damage induced by free radicals. RK exhibits, even more potently, therapeutic activity through anti-apoptotic and anti-inflammatory properties.

CONCLUSION

In conclusion, to the best of our knowledge this is the first study to demonstrate the hepatoprotective effect of RK against liver injury induced by CCl_4. RK showed dose-dependent cytoprotective effects against liver injury induced by CCl_4 at least partly through the inhibition of the proinflammatory NF-κB pathway and the reduction in TNF-α levels. Moreover, treatment with RK reduced apoptosis, as indicated by a decrease in caspase-9 activity, DNA fragmentation, and cytochrome c expression and ameliorated oxidative stress changes in the liver. Our data suggested that RK protects the rat's liver from CCl_4-induced injury and can be considered as a potential therapeutic antioxidant agents against hepatotoxicity.

AUTHOR CONTRIBUTIONS

All authors listed have contributed sufficiently to this study. "D.F. and A.B conceived and designed the work, per-form the practical experiments, analyzed and revised the data.

CONFLICTS OF INTEREST

To the best of our knowledge, no conflict of interest, financial or others, exists. All authors are fully aware of this submission. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results".

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